STUDIES ON THE ANTIBIOTICS FROM *STREPTOMYCES SPINICHROMOGENES* VAR. *KUJIMYCETICUS*. V

SOME ANTIMICROBIAL CHARACTERISTICS OF KUJIMYCIN A AND KUJIMYCIN B AGAINST MACROLIDE RESISTANT STAPHYLOCOCCI

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Two neutral macrolide antibiotics, kujimycin A (desacetyl lankamycin) and kujimycin B (lankamycin) inhibited the growth of certain macrolide resistant strains of staphylococci. The antimicrobial activities of kujimycins A and B against the strains of clinically isolated staphylococci which were resistant to other antibiotics were examined. Kujimycins A and B inhibited the growth of strains resistant to penicillin (PC), tetracycline (TC), streptomycin (SM), kanamycin (KM) and chloramphenicol (CP) as well as strains of an unknown type of staphylococcus resistant to erythromycin (EM) and oleandomycin (OM) constitutively. Kujimycins A and B did not inhibit the growth of group A strains (EM, OM, leucomycin (LM), spiramycin (SPM) resistant or EM, OM, LM, SPM, lincomycin (LCM) resistant), group B strains (EM, OM-resistant) and group C strains (EM, OM, LM, SPM, LCM-resistant) carrying induced resistance. Kujimycins A and B were found to be capable of inducing macrolide resistance as do EM and OM in inducible resistant strains. *Staphylococcus aureus* TPR-4B, a laboratory-developed strain, was constitutively resistant to LM and SPM at the concentration of 100 mcg/ml or more but it was sensitive to kujimycins A and B at the concentration of 12.5 mcg/ml. The minimal inhibitory concentrations of kujimycins A and B against *Staphylococcus aureus* FDA 209P did not change at the range of pH 5.8~8.2.

KONO *et al.*[1], in 1966, classified three types of macrolide (Mac) antibiotic-resistant staphylococci isolated from patients. These are: group A strains (resistant constitutively to erythromycin (EM), oleandomycin (OM), leucomycin (LM), spiramycin (SPM) and sometimes to lincomycin (LCM); group B strains (resistant to EM and OM); and group C strains (resistant inducibly to EM, OM, LM, SPM and LCM). Recently, group B strains were also found to be inducible[2]. The resistance of group B strains and group C strains is effectively induced by EM or OM. However, the antimicrobial spectra of neutral Mac antibiotics against the resistant strains of each group have never been reported.

In the investigation of the antimicrobial properties of kujimycins A and B which were prviousley reported to be neutral Mac antibiotics[3], they showed some different antimicrobial spectra from those of EM and OM. Kujimycins A and B were employed to examine the cross resistance with EM, OM, LM, SPM, tylosin (TYL)

and LCM in each group of staphylococci. It was found that kujimycins A and B are capable of inducing Mac resistance and they were then compared with EM, OM and some related compounds. The effect of medium pH on minimal inhibitory concentrations (MIC) of the kujimycins were compared with those of the basic Mac antibiotics and LCM. The present paper reports the results of these examinations.

Materials and Methods

Chemicals: Kujimycins A and B were prepared by the procedure described in the previous report[3]. The standard samples of EM, OM, LM and SPM were obtained from the National Institute of Health of Japan, Tokyo. TYL is a gift from Dr. R. H. WILLIAMS, Eli Lilly and Co. Triacetyl OM and LCM were purchased commercially and purified. Acetyl EM was prepared by acetylation of EM with pyridine and acetic anhydride. Methyl arcanoside and darcanolide were prepared by the methanolysis of kujimycin A and subsequently by the chromatography on a column of silica gel as reported previously[4]. Erythralosamine and cladinose were prepared by the acid hydrolysis of EM with 0.75 N HCl as reported by E. A. FLYNN *et al*[5]. A kit of three concentration disks obtained from Eiken-kagaku Co., Ltd., Tokyo, was employed for the sensitivity test of the drug-resistant staphylococci. For the experiments of resistance induction on agar plate, Showa Sensitivity Disks obtained from Showa Yakuhin Kako Co., Ltd., Tokyo were used.

Media: Nutrient broth or nutrient agar was employed for most experiments. MUELLER-HINTON agar (Eiken-kagaku Co.) was used for sulfonamide sensitivity test.

Bacterial strains and culture conditions: Most Mac-resistant strains of staphylococci were kindly provided by the Department of Clinical Microbiology, Toranomon Hospital, Tokyo and the Division of Biological Activity, the Institute of Applied Microbiology, University of Tokyo. *Staphylococcus aureus* TPR-4B is a mutant strain of *S. aureus* BR4 which was developed resistance to EM and carbomycin in laboratory[6].

For the preparation of standard inoculum, an overnight culture on nutrient agar was inoculated in nutrient broth and the culture was incubated for 18 hours at 37°C. The resistance inducibilities of kujimycins A and B, EM and related compounds were tested with *S. aureus* TPR-20 and *S. epidermidis* TPR-25.

The determination of resistance patterns of test organisms: Nineteen strains of clinically isolated drug-resistant staphylococci and strain TPR-4B were employed to determine the resistant patterns against sulfisoxazol (SA), benzyl penicillin (PC), tetracyclines (TC), streptomycin (SM), kanamycin (KM), chloramphenicol (CP), EM, OM, LM, SPM, TYL and LCM by sensitivity testing with three concentration disks. An overnight culture of each strain (containing about 1×10^9 cells/ml) was served as the standard inoculum. For the sensitivity test, one tenth ml of the standard inoculum was spread on an agar plate and the plate was incubated for 24 hours at 37°C. The strain showing no inhibitory zone around the disk of the highest concentration of the antibiotic was regarded as the resistant one. Disks of TYL (30 mcg/disk) and LCM (30 mcg/disk) were prepared in our laboratory. The criterion for resistance to SA was fixed at above 12.5 mcg/ml by disk method.

The resistant pattern of *S. aureus* TPR-4B to Mac antibiotics: The MIC of EM, OM, LM, SPM, TYL and LCM against *S. aureus* TPR-4B were determined by two-fold broth dilution method. One twentieth ml of an overnight culture of the strain was inoculated in 5.0 ml of nutrient broth containing various concentrations of the antibiotic to be examined. After the incubation for 24 hours, the growth responses were determined visually.

The physiological tests of resistant staphylococci: Tests of the physiological characteristics of the strains were carried out by the method described by MARUYAMA *et al.*[7] *S. aureus* and *S. epidermidis* were mainly differentiated by the coagulase test and the DNase test.

The determination of MIC of kujimycins A and B against the resistant staphylococci: After the standard inoculum was diluted to 1 : 10, it was streaked with a loop on agar containing the antibiotic at the concentration of 0.2~100 mcg/ml, the plate was incubated for 24 hours at 37°C. The MIC was defined as the lowest concentration of the antibiotic which completely inhibited the growth of the test organism.

The determination of optimal concentration of kujimycin A for resistance induction: One tenth ml of the standard inoculum of the strain TPR-20 was inoculated in 1.9 ml of nutrient broth containing an inducer at several concentrations and shaken at 37°C. After incubation for 3 hours, the induction mixture was transferred into 3 ml of nutrient broth containing 100 mcg of LM per ml adjusting the optical density at about 0.05 and the broth was then shaken for 10 hours at 37°C. The optical density at 535 mμ was read at 2-hour intervals with Bausch & Lomb Spectronic-20 Colorimeter. EM was employed as a standard inducer. Each growth curve in figures was shown by absorbancy without any dilutions.

The induction of EM and LM resistance with kujimycin A, kujimycin B and oleandomycin: The induction mixture was provided by the above method. The induction with kujimycin A was compared with those of kujimycin B and oleandomycin. Each 5 mcg/ml of kujimycin A, 5 mcg/ml of kujimycin B or 0.2 mcg/ml of oleandomycin was used as an inducer respectively. After 1 hour and 3 hours of induction, the mixture was transferred into 3 ml of nutrient broth containing 100 mcg of EM or LM per ml adjusting the optical density at about 0.05 and the culture was shaken for 10 hours at 37°C.

The MIC of some Mac antibiotics against induced resistant strain: A half ml of an overnight culture broth of the strain TPR-20 was inoculated in 9.5 ml of nutrient broth containing an inducer (kujimycin A 5 mcg/ml, kujimycin B 5 mcg/ml, EM 0.1 mcg/ml or OM 0.2 mcg/ml). The culture was shaken for 1 hour and 3 hours at 37°C. One twentieth ml of the induction mixture was then inoculated in 5 ml of nutrient broth containing several two-fold dilutions of the antibiotics. After 12, 22 and 32 hours of incubation at 37°C, MIC of the antibiotic was scored. The antibiotics used in this experiment are kujimycin A, kujimycin B, EM, OM and LM.

The comparison of resistance inducibility between strain TPR-20 and TPR-25: The induction mixture of strain TPR-20 or TPR-25 was inoculated by 1×10^5 cells/ml in 5 ml of nutrient broth containing several two-fold dilutions of EM, OM and kujimycin B. After 16, 19, 22, 25 and 32 hours of incubation at 37°C, MIC of the antibiotic was scored. OM was used as an inducer.

The induction test of LM, SPM and LCM resistance with kujimycin A, kujimycin B, acetyl kujimycin, methyl arcanoside, darcanolide, cladinose, erythralosamine, acetyl erythromycin and acetyl oleandomycin: This experiment was performed by the disk method reported by GRIFFITH *et al.*[8] Each of disks containing 1, 10, 100 and 500 mcg of inducers was put on the center of agar plate inoculated with the strain TPR-20 and the disks containing the antibiotics and their related compounds were put on around the disk of inducer. Kujimycin A, kujimycin B, acetyl kujimycin, methyl arcanoside, darcanolide, cladinose, erythralosamine, acetyl erythromycin and acetyl oleandomycin were employed as the inducers and EM was employed as a positive control of the inducer. LM 30 mcg, SPM 30 mcg and LCM 30 mcg per disk were used as the antibiotics. After the plate was incubated for 24 hours at 37°C, the induction of resistance was scored by a decreased radius of the inhibition zone on the side of the test disk proximal to the disk of inducer.

The effect of pH of media on MIC of kujimycins A and B: The nutrient broth was adjusted to pH 5.8, 6.8, 7.5 and 8.2 after the sterilization. The antibiotic was serially diluted from 100 mcg to 0.01 mcg per ml by the medium. One twentieth ml of cell suspension containing 1×10^8 cells/ml which had been prepared by 10-fold dilution of an overnight culture broth of *S. aureus* FDA 209P was added to each of the test tube containing 2 ml of the above medium. EM, OM, LM, SPM, TYL and LCM were used as

references. The MIC of each antibiotic was determined after the incubation for 24 hours at 37°C.

Results

The Resistant Patterns and the Physiological Properties of Test Organisms

Nineteen strains of clinical isolates and a laboratory-developed resistant strain of staphylococci were selected for the test. They were found to show the resistance patterns and the physiological properties as shown in Tables 1 and 2. From the physiological studies of 20 staphylococcal strains, 11 strains belonged to *S. aureus* and 8 strains belonged to *S. epidermidis*. Fourteen strains of them were Mac-resistant strains which were divided into 6 strains of A group, 1 strain of B group, 3 strains of C group, TPR-4B and 3 strains of an unknown type. The last 3 strains, *S. epidermidis*, were constitutive resistant to EM and OM. Hence, these strains could not be assigned into any group of Mac-resistance according to the previous classification[1].

The growth of *S. aureus* TPR-4B was inhibited at the minimal concentration of SPM 200 mcg, LM 100 mcg, TYL 25 mcg, OM 6.25 mcg, EM 0.4 mcg and LCM 0.75 mcg per ml. *S. aureus* FDA 209P which was employed as a reference of sensitive strain to all samples of the antibiotics was inhibited at the minimal concentration of SPM 1.6 mcg, LM 0.4 mcg, TYL 1.6 mcg, OM 0.4 mcg, EM 0.2 mcg and LCM 0.75 mcg per ml. Most Mac-resistant strains were also resistant to a variety of other antibiotics including SA, PC, TC, SM, KM and CP. Six strains of A group, 3 strains of C group and a strain of B group are resistant to SA, PC, TC or SA, PC, TC, SM. These results agree with the observation which was reported previously by KONO *et al.* that Mac-resistant staphylococci are resistant to PC, SM, TC, SA or to combinations of three antimicrobial reagents (PC, SM, SA or PC, TC, SA)[9]. However, two strains of an unknown type (TPR-13, TPR-15) are resistant only to SA, PC and SA, PC, CP

Table 1. Physiological properties

Physiological properties	Staphylococcal strain																					
	aureus 209P	*aureus* Smith	TPR-5	TPR-14	TPR-7	TPR-1	TPR-16	TPR-3	TPR-4B	TPR-13	TPR-15	TPR-17	TPR-20	TPR-22	TPR-23	TPR-25	TPR-12	TPR-18	TPR-21	TPR-26	TPR-27	TPR-28
Coagulase test	+	+	+	−	+	+	−	+	+	−	−	−	+	−	+	−	+	+	+	+	+	−
DNase test	+	+	+	−	+	+	−	+	+	−	−	−	+	−	+	−	+	+	+	+	+	−
Egg yolk test	+	−	+	−	+	+	−	+	+	−	−	−	+	−	+	−	−	−	+	±	−	−
Phosphatase test	+	+	+	−	+	+	−	+	+	−	+	−	+	−	+	+	+	+	+	+	+	+
Protease test	+	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	±	−	−	−	−
Gelatinase test	+	+	+	−	+	+	+	+	−	−	+	−	+	−	+	+	+	+	+	+	+	−
Pigment production	Y	Y	Y	W	Y	Y	W	YW	W	W	W	W	Y	W	Y	W	Y	Y	Y	YW	YW	W
Mannitol utilization	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	−	+	+	+	+	+	+
Tellurite reduction	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+	+	+	+	+
α-Hemolysin	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+
Phage type			NT		I	NT		I	NT				NT		NT		NT	I	III	NT	NT	

Abbreviations : Y; yellow, W; white, YW; yellowish white, NT; non typable

Table 2. Drug resistance patterns of staphylococci and MIC of kujimycins A and B

	Group	Strain	Antibiotics													KJM-A MIC (mcg/ml)	KJM-B MIC (mcg/ml)
			SA	PC	TC	SM	KM	CP	EM	OM	LM	SPM	TYL	LCM			
		aureus 209P														3.0	6.0
		aureus Smith														3.0	6.0
		TPR-5	+	+												3.0	6.0
Mac		TPR-14		+				+								12.0	12.0
sensitive		TPR-7	+		+											6.0	12.0
		TPR-1	+	+	+											3.0	6.0
		TPR-16	+	+	+			+								6.0	6.0
		TPR-3	+	+	+	+										6.0	12.0
		TPR-12	+	+	+			+	+	+	+	+	+	+		>100	>100
		TPR-18	+	+	+		+	+	+	+	+	+	+	+		>100	>100
	A	TPR-21	+	+	+	+			+	+	+	+	+	+		>100	>100
		TPR-26	+	+	+	+		+	+	+	+	+	+	+		>100	>100
Constitutive		TPR-27	+	+	+	+	+	+	+	+	+	+	+			>100	>100
Mac-		TPR-28	+	+	+	+	+	+	+	+	+	+	+	+		>100	>100
resistant		TPR-13	+	+				+	+	+						6.0	12.0
	Others	TPR-15	+	+					+	+						6.0	12.0
		TPR-17	+	+	+				+	+						12.0	12.0
		TPR-4B	+	+	+					±	+	+				6.0	6.0
		TPR-20	+	+	+	+	+		(+)	(+)	(+)	(+)	(+)	(+)		100	12.0
Inducible	C	TPR-22	+	+	+	+		+	(+)	(+)	(+)	(+)	(+)	(+)		100	12.0
Mac- resistant		TPR-23	+	+	+	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)		50	12.0
	B	TPR-25	+	+	+	+	+	+	+	+	(+)	(+)	(+)	(+)		100	6.0

Abbreviation : +; resistant, (+); inducible resistant, KJM-A; kujimycin A, KJM-B; kujimycin B

respectively. These patterns of resistance seem to be different from those of group A, group B and group C strains.

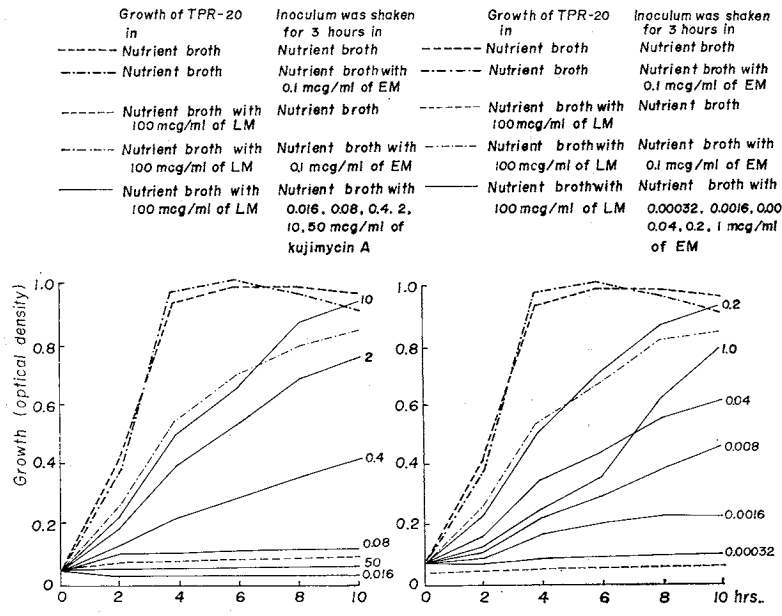
The Antimicrobial Spectra of Kujimycins A and B against the Resistant Staphylococci

Kujimycins A and B were found to have MIC at the range of 3.0 to 12.0 mcg/ml against the sensitive strains, similar to those of other known neutral Mac antibiotics. Antimicrobial activities of kujimycins A and B against drug-resistant staphylococci are shown in Table 2. Kujimycins A and B were not effective against all of group A strains at the concentration of 100 mcg/ml, and kujimycin A showed so weak inhibitory activity against group B strain and group C strains as 1/4 to 1/16 times of that of kujimycin B on nutrient agar. They inhibited the growth of all the strains which are resistant to antibiotics other than Mac antibiotics and also inhibited the growth of EM- and OM-resistant strains of an unknown type at lower concentrations. The growth of *S. aureus* TPR-4B was inhibited by kujimycins A and B at the concentration of 12.5 mcg/ml by broth dilution method.

The Determination of Optimal Concentration of Kujimycin A for Resistance Induction

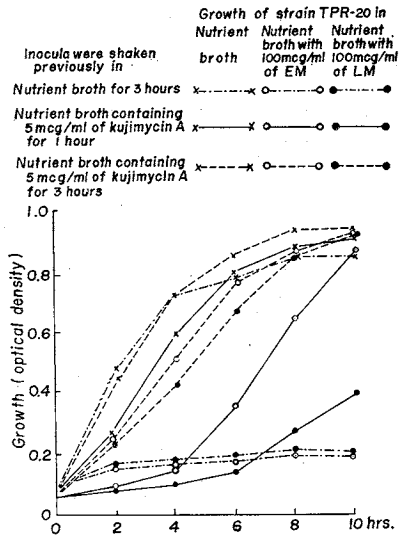
Fig. 1. Effect of kujimycin A concentration in induction mixture on induction of LM resistance.
Inoculum size : 1×10^7 cells/ml.

Fig. 2. Effect of EM concentration in induction mixture on induction of LM resistance.
Inoculum size : 1×10^7 cells/ml.



Since kujimycin A was found to exhibit the inducibility of Mac-resistance like EM and OM by the disk method, the optimal concentration of kujimycin A for resistance induction was examined[10]. As shown in Fig. 1, kujimycin A was found to induce the LM resistance in *S. aureus* TPR-20 at the concentration range of 0.4~10.0 mcg/ml. On the other hand, the apparent MIC and the proper concentration for the induction of resistance of EM (Fig. 2) were 2 mcg/ml (Table 3) and 0.0016~1.0 mcg/ml respectively.

Fig. 3. The effect of induction time with kujimycin A on induction of EM and LM resistance.
Inoculum size : 1×10^7 cells/ml.



The Induction of EM and LM Resistance with Kujimycin A, Kujimycin B or Oleandomycin

As shown in Fig. 3, EM and LM resistances were more effectively induced by the incubation with kujimycin A for 3 hours than 1 hour. It was also found that kujimycin B was capable of inducing EM and LM resistances in TPR-20 but the induction with kujimycin B was not so effective as that with kujimycin A (Fig. 4). Growth curves of the strain after induction with kujimycin B is similar with that of OM as shown in Fig. 5. The induction of EM resistance was occurred more easily than that of LM resistance in all cases. It is of interest that

Table 3. The MIC of some Mac antibiotics against resistance induced strain TPR-20

Inducer	Induction time (hrs.)	Incubation time (hrs.)	MIC of inhibitor (mcg/ml)				
			Kujimycin A	Kujimycin B	EM	OM	LM
Control		12	32	32	2	1	2
		22	64	64	32	1	2
		32	>1,000	64	>1,000	4	4
Kujimycin A 5 mcg/ml	1	12	500	64	>1,000	4	2
		22	1,000	250	>1,000	16	2
		32	>1,000	>500	>1,000	>1,000	4
	3	12	>1,000	250	>1,000	32	2
		22	>1,000	500	>1,000	>1,000	2
		32	>1,000	>500	>1,000	>1,000	4
Kujimycin B 5 mcg/ml	1	12	32	32	2	2	2
		22	64	32	125	2	2
		32	>1,000	125	>1,000	>1,000	4
	3	12	1,000	32	>1,000	2	2
		22	>1,000	32	>1,000	8	2
		32	>1,000	>1,000	>1,000	>1,000	4
EM 0.1 mcg/ml	1	12	>1,000	500	>1,000	>1,000	4
		22	>1,000	500	>1,000	>1,000	8
		32	>1,000	>500	>1,000	>1,000	8
	3	12	>1,000	500	>1,000	>1,000	4
		22	>1,000	500	>1,000	>1,000	4
		32	>1,000	>500	>1,000	>1,000	8
OM 0.2 mcg/ml	1	12	64	32	< 4	< 2	2
		22	1,000	32	>1,000	< 2	2
		32	>1,000	64	>1,000	4	4
	3	12	1,000	32	>1,000	< 2	2
		22	>1,000	32	>1,000	2	2
		32	>1,000	125	>1,000	16	4

Inoculum size; 1×10^6 cells/ml

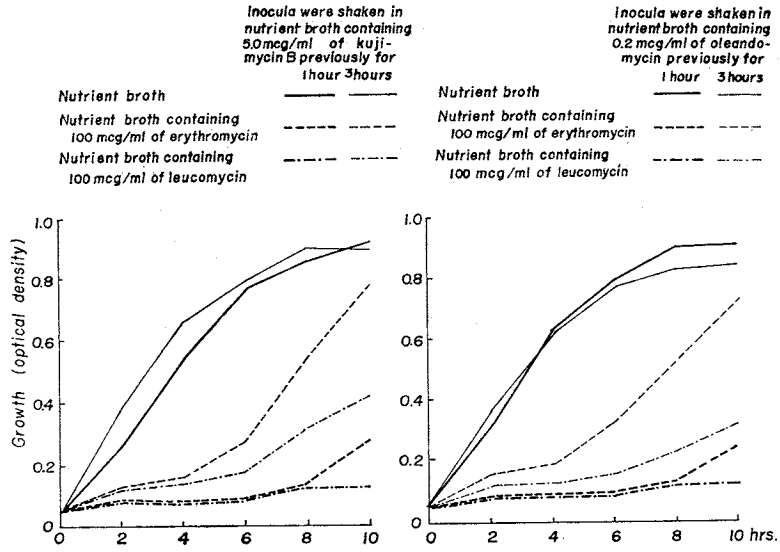
the degree of inducibility is different with kujimycin A and kujimycin B as with EM and OM.

The MIC of Some Mac Antibiotics against Induced Resistant Strain TPR-20

It was previously reported that EM is capable of inducing OM resistance in some strains of C group more than OM is[1]. The inducibilities of kujimycin A, kujimycin B and oleandomycin were compared with that of EM, after incubation for 1 hour and 3 hours at optimal concentration of each inducer. The increase of resistance to kujimycin A, kujimycin B, EM, OM and LM was measured in several intervals by the broth dilution method of MIC determination. As shown in Table 3, the induction of resistance to kujimycin A and EM occurred easily by use of kujimycin A or EM as an inducer, whereas resistance to kujimycin B or OM was not induced so easily with kujimycin B or OM. The induction of LM resistance was not observed with any inducer in this experiment. The reason why LM resistance was not induced may be due to a smaller inoculum size of 10^6 cells/ml. Consequently, it was noted that the strain TPR-20 acquired high degree of resistance to kujimycin A and EM in a shorter period by incubation with the inducer. Also, high resistance to kujimycin B and OM

Fig. 4. The effect of induction time with kujimycin B on induction of EM and LM resistance.
Inoculum size : 1×10^7 cells/ml.

Fig. 5. The effect of induction time with OM on induction of EM and LM resistance.
Inoculum size : 1×10^7 cells/ml.



generally seems to be induced by incubation for a longer time. The inducibility of LM resistance seems to be less than resistance to others. As seen in Table 3, the MICs of uninduced cultures as references were elevated in proportion to elongation of incubation time. This suggests that the resistance induction was occurred slowly with higher inhibitory concentrations of Mac antibiotics without pretreatment with inducer during the longer period of incubation.

The Comparison of Resistance Inducibility between Strains TPR-20 and TPR-25

It was found that the degree of development of MIC with incubation periods was different among some strains inducibly resistant to Mac antibiotics. When TPR-20

Table 4. The MIC of EM, OM and kujimycin B against TPR-20 and TPR-25 at various incubation time

		MIC (mcg/ml)					
		Incubation time	16 hrs.	19 hrs.	22 hrs.	25 hrs.	32 hrs.
TPR-20	No Induction	EM	0.5	2	>1,000	>1,000	>1,000
		OM	0.5	0.5	0.5	0.5	2
		KJM-B	16	16	32	32	63
	Induction	EM	>1,000	>1,000	>1,000	>1,000	>1,000
		OM	2	2	4	4	8
		KJM-B	16	16	32	32	125
TPR-25	No Induction	EM	8	>1,000	>1,000	>1,000	>1,000
		OM	2	16	63	>1,000	>1,000
		KJM-B	16	32	63	>1,000	>1,000
	Induction	EM	>1,000	>1,000	>1,000	>1,000	>1,000
		OM	8	>1,000	>1,000	>1,000	>1,000
		KJM-B	16	>1,000	>1,000	>1,000	>1,000

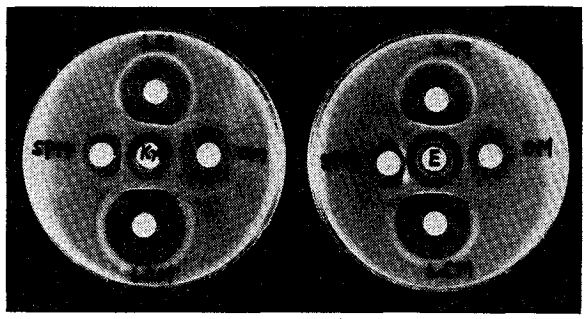
Inoculum size; 1×10^5 cells/ml. Induction; OM 0.2 mcg/ml for 3 hours. KJM-B; kujimycin B.

and TPR-25 were compared with each other, TPR-25 acquired more easily and quickly OM resistance either by preincubation with OM or without OM than TPR-20 as shown in Table 4. However, the development of the inducible resistance was generally faster by preincubation with the inducer. TPR-20 resembles *S. aureus* MS 537 reported by KONO *et al.* in inducibility of OM resistance[1]. TPR-25 also resembles *S. aureus* MS 537-1 reported to be selected from MS 537 on agar plate containing OM[11].

The Induction of LM, SPM and LCM Resistance with Kujimycin A, Kujimycin B, Acetyl Kujimycin, Methyl Arcanoside, Darcanolide, Cladinose, Erythralosamine, Acetyl EM and Acetyl OM

The inducibility of these drugs was examined by the disk method using strain TPR-20. No inducibility was observed with the compounds except kujimycin A, acetyl EM and acetyl OM. Kujimycin B showed weak inducibility. The result of this experiment indicates that compounds possessing resistance inducibility properties must have growth inhibition capability. Resistance induction with kujimycin A and EM was shown in Plate 1.

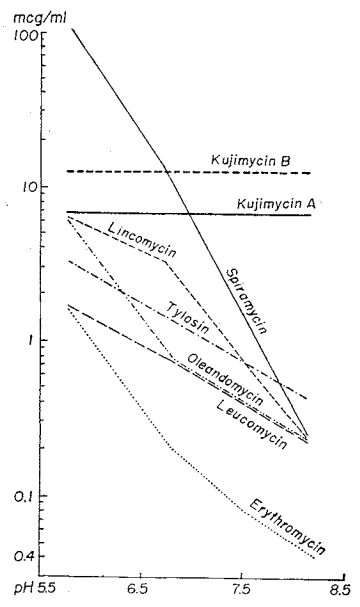
Plate 1. The resistance induction with kujimycin A and EM.
Inducer : the center of the left plate···kujimycin A, the center of the right plate···EM.
Inhibitors : from the top of the plate to right side··· LM, OM, LCM and SPM.



The Effect of pH of Media on MIC of Kujimycins A and B

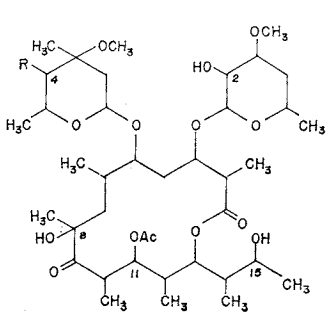
Basic Mac antibiotics are known to show a larger growth inhibition in a high pH medium than in one of lower pH[12]. But the activity of neutral Mac antibiotics seems not to be effected by varied pH of the medium, as there are no ionized groups in the molecule. The effect of pH of the medium on the growth inhibitory activity of kujimycins and some related antibiotics was tested by the broth dilution method in the range from pH 5.8 to 8.2. The MIC of kujimycins A and B were changed slightly with the varied pH of media, whereas those of five basic Mac antibiotics and LCM employed as references were notably changed as shown in Fig. 6. These observetions seem to be correlate with the finding by MAO[13] that EM and OM inhibited polyphenylalanine synthesis in cell-free system of *S. aureus* to a great extent at higher pH than at lower pH but chalcomycin (a neutral Mac antibiotic) did not show any significant difference in the extent of inhibition produced at

Fig. 6. The effect of pH of media on MIC of Mac antibiotics and LCM.
(Test organism : *Staph. aureus* FDA 209P)

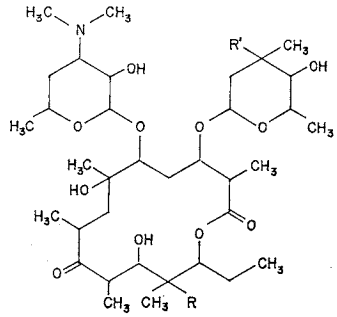


various pH values. MIC of Mac antibiotics possessing high pK value such as EM and OM (pK 8.5~8.6) seem to be effected by medium pH more than those possessing low pK value such as LM (pK 6.6~6.7) and TYL (pK 7.1) except SPM (pK 7.6~7.7). The magnitude of change of MIC of the five basic Mac antibiotics at various pH was not proportional to pK values of these antibiotics but was almost proportional to mobilities in high-voltage paper electrophoresis which was reported by MAEDA and his co-workers as relative mobility (*RM*; SPM 0.88, OM 0.57, EM 0.50, LM 0.46, TYL 0.45). The mobilities of the antibiotics relative to alanine were shown as *RM* values.)[14].

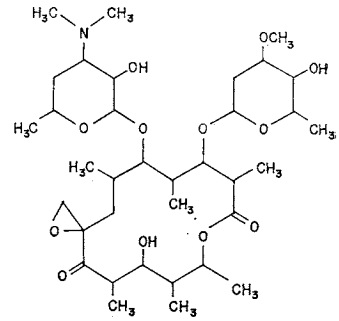
Fig. 7. The structures of kujimycins, EM and OM.



Kujimycins
Kujimycin A : R=-OH
Kujimycin B : R=-OCOCH$_3$



Erythromycins
Erythromycin : R=-OH, R'=-OCH$_3$
Erythromycin B : R=-H, R'=-OCH$_3$
Erythromycin C : R=-OH, R'=-OH



Oleandomycin

Discussion

GARROD and WATERWORTH reported that staphylococcal strains with dissociated resistance were converted to strains resistant not only to EM, but also to other Mac antibiotics in the presence of subinhibitory concentration of EM[15,16]. PATTEE and BALDWIN described that this enhanced resistance of dissociated strain was acquired by induction with low concentrations of EM[17]. OM was found to be also capable of inducing resistance as well as EM[1,10]. Recently, the inducibility with LM and LCM was recognized in certain mutant strains of C group *Staphylococcus*[11,18].

According to studies by several investigators, the antibiotics to which resistance is induced by EM include some Mac antibiotics (EM, OM, SPM, LM, carbomycin, niddamycin and TYL) as well as some other antibiotics affecting on ribosomal 50S subunit, which are lincosaminide (LCM, 7-chloro-7-deoxy-LCM) and the group B antibiotics of the streptogramin type (streptogramin B, pristinamycin I, vernamycin B$_\alpha$ and viridogrisein[8,15,17,19,20,21,22].

In addition, it was found in the present studies that kujimycins A and B, which were found to be neutral Mac antibiotics, were as capable as EM of inducing resistance in inducible strains. EM and OM were also capable of inducing resistance to kujimycins A and B. Moreover, it was noted that kujimycin A is capable of inducing Mac resistance in TPR-20 more easily than kujimycin B. Kujimycin B contains an acetyl group at C-4 of the arcanose, and is otherwise identical to kujimycin A. This shows that the degree of inducibility with Mac antibiotics is dependent upon minor structural changes in the antibiotic, although no marked variation was seen in the antimicrobial activity against a sensitive strain. A similar investigation was reported by SAITO *et al.* in respect to the resistance inducibility of several LM derivatives[11]. The strain used for their experiment

was *S. aureus* MS 537-59, a mutant strain of MS 537, in which resistance was induced with LM. One derivative of LM having no acyl group at C-4 position of mycarose was not effective for resistance induction. Other derivatives having acetyl, propionyl, butyl and isovaleryl groups induced resistance. The one having propionyl side chain was most effective. These results seem to be somewhat different from the result of our experiment with kujimycins. NAKAJIMA *et al.* reported the fact that the resistance inducibility and the antimicrobial activity of EM could not be separated using EM, acetyl-EM and their chemical degradation products[18]. In our experiment with kujimycins A and B, acetyl kujimycin and their partial degradation products, kujimycins A and B only showed resistance inducibility and antimicrobial activity. Similar results are obtained with compounds derived from EM. Mac antibiotics exhibiting inducibility generally seem to have in common a macrocyclic 14-membered ring lactone and two sugar moieties forming glycosidic linkages directly to the lactone. A 9-carbonyl group on the lactone is reported to be important for resistance induction[18]. The 2-hydroxyl and 3-dimethyl amino groups of desosamine (EM, OM), the 6, 11, 12-hydroxyl groups of the lactone (EM, OM) and the 4-hydroxyl group of arcanose (kujimycin A) and oleandrose (OM) are not obligate for induction of resistance as these groups are not common in EM, OM, their acetyl derivatives and kujimycins. The lack of inducibility with acetyl kujimycin may indicate the importance of a free hydroxyl group for resistance induction.

MAO and PUTTERMAN studied the binding of EM-A to ribosomes of *S. aureus* and proposed that some important groups of EM, such as 2'-, 11-, 12-hydroxyl, 3'-dimethylamino, 9-keto and 3''-methoxyl groups, form hydrogen bond with primary amino groups and the nitrogen of nucleotide bases of ribosomal RNA and that ribosomal protein stabilizes the complex by forming hydrophobic bonds with hydrophobic region of EM[23]. From the experiment with kujimycins it appears that the important groups for binding of kujimycins to ribsosmes are the C-2 hydroxyl group of lankavose and/or C-15 hydroxyl group of lankolide since kujimycins lose their antimicrobial activities by acetylation.

It has been reported that a decrease of the binding ability of the ribosome of strains resistant to EM[1,24] accounts for some of the resistance to Mac antibiotics in *S. aureus* rather than inactivation of Mac antibiotics. This explanation had also been made in the case of resistant strains of *Bacillus subtilis* and *Escherichia coli* developed *in vitro*[25,26]. TANAKA *et al.* suggested that a change of an amino acid component in one of 50S ribosomal proteins caused the decrease in affinity of ribosomes for EM seen with a resistant strain of *E. coli* WILHELM *et al.* showed that the genetic mutation leading to EM-A resistance in *B. subtilis* might affect the binding affinity of ribosomes for the neutral sugar of the antibiotics, since the Mac antibiotics of monoglycosides (methymycin and LCM) bind equally well to both sensitive and resistant ribosomes of *B. subtilis*[27]. But with inducible resistant strains the situation appears to be more complete because the resistance of these strains arises quickly after incubation with inducer and disappears in several generations after removal of the inducer. Further, the induced resistant strains are resistant not only to Mac antibiotics possessing two sugar moieties but also to methymycin and LCM possessing a basic monoglycoside. It is suggested from these investigations that resistance in inducible resistant strains of staphylococci is accomplished by an unknown induced alteration in the 50S ribosomes and consequently a decreased binding affinity of the ribosome to Mac antibiotics and LCM is observed.

Among the Mac antibiotics which were employed for the present study, kujimycins were closely related to EM and OM in their ability to induce resistance. But it is interesting that the strains of an unknown type, *S. epidermidis* resistant to EM and OM, are sensitive to kujimycins A and B. The antimicrobial spectra in the strains of this group, kujimycins A and B resemble LM and SPM rather than EM and OM (Table 5). This study indicated four types (A, B, C and the unknown group) of Mac resistant strains of *S. epidermidis* from clinical isolates. The main questions which arise from these

results are as follows: (1) What is the mechanism of constitutive resistance to EM and OM in the strains of the unknown type? (2) Are there genetic difference in EM and OM resistance between the strains of the unknown type and group A strains? (3) Can strains of *S. aureus* belonging to the unknown type, resistant constitutively to EM and OM, be isolated clinically?

Table 5. Types of Mac resistance in staphylococci

Antibiotics	Group				
	A	B	C	Unknown	TPR-4B
EM	+	(++)	(++)	+	−
OM	+	(++)	(+)	+	±
Kujimycin-A	+	(++)	(++)	−	−
Kujimycin-B	+	(++)	(+)	−	−
LM	+	(−)	(−)	−	+
SPM	+	(−)	(−)	−	+

(++); strong inducibility (+); weak inducibility
(−); no inducibility +; resistant −; sensitive

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References

1) KONO, M.; H. HASHIMOTO & S. MITSUHASHI: Drug resistance of staphylococci. III. Resistance to some macrolide antibiotics and inducible system. Jap. J. Microbiol. 10: 59~66, 1966
2) SAITO, T.; H. OSHIMA, M. SHIMIZU, H. HASHIMOTO & S. MITSUHASHI: Macrolide resistance in *Staphylococcus aureus*. Progress in antimicrobial and anticancer chemotherapy (Proceedings of the 6th International Congress of Chemotherapy). II. pp. 572~578, University of Tokyo Press. 1970
3) OMURA, S.; S. NAMIKI, M. SHIBATA, T. MURO, H. NAKAYOSHI & J. SAWADA: Studies on the antibiotics from *Streptomyces spinichromogenes* var. *kujimyceticus*. II. Isolation and characterization of kujimycins A and B. J. Antibiotics 22: 500~505, 1969
4) OMURA, S.; T. MURO, S. NAMIKI, M. SHIBATA & J. SAWADA: Studies on the antibiotics from *Streptomyces spinichromogenes* var. *kujimyceticus*. III. The structures of kujimycin A and kujimycin B. J. Antibiotics 22: 629~634, 1969
5) FLYNN, E. H.; M. V. SIGAR, Jr., P. F. WILEY & K. GERZON: Erythromycin. I. Properties and degradation studies. J. Am. Chem. Soc. 76: 3121~3131, June 20, 1954
6) TANAKA, N.; N. MIYAIRI, T. NISHIMURA & H. UMEZAWA: Activity of mikamycins, angustmycins and emimycin against antibiotic-resistant staphylococci. J. Antibiotics, Ser. A 14: 18~22, 1961
7) MARUYAMA, T.; Y. NAOI, K. KITAMURA, Y. KOKUBO & S. MATSUMOTO: Studies on the biological properties of staphylococci isolated from foods (in Japanese). Annual Report of Tokyo-To Laboratories for Medical Sciences 17: 135~141, 1965
8) GRIFFITH, L. J.; W. E. OSTRANDER, C. G. MULLINS & D. E. BESWICK: Drug antagonism between lincomycin and erythromycin. Science 147: 746~747, 1965
9) KONO, M.; T. KASUGA & S. MITSUHASHI: Drug resistance of staphylococci. IV. Resistance patterns to some macrolide antibiotics in *Staphylococcus aureus* isolated in the United States. Jap. J. Microbiol. 10: 109~113, 1966
10) WEAVER, J. R. & P. A. PATTEE: Inducible resistance to erythromycin in *Staphylococcus aureus*. J. Bact. 88: 574~580, 1964
11) SAITO, T.; H. OSHIMA, H. HASHIMOTO & S. MITSUHASHI: Drug resistance of staphylococci. Jap. J. Bact. 23: 590~591, 1968
12) SANO, Y.; T. HOSHI & T. HATA: Studies on leucomycin. IV. Comparison of leucomycin with erythromycin and carbomycin. J. Antibiotics, Ser. A 7: 88~92, 1954
13) MAO, J. C. H. & R. G. WIEGAND: Mode of action of macrolides. Biochim. Biophys. Acta 157: 404~413, 1969
14) MAEDA, K.; A. YAGI, H. NAGANAWA, S. KONDO & H. UMEZAWA: Application of high-voltage paper electrophoresis for separation and identification of antibiotics. J. Antibiotics 22: 635~636, 1969
15) GARROD, L. P.: The erythromycin group of antibiotics. Brit. Med. J. 1957-2: 57~63, 1957
16) WATERWORTH, P. M.: The antibacterial properties of leucomycin. Antibiot. & Chemoth. 10: 101~108, 1960

17) PATTEE, P. A. & J. N. BALDWIN : Transduction of resistance to some macrolide antibiotics in *Staphylococcus aureus*. J. Bact. 84 : 1049～1055, 1962
18) NAKAJIMA, Y.; M. INOUE, Y. OKA & S. YAMAGISHI : An elucidation on mechanism of resistance to macrolide antibiotics in *Staphylococcus aureus* (in Japanese). Proc. 87th (No. 24) Ann. Meet. Pharmaceut. Soc. Japan, p. 272, 1967
19) CHABBERT, Y. : Antagonisme *in vitro* entre l'erythromycine et la spiramycine. Ann. Inst. Pasteur (Paris) 90 : 787～790, 1956
20) BARBER, M. & P. M. WATERWORTH : Antibacterial activity of lincomycin and pristinamycin : a comparison with erythromycin. Brit. Med. J. 1964-2 : 603～606, 1964
21) BOURSE, R. & J. MONNIER : Effet de l'erythromycine sur la croissance de *Staph. aureus* "résistant dissocié" en bactériostase par un autre macrolide ou un antibiotique apparente. Ann. Inst. Pasteur (Paris) 110 : 67～79, 1968
22) WEISBLUM, B. & V. DEMOHN : Erythromycin-inducible resistance in *Staphylococcus aureus* : Survey of antibiotic classes involved. J. Bact. 98 : 447～452, 1969
23) MAO, J. C. H. & M. PUTTERMAN : The intermolecular complex of erythromycin and ribosome. J. Mol. Biol. 44 : 347～361, 1969
24) SAITO, T.; H. HASHIMOTO & S. MITSUHASHI : Drug resistance of staphylococci. Formation of erythromycin-ribosome complex. Decrease in the formation of erythromycin-ribosome complex in erythromycin resistant strains of *Staphylococcus aureus*. Jap. J. Microbiol. 13 : 119～121, 1969
25) TANAKA, K.; H. TERAOKA, M. TAMAKI, E. OTAKA & S. OSAWA : Erythromycin-resistant mutant of *Escherichia coli* with altered ribosomal protein component. Science 162 : 576～578, 1968
26) TAUBMAN, S. B.; N. R. JONES, F. E. YOUNG & J. W. CORCORAN : Sensitivity and resistance to erythromycin in *Bacillus subtilis* 168 : the ribosomal binding of erythromycin and chloramphenicol. Biochim. Biophys. Acta 123 : 438～440, 1966
27) WILHELM, J. M.; N. L. OLEINICK & J. W. CORCORAN : Interaction of antibiotics with ribosomes : Structure-function relationships and a possible common mechanism for the antibacterial action of the macrolides and lincomycin. Antimicr. Agents & Chemoth.-1967 : 236～250, 1968