RESISTANCE TO MACROLIDE ANTIBIOTICS IN *STAPHYLOCOCCUS AUREUS* SUSCEPTIBLE TO LINCOMYCIN AND MIKAMYCIN B

Sir:

Clinical isolates of *Staphylococcus aureus* that exhibit resistance to macrolide antibiotics have been divided into two major phenotypic classes: (1) a class of constitutive-resistant strains able to grow in the presence of a high concentration (more than 100 μg/ml) of macrolide, and (2) a class of inducibly resistant strains whose resistance to macrolides can be induced by exposure to subinhibitory concentrations of erythromycin (EM: 0.01~0.1 μg/ml). The constitutively-resistant and inducibly-resistant strains often show cross resistance to lincosamide and streptogramin type B antibiotics: the former has been referred to as constitutively MLS-coresistant and the latter as inducibly MLS-coresistant. Resistance to macrolide antibiotics in coresistant strains has been shown to depend on an alteration in the 23S ribosomal RNA of the 50S subunit[1,2]: an inducible, plasmid-mediated ribosomal RNA methylase is responsible for N^6,N^6-dimethylation of two specific adenine moieties in the 23S rRNA[3~5]. The modified ribosomes have lowered affinity for macrolide, lincosamide, and streptogramin type B (MLS) antibiotics, and consequently the cells become resistant to them.

Staphylococcus aureus strains S704, S1198, S1279, S1280, and TPR-27 are clinical isolates which are phenotypically, constitutively resistant to macrolide antibiotics, but susceptible to lincosamide and mikamycin B; the latter belongs to the same group as streptogramin B antibiotics (Fig. 1A and B). Strains of type TPR-27 have appeared, as isolated examples, at a frequency of about 20% of constitutively resistant *S. aureus* in Japan[6]. We have studied the mechanism of resistance to macrolide antibiotics in *S. aureus* S704 (Fig. 1B and Table 1), to investigate whether the resistance mechanism is the same as that in the inducibly coresistant strains.

As a part of studies of phenotypic characterization of resistant strain S704, we tried to isolate a sensitive mutant (as a control strain) from S704 exposed to nitrosoguanidine. However, we have not succeeded in finding any derivative susceptible to the macrolide antibiotics used in these tests. However, we obtained a mutant strain S704-60S resistant to spiramycin (SPM), but susceptible to the other macrolide antibiotics (Fig. 1B and Table 1). Hereafter this mutant is referred to as a partially sensitive strain. A revertant strain S704-60S-9 showed the same phenotype as strain S704 (Fig. 1B and Table 1). The phage types of the three strains (S704, S704-60S, and S704-60S-9) were 80/81. Assuming that mutant S704-60S is a point mutant, there are two possible explanations; (1)

Table 1. Minimum inhibitory concentrations of macrolides and lincosamides, and mikamycin B against *Staphylococcus aureus*[a].

Strain	Macrolide (μg/ml)					Lincosamide (μg/ml)		Mikamycin B (μg/ml)
	EM	OM	SPM	LM	CBM	LCM	CLDM	
S704	>800	>800	400	400	400	2	0.06	10
S704-60S	2	12.5	200	5	3	1	0.06	10
S704-60S-9	>800	>800	400	400	400	1	0.06	5
U9	2.5	0.8	2.5	0.63	0.63	0.5	0.06	5
209P	0.25	0.4	1	0.5	0.16	0.5	0.03	5

[a] The MIC of the drugs were determined by the agar dilution technique. A two-fold dilution of the antibiotics, from 0.03 to 800 μg/ml, was distributed into Sensitivity Test Agar (Eiken, Tokyo). The bacteria were grown overnight in Sensitivity Test Broth. The concentrations of the cell cultures were adjusted to 10^6 cells/ml. Five μl of the cell suspension (5×10^3 cells) was inoculated onto the drug-containing plates with a Microplanter (Sakuma Seisakujo, Tokyo). The plates were incubated for 18 to 20 hours at 37°C. The inhibitory concentration was the lowest concentration of antibiotics that prevented visible growth.

Abbreviations: EM, erythromycin; OM, oleandomycin; SPM, spiramycin; LM, leucomycin; CBM, carbomycin; LCM, lincomycin; CLDM, clindamycin.

this single gene might be responsible for a pleiotropic phenotype; (2) since strain S704-60S was derived from parent strain S704, S704 might possess two genes responsible for resistance to macrolide antibiotics: one for SPM and the other for resistance to macrolide antibiotics other than SPM.

As shown in Fig. 1B, strain U9, which belongs to the same phenotypic group as inducibly MLS-coresistant strains[8] (data not shown) has the

Fig. 1. Effect of erythromycin on the sensitivity of *S. aureus* to spiramycin, leucomycin, lincomycin, and mikamycin B.

The effect was assayed by layering strain U9 (the upper half of each plate) over the other strains (the lower half) in soft agar on agar plates prepared from a medium containing (in g/liter): peptone 5, yeast extract 5, K_2HPO_4 1, glucose 2, agar 20. The soft agar had the same composition except that 5 g of agar was used instead of 20 g. After the soft agar layer was hardened, discs (8 mm paper disc, Toyo Seisakusho Co.) containing antibiotics were applied. The level of antibiotics contained is listed below Table.

The plates were then incubated at 37°C for 20 hours. Strain S704-60S, a mutant (occurring at a frequency of about 10^{-5}), derived from strain S704 by treatment with *N*-methyl-*N'*-nitrosoguanidine (Aldrich Chemical Co.)[7]. Strain S704-60S-9, a revertant strain (occurring at a frequency of about 10^{-4}), selected in the presence of 10 μg EM/ml after treatment of strain S704-60S with ethyl methanesulfonate (EMS: Tokyo Kasei Kogyo Co.). U9[8] and 209P were inducibly MLS-coresistant and a standard antibiotic-sensitive strains, respectively. Strains in the lower part of each plate were S1198, S1279, S1280, and TPR-27 (Fig. 1A), and S704, S704-60S, S704-60S-9, and 209P (Fig. 1B).

Symbols: LC, lincomycin; S, spiramycin; M, mikamycin B; L, leucomycin; E, erythromycin.

Strain	Antibiotic (μg/disc)				
	E	S	L	LC	M
S704, S1198, S1279, S1280, TPR-27, S704-60S, S704-60S-9	10	50	50	10	50
U9	10	40	30	10	40
209P	10	20	10	5	20

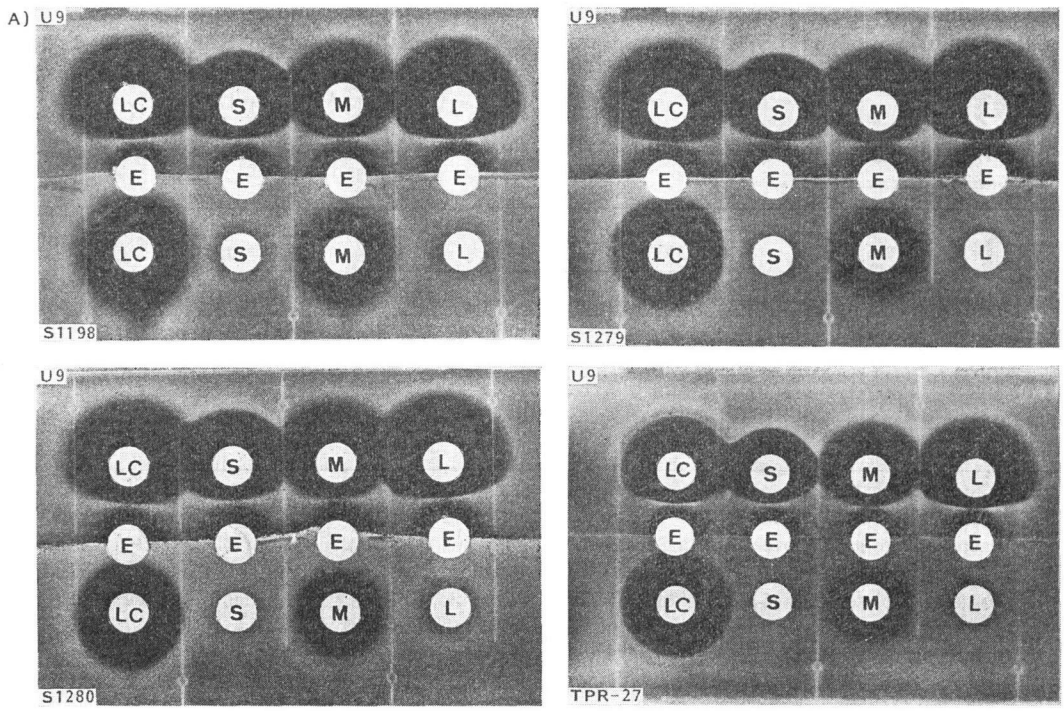


Fig. 1. (continued)

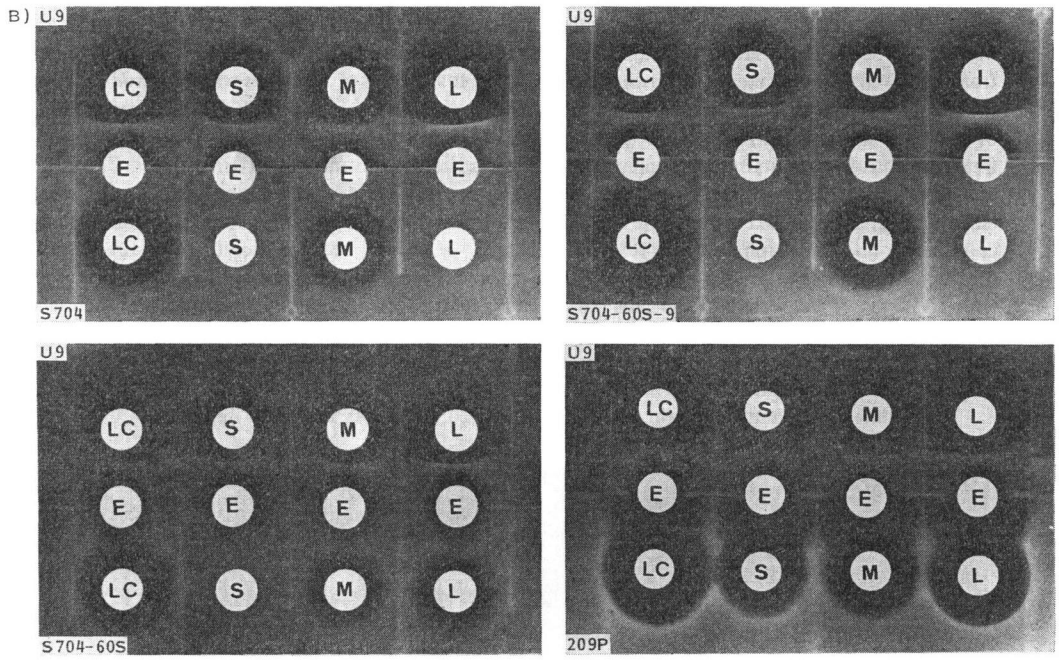


Table 2. Accumulation of antibiotics by staphylococcal cells.

Strain	Accumulated ($\times 10^{-2}$ μg/mg)[a]		
	EM	SPM	LCM
209P	6.5	7.0	2.0
S704-60S	5.8	0.5	2.0
U9	5.5	7.0	2.2
S704	<0.3	0.7	1.9
U9 induced	<0.2	<0.7	<0.1

[a] The experiment was performed as follows: cells were grown exponentially (O.D. 0.5~0.7 at 560 nm) in Trypticase Soy Broth were exposed to EM (100 μg/ml), SPM (10 μg/ml) or LCM (0.1 μg/ml: this was an LCM level unable to show antibiotic activity, *i.e.* formation of an inhibition zone by the cylinder plate method) with shaking at 37°C for 1 hour, chilled rapidly to 0°C, and washed 5 times with physiological saline (5 ml) by centrifugation (8,000×*g* for 10 minutes) except that the cells exposed to LCM were harvested without the saline wash. Cells pellets were suspended in 2~3 ml of 100 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 8.0). The antibiotic accumulated by cells was extracted by immersion in boiling water for 5~20 minutes.

Biological activity of the drug was determined by the cylinder plate method used with *M. luteus* ATCC 9341 as an indicator organism. The loss of drug activity during heat extraction was corrected. The accumulated antibiotic was represented as the drug amount (μg) per dry weight of the cells (mg) determined turbidimetrically (at 560 nm)[10].

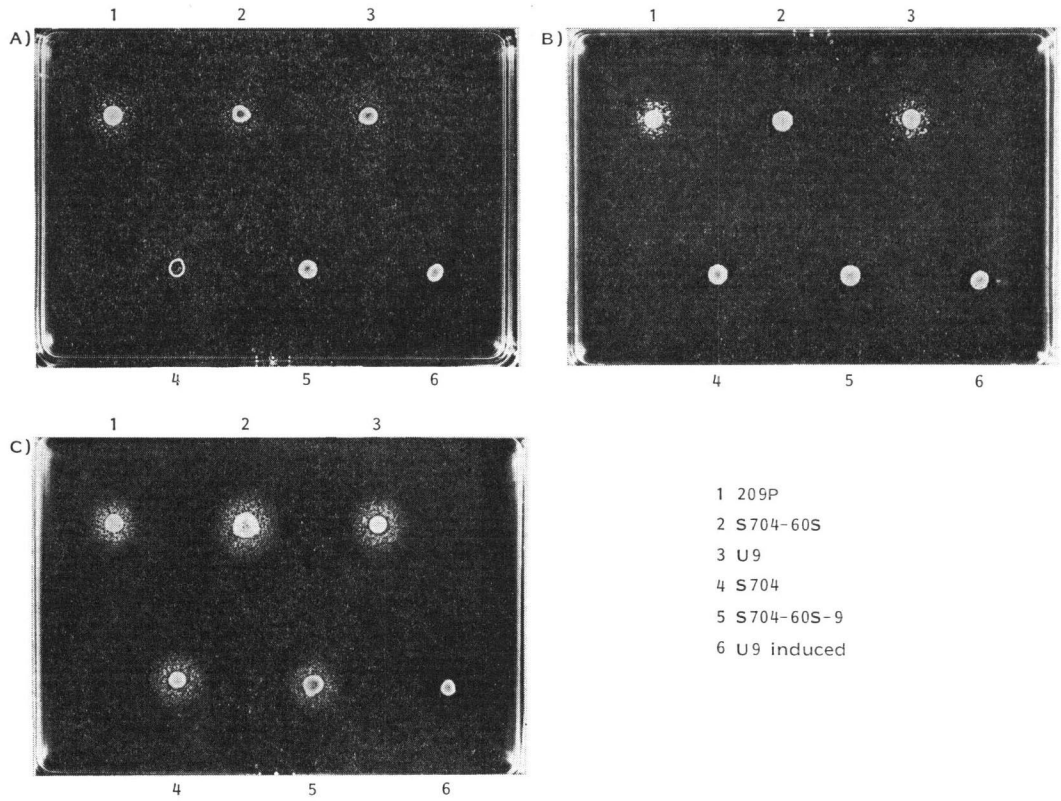
characteristic "D"-shaped inhibition zone around the MLS antibiotic discs. Strain S704 appears to be different from constitutively and partial-constitutively MLS-coresistant mutants which were spontaneously derived from an inducibly MLS-coresistant strain 1206^{+} [9].

This conclusion is also supported by the following observation. Using methods described previously[10], we compared drug uptake in strains S704 and S704-60S with that in strain U9. If the satellite growth of *Micrococcus luteus* appeared around *S. aureus* susceptible to the drug

Fig. 2. Accumulation of antibiotics by *S. aureus* on agar plates containing *M. luteus* ATCC 9341.

Procedures for the accumulation test were as follows: in the case of erythromycin (EM) and spiramycin (SPM), a 0.05-ml aliquot of an overnight culture of *M. luteus* in Trypticase Soy Broth (BBL) was mixed with 50 ml Brain Heart Infusion (BBL) containing glucose (1%) and agar (1% in final concentration: BHGA). In the case of lincomycin (LCM), a suspension of *M. luteus* was prepared by a similar manner except that the BHGA medium contained yeast extract (1%: BHGYA). Fifty milliliters of sterilized sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 7.9~8.0: 50 mM in a final concentration) containing an appropriate concentration of the antibiotics (EM 20, SPM 100, LCM 100 ng/ml) was added to BHGA or BHGYA. The mixture (15 ml) was poured into a plastic plate (square type, No. 2, Eiken Co., Tokyo). The plate was inoculated with a loopful of an overnight culture (*ca*, 5 μl) of staphylococci ($10^8 \sim 10^9$ cells/ml). However, because growth of strain 209P was greatly inhibited in the presence of 100 ng LCM/ml, the plate containing the antibiotic was inoculated with 1 to 2 mg of wet weight of the cells harvested by centrifuge. After various intervals of incubation (2~4 days) at 37°C, satellite growth of *M. luteus* around the test staphylococci was observed. Induction of resistance to macrolide antibiotics in strain U9 was performed by exposure of U9 cells to a low concentration (1 μg/ml) of EM.

Agar plates A, B, and C contain, respectively, EM 20, SPM 100, LCM 100 ng/ml.



(Fig. 2), this result will be interpreted as an evidence for the accumulation of the drug, because a decrease in the drug concentration around the susceptible strain will allow *M. luteus* to grow. In contrast, if *M. luteus* grew around resistant strain of *S. aureus*, degradation or conversion of drug to an inactive derivative can be assumed to have taken place. Strain S704 accumulated EM very slightly (A in Fig. 2) and SPM (B), except lincomycin (LCM) (C), whereas strain U9 accumulated EM (A), SPM (B), and LCM (C), though cells of U9 induced by EM did not accumulate these antibiotics (A, B, C). Strain S704-60S accumulated more EM than did strains S704 and S704-60S-9 (A in Fig. 2) but no SPM was taken up by the cells (B). After a 4-hour incubation of cultures at 37°C in the presence of 20 μg macrolide antibiotic per ml,

no inactivation of macrolide antibiotics by resistant strains could be found, since no significant decrease of the drugs in the supernatant was observed. In addition, the biological activity of antibiotics accumulated in the cells was determined as described previously[10] (Table 2). These results shown in Table 2 and Fig. 2 suggest that profiles in the level of susceptibility to macrolide and lincosamide antibiotics (Table 1 and Fig. 1B) are closely related to profiles in ability to accumulate the antibiotics quantitatively (Table 2) and qualitatively (Fig. 2). Thus with respect to drug accumulation, S704 is greatly different from the inducibly MLS-coresistant strain U9. Accordingly, strains S704, S1198, S1279, S1280, and TPR-27 appear to be a new class of staphylococci constitutively resistant to macrolide antibiotics. A comparison of the resistance mechanisms to macrolide antibiotics in strains S704, S704-60S, and U9 is now in progress.

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YOSHINORI NAKAJIMA*
HIDEKO ABE
KIKUTAROU ENDOU
MAYUMI MATSUOKA

Division of Microbiology,
Hokkaido Institute of
Pharmaceutical Sciences,
7-1 Katsuraoka-cho, Otaru,
Hokkaido, 047-02 Japan

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References

1) LAI, C. J. & B. WEISBLUM: Altered methylation of ribosomal RNA in an erythromycin-resistant strain of *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. 68: 856~860, 1971
2) LAI, C. J.; B. WEISBLUM, S. R. FAHNESTOCK & M. NOMURA: Alteration of 23S ribosomal RNA and erythromycin-induced resistance to lincomycin and spiramycin in *Staphylococcus aureus*. J. Mol. Biol. 74: 67~72, 1973
3) HORINOUCHI, S. & B. WEISBLUM: Posttranscriptional modification of mRNA conformation: Mechanism that regulates erythromycin-induced resistance. Proc. Natl. Acad. Sci. U.S.A. 77: 7079~7083, 1980
4) HORINOUCHI, S. & B. WEISBLUM: Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. 150: 804~814, 1982
5) SHIVAKUMAR, A. G.; J. HAHN, G. GRANDI, Y. KOZOLOV & D. DUBNAU: Posttranscriptional regulation of an erythromycin resistance protein specified by plasmid pE194. Proc. Natl. Acad. Sci. U.S.A. 77: 3903~3907, 1980
6) OMURA, S.; S. NAMIKI, M. SHIBATA, T. MURO & J. SAWADA: Studies on the antibiotics from *Streptomyces spinichromogenes* var. *kujimyceticus*. V. Some antimicrobial characteristics of kujimycin A and kujimycin B against macrolide resistant staphylococci. J. Antibiotics 23: 448~460, 1970
7) OMENN, G. S. & J. FRIEDMAN: Isolation of mutants of *Staphylococcus aureus* lacking extracellular nuclease activity. J. Bacteriol. 101: 921~924, 1970
8) WEAVER, J. R. & P. A. PATTEE: Inducible resistance to erythromycin in *Staphylococcus aureus*. J. Bacteriol. 88: 574~580, 1964
9) WEISBLUM, B. & V. DEMOHN: Erythromycin-inducible resistance in *Staphylococcus aureus*: Requirements for induction. J. Bacteriol. 106: 835~847, 1971
10) YAMAGISHI, S.; Y. NAKAJIMA, M. INOUE & Y. OKA: Decrease in accumulation of macrolide antibiotics as a mechanism of resistance in *Staphylococcus aureus*. Jpn. J. Microbiol. 15: 39~52, 1971