SPIRAMYCIN RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

DECREASE IN SPIRAMYCIN-ACCUMULATION AND THE RIBOSOMAL AFFINITY OF SPIRAMYCIN IN RESISTANT STAPHYLOCOCCI

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Spiramycin-uptake by *Staphylococcus aureus* MS537 was not affected by the presence or absence of glucose and Mg in media and was not inhibited by uncoupling agents of oxidative-phosphorylation. Maximal spiramycin-uptake by MS537 took place at pH 7 and within 20 minutes of addition of the drug. Spiramycin-uptake and growth inhibition curves with MS537 increased in parallel from pH 5 to 7. Despite a decrease in spiramycin-uptake from pH 7 to 9, growth inhibition remained constant. Spiramycin-uptake by MS537 after induction of spiramycin-resistance was half the uptake before induction. Spiramycin-uptake by constitutively resistant mutant of MS537 was the same level as that of induced population of MS537. The affinity of ribosomes for spiramycin, derived from MS537 after induction or from a constitutively resistant mutant of MS537, was one fifth to one tenth that of ribosomes prepared from MS537 before induction.

In the previous papers[2,4,5,9], it was reported that there are two types of resistance to macrolide antibiotics (Mac) in *Staphylococcus aureus*; inducible and constitutive resistance. In the former type of resistance, erythromycin (EM) and oleandomycin (OM) were found to be good inducers for Mac resistance and the induced population acquired a high resistance to all Mac and lincomycin (LCM). The induced cells or their extract were incapable of EM inactivation. Similarly, staphylococcal strains carrying constitutive resistance to Mac were found to be incapable of inactivation of EM, although they are highly resistant to Mac.

It was reported[6,7,8] that EM has a high affinity for complex formation with ribosomes obtained from EM-sensitive strains of *Escherichia coli*, *Bacillus subtilis* and *S. aureus* and that the ribosomes from EM-resistant strains reduce affinity for EM.

This paper deals with the mechanism of spiramycin-resistance in *S. aureus*—spiramycin-accumulation in staphylococcal cells and spiramycin-binding to their ribosomes.

Materials and Methods

Bacterial Strains: *S. aureus* MS537 is a strain inducible to Mac resistance and which becomes resistant to all Mac when pretreated with subinhibitory concentrations of EM.

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S. aureus MS537-R is a MS537 mutant obtained from a plate containing 100 mcg/ml of spiramycin (SP). It has high stable resistance to Mac.

Chemicals: EM and ^{14}C-SP (0.24 μCi/mg) were supplied by Japan Upjohn Co., Ltd. and Rhône-Poulenc Co., Ltd., respectively. SP and bacteriolytic enzyme (LE-2) were supplied by Kyowa Hakko Co., Ltd. All other chemicals were of reagent quality and were purchased from commercial sources.

Media: Brain heart infusion broth (BHI, Difco), medium A (Na_2HPO_4 7 g, KH_2PO_4 2 g, $(NH_4)_2SO_4$ 1.2 g, H_2O 1,000 ml, pH 7.4) and medium B (medium A containing yeast extract 2 g, peptone 10 g, glucose 2 g and $MgSO_4 \cdot 7H_2O$ 0.4 g) were employed for liquid culture and for the determination of SP-uptake by bacteria.

Induction of Mac Resistance: One tenth ml of an overnight broth culture of MS537 was inoculated into 30 ml of BHI or nutrient broth containing a subinhibitory concentration (0.05 mcg/ml) of EM and shaken in a water bath attached to the shaker. After 1 hour of incubation at 37°C, cultures were tested for drug resistance and SP-uptake.

Determination of Drug Resistance: Overnight cultures of *S. aureus* were spotted by small capillaries (10 cm × 0.1 cm) on heart infusion agar (HIA) plates which contained twofold dilutions of drugs. After 24 hours of incubation at 37°C, plates were examined for the maximum concentration of drug in the plate which allowed visible growth of bacteria.

Uptake of Spiramycin: One ml of SP solution was added to 100 ml of broth culture of *S. aureus* at exponential growth. After appropriate time intervals of incubation at 37°C with shaking, cultured cells were washed several times with 30 ml of cold saline by centrifugation. The washed cells were suspended in distilled water and heated in a boiling water bath (100°C) for 5 minutes. SP contained in boiled cells was determined by cup methods with *B. subtilis* ATCC 6633 as assay organism.

Uptake of ^{14}C-SP: Various concentrations of ^{14}C-SP were added to 30 ml of broth culture of *S. aureus* in exponential growth. After appropriate time intervals of incubation at 37°C with shaking, cultured cells were washed with cold saline by centrifugation until the supernatant had no radioactivity. The washed cells were suspended in 1 ml of distilled water and 0.4 ml of 10 % hyamine hydroxide, incubated at 60°C for 5 hours, cooled to room-temperature, and then 10 ml of scintillator (PPO 10 g, POPOP 250 g, naphthalene 100 g and dioxane 1,000 ml) was added. Radioactivity was determined by Packard Tri-Carb liquid scintillation spectrometer. SP-uptake was expressed as mcg of SP per mg (dry wt.) of microorganisms.

Preparation of Ribosomes: Except for the proteolytic digestion, all treatments were at 2~5°C. Six hundred ml of BHI culture of MS537 in exponential growth was used for the preparation of ribosomes. Bacterial cells were harvested by centrifugation, washed once with distilled water by centrifugation and suspended in 40 ml of buffer A (0.075 M Tris-HCl containing 0.016 M $Mg(OAc)_2$ and 0.05 M NH_4Cl, pH 7.4) containing bacteriolytic enzyme (LE-2 of 100 mcg/ml). The suspended cells were partially lysed by incubation at 37°C for 60 minutes and thereafter centrifuged at 8,000 rpm for 10 minutes. The precipitate (spheroplasts) was broken in 10 ml of buffer B (0.005 M Tris-HCl containing 0.016 M $Mg(OAc)_2$ and 0.05 M NH_4Cl, pH 7.4) containing 0.5 % Emalgen 220 (Kao-Atlas) at 0°C for 15 minutes and centrifuged at 10,000 rpm for 20 minutes. The obtained supernatant was centrifuged at 50,000 rpm for 50 minutes and the precipitate (ribosomes) was washed with 10 ml of buffer B by centrifugation and stored frozen at −80°C until needed.

Detection of ^{14}C-SP-Ribosome Complex: ^{14}C-SP-Ribosome complexes were separated by gel filtration on Sephadex G-100 columns. Reaction mixtures containing 46.8 A-260 mμ units of ribosomes and 7.0 mcg of ^{14}C-SP in 0.35 ml buffer B were incubated at 30°C. After incubation for 30 minutes, 0.3 ml of sample was placed on pre-equilibrated Sephadex G-100 column (0.9 × 20 cm) and eluted with buffer B at 4°C. Ten drop (0.6 ml) samples

were collected and optical density was read at 260 mμ. Radioactivity was determined as indicated above.

Results

No significant differences in SP-uptake were detected when MS537 was grown in BHI broth, media A and B. The presence or absence of glucose and Mg in media A and B did not affect SP-uptake (Table 1).

Inhibition of growth of MS537 by SP increased with increasing pH 5 to pH 7 and then remained constant to pH 9. SP-uptake was optimal at pH 7 (Fig. 1 and Table 2).

Optimal SP-uptake was observed 20 minutes after addition of the drug (Fig. 2). SP-Uptake increased with increasing concentrations of SP to a concentration of 1 mcg/ml (Fig. 3). It was found that SP-uptake in SP-resistant strain MS537-R was one half that in SP-sensitive strain MS537 (Fig. 3).

2, 4-Dinitrophenol and sodium azide were used as uncoupling agents of oxidative phosphorylation. These drugs inhibited the growth of MS537 (Fig. 4) but SP-uptake was not affected by them (Table 3).

MS537-R and an induced population of MS537 showed high resistance to SP and almost the same level of SP-uptake. SP-Uptake of the parent MS537 was, however, approximately double that of induced population. No significant differences in SP-uptake was observed with cells cultured in BHI broth containing 20~200 mcg/ml of

Table 1. Effect of media on SP-uptake by *S. aureus* MS537

Media (pH 7.4)	^{14}C-SP Uptake (mcg/mg dry wt. cells)
BHI	0.21
Medium B	0.18
−glucose [a]	0.16
−glucose−Mg	0.18
Medium A	0.17
+glucose	0.21
+glucose+Mg	0.17

Cultured cells of MS537 at exponential growth in BHI broth were collected, washed with cold distilled water and suspended in various media (30 ml) containing 10 mcg/ml of ^{14}C-SP. ^{14}C-SP uptake was determined after incubation of 1 hour at 37°C with shaking.
a) −, without; +, with.

Table 2. Growth inhibition of MS537 by SP in the media at various pH

pH	Growth (550 mμ)		Growth inhibition (%)
	Control [a]	SP (10 mcg/ml)	
5	0.280	0.225	20
6	0.530	0.265	50
7	0.620	0.000	100
8	0.635	0.000	100
9	0.472	0.000	100

An overnight broth culture of MS537, 0.5 ml, was inoculated in 10 ml of BHI broth (at various pH) containing SP (10 mcg/ml). Bacterial growth was assayed photometrically, after 3-hour incubation at 37°C in a shaker.
a) Control without SP.

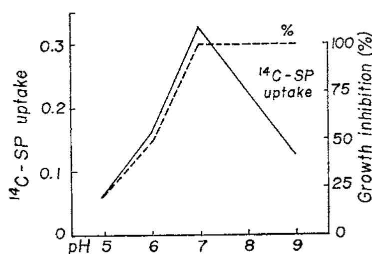


Fig. 1. Effect of pH on SP-uptake and growth inhibition by SP in medium A.

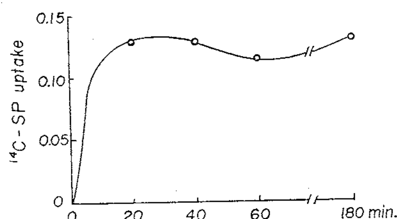


Fig. 2. Time course of SP-uptake by MS537. 300 mcg of ^{14}C-SP was added to 30 ml of BHI culture of MS537 at exponential growth. ^{14}C-SP uptake was determined after incubation at 37°C with shaking.

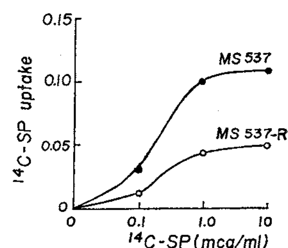


Fig. 3. Relation between SP-uptake and its concentration in culture medium. SP-uptake was assayed after 1 hour of incubation.

Fig. 4. Inhibition of growth of MS537 by uncoupling agents. Bacteria were inoculated in L-shaped tube containing 10 ml of BHI broth and incubated at 37°C with shaking. Agents were added at 2 hours. Bacterial growth was determined photometrically.

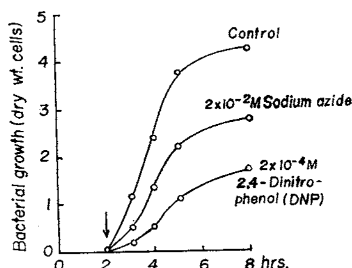


Table 3. Effect of uncoupling agents on ^{14}C-SP uptake by MS537

Agent	Concn. (M)	^{14}C-SP uptake (mcg/mg dry wt. cells)	Inhibition (%)
—	—	0.11	0
DNP	2×10^{-4}	0.13	<0
NaN_3	2×10^{-2}	0.15	<0

^{14}C-SP and uncoupling agent were added to BHI culture of MS537 at exponential growth. ^{14}C-SP-uptake was determined after incubation for 1 hour at 37°C with shaking.

Table 4. Comparison of SP uptake between Mac-sensitive and Mac-resistant strains of *S. aureus*

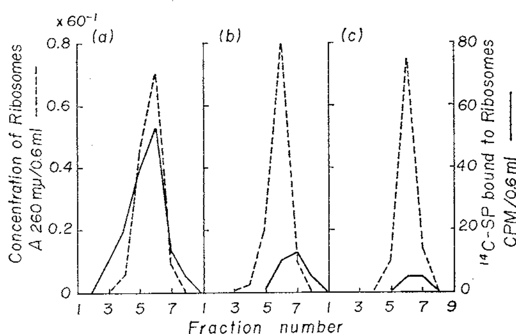
Strain	Induction a)	SP resistance (mcg/ml)	SP-uptake (mcg/mg dry wt. cells) Exp. 1 b)	Exp. 2 c)	Exp. 3 d)
MS537	—	0.8	0.24	0.24	0.18
MS537	+	>400.0	0.14	0.10	0.12
MS537-R	—	>400.0	0.10	0.10	0.11

a) Cells were pretreated with EM for 1 hour at 37°C.
b) Cultured in BHI broth containing 200 mcg/ml of SP. SP-uptake was assayed against *B. subtilis* ATCC6633.
c) and d) Cultured in BHI broth containing 20 and 40 mcg/ml of ^{14}C-SP, respectively.

SP (Table 4).

Binding of SP to *S. aureus* ribosomes was compared in both SP-sensitive and resistant strains. ^{14}C-SP-Ribosome complex was eluted from Sephadex G-100 column according to the procedure described under Materials and Methods. Ribosomes from both induced populations of MS537 and MS537-R had a lower affinity for SP as compared with those prepared from the SP-sensitive strain of MS537 not subjected to induction (Fig. 5).

Fig. 5. Binding of ^{14}C-SP to *S. aureus* ribosomes.
(a): MS537 before induction, (b): MS537 after induction, (c): MS537-R.



Discussion

This laboratory previously[6] reported that EM-accumulation by uninduced populations of *S. aureus* was about 5-fold larger than that of induced populations of the same organism. It was also reported that EM-binding to ribosomes of induced populations and EM-resistant strain was of the same order and about one tenth that observed with ribosomes from uninduced populations[6]. These findings are in agreement with those reported by TAUBMAN *et al.*[8] in EM-resistant mutant strain of *B. subtilis*. Our results indicate that SP-accumulation by an uninduced culture of *S. aureus* MS 537 was approximately double that of induced populations of the same oragnism or that of the SP-resistant mutant MS537-R. SP-Binding to ribosomes from a non-induced sensitive populations was approximately five times that observed with ribosomes from induced populations or the SP-resistant mutant MS 537-R. These results strongly suggest that decreased SP-accumulation in induced populations is due to the decreased affinity of ribosomes to SP, as reported for EM-accumulation, and that the affinity of ribosomes to Mac is altered not only by mutation but also by induction. AHMED[1]

reported that sensitive and resistant ribosomes of *B. subtilis* bound the same amount of SP. Differences between AHMED's results and ours may depend on the difference in experimental conditions or resistance mechanisms between (a) species, or (b) resistant bacteria obtained *in vitro* and those obtained naturally, or (c) intermediate-resistance and high-resistance.

SP-Uptake by *S. aureus* was not inhibited by uncoupling agents and not affected by presence or absence of glucose and Mg. The mechanism of SP-resistance was thus found to be different from that of tetracycline resistance which is remarkably affected by such agents[3].

SP-Uptake and growth inhibition curves with *S. aureus* were parallel from pH 5 to 7. In more alkaline media, the antibacterial activity remained constant even though SP-uptake decreased. An explanation for this is not apparent.

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