PHOSPHORYLATION AND INACTIVATION OF STREPTOMYCIN BY PLANT PATHOGENIC *PSEUDOMONAS LACHRYMANS*

Sir:

Inactivation of aminoglycoside antibiotics in clinical isolates has been studied by many research workers[1]. Streptomycin(SM)-inactivating enzymes were reported to exist in SM-resistant strains of *Staphylococcus aureus*[2~4], *Escherichia coli*[5~10], and *Pseudomonas aeruginosa*[11~13], in which are operative 2 mechanisms, phosphorylation and adenylylation, *i.e.*, SM 3″-phosphotransferase; APH(3″), SM 6-phosphotransferase; APH(6), SM 3″-adenylyltransferase; AAD (3″), SM 6-adenylyltransferase; AAD(6). *Pseudomonas lachrymans* is known to be the cucumber angular leaf spot bacterium and SM-resistant strains have been recently isolated[14]. However, the mechanisms of SM-resistance in plant pathogenic *P. lachrymans* have not been studied. Thirteen SM-resistant *P. lachrymans* were selected and biochemical mechanisms of SM-resistance in these strains were investigated.

Peptone water was used for liquid culture, which consisted of 10 g peptone, 5 g NaCl, and 1,000 ml of deionized water. Bacteria were inoculated in peptone water and shaken at 27°C. After 20 hours of incubation, cells were harvested. The S-30 fraction, the supernatant of 30,000 *g* centrifugation, was prepared as described in a previous paper[3]. An incubation reaction was carried out at 27°C for 1 hour and then stopped by heating in boiling water for 3 minutes. Antibiotic activity remaining in the reaction mixture was determined by bioassay using *Bacillus subtilis* ATCC6633 as test organism. As shown in Table 1, five *P. lachrymans* strains inactivated SM but the remaining 8 strains did not inactivate the drug under these conditions. The enzymatic transfer of the γ-^{32}P or ^{14}C-AMP from isotope-labeled ATP into SM was carried out as described previously[3]. From the result, we concluded that 5 strains inactivated SM by phosphorylation. γ-^{32}P-SM was prepared by the inactivation reaction using the extract of one of the 5 strains N-7554. The reaction mixture contained: 100 μl of S-30 fraction (5 mg of protein/ml), 20 μl of 1 μCi of γ-^{32}P-ATP (515 mCi/mmol), 20 μl of 1 mM of SM, 20 μl of 0.02 M $MgCl_2$, and 40 μl of 0.2 M tris-HCl buffer (pH 7.0). After 60 minutes of incubation at 27°C, the reaction was stopped by heating in boiling water and centrifuged. The supernatant was pipetted onto a phosphocellulose paper and washed with deionized water, extracted with 10 ml of 0.5 N HCl and lyophilized. ^{32}P-labeled phosphoryl SM thus obtained, the authentic samples of SM-3″-phosphate prepared from *P. aeruginosa* TI-13 and SM-6-phosphate from *P. aeruginosa* GN573 were developed with the following solvent system on a thin-layer of silica gel (Tokyo Kasei). The radioactive spot of ^{32}P-phosphoryl SM coincided exactly with the SAKAGUCHI reaction[15]-positive spot of SM-3″-phosphate (Rf 0.36), while Rf value of SM-6-phosphate was 0.23, on thin-layer chromatography using CH_3OH - H_2O - 15% NaCl (9: 1: 5 in volume). On high-voltage paper electrophoresis under 3,500 volts for 20 minutes using formic acid - acetic acid - water (25: 75: 900 in volume), the ^{32}P-phosphoryl SM and SM-3″-phosphate moved toward the cathode 9.4 cm. From these results, it was concluded that the chemical structure of SM inactivated by *P. lachrymans* was SM-3″-phosphate. The biochemical mechanisms of SM resistance in the remaining 8 strains which could not inactivate the drug will be described elsewhere.

Table 1. Inactivation of SM by SM-resistant *P. lachrymans* strains.

Bacterial strains	SM-resistance (μg/ml)	Inactivation of SM
N-7401	>400	−
N-7403	100	+
N-7504	200	+
N-7506	200	+
N-7513	200	−
N-7554	>400	+
N-7582	50	−
N-7585	>400	−
N-7588	50	−
N-7590	50	−
N-7595	100	+
N-7598	>400	−
T-7445	12.5	−

We are notified after submitting for publication that the same mechanism of SM resistance in *P. lachrymans* has been reported earlier by YANO *et al.*[16].

Acknowledgements

We wish to thank Dr. A. OHUCHI, National Institute of Agricultural Science, Tokyo, for providing bacterial cultures.

HARUHIDE KAWABE*
HISASHI SAKURAI**
KEIJI FUKASAWA
SHOJI SHIMIZU
KATSUMI HASUDA
SHIZUKO IYOBE
SUSUMU MITSUHASHI

Department of Microbiology, School of Medicine, Gunma University, Maebashi
*Episome Institute, Fujimi, Seta, Gunma
**Soil and Pesticide Section, Environment Agency, Tokyo, Japan

(Received January 16, 1979)

References

1) MITSUHASHI, S.; S. YAMAGISHI, T. SAWAI & H. KAWABE: Biochemical mechanisms of plasmid-mediated resistance. *in* R Factor-Drug Resistance Plasmid. pp. 195~251, *Ed.* by S. MITSUHASHI, University of Tokyo Press, Tokyo, 1977
2) DOI, O.; M. MIYAMOTO, N. TANAKA & H. UMEZAWA: Inactivation and phosphorylation of kanamycin by drug-resistant *Staphylococcus aureus*. Appl. Microbiol. 16: 1282~1284, 1968
3) KAWABE, H.; M. INOUE & S. MITSUHASHI: Inactivation of dihydrostreptomycin and spectinomycin by *Staphylococcus aureus*. Antimicr. Agents & Chemoth. 5: 553~557, 1974
4) SUZUKI, I.; N. TAKAHASHI, S. SHIRATO, H. KAWABE & S. MITSUHASHI: Adenylylation of streptomycin by *Staphylococcus aureus*: A new streptomycin adenylyltransferase. Microbial Drug Resistance. pp. 463~473, *Ed.* by S. MITSUHASHI & H. HASHIMOTO, University of Tokyo Press, Tokyo, 1975
5) BENVENISTE, R.; Y. YAMADA & J. DAVIES: Enzymatic adenylylation of streptomycin and spectinomycin by R-factor-resistant *Escherichia coli*. Infect. Immunity 1: 109~119, 1970
6) HARWOOD, J. & D. H. SMITH: Resistance factor-mediated streptomycin resistance. J. Bacteriol. 97: 1262~1271, 1969
7) OZANNE, B.; R. BENVENISTE, D. TIPPER & J. DAVIES: Aminoglycoside antibiotics: Inactivation by phosphorylation in *Escherichia coli* carrying R factors. J. Bacteriol. 100: 1144~1146, 1969
8) TAKASAWA, S.; R. UTAHARA, M. OKANISHI, K. MAEDA & H. UMEZAWA: Studies on adenylyl streptomycin, a product of streptomycin inactivation by *E. coli* carrying R factor. J. Antibiotics 21: 477~484, 1968
9) UMEZAWA, H.; S. TAKASAWA, M. OKANISHI & R. UTAHARA: Adenylylstreptomycin, a product of streptomycin inactivated by *E. coli* carrying R factor. J. Antibiotics 21: 81~82, 1968
10) YAMADA, T.; D. TIPPER & J. DAVIES: Enzymatic inactivation of streptomycin by R-factor resistant *Escherichia coli*. Nature 219: 288~291, 1968
11) DOI, O.; M. OGURA, N. TANAKA & H. UMEZAWA: Inactivation of kanamycin and streptomycin by enzymes obtained in cells of *Pseudomonas aeruginosa*. Appl. Microbiol. 16: 1276~1281, 1968
12) KAWABE, H.; F. KOBAYASHI, M. YAMAGUCHI, R. UTAHARA & S. MITSUHASHI: 3″-Phosphoryldihydrostreptomycin produced by the inactivating enzyme of *Pseudomonas aeruginosa*. J. Antibiotics 24: 651~652, 1971
13) KIDA, M.; T. ASAKO, M. YONEDA & S. MITSUHASHI: Phosphorylation of dihydrostreptomycin by *Pseudomonas aeruginosa*. Microbial Drug Resistance. pp. 441~448, *Ed.* by S. MITSUHASHI & H. HASHIMOTO, University of Tokyo Press, Tokyo, 1975
14) HASUDA, K. & H. SAKURAI: Drug-resistance of plant pathogenic *Pseudomonas aeruginosa* strains. Medicine and Biology (in Japanese) 95: 203~206, 1977
15) SAKAGUCHI, S.: Über eine neue Farbenreaktion von Protein und Arginin. J. Biochem. 5: 25~31, 1925
16) YANO, H.; H. FUJII, H. MUKOO, M. SHIMURA, T. WATANABE & Y. SEKIZAWA: On the enzymatic inactivation of dihydrostreptomycin by *Pseudomonas lachrymans*, cucumber angular leaf spot bacterium: isolation and structural resolution of the inactivated product. Ann. Phytopath. Soc. (Japan) 44: 413~419, 1978