Journal of Microbiology and Biotechnology
Vol. 7. No. 6, 397-401, 1997

Characterization of *Streptomyces* sp. AMLK-135 Producing Anti-MRSA Antibiotics

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The present research program was conducted to characterize a strain of actinomycetes producing an anti methicillin-resistant *Staphylococcus aureus* (MRSA) antibiotic. Soil samples were collected from various sites in Korea and a number of actinomycetes were isolated from the soil samples by applying selective agar for actinomycetes. Among over 400 isolates, a strain (AMLK-135) producing anti-MRSA antibiotic against *S. aureus* TK 784 was selected. According to the morphological and physiological characteristics, the strain AMLK-135 was confirmed to belong to the genus *Streptomyces*. From the results of species identification with the TAXON program, the strain AMLK-135 was shown to belong to major cluster 5 (*Streptomyces exfoliatus*), but it had a low simple matching coefficient (S_{SM}) value to member organisms of major cluster 5. Percentage (%) of strain further away of the strain AMLK-135 was low (1.9400) and it was placed further away than the outer-most members in major cluster 5. Therefore, the strain AMLK-135 was identified as a new species of the genus *Streptomyces*.

Gram-positive *Staphylococcus aureus commonly* exists in the human body (skin surface etc.), and is recovered from a variety of infections including skin lesions, such as furuncles and carbuncles, abscesses, wound infections, pneumonia, osteomyelitis, and others. From these sites organisms can invade the bloodstream and seed metastatically, appearing in the urine or forming abscesses in various body organs, or producing septic shock or endocarditis. The organism can also be recovered from the anterior nares, perineum, and other skin sites from as many as 10% to 15% of healthy people and a significantly greater percentage of people in the hospital setting (6, 12, 18). Most strains of *S. aureus*, even those acquired in the community, are penicillin resistant. In most cases this resistance is attributable to β-lactamase production due to genes located on extrachromosomal plasmids. Some staphylococci that are penicillin resistant are also resistant to the newer β-lactamase-resistant semisynthetic penicillins such as methicillin, oxacillin, and nafcillin. This resistance is due primarily to the presence of an unusual penicillin-binding protein in the cell wall of resistant strains (3, 7). Clinically significant methicillin-resistant *S. aureus* (MRSA) is being isolated with greater frequency in the United States, often posing problems as causes of nosocomial infections (1, 2). Numerous hospitals have begun active screening for MRSA, since infections caused by these strains significantly affect patients' morbidity and reimbursements by hospitals. Studies on development of remedial agents for MRSA are going through development of new anti-MRSA antibiotics (26, 8) and combination administration of known antibiotics (17, 5), but, till now, these treatments have not taken a significant effect. Consequently, in the present studies, we numerically identified *Streptomyces* sp. AMLK-135 active against *S. aureus* TK 784 (MRSA) using the TAXON program.

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Key words: methicillin-resistant *Staphylococcus* aureus, numerical identification, *Streptomyces exfoliatus*

MATERIALS AND METHODS

Antibiotic-producing Actinomycete Strain AMLK-135

Actinomycete strain AMLK-135 producing an anti-MRSA antibiotic was isolated in a medium designed for actinomycete isolation (11) from a soil sample collected in Mt. Sokri, Chungcheong Province. The organism was grown at 28°C on the modified Bennett's agar (11) slant and stored at 4°C for future use.

Identification of Actinomycete Strain AMLK-135

To determine the genus of actinomycete strain AMLK-135, the type of 2,6-diaminopimelic acid (DAP), known as one of the components of cell wall of actinomycete mycelia, was analyzed using the methods of the ISP (International Streptomyces Project) suggested by Shirling and Gottlieb (16), and Bergey's Manual of Systematic Bacteriology (21). Actinomycete strain AMLK-135 was cultured on the tryptic soy broth (17.0 g pancreatic digest of casein, 3.0 g papaic digest of soybean meal, 5.0 g sodium chloride, 2.5 g dipotassium phosphate, 2.5 g dextrose and 1 liter H_2O, adjusted to pH 7.3 before autoclaving) for 7 days at 28°C using a rotary-shaking incubator. Cultured broth was filtered with Whatman No. 1 paper, washed with sterilized distilled water, and freeze-dried. Dried cells (20 mg) were placed into a cap-tube (13×100 mm) containing 5 ml 6 N HCl, sealed compactly, and hydrolyzed by heating the tube for 18 h in a boiling water bath. The hydrolysate was filtered with Whatman No. 1 paper and evaporated to dryness to remove residual HCl. This residue was dissolved in 1 ml distilled water and loaded on TLC plate (10×10 cm, HFTLC Cellulose, Merck Co.). Five μl of 0.01 M DL-DAP (Sigma) containing meso- and LL-DAP isomers and amino acids (alanine, glycine and glutamate) were also loaded on plates as standards (25).

To examine spore chain morphology, actinomycete strain AMLK-135 was incubated for 14 days on yeast extract-malt extract agar (ISP medium 2) (4.0 g yeast extract, 10.0 g malt extract, 4.0 g dextrose, 20.0 g agar and 1 liter H_2O, adjusted to pH 7.3 before autoclaving). Spore chain morphology of the strain AMLK-135 was examined using light and scanning electron microscopy (SEM) (Model S-800, Hitachi, Japan). Spore chain morphology was examined by light microscopy at ×400 magnification. Specimen for SEM was prepared by the method of Williams and Davies (22). Among morphological categories suggested by Pridham *et al.* (15), the two categories, *Rectiflexibles* and *Spirales* were employed for evaluation of spore chain morphology.

Numerical Identification of Actinomycete Strain AMLK-135 Using the TAXON Program

Numerical identification of actinomycete strain AMLK-135 was conducted using the TAXON program (19) to determine the species of the strain AMLK-135. TAXON is a computer program that identifies unknown strains by testing 50 unit characters and analyzing the results numerically based on data collected by Williams *et al.* (23). Tests of 50 unit characters for numerical identification were done following the method of Williams *et al.* (23, 24) and Langham *et al.* (9). Taxonomic 50 unit characters and their code name for the computer are presented in Table 1. Data for unit characters was recorded as + or -, and identification scores of actinomycete strain AMLK-135 were then determined using the TAXON program.

Medium for Antibiotic Production and Test Pathogen (MRSA)

Medium used for preculture (for 2 days) of the strain AMLK-135 was PC II (10 g dextrose, 2 g polypeptone, 1 g yeast extract, 1 g meat extract, 0.5 g asparagine, 0.1 g thiamine·HCl and 1 liter H_2O, adjusted to pH 7.0 before autoclaving), and the medium used for main culture (for 4 days) was PY (5 g dextrose, 3 g polypeptone, 2 g yeast extract, 5 g meat extract, 10 g soluble starch, 10 g glycerol, 1 g casein [from milk], 2 g $CaCO_3$, 0.01 g thiamine·HCl and 1 liter H_2O, adjusted to pH 7.0 before autoclaving). Medium used for methicillin-resistant *Staphylococcus aureus* TK 784 (MRSA) was glucose bouillon agar (3 g dextrose, 10 g polypeptone, 5 g NaCl, 10 g meat extract, 10 g agar and 1 liter H_2O, adjusted to pH 7.0 before autoclaving). The method for test plate manufacture of the pathogen and measurement of anti-MRSA activity were those of Lim *et al.* (11).

RESULTS AND DISCUSSION

Analysis of Diaminopimelic Acid (DAP) Type and Morphological Characteristics

Cell wall hydrolysates of actinomycete strain AMLK-135 were developed on cellulose TLC plate. Diaminopimelic acid present in the cell wall turned out to be LL-DAP. Spore chains of strain AMLK-135 formed rectiflexible type, as observed by light microscopy. Under the scanning electron microscope, actinomycete strain AMLK-135 was observed to have long and straight spores of fractionized rod form on aerial mycelia, and spore surface ornamentation was smooth among the five groups classified by Dietz and Mathews (4) (Fig. 1). Special structures, such as zoospore and sporangium, were found in strain AMLK-135. Based on the DAP type of cell wall and the morphological characteristics, strain AMLK-135 belonged to the genus *Streptomyces*.

Numerical Identification of *Streptomyces* sp. Strain AMLK-135 Using the TAXON Program

Numerical identification of *Streptomyces* sp. strain AMLK-135 was conducted using the TAXON program based on the data of characters of *Streptomyces* spp. classified by Williams *et al.* (23) (Table 1). Results of *Streptomyces* sp. strain AMLK-135 obtained from the tests of 50 unit characters were input into the TAXON program to compare with the data of the other strains. Generally, correct identification of an unknown strain requires that the Willcox probability approaches 1.0, that taxonomic distance is included within 95% taxon radius and has a low score, and that % probability of strain further away has a high score. Numerical identification was conducted using the TAXON program to determine which major clusters *Streptomyces* sp. strain AMLK-135 belongs to

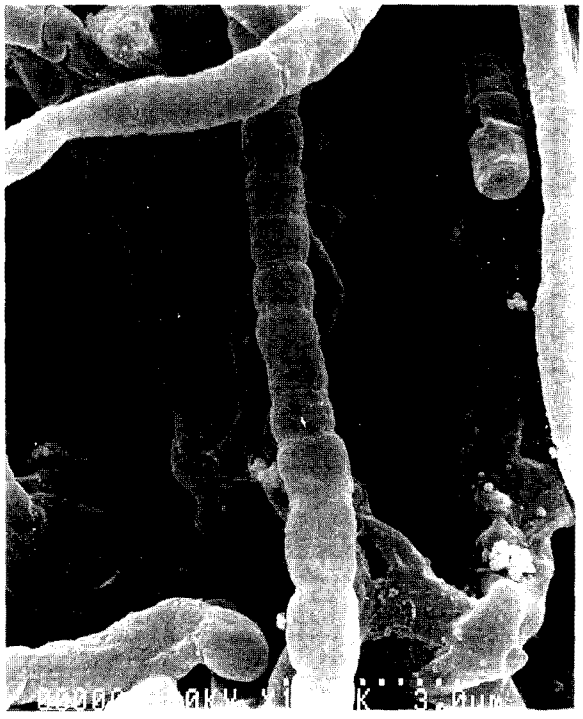

Fig. 1. Scanning electron microphotograph of spore surface of strain AMLK-135.
Medium: Yeast extract-malt extract agar, Cultivation: 28°C for 14 days.

(Table 2). The Willcox probability which strain AMLK-135 might fall into the taxon major clusters was 0.996638, which was much higher than the 0.003361 of the Willcox probability to the taxon major cluster 61. But taxonomic distance value (0.4617) of strain AMLK-135 existed out of its 95% taxon radius (0.4455). Percent (%) probability of strain further away of strain AMLK-135 (1.9400) to the taxon major cluster 5 was distinctly higher than that of the other major clusters, but absolutely low. Therefore, it might fall into the taxon major cluster 5, but exist in regions further away from the center of major cluster 5.

The numerical identification scores of *Streptomyces* sp. strain AMLK-135 were compared with those of a hypothetical median organism (HMO), centrotype (*Streptomyces roselous*), the outer-most member (*Streptomyces umbrinus*), and the best match strain (*Streptomyces gardneri*) among the taxon major cluster 5 members (Table 3). Taxonomic distance (0.4617) of strain AMLK-135 wasn't within 95% taxon radius (0.4455), and % probability of strain further away meaning the probability of being placed in the center of cluster was 1.9400, which was lower than that of either the centrotype (93.4908) or the best match strain (23.5300). Thus, it was supposed that the strain AMLK-135 was placed in regions further away from the center of major cluster 5.

Table 1. Unit characters of strain AMLK-135.

1. Morphology and pigmentation
   Spore chain morphology: rectiflexibilis (RFS), +; spirales (SPI), -
   Color of spore mass : red (RED), -; grey (GRY), +
   Mycelial pigment : red/orange
   Diffusible pigment : production (PIG), -; yellow/brown (YBP), -
   Melanin production on : PYI medium (MPI), -; tyrosine medium (MTY), -

2. Antimicrobial activity
   Bacillus subtilis (SUB), +;
   Micrococcus luteus (LUT), +;
   Candida albicans (ALB), +;
   Saccharomyces cerevisiae (CER), +;
   Streptomyces murinus (MUR), -;
   Aspergillus niger (NIG), -

3. Biochemical tests
   Lecithinase (LEC), +; Lipolysis (LIP), +;
   Pectin hydrolysis (PEC), -; Nitrate reduction (NO3), -;
   H_2S production (H2S), +; Hippurate hydrolysis (HIP), -

4. Degradative tests
   Elastin (ELA), +; Xanthine (XAN), -;
   Arbutin (ARB), +

5. Antibiotic resistance
   Neomycin (NEO), +; Rifampicin (RIF), -;
   Oleandomycin (OLE), -; Penicillin (PEN), -

6. Growth tests
   45°C (45C), -; NaCl (7NA), -;
   Sodium azide (01Z), -; Phenol (PHN), +;
   Potassium tellurite (01T), +; Thallus acetate (T01), -

7. Compounds as sole source of nitrogen
   DL-α-amino-*n*-bytyric acid (BUT), +;
   L-Cysteine (CYS), -; L-Valine (VAL), +;
   L-Phenylalanine (PHE), +; L-Histidine (HIS), +;
   L-Hydroxyproline (HYD), +

8. Organic compounds as sole source of carbon
   Sucrose (SUC), +; meso-Inositol (INO), -;
   Mannitol (MAN), -; L-Rhamnose (RHA), -;
   Raffinose (RAF), +; D-Melezitose (MEZ), -;
   Adonitol (ADO), -; Dextran (DEX), -;
   D-Melibiose (MEB), +; Xylitol (XYT), -

*The three letters in parenthesis are the code names for computer analysis.

Simple matching coefficients (S_{SM}) of *Streptomyces* sp. strain AMLK-135 to 18 strains in the taxon major cluster 5 were calculated based on the data of fifty unit characters (Table 4). The greater this value is, the higher the similarity level is. S_{SM} of the best match strain *S. gardneri* (76.00%) was highest among those of the 18 strains.

In conclusion, the results of numerical identification

Table 2. Identification of strain AMLK-135 to the major cluster of *Streptomyces* by TAXON program.

TAXON Major cluster (Centrotype member)	TAXON distance	95% TAXON radius	% Probability of strain further away	Willcox probability
5 (*Streptomyces exfoliatus*)	0.4617	0.4455	1.9400	0.996638
61 (*Streptomyces lavendulas*)	0.5071	0.4118	0.0008	0.003361
10 (*Streptomyces fulvissimus*)	0.5367	0.4036	0.0000	0.000000
32 (*Streptomyces violaceoniger*)	0.5368	0.3854	0.0000	0.000000
23 (*Streptomyces microflavus*)	0.5430	0.3931	0.0000	0.000000
19 (*Streptomyces diastaticus*)	0.5469	0.4508	0.0019	0.000000
6 (*Streptomyces violaceus*)	0.5493	0.4126	0.0000	0.000000
33 (*Streptomyces chromogenus*)	0.5557	0.3955	0.0000	0.000000
20 (*Streptomyces olivaceoviridis*)	0.5604	0.3720	0.0000	0.000000
1C (*Streptomyces halstedii*)	0.5616	0.3883	0.0000	0.000000
1A (*Streptomyces albidoflavus*)	0.5616	0.3782	0.0000	0.000000
1B (*Streptomyces anulatus*)	0.5700	0.4404	0.0000	0.000000
3 (*Streptomyces atroolivaceus*)	0.5713	0.3631	0.0000	0.000000
29 (*Streptomyces lydicus*)	0.5748	0.3831	0.0000	0.000000
15 (*Streptomyces chromofuscus*)	0.5756	0.4271	0.0000	0.000000
31 (*Streptomyces antibioticus*)	0.5787	0.4131	0.0000	0.000000
30 (*Streptomyces filifinensis*)	0.5837	0.3845	0.0000	0.000000
18 (*Streptomyces cyaneus*)	0.6020	0.4497	0.0000	0.000000
42 (*Streptomyces rimosus*)	0.6028	0.3507	0.0000	0.000000
12 (*Streptomyces rochei*)	0.6087	0.4173	0.0000	0.000000
40 (*S. phaeochromogenes*)	0.6098	0.3805	0.0000	0.000000
37 (*Streptomyces greceoflavus*)	0.6310	0.3658	0.0000	0.000000
21 (*Streptomyces greceoruber*)	0.6406	0.3709	0.0000	0.000000
65 (*Kitassatoa* spp.)	0.6480	0.3374	0.0000	0.000000
17 (*Streptomyces griceoviridis*)	0.6638	0.3943	0.0000	0.000000
16 (*Streptomyces albus*)	0.6658	0.3347	0.0000	0.000000

Table 3. Comparisons of taxonomic scores between hypothetical median organism (HMO), centrotype, outer-most member strain (OMM), best matched organism (BMO) and strain AMLK-135 in cluster 5.

Member strain in cluster 5	TAXON distance	95% TAXON radius	% Probability of strain further away	Willcox probability
HMO	0.2800	0.4455	99.6117	>0.999999
Centrotype (*Streptomyces roselous*)	0.3242	0.4455	93.4908	0.999529
OMM (*Streptomyces umbrinus*)	0.4837	0.4455	0.4159	0.986515
BMO (*Streptomyces gardneri*)	0.4632	0.4455	23.5300	0.995618
Isolate (stain AMLK-135)	0.4617	0.4455	1.9400	0.996638

Table 4. Simple matching coefficient (S_{SM}) of strain AMLK-135 to member organisms in *Streptomyces* cluster 5.

ISP No.	Strain	ATCC No.	S_{SM} (%)
5011	*Streptomyces cenereoruber*	19740	66
5060	*Streptomyces exfoliatus*	12627	64
5022	*Streptomyces filamentosus*	19753	64
5541	*Streptomyces filavochromogenes*	14841	64
5064	*Streptomyces gardneri*	23911	76
5086	*Streptomyces hydrogenans*	19631	60
0164	*Streptomyces litimcidini*	19914	70
5016	*Streptomyces narbonensis*	19790	70
0314	*Streptomyces nashivillensis*	27476	68
5552	*Streptomyces omieyensis*	27454	68
5174	*Streptomyces roselous*	23210	66
5122	*Streptomyces roceosporus*	23958	58
5175	*Streptomyces roceoviridis*	23959	54
5329	*Streptomyces lermitus*	25499	70
5278	*Streptomyces umbrinus*	19929	50
5279	*Streptomyces violaceorectus*	25514	64
5196	*Streptomyces zaomyceticus*	27482	60
T1	*Streptomyces* sp.	T1	54

using the TAXON program and the similarity level indicated that *Streptomyces* sp. strain AMLK-135 was similar to a strain of *Streptomyces gardneri* included in taxon major cluster 5 (*Streptomyces exfoliatus*). However, since percent (%) of strain further away of the strain AMLK-135 was low (1.9400) and it was placed further away than outer-most members in major cluster 5, the strain AMLK-135 was identified as a new species within the major cluster 5. Consequently, we are hoping to obtain new anti-MRSA antibiotics from it.

Acknowledgement

This study was supported by Konkuk University and the authors deeply appreciated their financial supports.

REFERENCES

1. Barber, M. 1961. Methicillin-resistance staphylococci. *J. Clin. Pathol.* **14**: 385-393.
2. Baron, E. J. 1992. The detection, significance, and rationale for control of methicillin-resistant *Staphylococcus aureus*. *Clin. Microbiol. Newsletter*. **14**: 129.
3. Bryan, L. E. 1982. The control of antibiotic resistance. pp. 161-210. *Bacterial resistance and susceptibility to chemotherapeutic agents*. Cambrige University Press, Cambrige, U. K.
4. Dietz, A. and J. Mathew. 1971. Classification of *Streptomyces* spore surfaces into five groups. *Appl. Microbiol.* **21**: 527-533.
5. Ishii, T., Y. Takayama, Y. Takase, and Y. Orikasa. 1994. Antibacteriological activities of arbekacin and vancomycin against strain of methicillin-resistant *Staphylococcus aureus*. *Jpn. J. Antibiot.* **47**: 647-654.
6. Jawetz, E., J. L. Melnick, and E. A. Alderson. 1987. The staphylococci. pp. 217-222. *Review of medical microbiology*. 17th ed.
7. Jorgensen, J. H. 1991. Mechanisms of methicillin resistance in *Staphylococcus aureus* and the methods for laboratory detection. *Infect. Control Hosp. Epidemiol.* **12**: 14.
8. Kimiko, U., H. Muneo, Y. Masuhito, N. Katsuyuki, F. Yasuo, T. Keiko, K. Masatoshi, and M. Susumu. 1990. *In vitro* activity of LJC 10627, a new cabapenem antibiotics with high stability to dehydropeptidase. *Antimicrob. Agents chemother.* **34**: 794-1000.
9. Langham, C. D., S. T. Williams, P. H. A. Sneath, and A. M. Mortimer. 1989. New probability matrices for identification of *Streptomyces*. *J. Gen. Microbiol.* **135**: 121-133.
10. Lee, J. Y., B. S. Kim, and B. K. Hwang. 1995. Numerical identification of *Streptomyces flaveus* producing antibiotic substances inhibitory to plant pathogenic fungi. *J. Microbiol. Biotechnol.* **5**: 324-334.
11. Lim, D. S., S. K. Yoon, M. S. Lee, W. H. Yoon, and C. H. Kim. 1996. Isolation and identification of *Streptoverticillium* sp. NA-4803 producing antifungal substance. *Kor. J. Appl. Microbiol. Biotechnol.* **24**: 664-670.
12. Lyon, B. R., W. M. Jhon, and R. A. Skurray. 1983. Analysis of plasmid in nosocomial strain of multiple-antibiotics resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **23**: 817-826.
13. Okami, Y. and M. Suzuki. 1958. A simple method for microscopical observation of streptomycetes and critique of *Streptomyces* grouping with reference to aerial structure. *J. Antibiot.* **11**: 250-253.
14. Porter, J. N. 1975. Cultural conditions for antibiotic-producing microorganisms. pp. 3-23. *In* J. H. Hash (ed.), *Method in Enzymology*. vol. 63. Academic Press.
15. Pridham, T. G., C. W. Hesseltine, and R. G. Benedict. 1958. A guide for classification of streptomycetes according to selected groups placement of strains in morphological sections. *Appl. Microbiol.* **6**: 52-79.
16. Shirling, E. B. and D. Gottlieb. 1966. Methods for characterization of *Streptomyces species*. *Int. J. Syst. Bacteriol.* **16**: 313-340.
17. Simpson, W. J., J. R. M. Hammond, and R. B. Miller. 1988. Avoparcin and vancomycin: useful antibiotics for the isolation of brewery lactic acid bacteria. *J. Appl. Bacteriol.* **64**: 299-309.
18. Varald, P. E., P. Cipriani, A. Foca, C. Greaci, A. Giordano, M. A. Madeduu, A. Oris, and P. Roselli. 1984. Identification, clinical distribution and 18 additional antibiotics of clinical *Staphylococcus* isolate. nationwide investigation in Italy. *J. Clin. Microbiol.* **19**: 838-843.
19. Ward, A. C. 1991. TAXON-Data input and analysis for binary data from numerical taxonomic studies. pp. 723-823. *In* M. Goodfellow (ed.), *Selected readings in microbial systematics*, Research Center for Molecular Microbiology, Seoul National University.
20. Willcox, W. R., S. P. Lapage, S. Bascomb, and M. A. Curtis. 1972. Identification of bacteria by computer: theory and programming. *J. Gen. Microbiol.* **77**: 317-330.
21. Williams, S. T., M. E. Sharpe, J. G. Holt, R. G. E. Murray, D. J. Brener, N. R. Krieg, J. W. Mouldar, N. Pfennig, P. H. A. Sneath, and J. T. Staley. 1989. *Bergey's Manual of Systematic Bacteriology*, vol. 4. William & Willkins, Baltimore.
22. Williams, S. T. and F. L. Davies. 1967. Use of a scanning electron microscope for the examination of actinomycetes. *J. Gen. Microbiol.* **48**: 171-177.
23. Williams, S. T., M. Goodfellow, G. Alderson, E. M. H. Wellington, P. H. A. Sneath, and M. J. Sackin. 1983a. Numerical classification of *Streptomyces* and related genera. *J. Gen. Microbiol.* **129**: 1743-1813.
24. Williams, S. T., M. Goodfellow, E. M. H. Wellington, J. C. Vickers, G. Alderson, P. H. A. Sneath, M. J. Sackin, and A. M. Mortimer. 1983. A probability matrix for identification of some streptomycetes. *J. Gen. Microbiol.* **129**: 1815-1830.
25. Yamada, K. and K. Kamagata. 1970. Taxonomic studies on coryneform bacteria. II. Principle amino acids in the cell wall and their taxonomic significance. *J. Gen. Appl. Microbiol.* **16**: 103-113.
26. Yasuhiro, M., W. Yuji, S. Hiroshi, H. Kazuo, K. Kyoichiro, T. Shuichi, and M. Yoshimi. 1993. Excellent activity of FK 037, a novel parrental broad-spectrum cephalosporin against methicillin-resistant staphylococci. *J. Antibiot.* **46**: 99-119.

(Received June 28, 1997)