# YM-47522, a Novel Antifungal Antibiotic Produced by *Bacillus* sp.

## I. Taxonomy, Fermentation, Isolation and Biological Properties

MITSUYOSHI SHIBAZAKI, TAKEO SUGAWARA†, KOJI NAGAI, YASUYO SHIMIZU, HIROSHI YAMAGUCHI and KENICHI SUZUKI

Microbiology Research Department, Drug Serendipity Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd., 1-1-8 Azusawa, Itabashi-ku, Tokyo 174, Japan
†Drug Serendipity Research Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba, Ibaraki 305, Japan

(Received for publication October 18, 1995)

YM-47522, a novel antibiotic, was isolated from the culture broth of *Bacillus* sp. YL-03709B. The antibiotic was purified by centrifugal partition chromatography and ODS column chromatography. It exhibited potent *in vitro* antifungal activity especially against *Rhodotorula acuta* and *Pichia angusta* (MIC: 0.05 and 0.75 μg/ml, respectively). It also showed moderate or weak antifungal activity against *Candida albicans* and *Cryptococcus neoformans* (MIC: 25 and 6.25 μg/ml, respectively), whereas it was inactive against filamentous fungi and bacteria.

In the course of our screening for antifungal substances, a novel antibiotic was found from the culture broth of *Bacillus* sp. YL-03709B and designated as YM-47522 (Fig. 1). In this paper, we describe the taxonomy of the producing organism, fermentation, isolation and biological properties of the antibiotic. Physicochemical properties and the structure elucidation of the compound are reported in the following paper.

### Materials and Methods

#### Isolation of Producing Organism

Strain YL-03709B was isolated from the reddish brown lateritic soil collected at Mt. Omotodake, Ishigaki Island, Okinawa Prefecture, Japan. Microorganisms were isolated by the dilution-plate method using the medium consisted of potato starch 2.0%, L-asparagine 0.1%, $K_2HPO_4$ 0.05%, agar 1.5%, pH 8.0 before sterilization. 50 μg/ml of cefotetan (Yamatetan: Yamanouchi Pharmaceutical Co., Ltd.) and 50 μg/ml of nalidixic acid were added to exclude undesirable microorganisms. Nystatin and actidione (50 μg/ml, each) were also added to inhibit the growth of fungi.

The plate was incubated at 32°C for 3 weeks and colonies were classified preliminarily, and picked onto YS-agar slants (yeast extract 0.2%, potato starch 1.0%, agar 1.5%, pH 7.6 before sterilization).

#### Morphological Characterization

Nikon Microphoto-FXA and Jeol T220 scanning electron microscope were used for microscopic evaluation. For electron microscopic observation bacteria in submerged culture were fixed with 2% glutaraldehyde, post-fixed with 1% osmium tetroxide, dried in air conditions and coated with gold by a sputter.

#### Cultural Characterization and Physiological Tests

Observation of growth on various media and tests for physiological characteristics were made on the basis of methods of COWAN[1], CHRISTENSEN and COOK[2], and GILARDI[3] during incubation at 10 to 50°C for 21 days unless otherwise mentioned. Deoxyribonucleic acid was prepared by the method of "Genetic Manipulation of Streptomyces"[4]. The guanine-plus-cytosine content of the deoxyribonucleic acid of the strain YL-03709B was determined by the method of MARMUR[5], and MARMUR and DOTY[6].

#### Fermentation

A thawed suspension of the producing organism was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of glucose 1%, potato starch 2%, yeast extract 0.5%, Polypepton

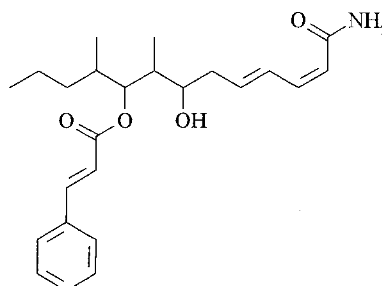



Fig. 1. The chemical structure of YM-47522.

(Nihon Pharmaceutical Co., Ltd.) 0.5%, $CaCO_3$ 0.4% and NKL-5430 (NOF Corporation) 0.03% (pH 7.3). The flask was incubated at 28°C for 3 days on a rotary shaker at 220 rpm. The seed culture (8 ml) was transferred to a 2-liter flask containing 400 ml of the same medium. After 3 days incubation at 28°C, the entire volume of the seed was used to inoculate a 30-liter jar fermentor containing 18 liters of the same medium. The fermentation was carried out at 28°C for 3 days under agitation of 150 rpm and aeration of 9 liters/minute.

Isolation

The fermentation broth of *Bacillus* sp. YL-03709B (17 liters) was centrifuged, and the supernatant was applied to a Diaion HP-20 column (700 ml), which was washed with $H_2O$ and MeOH - $H_2O$ (4:6), and eluted with MeOH. The MeOH elute was concentrated to an aqueous solution, adjusted to pH 3 with 4 N HCl, and extracted with EtOAc. The extract was fractionated by ODS flash chromatography on YMC gel ODS-A with increasing amounts of MeOH in water. Fractions eluted with MeOH - $H_2O$ (8:2) and MeOH were subjected to centrifugal partition chromatography with $CHCl_3$ - MeOH - $H_2O$ (2:2:1) system using the upper layer as the mobile phase. Combined active fractions were finally purified by ODS column chromatography on Cosmosil 140 $C_{18}$-OPN with MeOH - $H_2O$ (85:15) to give 291.1 mg of YM-47522.

*In Vitro* Antifungal Activity

The antifungal activity of YM-47522 was determined on yeast nitrogen base (Difco) supplemented with 2% glucose and 1.5% agar (Difco, Bacto agar). A conventional agar dilution method was used. The MIC for filamentous fungi and yeasts was expressed in terms of μg/ml for 48 to 72 hours at 28°C.

Cytotoxic Activity on Tumor Cells

L1210 cells were maintained in the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). To determine the cytotoxicity of YM-47522, L1210 cells ($1 \times 10^4$) in 1 ml of the medium (RPMI-1640 + 10% FBS) containing various concentration of the antibiotic were placed in a tissue culture plate (Falcon, 24-well) and incubated for 72 hours at 37°C in a 5% $CO_2$ - 95% air atmosphere. At the end of the incubation period, the cells were counted by a hemocytometer. Concentration of the antibiotic required for 50% inhibition of cell growth ($IC_{50}$, μg/ml) was examined by plotting the logarithms of the treated cells.

## Results and Discussion

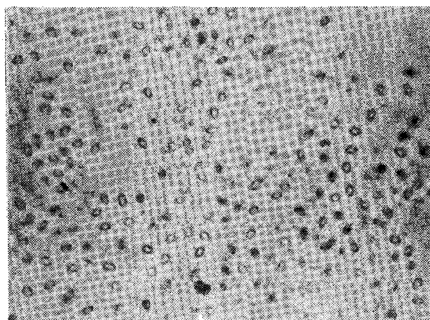
### Taxonomic studies of the Producing Strain

The cultural and physiological characteristics are listed in Table 1. Colony of strain YL-03709B was opaque, circular with irregular circumference, pale yellow to pale brown with rugose surface after 3 days incubation. No diffusible pigment was produced. By light and electron microscopic observation, strain YL-03709B was Gram-positive, slender rods, usually 0.6 to 1.0 μm in width, 2.0 to 7.0 μm in length, without flagella (Fig. 2). Highly refractile endospores are produced under aerobic conditions. Spores were oval to spherical, mostly in central to terminal position. It did not require any growth factors and did not form fruiting bodies. The GC-content of the DNA of strain YL-03709B was determined to be 41.5 mol%. The major isoprenoid quinone was unsaturated menaquinone with seven isoprenoid units. Nitrate reduction, casein hydrolysis, citrate utilization (KOSER's and CHRISTENSEN's media), and oxidase and catalase formation were positive. Denitrification, methyl red test (glucose peptone broth), acetylmethylcarbinol produc-

Table 1. Morphological and physiological characteristics of strain YL-03709B.

| | | | |
|---|---|---|---|
| Cell shape | Non-vacuolated straight rod | Growth factor requirement | Negative |
| Cell size (μm) | 0.6 - 1.0 x 2.0 - 7.0 | Urease | Negative |
| Motility | Positive | Oxidase | Positive |
| Form of spore | Ellipsoidal | Catalase | Positive |
| Swelling of cell at spore formation | Positive | Tyrosinase | Negative |
| Position of spore | Central to terminal | OF-test | Not reactive |
| Gram stain | Positive | Range of growth: | |
| Reduction of nitrate | Positive | pH | 5.5 - 9.0 |
| Denitrification | Negative | (Optimum) | (6.0 - 8.0) |
| Methyl red test | Negative | Temperature | 15 - 40°C |
| Voges-Proskauer test | Negative | (Optimum) | (24 - 37°C) |
| Production of: | | Degradation of: | |
| Acetylmethylcarbinol | Negative | Colloidal chitin | Negative |
| Indole | Negative | Agar | Negative |
| $H_2S$ | Negative | Cellulose | Negative |
| Utilization of | | Hydrolysis of starch | Negative |
| Citrate | Positive | Liquefaction of gelatin | Positive |
| $KNO_3$ | Positive | Tolerance to NaCl | 0 - 3% |
| $(NH_4)_2SO_4$ | Doubtful | Lysozyme sensitivity (0.001%) | Negative |
| | | Mol% G + C of DNA | 41.5 |

Fig. 2. Phasecontrast micrograph of strain YL-03709B.



Bar represents 20μm

tion (glucose peptone broth), $H_2S$ production, indole production, starch hydrolysis and urease production were negative. Potassium nitrate was utilized as a sole nitrogen source but the utilization of ammonium sulfate as a sole nitrogen source was doubtful. It was facultatively anaerobic and not sensitive to lysozyme (peptone 1%, meat extract 1%, NaCl 0.15%, and lysozyme 0.001%, pH 7.0). Hugh-leifson's OF test was the non reactive type. The temperature range for growth was 15 to 40°C with an optimum temperature range of 24 to 37°C. The pH range for growth was 5.5 to 9.0 with an optimum pH range for growth of 6.0 to 8.0. Carbon utilization and acid production were examined using the medium containing sugar 1.0%, yeast extract 0.02%, inorganic salts 0.14% and agar 1.5% with bromocresol purple as pH indicator. The result is given in Table 2.

On the basis of the above characteristics such as the morphological, cultural and physiological properties, the strain YL-03709B was identified as a bacterium of the genus *Bacillus*. Therefore, the characteristics of the strain YL-03709B were compared with the known species of *Bacillus* described in BERGEY's Manual of Determinative Bacteriology (8th Ed.), BERGEY's Manual of Systematic Bacteriology (Vol. 2) and other reports. As a results the strain YL-03709B was presumed to be close to two species of *Bacillus*: *Bacillus badius* and *Bacillus brevis*. Therefore, we carried out the direct comparison of the strain YL-03709B with the type strains of the two species described above and the result is shown in Table 3. Strain YL-03709B differed from *Bacillus badius* in reference to the growth temperature (Strain YL-03709B cannot grow at 50°C but *Bacillus badius* can grow at 50°C.) and NaCl tolerance (Strain YL-03709B cannot grow in the medium containing 5% of NaCl, but *Bacillus badius* can grow in the same medium). On the other hand, *Bacillus brevis* differed from strain YL-03709B in reference to gelatin hydrolysis and utilization of citric acid. However, these differences were considered to be insufficient to propose a new species or a new subspecies. The detailed taxonomic examinations are in progress. Therefore, strain YL-03709B was classified and designated as *Bacillus* sp. YL-03709B.

Strain, YL-03709B has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaraki prefecture, Japan, with accession No. FERM P-14126.

Table 2. Utilization of carbon sources by strain YL-03709B.

| | Acid | Growth |
|---|---|---|
| L-Arabinose | − | − |
| D-Xylose | − | − |
| D-Rhamnose | − | − |
| D-Fructose | + | + |
| D-Galactose | − | − |
| D-Glucose | + | + |
| D-Mannose | − | + |
| Cellobiose | − | − |
| Lactose | − | − |
| Maltose | ± | + |
| Sucrose | − | − |
| Trehalose | − | − |
| D-Raffinose | − | − |
| D-Sorbitol | − | − |
| D-Mannitol | − | + |
| Inositol | − | − |
| Glycerol | − | + |
| Starch | − | − |

1) Carbon utilization and acid production were examined using the medium containing sugar 1.0%, yeast extract 0.02%, inorganic salts 0.14% and agar 1.5% with bromocresol purple as pH indicator.
2) +: positive, +−: doubtful, −: negative.

Table 3. Comparison of strain YL-03709B with the known species of the genus *Bacillus*.

| | YL-03709B | *B. badius* | *B. brevis* |
|---|---|---|---|
| Cell size(μm) | 0.6-1.0 x 2.0-7.0 | 0.8-1.2 x1.5-4.0 | 0.5-0.8 x1.5-6.0 |
| Oxidase | + | + | + |
| Catalase | + | + | + |
| Tyrosinase reaction | − | ± | − |
| Liquefaction of gelatin | + | − | ± |
| Hydrolysis of starch | − | − | − |
| Utilization of citrate | + | − | − |
| Growth at 50°C | − | + | − |
| Carbon utilization | | | |
| D-Fructose | + | − | + |
| D-Glucose | + | + | + |
| D-Galactose | − | − | − |
| Maltose | + | ± | ± |
| Glycerol | + | + | + |

+: positive, +−: doubtful, −: negative.

Fig. 3. Time course of YM47522 production.
○ pH, ● YM47522.

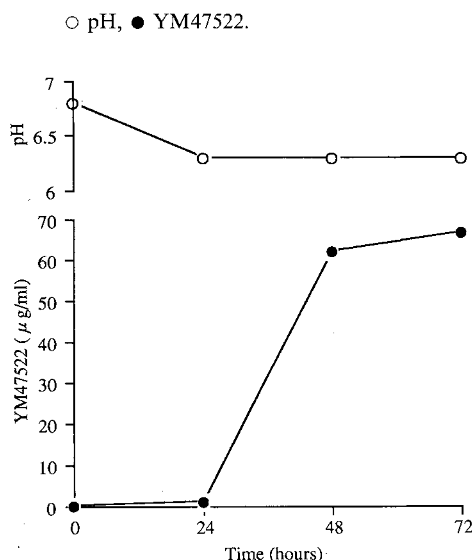
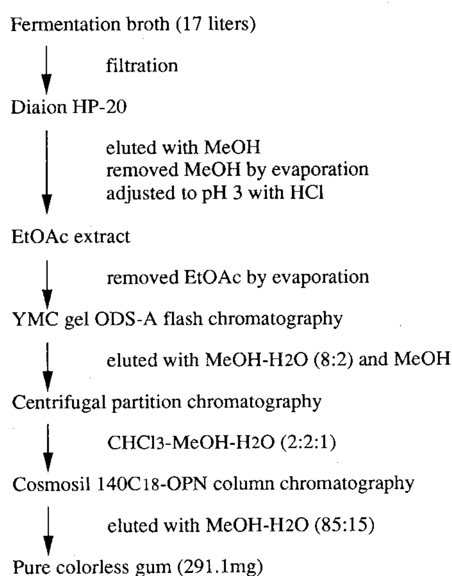



Fig. 4. Purification procedure.




## Production and Isolation

The fermentation of YM-47522 was carried out as described in Materials and Methods. Strain YL-03709B was fermented using a 30-liter jar fermentor. The pH of the broth was kept at 6.3 after reaching this value. A typical time course of the fermentation is shown in Fig. 3. The production of YM-47522 started at 24 hours after inoculation, then increased and reached a maximum (66.6 μg/ml) at 72 hours. From the culture filtrate (17 liters), the antibiotic was isolated as shown in Fig. 4. The total yield of YM-47522 was 291.1 mg.

## Antifungal Activity and Cytotoxicity

The antifungal activity of YM-47522 is shown in Table 4. The antibiotic exhibited potent antifungal activity especially against *Rhodotorula acuta* and *Pichia angusta* with MIC value of 0.05 and 0.75 μg/ml, respectively. It also showed moderate or weak antifungal activity against the other yeasts described in Table 4 with MIC ranging from 6 to 25 μg/ml. However, no activity was observed against filamentous fungi and bacteria. The further examinations revealed that the antibiotic exhibited strong antifungal activity against *Rhodotorula* species such as *R. acuta*, *R. aurantiaca*, *R. bogoriensis*, *R. bogiriensis* and *R. rubra* with MIC of 0.05 to 0.1 μg/ml, whereas it showed moderate activity against several strains of *Candida albicans* and *C. tropicalis* with MIC of 25 to 50 μg/ml (data not shown).

The cytotoxic activity *in vitro* of YM-47522 was determined as described in Materials and Methods. It exhibited cytotoxic activity against lymphoid leukemia L1210 with $IC_{50}$ of 0.41 μg/ml.

To evaluate the reason why it shows the specific antifungal activity against *Rhodotorula* species, the further examinations are now in progress. In addition, we carried out the chemical and biological modification of the compound. We found seven novel compounds

Table 4. Antifungal spectrum of YM-47522.

| Test organisms | AMPH (μg/ml) | MCZ (μg/ml) | YM-47522 (μg/ml) |
|---|---|---|---|
| *Candida albicans* ATCC10231 | 0.2 | 1.95 | 25 |
| *C. parapsilosis* IFO0585 | 0.2 | 1.95 | 25 |
| *Pichia angusta* JCM3620 | 7.8 | 0.98 | 0.75 |
| *Rhodotorula acuta* JCM1602 | 1.56 | 3.13 | 0.05 |
| *Trigonopsis variabilis* IFO0755 | 0.1 | 15.6 | 6.25 |
| *Saccharomyces cerevisiae* ATCC26108 | 0.1 | 0.98 | 50 |
| *S. sake* YFC257 | 0.1 | 0.98 | 50 |
| *Cryptococcus* sp. YFC75 | 1.56 | 7.8 | 6.25 |
| *Aspergillus niger* ATCC9642 | 15.6 | 15.6 | >50 |
| *Mucor hiemalis* IFO9401 | 7.8 | 15.6 | >50 |
| *Trichophyton interdigitale* YFC284 | 3.13 | 1.95 | >50 |

using the directed biosynthetic method. Details of the experimental results will be reported later.

Finally, in the course of preparation of this paper, we found a group of Takeda Chem. Ind. Ltd. had made a patent application on the same antibiotic as YM-47522.[7]

Acknowledgments

The authors are grateful to the members of Microbiology Research Department, Drug Serendipity Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd. for their valuable collaborations on the whole research.

## References

1) COWAN, S. T. (*Ed.*): Manual for the Identification of Medical Bacteria. Cambridge University Press, 1974
2) CHRISTENSEN, P. J. & F. D. COOK: The isolation and enumeration of cytophagas. Can. J. Microbiol. 18: 1933~1940, 1972
3) GILARDI, G. L. (*Ed.*): Glucose Nonfermenting Gram-negative Bacteria in Clinical Microbiology. CRC Press, Inc., 1978
4) RIBB, M. J. (*Ed.*): Genetic manipulation of Streptomyces Section III, 1983. European Molecular Biology Oganization, 1983
5) MARMUR, J.: A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. 3: 208~218, 1961
6) MARMUR, J. & P. DOTY: Determination of the base composition of deoxynucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5: 109~118, 1962
7) FUKUDA, K.; M. SHIRASAKI & S. TSUBOTANI (Takeda Chem. Ind. Ltd.): TAN-1771 related compounds. Jpn. Kokai 206797 ('95), Aug. 8, 1995