

Identification of Soil *Streptomyces* spp. Producing Antifungal Substance

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A soil isolate, CM 001 producing antifungal substance, was identified by morphological, physiological, and chemotaxonomic methods. The strain formed aerial and substrate mycelia on various media. It contained L-diaminopimelic acid and glycine in its cell wall without any characteristic sugar. Saturated branched-chain fatty acids of iso-/anteiso types were shown as major cellular fatty acids. From a number of morphological, physiological, and chemotaxonomic characteristics, the isolate was identified as genus *Streptomyces* (similarity index 0.445 with *Streptomyces californicus*). The antifungal activity of the culture supernatant of the isolate was detected against *Candida albicans* and *Saccharomyces cerevisiae*. The antifungal substance was water soluble, thermostable, and active in wide range of pH.

KEY WORDS □ identification, chemotaxonomy, *Streptomyces* spp., antifungal activity

Actinomycetes are the most abundant bacterial group in terrestrial environments, and constitute a significant component of microbial population in most of soils (6). Since 1940's, actinomycetes, especially *Streptomyces*, have drawn great interests of scientists, because of their value of antibiotic and other useful metabolite producers and their roles in nature as good decomposers and nitrogen fixers.

In general, actinomycetes, the Gram positive bacteria with high G+C molar ratio, shares a common character of mycelial growth (3), but some genera of *Corynebacterium*, *Mycobacterium*, *Rhodococcus* and *Nocardia* have rudimentary mycelium.

Nowadays, the number of patients suffering from fungal diseases is increasing. Pathogenic as well as opportunistic fungi may cause various of internal or external fungal diseases, especially in immune system-debilitated host, for an example, AIDS patients (14).

As to its eucaryotic nature of fungus, it is not easy to obtain antifungal drugs with great selective toxicity. Most of the antifungal drugs in clinical use accompany side effects to a certain extent. Despite numerous efforts for obtaining antifungal substances originated from microorganisms for the past few decades, most of the antifungal drugs searched were discarded, due to mainly their human toxicity. Nowadays, only a few of antifungal drugs such as amphotericine B, nystatin and griseofulvin are in practical use.

Consequently, new antifungal agents with better effectiveness and less host toxicity are urgently needed.

This study aimed to identify the soil bacterial isolate producing antifungal substances by morphological, physiological, cultural characteristics and chemotaxonomical approach. Then, antifungal activity of the culture supernatant of the isolate was investigated.

MATERIALS AND METHODS

Selection of actinomycetes from soil samples

Soil samples collected from surroundings of the Chungbuk National University campus were dried at room temperature and heated at 100°C for 40 min (11). One gram of heat-treated soil was homogenized with 10 ml of sterile saline solution. One-tenth ml of each diluent prepared by ten-fold serial dilution was spread on Bennet's agar containing 0.01% 2,4-dinitrophenol (DNP) to isolate the DNP-resistant soil bacteria and incubated at 25~30°C until colonies appeared. The colonies assumed as *Actinomyces* by naked eye or dissecting microscope were restreaked on Bennet's agar until the pure single colony was obtained.

Bacterial strain and media

Among many soil isolates, CM 001 showing antifungal activity, was chosen in this study. Media used in this study were as recommended by the International *Streptomyces* Project (ISP)

including nutrient agar, *Actinomyces* isolation agar (AI agar), oatmeal agar (ISP medium-3), inorganic salts starch agar (ISP medium-4), and glycerol-asparagine agar (ISP medium-5) (1, 13).

Morphological observation

The isolate grown on many media was examined for growth, pigmentation, the formation of aerial mycelium, reverse color of colony, and other morphological features. Observation of cultural characteristics of the soil isolate at various media was made at weekly intervals for 2~3 weeks of cultivation period. Air-dried smears of colony on Bennet's medium were subjected to Gram staining. The study of micromorphology was made with a 14-day old culture on Bennet's agar grown at 30°C. Preparations of whole cells were stained with safranin and examined aerial and/or substrate mycelium under a light microscope at 40~100 \times . The specimens for scanning electron microscopy were prepared by cutting agar blocks of colony from the ISP medium-4, dehydrating by gradually increasing ethanol series (17). The dehydrated agar blocks mounted on aluminum stubs, were dried with critical point drier and coated with gold palladium. Scanning electron micrographs of the preparations were taken with a Hitachi model S-2500C.

Physiological tests

The media and procedures for the cultural-physiological characteristics and carbon source utilization of the strain were described previously (13). The tests for the utilization of carbon sources were carried out in ISP medium-4 omitted soluble starch at 30°C for 7~14 days. In order to confirm the utilization of DNP as nutritional substance, the growth of isolate on modified ISP medium-4 (omitted soluble starch and ammonium sulfate as C and N source but supplemented with 0.01~0.03% DNP) at 30°C for 7~14 days. Catalase test was done with one-week-old colony on Bennet's agar (5).

Analysis of cellular components

Analysis of cell wall amino acids and whole-cell sugars was performed by the methods described previously (15) but with some modification. The method of acid hydrolysate preparation and amino acid visualization was published somewhere else (18). Sugars were detected with acid aniline phthalate (0.332 g of phthalic acid dissolved in 20 ml of water saturated butanol and 0.186 g aniline) (4). Cellular fatty acids were extracted as methylated form (MIDI Technical Note 101, May, 1990) and analyzed by flame ionization detector-gas chromatography (Analytical Service Inc.).

Screening and production of antifungal substance

To screen the antibiotic activity of the soil isolate, the isolate, CM 001 strain was streaked on the center of Bennet's agar plate and incubated

at 30°C for 3~6 days. Then, the test organisms, one-day-old culture of *C. albicans* and *S. cerevisiae* grown in YM broth at 30°C, were cross-streaked perpendicularly on the same plate and the growth inhibition of yeasts was observed after 24 hours. In disc diffusion method, the disc (made with Whatman no. 2 filter paper, 5 mm in diameter) impregnated with 70~80 μ l of culture supernatant of the soil isolate in Bennet's medium was placed on the YM agar plate which was overlaid with either *C. albicans* or *S. cerevisiae* and incubated at 30°C for 1~2 days. Then, the antifungal activity was examined by measuring the growth inhibition zone of yeasts.

To investigate cultivation time showing maximum production of antifungal substances in 250 ml Bennet's broth (30°C, 140 rpm, 1~14 days), the culture supernatant was subjected to antibiotic assay by disc diffusion method at time interval of 24 hrs.

Physical and chemical properties of antifungal substance

The physical and chemical properties of antifungal substance were studied with the culture supernatant of isolate in Bennet's medium at 30°C for 4~6 days at 140~160 rpm.

Solubility: The residue obtained from 10 ml of supernatant freeze-dried at -70°C was dissolved in either 1 ml of n-hexane, chloroform, dimethylsulfoxide (DMSO), methanol, or distilled water. After vacuum-drying the extracts, the residue of water soluble solvent was redissolved in 1 ml of water, and that of water insoluble in 1 ml of DMSO, 20~30 μ l of each extract was used for antifungal assay.

Thermostability: An aliquot of the culture supernatant either incubated at 10, 30, 50, 70, and 100°C for 1 hr, or autoclaved at 121°C, 15 psi was subjected to antifungal assay.

pH effects on antifungal activity: The culture supernatant of isolate was adjusted to pH 1, 4, 7, 10, and 13, and kept at room temperature for 1 hr. After neutralized the supernatant to pH 7, antifungal activity (of 100 μ l) was assayed.

Antifungal activity of the culture supernatant of CM 001 in acidic condition: The acid precipitate obtained by centrifugation of culture supernatant adjusted to pH 1 was redissolved in small quantity of water. This preparation was neutralized to pH 7 for antifungal assay.

RESULTS AND DISCUSSION

Bacterial identification is generally carried out by morphological, physiological and biochemical characteristics. Nowadays, the methods for bacterial identification have been more diversified by chemical analysis of various cellular components (8). Especially in chemotaxonomical identification, the compositions of cell wall amino acids

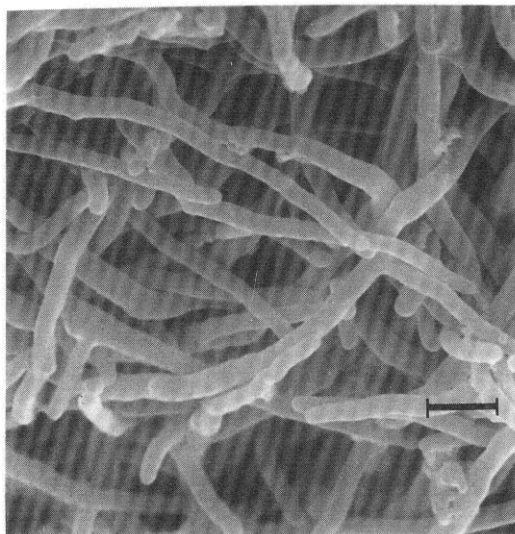


Fig. 1. Scanning electron micrograph of aerial mycelia of CM 001 grown on ISP-4 agar at 30°C for 14 days.
Bar = 1 μ m.

and whole cell sugars are useful with a great value (12). Cellular fatty acid profiles have been also widely applied in bacterial identification (2, 9). A number of new actinomycetes spp. had been identified on the basis of various methods mentioned above (7, 10).

Morphological characteristics of the soil isolate CM 001

The colonies of the isolate CM 001 after culturing appeared very tough in their texture. The bacterium was Gram positive. The isolate was an absolute aerobics since there is no colony formation in Gas-pack anaerobic jar. The aerial mycelium of the isolate looked very sparse in many cases, but substrate mycelium looked rather dense. The vegetative hypha was well developed, long and irregularly branched. Numerous bundles of hypha forming thick fibers were readily visible.

Chain of arthrospore with smooth surface was observed in CM 001 (Fig. 1). Some of these characteristics observed in the soil isolate share the characteristics of actinomycetes (16).

Cultural and physiological characteristics of the isolate

As depicted in Table 1, the isolate grew well on both complex (Bennets, nutrient, and AI agar) and chemically defined media (ISP media-3, 4, and 5) and showed colored colonies. The aerial mycelium of the isolate, white to gray color, were well developed on most media.

The colony color of reverse side of CM 001 was almost same as that of colony (Table 1). The strain produced diffusible pigment, whose color was somewhat different, depending upon medium composition. The strain grew over 25~50°C, but growth optimum temperature was about 30°C. Like other aerobic organisms, the bacterium produced catalase. The isolate utilized L-histidine, L-phenylalanine, D-melibiose, raffinose, sucrose, meso-inositol, and mannitol as the sole carbon source for growth. The isolate could grow in the presence of growth inhibiting substance such as potassium tellurite and 2,4-dinitrophenol (Table 2). This fact suggested that either the soil isolate is resistant to 2, 4-DNP, or utilizes it as a nutrient. Subsequently, it turned out to be utilizer of 2,4-DNP as C or N source.

Chemotaxonomy of the isolate

The presence of L-diaminopimelic acid and glycine without any characteristic sugars in hydrolyzed whole cells suggests that the isolate belongs to type I cell wall and type C sugar pattern. Saturated branched-chain fatty acids of iso-/anteisotypes, examples of 12-methyltetradecanoic acid and 14-methylhexadecanoic acid, were major forms occurred in the isolate. There was no 10-methyl branched fatty acid detected and unsaturated fatty acid (palmitoleic acid) was rather minute. A detail of this is shown in Table 3.

Identification of the isolate CM 001

The morphological, cultural, and chemotaxonomical characteristics described above suggest that

Table 1. Cultural characteristics of the CM 001 on various media^a.

Media	Color of			Production of aerial mycelium (color)
	Colony	Reverse	Soluble pigment	
Bennet agar	Violet	Violet	Violet	Trace (White)
Nutrient agar	Brown	Brown	Absent	None
AI agar ^b	White	Ivory	Absent	Moderate (White)
ISP medium-3	Violet	Violet	Brown (trace)	Good (white-gray)
ISP medium-4	Black-red	Black-red	Brown	Good (white-gray)
ISP medium-5	Violet	Violet	Brown	None

^a Color description was made after incubation for 14 days at 30°C.

^b *Actinomyces* isolation agar.

Table 2. Morphological and physiological characteristics of CM 001^a.

Characteristics	CM 001
Gram stain	+
Catalase production	+
Spore color	Gray
Chain morphology	Rectiflexus
Spore ornamentation	Smooth
Diffusible pigment ^b	+
Growth at 45°C	+
pH range of growth	5~10
Utilization of ^c	
L-histidine (1%)	+
L-phenylalanine (1%)	+
meso-inositol (1%)	+
mannitol (1%)	+
D-melibiose (1%)	+
raffinose (1%)	+
sucrose (1%)	+
Growth with ^d	
potassium tellurite (0.001%)	+
2,4-dinitrophenol (0.01%)	+
2,4-dinitrophenol (0.03%)	+

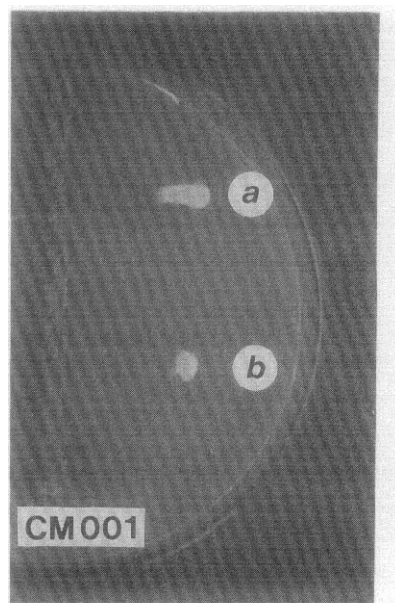
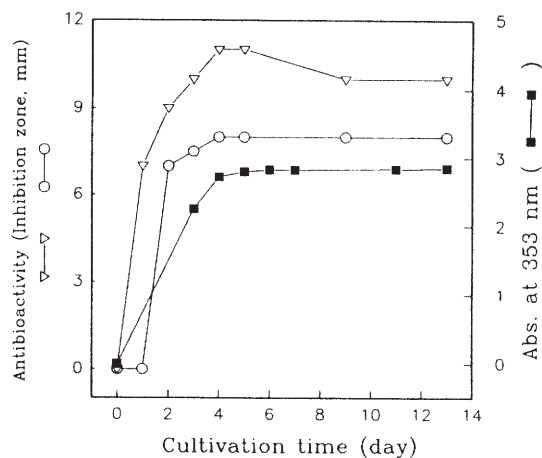
^a Bacteria were cultured on Bennet's agar or borth.^b ISP-5 agar.^c Positive reaction.^d Modified ISP-4 medium without carbon or nitrogen source for 7~14 days at 30°C.**Table 3.** Cellular fatty acid composition of the soil isolate CM 001.

Fatty acids	Amount (%) ^a
	CM 001
C15:0 anteiso	41.41
C17:0 anteiso	15.40
C16:0 iso	11.36
C16:0	9.02
C15:0 iso	6.84
C17:0 iso	3.11
C16:1 cis 9	2.79

^a Percentage of the total peak area from gas-liquid chromatography of methyl esters.

the isolate may belong to the genus *Streptomyces* (16). According to Microbiological Identification System (MIS) database (Analytical Service, Inc.), CM 001 showed 0.445 similarity index with *Streptomyces californicus*, so our soil isolate seemed to be good match with *S. californicus* (SI 0.400 or higher, very good match; 0.250 or lower, poor match). And the isolate showed SI. 0.337 and 0.293 with *S. halstedii* and *S. rochei*, respectively.

Antifungal activity of the culture supernatant of the isolate

**Fig. 2.** Antifungal activity of the isolate CM 001 against *C. albicans* (a) and *S. cerevisiae* (b).**Fig. 3.** Time-dependent production of antifungal substance and soluble pigment during the growth of CM 001.

The bacterium was grown in Bennet's medium at 30°C. *C. albicans* and *S. cerevisiae* were used as antibiotic test strains. ○, *C. albicans*; ▽, *S. cerevisiae*; ■, soluble pigment.

The culture supernatant of the isolate showed antifungal activity against *C. albicans* and *S. cerevisiae* (Fig. 2). The production of antifungal substance became greater after 4~5 days. The soluble pigment in culture supernatant of the strain CM 001 cultured in Bennet's broth showed

its absorbance peaks at 323, 353, and 392~437 nm. The absorbance at 353 nm was coincidentally increased along with the production of antifungal substance. The production pattern of antifungal substance and pigment in culture supernatant was similar (Fig. 3). This fact suggests that the antifungal activity of the isolate against *C. albicans* and *S. cerevisiae* seems to be associated with its pigment production. Since the increase of antifungal activity against *C. albicans* and *S. cerevisiae* was parallel with the pigment production after 2~3 day cultivation of the isolate, it would make us to monitor the production of antifungal substance by the soil isolate.

Physical and chemical properties of antifungal substance

Antifungal substance of the soil isolate was soluble in distilled water and methanol, but insoluble in n-hexane, chloroform, and DMSO. The antifungal activity of the supernatant of CM 001 was fully maintained even after autoclaving. These data suggest that the antifungal substance of CM 001 may have an advantage to obtain aseptic preparation to a certain extent. The antifungal activity of supernatant of CM 001 was highly stable in range of pH 1~13. Most antifungal activity of CM 001 culture supernatant was retained in the acid precipitate at pH 1. The acid precipitability of the antifungal substance of the CM 001 may be useful in purification of antifungal substance in large quantities.

ACKNOWLEDGEMENT

This work was partially supported by SRC research grant (Research Center for Molecular Microbiology, SNU) from KOSEF.

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(Received August 18, 1994)

(Accepted November 17, 1994)

초 록: 항진균성물질을 생산하는 토양 *Streptomyces* spp.의 동정**윤나래 · 이영남*** (충북대학교 자연과학대학 미생물학과)

토양시료로부터 항진균성 물질을 생산하는 CM 001 균주를 분리하여 형태학적, 생리학적, 화학분석적 방법을 통하여 동정하였다. 그람양성, 절대 호기성, 중온성 세균으로 기균사와 배지속 균사를 형성하였다. 이 균은 호기성 생명체의 에너지 대사 저해제인 2,4-dinitrophenol에 의하여 성장이 저해되지 않고 내성을 보였다. 균주의 세포벽에서는 L-diaminopimelic acid와 glycine이 검출되었으나 특징적인 당은 발견되지 않아 방선균류의 chemotype I과 sugar pattern C에 해당된다고 사료되었다. 세포 구성 지방산으로 iso-/anteiso 형태의 측쇄 지방산이 주량을 이루었고 불포화 지방산은 미량 있음을 알 수 있었다. 상기의 형태학적, 생리학적, 화학분석적인 특징들을 종합하여, CM 001이 *Streptomyces*속의 방선균으로 동정되었는데, Microbial Identification System database에 근거하면 분리균은 *S. californicus*와 0.445의 similarity index를 지니 양호한 적합성(0.400 또는 이상)을 지닌 것으로 판정되었다. DNP 내성 토양 방선균의 배양상등액은 *Candida albicans*와 *Saccharomyces cerevisiae*에 대한 항진균활성을 보였다. 분리된 *Streptomyces*균의 배양상등액에 있는 항진균성 물질은 수용성으로 열과 넓은 범위의 pH에 대해 매우 안정하였다.