Cordycepin: Selective Growth Inhibitor Derived from Liquid Culture of *Cordyceps militaris* against *Clostridium* spp.

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The growth responses of nine human intestinal bacteria to liquid culture of *Cordyceps militaris* Link. Pt. (Ascomycotina: Clavicipitaceae) collected from a pupa of *Bombyx mori* L. (Lepidoptera: Bombycidae) were examined using spectrophotometric and impregnated paper disk methods and compared to those of tetracycline and chloramphenicol, as well as those of *Coptis japonica* root-derived berberine chloride. The biologically active constituent of the cultures was characterized as cordycepin (3′-deoxyadenosine) by spectroscopic analysis. This compound revealed potent growth-inhibiting activity toward *Clostridium paraputrificum* and *Clostridium perfringens* at 10 μg/disk without adverse effects on the growth of *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Lactobacillus acidophilus*, and *Lactobacillus casei*, whereas tetracycline and chloramphenicol inhibited the growth of these lactic acid-producing bacteria, clostridia and *Escherichia coli*. However, *C. militaris*-derived materials revealed no growth stimulation on the bifidobacteria and lactobacilli. These results may be an indication of at least one of the pharmacological actions of *C. militaris*. As a naturally occurring antibacterial agent, cordycepin could be useful as a new preventive agent against various diseases caused by clostridia.

Keywords: *Cordyceps militaris; Clavicipitaceae; Bombyx mori; intestinal bacteria; growth inhibition; Clostridium; cordycepin; 3′-deoxyadenosine*

INTRODUCTION

Various microorganisms are resident in the human intestinal tract as a highly complex ecosystem and contain a variety of enzymes that perform extremely various types of metabolisms in the intestine. These bacteria participate in normal physiological functions such as lactic acid-producing bacteria and also contribute to the genesis of various disease states by biotransforming a variety of ingested or endogenously formed compounds to harmful agents such as *E. coli*, *Staphylococcus*, *Veillonella*, and *Clostridium*, or by protecting against diseases by generation of beneficial products (Finegold et al., 1975; Modler et al., 1990; Hoover, 1993). Gastrointestinal ecological investigations have demonstrated that there are some differences in intestinal bacteria between cancer patients and healthy control subjects, between young and elderly subjects, as well as between breast- and bottle-fed infants (Finegold et al., 1975; Mitsuoka, 1984; Modler et al., 1990; Moore and Moore, 1995). The microbiota of cancer patients or elderly subjects is known to be mainly composed of *Clostridium*, with few lactic acid-producing bacteria. The composition of the biota may also be influenced by factors such as diet and stress (Hentges, 1983; Mitsuoka, 1984). Disturbance of the microbiota may cause a variety of diseases or abnormal physiological states.

In relation to human health, much current concern has been focused on naturally occurring bifidus factors and growth inhibitors against harmful bacteria such as *Clostridium* and *E. coli* because natural resources such as plants, animals, and microorganisms constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (Tang and Eisenbrand, 1992; Namba, 1993). Of the natural products, *Cordyceps* spp. belonging to Clavicipitaceae, Ascomycotina have long been considered to have natural medicinal properties such as acting as a hemostatic, a mycolytic, an antiasthmatic, and an expectorant (Tang and Eisenbrand, 1992; Namba, 1993) and have been discovered as a parasite to a variety of insect species under humid ground in the mountains of China, Korea, and Japan (Namba, 1993). However, little work has been carried out on the effect of *Cordyceps* spp.-derived materials on the growth of intestinal microorganisms despite their excellent pharmacological actions.

In the laboratory study described herein, we assessed the growth-promoting and inhibitory responses of nine human intestinal bacteria to cordycepin derived from liquid cultures of *Cordyceps militaris*, tetracycline, chloramphenicol, and *Coptis japonica* root-derived isoquinoline alkaloid berberine chloride. The antitumor or anticarcinogenic action of *Cordyceps* spp. is also discussed in connection with the results obtained from the intestinal bacteria.

MATERIALS AND METHODS

Chemicals. Tetracycline and chloramphenicol were purchased from Sigma (St. Louis, MO). Berberine chloride was obtained from *Coptis japonica* roots as previously described (Chae et al., 1999). All other chemicals were of a reagent grade.

***Cordyceps* sp. and Culture Conditions.** *Cordyceps militaris* Link. Pt. (Ascomycotina: Clavicipitaceae) was collected from a pupa of *Bombyx mori* L. (Lepidoptera: Bombycidae).

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10.1021/jf990862n CCC: $19.00
Published on Web 06/14/2000

It has been stored on PDA media at −70 °C, and when required was subcultured on Hamada agar at 28 °C for 7 days as previously described (Park, 1996).

Bacterial Strains and Culture Conditions. The intestinal bacteria used in this study were *Bifidobacterium bifidum* ATCC 29521, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium longum* ATCC 15707, *Lactobacillus acidophilus* JCM 1028, *Lactobacillus casei* ATCC 14916, *Clostridium paraputrificum* ATCC 25780, *Clostridium perfringens* ATCC 13124, and *Escherichia coli* ATCC 11775. Stock cultures of these nine strains were routinely stored on Eggerth-Gagnon liver extract-Fieldes slants at −60 °C and when required were subcultured on Eggerth-Gagnon (EG) agar (Eiken Chemical, Japan). The plates were incubated at 37 °C for 2 days in an atmosphere of 5% H_2, 15% CO_2, and 80% N_2 in an anaerobic chamber (Coy Laboratory, Ann Arbor, MI). The bacteria were then grown in EG broth (pH 6.8). All cultures were checked by plating for contamination at the end of the growth cycle.

Isolation and Identification. The *Cordyceps* seed was homogenized by a mixer and 1 mL of the suspension was inoculated onto Hamada broth according to the method of Park (1996), and the cultures were incubated at 28 °C for 7 days. Then, the liquid culture (20 L) was filtrated in vacuo through filter paper (Whatman No. 2). The filtrate was applied to a column (5 cm i.d. × 60 cm) packed with Amberlite XAD-2 resins (20−50 mesh, 500 g) and eluted with distilled water (2 L) and methanol (2 L). The methanol eluate was concentrated to dryness using a rotary evaporator at 35 °C to give a yield of 9.5 g. The concentrate (9.5 g) was sequentially partitioned into ethyl acetate (0.8 g), butanol (2.3 g), and water-soluble (6.4 g) portions for subsequent bioassay. The organic solvent portions were concentrated in vacuo by rotary evaporator at 35 °C, and the water portion was freeze-dried. For isolation, 3 mg of each *C. militaris*-derived fraction in methanol was applied by an impregnated paper disk method described below.

The ethyl acetate portion (0.8 g) was chromatographed on a silica gel column (Merck 230−400 mesh, 20 g) and successively eluted with a stepwise gradient of chloroform/methanol (80/20, 50/50, 30/70, and 0/100). The growth-inhibiting fraction (30/70, 50 mg) was fractionated on a preparatory HPLC (Waters Delta Prep 4000). The column was 10 mm i.d. × 250 mm Lichrosorb RP-18 (E. Merck) using methanol/water (2:8, v/v) at a flow rate of 3 mL/min and detected at 254 nm. The active fraction (5 mg) was collected. Further separation of the biologically active constituent(s) was carried out with analytical HPLC (Eyela PLC 5D, Japan). An ODS column (4 mm i.d. × 250 mm) was used with an isocratic mobile phase consisting of methanol/water (15:85, v/v) at a flow rate of 1 mL/min. Eluates were monitored at 254 nm. Finally, a growth-inhibiting principle (4 mg) was isolated; R_f values of the isolate were 0.11 in chloroform/methanol (9:1, v/v), 0.39 in chloroform/methanol (3:1,v/v), 0.16 in ethyl acetate/acetone/water (5:2:1, v/v/v), and 0.64 in butanol/methanol/water (2:1:1, v/v/v).

Structural determination of the active isolate was made by spectroscopic analysis. ^{1}H- and ^{13}C NMR spectra were recorded with a Bruker AM-500 spectrometer and chemical shifts were given in δ (ppm). The unambiguous ^{1}H- and ^{13}C NMR chemical shifts were obtained using various two-dimensional NMR techniques such as the ^{1}H-^{1}H COSY spectrum, as well as a ^{13}C−^{1}H correlation spectrum and its long-range analogue. UV spectra were obtained on a Hitachi 340 spectrophotometer, IR spectra on a Biorad FT-80 spectrophotometer, and mass spectra on a JEOL GSX 400 spectrometer.

Growth-Inhibiting Assay. For assay of effects of test materials on the growth-inhibititing responses of the microorganisms used, one loopful of bacteria was suspended in 1 mL of sterile physiological saline. An aliquot (0.1 mL) of the bacterial suspensions was seeded on EG agar. A sample (0.1, 1, 10, 100, and 200 μg) in 100 μL of methanol solution was applied by Drummond glass microcapillary to a paper disk (Advantec, 8-mm diameter and 1-mm thickness). After evaporation of solvents, the paper disks were placed on the agar surface inoculated with test bacteria. All plates were incubated anaerobically at 37 °C for 2 days. Control disks received 100 μL of methanol, which exhibited no adverse effect against the organisms used. All tests on growth inhibition were repeated in triplicate.

The growth-inhibiting responses of the active isolate toward the nine bacterial strains were examined and compared with those of tetracycline, chloramphenicol, and *C. japonic*a root-derived berberine chloride, which is found to have strong inhibitory activity toward *C. perfringens* (Chae et al., 1999). The inhibitory responses were classified as previously described (Ahn et al., 1994): strong response, +++, zone diameter >20 mm; moderate response, ++, zone diameter 16−20 mm; weak response, +, zone diameter 10−15 mm; and no response, −, zone diameter <10 mm.

Growth-Promoting Assay. The growth-promoting responses of *C. militaris*-derived materials to the microorganisms used were spectrophotometically determined, as previously described (Lee and Ahn, 1997). In the experiments for growth-promoting factors derived from non-carbon and carbon sources (György et al., 1954), broth (pH 6.8) as modified by Yoshioka et al. (1968) and modified MRS broth for the lactobacilli and modified RCM broth for the other bacteria, respectively, were used. One percent of each culture was inoculated in the test media, and 0.01 and 0.1% of each filter-sterilized test material was added to the media in a final volume of 10 mL. Solutions of the test materials were prepared using methanol as a solvent. The methanol concentration in the solutions was 2%, which was found to be without adverse effect on the bacteria used. Samples from test and control solutions were assayed according to the membrane filter procedure (Ahn et al., 1990a). The media were anaerobically incubated at 37 °C for 2 days, and the bacterial growth was measured at 600 nm.

Growth-promoting responses were expressed as growth increase rate (GIR = A_{600} sample/A_{600} reference). The responses were classified as previously described (Chae et al., 1999): strong response, +++, GIR >2.0; moderate response, ++, 2.0> GIR >1.6; weak response, +, 1.5> GIR >1.0; and no response, −, GIR <1.0. Each assay was replicated three times.

RESULTS

Identification. Fractions obtained from liquid culture of *C. militaris* were assayed according to the impregnated paper disk method. At a rate of 3 mg/disk, the ethyl acetate fraction showed strong growth-inhibiting activity (+++) toward *C. perfringens*, whereas moderate and weak activity was produced from butanol and water fractions, respectively. Purification of the biologically active constituent(s) from the ethyl acetate fraction was done by silica gel column chromatography and HPLC, and the isolates were bioassayed. One active principle was isolated from the fraction. Structural determination of the isolate was made by spectroscopic methods including MS and NMR, and it was characterized as cordycepin (3′-deoxyadenosine). The compound was identified on the basis of the following evidence: HR-MS $C_{10}H_{13}N_5O_3$ (found 251.1050, calcd 251.2459); UV λ^{MeOH}_{max} nm (ϵ) 211 (6366); FT-IR (cm^{-1}) 3331, 3230, 3140, 1670, 1607, 1575; ^{1}H NMR (400 MHz, CD_3 OD) δ 2.07 (1H, m), 2.37 (1H, m), 3.67 (1H, m), 3.93 (1H, m), 4.51 (1H, m), 4.70 (1H, m), 5.92 (1H, d, J = 2.4 Hz), 7.01 (2H, s), 8.20 (1H, s), 8.41 (1H, s); ^{13}C NMR (100 MHz, CD_3OD) δ 157.3 s, 153.6 d, 149.8 d, 141.1 d, 120.6 d, 93.5 d, 82.5 d, 76.6 d, 64.2 t, 34.5 t.

Growth-Inhibiting Activity. The inhibitory activity of cordycepin toward harmful intestinal bacteria used was compared to that of tetracycline and chloramphenicol, as well as that of *C. japonica*-derived berberine chloride (Table 1). Responses varied with bacterial strain and chemicals tested. In tests with two strains of clostridia, potent growth-inhibiting activity (+++) was observed in 10 μg/disk of cordycepin, whereas

Table 1. Growth-Inhibiting Responses of Harmful Intestinal Bacteria to Test Materials

compound	bacterial strain[a]	dose (μ/disk) 0.1	1	10	100	200
cordycepin	*C. paraputrificum* ATCC 25780	−[b]	++	+++	+++	+++
	C. perfringens ATCC 13124	−	++	+++	+++	+++
	E. coli ATCC 11775	−	−	−	−	−
tetracycline	*C. paraputrificum* ATCC 25780	+++	+++	+++	+++	+++
	C. perfringens ATCC 13124	+++	+++	+++	+++	+++
	E. coli ATCC 11775	−	+	++	+++	+++
chloramphenicol	*C. paraputrificum* ATCC 25780	−	+	+++	+++	+++
	C. perfringens ATCC 13124	−	+	+++	+++	+++
	E. coli ATCC 11775	−	−	+++	+++	+++
berberine chloride	*C. paraputrificum* ATCC 25780	−	−	+	++	+++
	C. perfringens ATCC 13124	−	−	+	++	+++
	E. coli ATCC 11775	−	−	−	−	−

[a] They were cultured on Eggerth-Gagnon agar at 37 °C for 2 days in an atmosphere of 5% H_2, 15% CO_2, and 80% N_2. [b] Inhibitory zone diameter >20 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −.

Table 2. Growth-Inhibiting Responses of Lactic Acid-Producing Bacteria to Test Materials

compound	bacterial strain[a]	dose (μ/disk) 0.1	1	10	100	200
cordycepin	*B. bifidum* ATCC 29521	−[b]	−	−	−	−
	B. breve ATCC 15700	−	−	−	−	−
	B. adolescentis ATCC 15073	−	−	−	−	−
	B. longum ATCC 15707	−	−	−	−	−
	L. acidophilus JCM 1028	−	−	−	−	+
	L. casei ATCC 14916	−	−	−	−	−
tetracycline	*B. bifidum* ATCC 29521	−	+	+	++	+++
	B. breve ATCC 15700	−	+	+++	+++	+++
	B. adolescentis ATCC 15073	−	+	+++	+++	+++
	B. longum ATCC 15707	+	++	+++	+++	+++
	L. acidophilus JCM 1028	−	+	++	+++	+++
	L. casei ATCC 14916	−	+	++	+++	+++
chloram-phenicol	*B. bifidum* ATCC 29521	−	+	++	+++	+++
	B. breve ATCC 15700	−	−	+	++	+++
	B. adolescentis ATCC 15073	−	−	−	+	+++
	B. longum ATCC 15707	−	++	+++	+++	+++
	L. acidophilus JCM 1028	−	+	+++	+++	+++
	L. casei ATCC 14916	−	+	+++	+++	+++
berberine chloride	*B. bifidum* ATCC 29521	−	−	−	−	−
	B. breve ATCC 15700	−	−	−	−	−
	B. adolescentis ATCC 15073	−	−	−	−	−
	B. longum ATCC 15707	−	−	−	−	−
	L. acidophilus JCM 1028	−	−	−	−	−
	L. casei ATCC 14916	−	−	−	−	−

[a] They were cultured on Eggerth-Gagnon agar at 37 °C for 2 days in an atmosphere of 5% H_2, 15% CO_2, and 80% N_2. [b] Inhibitory zone diameter >20 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −.

berberine chloride revealed weak inhibition (+) at the same rate. At 1 μg/disk, cordycepin and tetracycline produced a very clear inhibitory effect on these bacterial strains, whereas weak or no growth inhibition was obtained in chloramphenicol and berberine chloride, respectively. Growth-inhibiting activity of cordycepin was comparable to that of the commercial antibiotics.

With *E. coli*, no growth inhibition was obtained from the application of cordycepin and berberine chloride even at as high as 200 μg/disk. However, tetracycline and chloramphenicol at 10 μg/disk showed strong growth inhibition (Table 1).

Table 2 shows growth-inhibiting responses of lactic acid-producing bacteria to test compounds. Responses were bacterial strain- and chemical-dependent. In the case of the bifidobacteria, at 200 μg/disk, cordycepin and berberine chloride did not cause growth inhibition of *B. bifidum*, *B. breve*, *B. adolescentis*, and *B. longum*. However, tetracycline and chloramphenicol strongly inhibited their growth even at as low as 10 μg/disk. Results similar to those produced in the test bifidobacteria were obtained in the lactobacilli used.

Growth-Promoting Activity. The effects of *C. militaris*-derived materials on the bacterial growth promotion are examined. For determination of bacterial growth, two kinds of media were used: modified György broth as a carbon source-containing medium, and MRS and RCM broths as a carbon source-free medium. At a concentration of 0.1%, *C. militaris*-derived fractions (ethyl acetate, butanol, and water) and cordycepin showed no growth-promoting activity for *C. perfringens* and *B. longum* in modified György, MRS, and RCM broths.

DISCUSSION

The intestinal microbiota in healthy subject remains relatively constant but is known to be greatly influenced by physical, biological, chemical, environmental or host factors (Hentges, 1983; Mitsuoka, 1984; Modler et al., 1990; Hoover, 1993). Alterations to the microbiota may cause abnormal physical conditions or diseases. In our study with nine human intestinal bacteria, *C. militaris*-derived materials showed selective growth inhibition toward clostridia used. Its active constituent was identified as cordycepin.

Among the various human intestinal microorganisms, bifidobacteria are often taken as useful indicators of human health under most environmental conditions, on the basis that they play important roles in metabolism such as amino acid and vitamin production, aid defense against infection, are associated with longevity, antitumor activity, pathogen inhibition, improvement of lactose tolerance of milk products, and immunopotentiation (Hentges, 1983; Rasic and Kurmann, 1983; Mitsuoka, 1984; Hoover, 1993). Bifidobacteria growth-promoting factors, usually called bifidus factor, are classified into lacteal secretions, derivatives of lactose, fructooligosaccharides, and xylooligosaccharides (Modler et al., 1990). On the contrary, clostridia are the most important causative agents of a wide variety of human diseases such as sudden death, toxicity, mutagenesis, carcinogenesis, or aging by biotransforming a variety of ingested or endogenously formed compounds to harmful agents such as *N*-nitroso compounds or aromatic steroids within the gastrointestinal tract (Finegold et al., 1975; Goldman, 1983; Mitsuoka, 1984; Moore and Moore, 1995). Therefore, selective growth inhibitors not only contribute to the understanding of the biochemical or molecular mechanisms of the bacterial

infection, but are also important in the prevention of human diseases.

It would therefore be desirable to both inhibit the growth of potential pathogens and/or increase the numbers of bifidobacteria in the human gut. Selective growth promoters for bifidobacteria or inhibitors for harmful bacteria are especially important for human health, because intake of these materials may normalize disturbed physiological functions that result in the prevention of diseases caused by pathogens in the gastrointestinal tract. In our microbial study, cordycepin showed potent growth-inhibiting activity toward *C. paraputrificum* and *C. perfringens* but no growth inhibition or stimulation on *B. bifidum*, *B. breve*, *B. adolescentis*, *B. longum*, *L. acidophilus*, and *L. casei*. However, tetracycline and chloramphenicol strongly inhibit growth of these lactic acid-producing bacteria, indicating alteration of intestinal microbiota which result in abnormal physiological state. This is the first report on *C. militaris*-derived cordycepin as a naturally occurring antibacterial compound toward these clostridia, although pharmacological investigations have proved that it has antiviral activity against HIV-1 in vitro (Mueller et al., 1991), antitumor activities (Penman et al., 1970), inhibition of RNA (Grahn and Loevtrup-Rein, 1971), and antiprotozoal activity (Trigg et al., 1971). Selective growth inhibition toward *C. perfringens* was also reported in oriental medicinal plant-derived materials such as polyphenols from *Thea sinensis* (Ahn et al., 1990b; Ahn et al., 1991) and *Panax ginseng* extract (Ahn et al., 1990a).

It has been reported that populations at risk for carcinoma of the gastrointestine had higher rates of carriage of clostridia (Finegold et al., 1975; Moore and Moore, 1995), suggesting that the organism may play large roles in the causation of cancer of the gastrointestine because of its ability to produce *N*-nitroso compounds or aromatic steroids which are highly carcinogenic (Goldman, 1983; Mitsuoka, 1984). Therefore, it is of great interest to investigate relationships between growth-inhibiting action of cordycepin against clostridia and human diseases caused by the bacteria but information is very limited, although cordycepin is found to have antitumor activities (Penman et al., 1970). Our results suggest that inhibitory effect of this compound on growth of *C. perfringens* may inhibit the formation of carcinogens which result in prevention of carcinogenesis. Epidemiological investigations of gastric cancer have reported a negative relationship between death related to gastric cancer and frequent intake of green tea (Oguni et al., 1983). Green tea components such as polyphenols may be responsible for the protective effect on the cancer and impede gastric carcinogenesis by inhibiting the formation of carcinogens (Kuwata et al., 1988). More recent in vivo investigations using human volunteers have shown that intake of ginseng extract or green tea extract affected favorably fecal microbiota and biochemical aspects of feces (Ahn et al., 1990a; Okubo et al., 1992).

In conclusion, daily intake of *C. militaris*-derived materials would be expected to alter the growth and composition of the intestinal flora and modulate the genesis of potentially harmful agents, thus maintaining optimal human health. Additionally, cordycepin may also have potential in the preservation of food and selective media for the propagation of bifidobacteria. On the basis of our data and these earlier findings, inhibitory action of cordycepin toward the clostridia used without any adverse effect on lactic acid-producing bacteria used may be an indication of at least one of the pharmacological actions of *C. militaris*, although many part of medical effects of *Cordyceps* spp. is regarded to be due to various components such as galactosaminoglycan, nucleic acids, and steroids (Lu et al., 1981; Xiao et al., 1983; Ohmori et al., 1989).

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Received for review August 3, 1999. Revised manuscript received April 22, 2000. Accepted April 26, 2000. This work was supported by grants from the Brain Korea 21 Project, and the Ministry of Agriculture and Forestry through the R & D Promotion Center for Agriculture and Forestry to Y.J.A.

JF990862N