TOPOSTIN, A NOVEL INHIBITOR OF MAMMALIAN DNA TOPOISOMERASE I FROM *FLEXIBACTER TOPOSTINUS* SP. NOV.

I. TAXONOMY, AND FERMENTATION OF PRODUCING STRAIN

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We found a new inhibitor of mammalian DNA topoisomerase I, named topostin, from a bacterial culture. The bacteria was identified as *Flexibacter topostinus* sp. nov., B-572. Morphological and physiological characteristics, and utilization of sugars were examined. Comparison of the strain with known species of the genus *Flexibacter* was made and indicates that the strain is a new species of the genus *Flexibacter*. The bacteria produced the inhibitor in parallel with their growth up to 72 hours.

In the course of our screening for new enzyme inhibitors, we found a new DNA topoisomerase (topo) I inhibitor, topostin, from a bacterial culture No. B-572. It was identified as *Flexibacter topostinus* sp. nov. This paper deals with the taxonomy and fermentation of the producing strain.

Materials and Methods

Isolation of Producing Strain

Strain B-572 was freshly isolated from a forest surface soil at Katsuta-city, Ibaraki Prefecture, Japan. The isolation method was as follows: Soil samples which were dried at room temperature overnight, were inoculated as a streak on the surface of GB-medium (L-asparagine 0.1%, $CaCl_2$ 0.1%, agar 1.5% in humus soil extract solution at pH 8.0). Nystatin (50 μg/ml) and cycloheximide (50 μg/ml) were added to the medium to reduce the growth of fungi. The plate was incubated at 25°C and checked in regular intervals of 1~2 days for gliding growth of organisms out of soil streaks on the surface. After 1 or 2 weeks, the gliding bacteria were observed and purified on the same medium. For storages the purified colonies were transferred to slants of modified GB-medium supplemented with 0.1% glucose and incubated at 25°C for several days.

Morphological Characterization

A Jeol T220 scanning electron microscope was used for microscopic evalution. Bacteria in submerged cultures were fixed with 2% glutaraldehyde, post-fixed with 1% osmium tetraoxide, dried at critical point and coated with gold by sputter.

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Cultural Characterization and Physiological Tests

Observation of growth on various media and tests for physiological characters were made on the basis of methods of COWAN[1], CHRISTENSEN and COOK[2], DWORKI and GIBSON[3], and GILARDI[4] during incubation at 10~50°C for 21 days unless otherwise mentioned.

DNA Base Composition

Deoxyribonucleic acid was prepared by the method of "Genetic Manipulation of *Streptomyces*"[5]. The guanine-plus-cytosine content of the deoxyribonucleic acids of strain B-572 were determined by the method of MARMUR[6], and MARMUR and DOTY[7].

Fermentation

Strain B-572 was grown on agar slants of modified GB-medium. Slants were incubated at 25°C for 10 days and stored at 4°C until needed. Seed culture was carried out in 60 ml of the following sterilized medium in 500-ml Erlenmeyer flasks: Defatted soybean meal 5.0%, sucrose 1.2%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% in distilled water. The medium was adjusted to pH 7.0 prior to sterilization at 121°C and 1.0 kg/cm^2 for 20 minutes. After 48 hours of growth at 28°C on a rotary shaker (220 rpm; 5 cm stroke), three percent vegetative inoculum was used to inoculate into a 30-liter jar fermenter (Biott Co. Ltd., BMD-30) charged to a volume of 20 liters of the same medium. The medium was prepared in distilled water and adjusted to pH 7.0 prior to sterilization at 121°C and 1.0 kg/cm^2 for 45 minutes. Fermentation was carried out at 28°C for 96 hours (aeration: 1 vol/vol/minute, working pressure: 0.5 kg/cm^2, agitation: 200 rpm). Packed cell volumes were determined by centrifugation at 1,200 × g for 10 minutes.

Assay of Topo I and Its Inhibitor

Enzymatic activity was assayed by relaxation of supercoiled plasmid ColE1 DNA as described previously[8] and one unit of the enzyme is defined as the minimum amount of enzyme giving complete relaxation of 0.2 μg supercoiled substrate under the reaction conditions. One inhibition unit of inhibitor is defined as the amount of inhibitor giving 50% inhibition of 3 U of the enzyme.

Results and Discussion

For the isolation of aerobic, yellow-pigmented gliding bacteria, we studied various media not only for suitability for the enrichment of gliding bacteria, but also to promote the spreading growth for gliding bacteria. Because there were no selective methods which were universally applicable for the enrichment

Table 1. Morphological and physiological characteristics of strain B-572.

Cell shape	Slender rods	Urease	Negative
Cell size (μm)	0.4~0.6 × 3~20	Oxidase	Positive
Motility	Gliding	Catalase	Positive
Flagella	None	Oxygen demand	Aerobic
Gram stain	Negative	O-F test	Not reactive
Microcyst formation	Not observed	Range of growth	
Reduction of nitrate	Negative	pH	5.5~8.0
Denitrification	Negative	(optimum)	(6.0~7.5)
Methyl red test	Negative	Temperature	10~33°C
Voges-Proskauer test	Negative	(optimum)	(15~27°C)
Production of:		Degradation of:	
Indole	Negative	Colloidal chitin	Negative
H_2S	Negative	Agar	Negative
Utilization of:		Cellulose	Negative
Citrate	Negative	Hydrolysis of starch	Positive
KNO_3	Negative	Liquefaction of gelatin	Positive
$(NH_4)_2SO_4$	Negative	Tolerance to NaCl	0~3%
Growth factor requirement	Negative	Mol% G+C of DNA	33.4

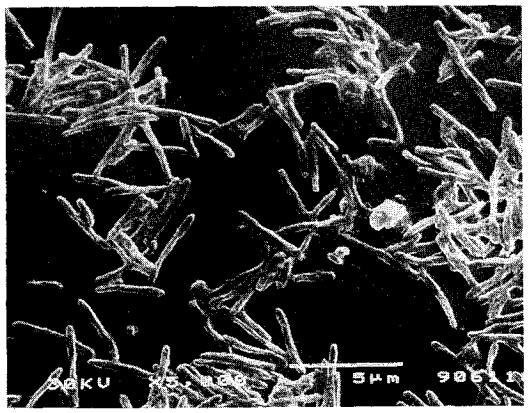
of this kind of microorganisms[9], we found GB-medium which promoted spreading-out-growth from soil streaks for gliding bacteria.

Colonies of strain B-572 were semi-transparent, white cream, circular, convex and entire-edged on nutrient agar. By light and electron microscopic observation, strain B-572 was Gram-negative, slender rods, usually 0.4 to 0.6 μm in width, 3 to 20 μm in length, without flagella (Fig. 1). Strain B-572 exhibited gliding motility, did not require any growth factors, and did not form fruiting bodies, intracellular granules, photosynthetic pigments, spores of microcysts.

The other cultural and physiological characteristics are listed in Table 1. The carbon source utilization pattern is given in Table 2.

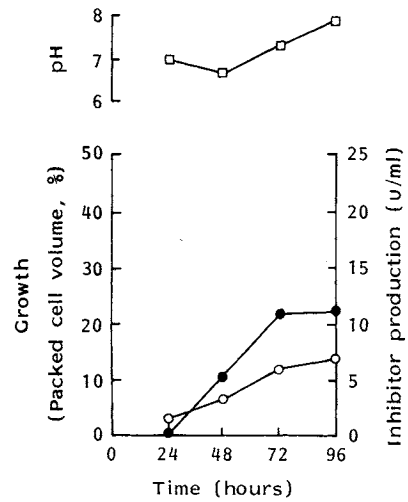
The key characters of strain B-572 were as follows; non fragmented slender rods; gliding motility; aerobic; yellow pigmented; microcyst-forming ability negative; intracellular granules negative; cellulose, chitin and agar decomposition negative; a low GC content of DNA. The morphology and cultural

Fig. 1. Electron micrograph of *Flexibacter* sp. B-572.
Bar represents 5 μm.



The bacteria were fixed with glutaraldehyde and post fixed with osmium tetraoxide, coated with gold by sputter and observed with a scanning electron microscope at 25 kilovolts and at a magnification of 5,000.

Fig. 2. Time course of topostin production.
○ Growth, ● inhibitor production, □ pH.



The bacteria were grown in a jar fermenter at 28°C with aeration. Growth, inhibitor production and pH of the culture broth were measured as described in Materials and Methods.

Table 2. Utilization of carbon sources by strain B-572.

	Acid[a]	Growth[b]		Acid[a]	Growth[b]
L-Arabinose	−	−	Lactose	−	−
D-Xylose	−	−	Trehalose	−	−
D-Glucose	±	+	D-Sorbitol	−	−
D-Mannose	−	−	D-Mannitol	−	+
D-Fructose	−	−	Inositol	−	+
D-Galactose	−	−	Glycerol	−	+
Maltose	−	−	Starch	−	+
Sucrose	±	+			

[a] Acid formation from sugars were examined in peptone-water containing 0.1% of a single carbon source. −: Acid production negative. +: acid production positive. ±: acid production doubtfull.
[b] The bacteria do (+) or do not use (−) the sugar indicated for growth.

Table 3. Comparison of strain B-572 with known species of the genus *Flexibacter*.

	F. flexilis	*F. aurantiacus*	B-572
Cell size (μm)	0.5 by 10~50	0.5 by 5~20	0.4~0.6 by 3~20
Pigment	Orange	Yellow	Yellow
Cellulose digestion	−	−	−
Starch hydrolysis	−	−	+
Gelatin hydrolysis	+	+	+
Catalase	−	+	+
Carbon utilization:			
D-Glucose	+	+	+
D-Galactose	−	+	−
Sucrose	+	+	+
Glycerol	−	−	+
Lactose	−	−	−
Na-acetate	−	−	−

characteristics of strain B-572 identified the bacteria to be of the genus *Flexibacter*.

The characteristics of strain B-572 were compared with those of six species of *Flexibacter* described in BERGEY's Manual of Determinative Bacteriology (8 th Ed.), BERGEY's Manual of Systematic Bacteriology (Vol. 1) and seven species cited in approved list[10]. Strain B-572 was close to two species of *Flexibacter*: *Flexibacter flexilis* and *Flexibacter aurantiacus*. There were some differences in cultural and physiological details and carbon utilization among them (Table 3).

Considering the results of comparative studies of strain B-572 with the strains described above we have concluded that it is suitable to recognize this taxon as a new species in the genus *Flexibacter*, named as *F. topostinus* sp. nov. The specific epithet is derived from its productivity of topostin.

The type strain, B-572, has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaraki Prefecture, Japan, with accession No. FERM P-8738. A typical time-course of topostin production in a 30-liter jar fermenter is shown in Fig. 2. Fermentation was carried out at 28°C for 96 hours. Topostin production started at 24 hours after inoculation, then gradually increase and reached a maximum at 72~96 hours.

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