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Naphthalecin, a Novel Antibiotic Produced by the Anaerobic Bacterium, *Sporotalea colonica* sp. nov.

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Abstract A novel antibiotic naphthalecin was purified and isolated from the cells of an anaerobic bacterium isolated from a soil sample. This antibiotic contained a naphthalene moiety, so named as naphthalecin, and showed antibacterial activity against gram positive species. The producing strain, an obligate anaerobe, was identified as a new species of the genus *Sporotalea*. Identification of the bacterium, cultivation, purification, structure determination, and antibacterial activity are shown.

Keywords antibacterial antibiotic, strictly anaerobic bacteria, naphthalene, naphthalecin, *Sporotalea colonica*

Introduction

During the screening of bacterial isolates from soil samples, an obligate anaerobic bacterium growing in a colony of an aerobic bacterium and a coculture of both strains with an antibacterially active mycelial form were found. After isolation of monocultures of both strains, we found that the anaerobe was the source of the antibiotic activity. As the reports on antibiotics produced by strictly anaerobic bacteria are few [1, 2], we tried to isolate the antibiotic from this anaerobic bacterium. The attempt resulted in the discovery of naphthalecin.

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Results

Taxonomic Study

Strain SYB was a strictly anaerobic Gram-positive bacterium. Microscopic observation showed long, straight rods occurring singly or in pairs. As shown in Fig. 1, cells measured 2.0～15.0 μm in length and 0.5～0.8 μm in diameter and were motile with peritrichous flagella. In older cultures, cells formed refractile terminal endospores which were heat resistant (80°C, 10 minutes). Growth on BP agar was poor; a 14-day culture yielded small, circular, yellow colonies about 2～3 mm in diameter. The temperature and pH ranges for growth were 15～35°C (optimal 25～30°C), and 6.0～9.0 (optimal 7.0～8.0), respectively.

The 16S rRNA gene sequence of strain SYB determined in this study was almost complete, comprising 1,419 nt. The sequence showed that strain SYB was most closely affiliated with a strain of the genus *Sporotalea* [3, 4]. A phylogenetic tree based on the neighbour-joining algorithm

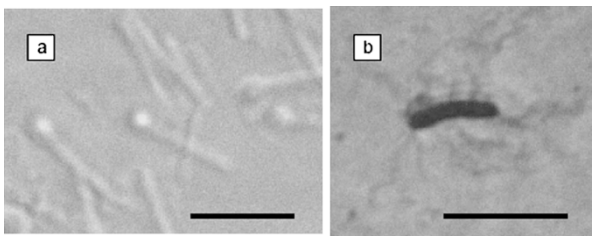

Fig. 1 Strain SYB grown on BP agar at 30°C.

(a) Endospores and vegetative cells after culturing for 10 days. Interference-contrast microscopy. (b) Flagella stain after culturing for 5 days. Phase-contrast microscopy. Bars represents 5.0 μm.

agreed with this determination; strain SYB fell within the cluster comprising the same genus (Fig. 2). In addition, strain SYB exhibited a 16S rRNA gene sequence similarity value of 99.0% for the type strain of *Sporotalea propionica*. This phylogenetic analysis confirmed that strain SYB belonged to the genus *Sporotalea*, as did morphological observation and the DNA G+C content analysis results (Table 1).

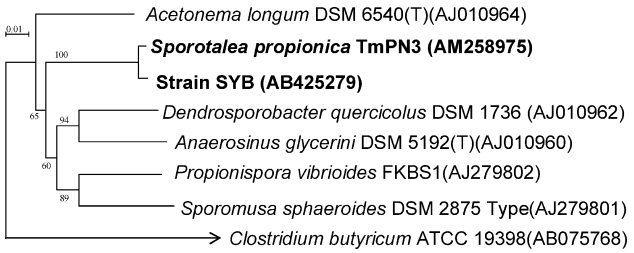

Fig. 2 Phylogenic tree derived from 16S rRNA gene sequence data of strain SYB and other related organisms.

The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1400 nt. Bootstrap percentages (based on 1000 replicates) greater than 50% are shown at branch points. Bar, 0.01 changes per nucleotide position.

The genus *Sporotalea* has been proposed only recently as a new genus of the family *Acidaminococcuseae* [5, 6], and comprises only one species, *S. propionica* [3]. As shown in Table 1, there were several differences between this species and strain SYB: temperature range for growth, pH range for growth, production of pigments, and acid production from several carbon sources. In addition, the mean DNA-DNA relatedness value between the strains was 54% (N=3, 51, 55, 56%), which supported the assertion that the two were different species [7]. Phenotypic and phylogenetic data indicate that strain SYB represents a novel species of the genus *Sporotalea*, for which the name *Sporotalea colonica* sp. nov. is now proposed. The type strain is SYB^T (NRBC 104305), and the GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SYB is AB425279.

Strain SYB was originally isolated from a colony of strictly aerobic bacterium, strain SYA, under aerobic conditions. When the strain was reinoculated into a growing colony of strain SYA under aerobic conditions, it grew again. The details of this symbiotic growth will be

Table 1 Comparison of strain SYB with *Sporotalea propionica* ATCC BAA-626^T

Characteristics	Strain SYB	Strain ATCC BAA-626^T
Cell shape	long rod	long rod
Cell size	0.5~0.8×2~15 μm	0.5~0.7×2~12 μm
Spore formation on BP agar	+	+
Motility	+	+
Flagellum	+, peritrichous	+, peritrichous
Temperature range for growth	15~35°C	10~35°C
	(25~30°C opt.)	(20~25°C opt.)
pH range for growth	6.0~9.0	5.5~9.0
Production of yellow pigments	+	−
Production of indole	−	−
Production of urease	−	−
Hydrolysis of gelatin	+	+
Acid production from D-glucose	+	−
sucrose	−	−
maltose	+	−
salicin	+	−
D-xylose	+	−
L-arabinose	+	−
glycerol	+	+
D-mannose	+	−
D-melezitose	−	−
D-raffinose	−	−
D-sorbitol	+	−
DNA GC%	40.4%	39.6%

Symbols represent +: positive, − : negative.

Table 2 Physico-chemical properties of naphthalecin

Appearance	Yellow powder
Molecular weight	274
Molecular formula	$C_{15}H_{14}O_5$
HRESI-MS	
detection	275.0871 $[M+H]^+$
calculated	275.0919 $[M+H]^+$
UV(MeOH) λ_{max} nm (ε)	280 (4615), 421 (2797)
IR ν_{max} (KBr) cm^{-1}	3200, 2950, 1630, 1590, 1530, 1430, 1400, 1270, 1170, 830
Solubility	
Soluble	MeOH, $CHCl_3$, $(CH_3)_2CO$
Slightly soluble	*n*-Hexane
Insoluble	H_2O
TLC (Rf value)*	0.24

*: Plate: Silica gel $60F_{254}$ (E. Merck Co.), *n*-Hexane : EtOAc=1 : 1.

Table 3 ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data of naphthalecin in $CDCl_3$

Position	δ_H	δ_C
1		170.4 (s)
2		109.6 (s)
3	7.49 (d, 8.5)	127.7 (d)
4	6.88 (d, 8.5)	117.6 (d)
4a		143.6 (s)
5	6.59 (s)	104.3 (d)
6		164.9 (s)
7		108.5 (s)
8		165.8 (s)
8a		107.4 (s)
Acetyl		
	2.82 (3H, s)	34.1 (q)
		205.2 (s)
Propionyl		
	1.31 (3H, t, 7)	8.9 (q)
	3.02 (2H, q, 7)	30.2 (t)
		204.8 (s)
1-OH	17.7 (s)	
6-OH	13.35 (s)	
8-OH	12.2 (s)	

presented elsewhere.

Isolation of Antibiotics

The stocked cell suspension of strain SYB was thawed and spread on BPM agar (25 ml, 1 plate). After cultivation at 30°C for 14 days under anaerobic conditions, the cells were collected and suspended in 10 ml of sterile water. Each 50-μl aliquot of this suspension was spread on BPY agar (25 ml×200 plates). After these cells were cultivated at 25°C for 14 days under anaerobic conditions, the cells were collected and suspended in 4.0 liters of 60% aq Me_2CO and agitated for a few hours. The filtrate of the extracts was concentrated *in vacuo* to remove Me_2CO. The aq solution was extracted twice with EtOAc. The EtOAc extract was concentrated *in vacuo* to give an oily residue. The oily substance was dissolved with DMSO (3.0 ml) and further purified by preparative HPLC (column: Mightysil RP-18 GP, 250×20 mm 5.0 μm, Kanto Chemical Co., Japan) developed with 70% aq CH_3CN containing 0.1% TFA (flow rate: 10 ml/minute; detection: UV 210 nm). The active fractions (60 ml) were collected and concentrated to remove acetonitrile *in vacuo*. The aqueous solution was extracted with EtOAc and concentrated *in vacuo* to give 4.0 mg of naphthalecin as a yellowish powder (Table 2).

Structure Determination

The molecular weight was derived from the observation of pseudomolecular ion peaks at *m/z* 275 $(M+H)^+$ in the positive-mode electrospray ionization (ESI)-MS and from that at *m/z* 273 $(M-H)^-$ in the negative-mode ESI-MS. High resolution (HR)-ESI-MS measurement on $(M+H)^+$ (found 275.0871; calcd. 275.0919) revealed the molecular formula to be $C_{15}H_{14}O_5$ which was consistent with ^{1}H- and ^{13}C-NMR data (Table 3). The ^{13}C-NMR spectrum exhibited 15 discrete carbon signals which were due to CH_3×2, CH_2×1, CH×3 and C×9, accounting for $C_{15}H_{11}$. The remaining three protons were attributed to exchangeable ones and seen in the ^{1}H-NMR spectrum at 17.7 (s), 13.35 (s) and 12.20 (s) ppm. A combined analysis of COSY and HSQC experiments indicated the presence of an acetyl, a propionyl, three phenolic OH, an ortho-coupled aromatic proton system and an isolated aromatic proton unit. Subtracting acetyl, propionyl and three hydroxyls from the molecular formula left $C_{10}H_3$ with requirement of seven degrees of unsaturation, inferring a naphthalene ring. The HMBC data allowed the assembly of the units into the 2-propionyl-7-acetylnaphthalene derivative as shown in Fig. 3. HMBC cross peaks from a chelated phenolic OH were an important clue for the attachment position. The phenolic hydroxy proton resonating at 13.35 ppm gave HMBC cross peaks to C-5, C-6 and C-7, indicating the placement of the OH at C-6. The HMBC correlations between OH proton at 12.20 ppm and C-7, C-8 and C-8a indicated the presence of OH at C-8. There is only one reasonable connection between C-1 and the remaining OH at 17.7 ppm, giving the complete structure. From the above information, the structure of naphthalecin was concluded to be 1-(7-acetyl-

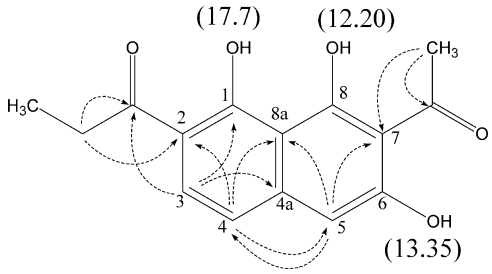

Fig. 3 Key HMBC of naphthalecin.

Numbers in parentheses indicate chemical shifts of relevant hydrogen-bonded OH groups.

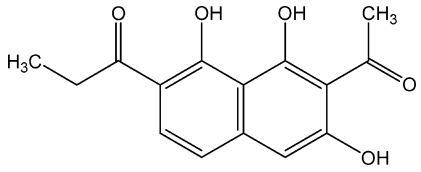

Fig. 4 Structure of naphthalecin.

1,6,8-trihydroxynaphthalen-2-yl)propan-1-one as depicted in Fig. 4.

Biological Activity

The antibiotic activity of naphthalecin is shown in Table 4. This compound showed broad antibacterial activity against Gram-positive bacteria but not against Gram-negative bacteria or fungi. The cytotoxicity (IC_{50}) of naphthalecin to EL-4 cells was 160 μg/ml.

Discussion

Here we showed clear proof of the existence of a novel, low-molecular-weight polyketide antibiotic produced by a strictly anaerobic bacterium. There have been several studies on virulent toxins [8], high-molecular-weight peptides, and lantibiotics [9] produced by anaerobes, which all seem to be direct ribosomal products or related substances. However, the only known example of a low-molecular-weight antibiotic is reutericyclin, which is produced by the lactate bacterium, *Lactobacillus reuteri* [10]. Naphthalecin is thus a rare case of low-molecular-weight antibiotic produced by an obligate anaerobe.

The producing strain, SYB, was identified as a new species of the genus *Sporotalea*. *Sporotalea propionica* was isolated from the termite gut, while strain SYB was isolated from a soil sample. Observed differences between the species, such as antibiotic production or acid production from carbon sources, seemed to reflect the environmental differences of each habitat. Bogga *et al.* explained that *Sporotalea propionica* (ATCC BAA-626^T) could survive under microaerobic conditions, as in the termite gut, by possessing the ability to reduce O_2 [3]. Strain SYB might possess a similar ability to avoid O_2 toxicity, as it was isolated from a symbiotic colony with an aerobic bacterium under aerobic conditions.

Table 4 Antimicrobial activity of naphthalecin

Microorganisms	MIC (μg/ml)
Strain SYA	3.1
Rhodococcus rhodochrous JCM3202	25.0
Bacillus subtilis IFO3301	25.0
Staphylococcus epidermidis ATCC12228	25.0
Micrococcus luteus IAM1056	3.1
Pseudomonas fluorescens IFO3903	>100
Pseudomonas putida IFO3738	>100
Escherichia coli IFO3301	>100
Saccharomyces cerevisiae IAM4017	>100
Aspergillus niger IFO2561	>100

Description of *Sporotalea colonica* sp. nov.

Sporotalea colonica sp. nov. (colo.ni.ca, L. fem. adj. *colonica* pertaining to colony, from which the organism was originally isolated, from an aerobe colony).

The cells are Gram-positive, spore forming, motile (peritrichous flagella) long rods that grow in creamy-yellow circular colonies. They are obligate anaerobes, catalase negative, and oxidase negative. The temperature range for growth is 15~35°C (optimum: 25~30°C); no growth occurs at 10°C and 37°C. The pH range for growth is 6.0~9.0 (optimum: 7.0~8.0). The colonies hydrolyze gelatin, and neither indole nor urease are produced. Acid is produced from glucose, glycerol, maltose, salicin, xylose, arabinose, mannose, and sorbitol, but not from sucrose, raffinose, or melizitose. The DNA G+C content is 43%.

Materials and Methods

Microorganisms

Strain SYB was isolated from a colony of aerobic strain SYA, which had been isolated on YM agar from a soil sample collected in Ibaraki, Japan. Strain SYB was isolated and cultivated as a pure culture on BP agar at 30°C under anaerobic conditions [N_2/CO_2 atmosphere 80 : 20 (v/v), AnaeroPack, Mitsubishi Gas Chemical Co. Japan]. After

cultivation for 14 days on BPM agar, colonies of this strain, which included not only cells but also many spores, were suspended in sterilized 10% glycerol solution and preserved at −80°C. The cells of *Sporotalea propionica* were prepared in a way similar to that for strain SYB. Strain SYA was isolated and cultivated on YM agar under aerobic conditions at 30°C.

Taxonomic Studies

Observation of growth and tests for physiological characteristics were made based on the methods in Bergey's manual [11] and those of Cowan [12]. The analysis of 16S rRNA was according to Muramatsu *et al.* [13]. In order to assess the morphology of this strain, it was cultured mainly on BP and ABCM agars at 30°C, followed by observation of the cells under an optical microscope (Leitz Aritoplan, Variophoto). The flagella were stained as described by Blenden *et al.* [14]. Enzymes and carbohydrate acid production were detected using the API 20A commercial kit (BioMéruex). Growth at various temperatures (5∼40°C) was observed on BP agar. The pH ranges for growth were determined on BP agar adjusted to pH values ranging from 4.5 to 10.5 (at intervals of 0.5) by the addition of HCl or Na_2CO_3 prior to sterilization. DNA G+C content [15] and DNA hybridization [16] were determined at TechnoSuruga Laboratory Co., Ltd.

Medium Components

BP agar contained 20 g nutrient broth (Kyokuto), 1.0 g sodium pyruvate (Nacalai Tesque), and 15 g agar (Difco) in 1.0 liter of distilled water. BPM agar contained 10 g BP agar with malt extract (Difco), and BPY agar contained 5.0 g BP with yeast extract (Difco). ABCM agar contained 46 g ABCM broth (Eiken) and 15 g agar (Difco) in 1.0 liter of distilled water. YM agar contained 4.0 g yeast extract (Difco), 10 g malt extract (Difco) and 4.0 g glucose in 1.0 liter distilled water. The pH of each medium was adjusted to 7.0 before sterilization.

HPLC Analysis

Naphthalecin was detected in the mycelium and fraction under purification at 280 nm using an HPLC equipped with a Mightysil RP-18 reverse-phase column (150×4.6 mm, 5 μm, Kanto Chemical Co.) and a mobile phase of 70% aq CH_3CN containing 0.1% TFA at a flow rate of 1.0 ml/minute. The retention time for naphthalecin was 6.9 minutes.

Analytical Measurement

The UV and IR spectra were obtained with a Shimadzu UV-2500PC spectrometer and a Jasco FT/IR-610 spectrometer, respectively. The HRESI mass spectrum was recorded with a Micromass-LG. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were obtained with a Bruker DRX 500 spectrometer.

Antimicrobial Test

The MIC was determined after 24 hours incubation at 30°C using the agar dilution method. Mueller Hinton agar (Eiken) and Sabouraud agar were used for bacteria and fungi, respectively.

Cell Cytotoxicity Assay

Cytotoxicity was examined using EL-4 cells. After 3 days of incubation with naphthalecin, cell viability was determined colorimetrically at 550 nm, using 660 nm as a reference, in accordance with the MTT method. Cytotoxicity was expressed as the concentration of compound needed to reduce EL-4 cell viability by 50%.

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References

1. Berdy J. Bioactive microbial metabolites. J Antibiot 58: 1–26 (2005)
2. Döhren HV, Gräfe U. General aspects of secondary metabolism. *In* Biotechnology, Vol. 7, second, completely revised edition, *Ed*., HJ Rehm *et al.*, pp. 1–55, A Wiley Company, Weinheim (1997)
3. Boga HI, Ji R, Ludwig W, Brune A. *Sporotalea propionica* gen. nov., a hydrogen-oxidizing, oxygen-reducing, propiogenic firmicute from the intestinal tract of a soil-feeding termite. Arch Microbiol 187: 15–27 (2007)
4. Euzéby J. List of new names and new combinations previously effectively, but not validly, published. Int J Syst Bacteriol 57: 893–897 (2007)
5. Rogosa M. *Acidaminococcus* gen. n., *Acidaminococcus fermentans* sp. n., anaerobic Gram-negative diplococci using amino acids as the sole energy source for growth. J Bacteriol 98: 756–766 (1969)
6. Garrity GM, Holt JH. Taxonomic outline of the Archaea and Bacteria. *In* Bergey's Manual of Systematic Bacteriology. 2nd edn, Volume 1, *Ed.*, DR Boone, RW Castenholz, pp. 156–166, Springer, New York (2001)
7. Stackebrandt E, Goebel BM. A place for DNA-DNA reassociation and 16S ribosomal RNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44: 846–849 (1994)
8. Riley MA, Wertz JE. Bacteriocins: Evolution, ecology and application. Annu Rev Microbiol 56: 117–137 (2002)

9. Sahl HG, Bierbaum G. Lantibiotcs: Biosynthesis and biological activities of uniquely modified peptides from Gram-positive bacteria. Annu Rev Microbiol 52: 41–79 (1998)
10. Ganzle MM, Holtzel A, Walter J, Gunther J, Hammes WP. Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl Env Microbiol 66: 4325–4333 (2000)
11. Sneath, PHA. Endospore-forming gram-positive rods and cocci. *In* Bergey's Manual Systematic Bacteriology. Volume 2. *Ed.*, PHA Sneath *et al.*, pp. 1104–1207, William & Wilkins (1986)
12. Cowan ST. Manual for the Identification of medical bacteria. Cambridge University Press, England (1974)
13. Muramatsu H, Mokhtar SI, Katsuoka M, Ezaki M. Phylogenic analysis of immunosuppressant FK506-producing streptomycete strains. Actinomycetologica 19: 33–39 (2005)
14. Blenden DC, Goldberg HS. Silver impregnation stain for *Lepitospira* and Flagella. J Bcteriol 89: 899–900 (1965)
15. Katayama FY, Komatsu Y, Kuraishi H, Kaneko T. Estimation of DNA base composition by high performance liquid chromatography of its nucease P1 hydrolysate. Agric Biol Chem 48: 3169–3172 (1984)
16. Ezaki T, Hashimoto Y, Yabuuchi E. Fluorometric DNA-DNA hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39: 224–229 (1989)