

Bacillus subtilis

AB89株の産生する抗クワ褐斑病菌物質の純化とその特性

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Purification and Characterization of Anti-*Phloeospora maculans* Substance Produced by *Bacillus subtilis* AB89

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Key words: *Bacillus subtilis* AB89, antimicrobial substance, *Phloeospora maculans*, mulberry leaf spot.

In 1992 mulberry leaf spot caused by *Phloeospora maculans* became endemic on the Goto Islands where sericulture has been major industry^{1,7,9}. Because chemical control is only available in the off-season, use of biological control has been much stressed. A micro-organism which is antagonistic to various phytopathogenic fungi and bacteria was isolated from the surface of a rice leaf in 1989 and designated as isolate AB89². From morphological, biological and biochemical data, this organism was identified as *Bacillus subtilis*⁴.

Pre-treatment of the mulberry leaves with the bacterial cells or the culture filtrate of isolate AB89 suppressed disease severity without any effect on the growth of silkworms^{3,5}. Thus, *B. subtilis* AB89 is considered to be an effective agent for biocontrol of mulberry leaf spot. In this experiment an anti-*P. maculans* substance produced by this bacterium was isolated. An abstract has been reported elsewhere⁴.

The producer *B. subtilis* AB89 was shake-cultured in potato semi-synthetic broth (PS broth), composed of 1000 ml of potato (300 g) decoction, 2 g Na₂HPO₄·12H₂O, 0.5 g Ca(NO₃)₂·4H₂O, 5 g peptone, 15 g sucrose, pH 7.0, in Sakaguchi flasks at 30°C for 3 days. The culture fluid was centrifuged at 4000×*g* for 20 min. The supernatant was shaken with an equal volume of 1-butanol. The extract was concentrated *in vacuo* by a rotary evaporator at less than 40°C. After drying with N₂ gas, the residue was dissolved in methanol and subjected to a preparative thin layer chromatography (TLC). Extracts were applied on silica gel TLC plate (Wako Chemicals, Wakogel B-10) and developed with a solvent mixture of 1-butanol-ethanol-28% ammonia water (1:2:1, v/v/v). The two active fractions (*R_f* 0.2, *R_f* 0.51) were scraped from the TLC plate and eluted with methanol. The eluate was dried with N₂ gas and dissolved in 75% aqueous methanol. Then, it was subjected to reverse phase high performance liquid chromatography (HPLC) with C-18 M&S column (20×250 mm) and eluted with 75% aqueous methanol at flow

rate 5 ml/min monitoring at 260 nm.

Fourteen strains of phytopathogenic bacteria and fungi were used as indicators for an antibiotic spectrum test (Table 1). The spectrum of the antibiotics produced by *B. subtilis* AB89 was determined by the paper disk method or cylinder cup diffusion method.

As can be seen in Table 1, the substances produced by *B. subtilis* AB89 showed a broad spectrum of activity against phytopathogenic fungi and bacteria. At least two kinds of antimicrobial substances were indicated by the fractionation with TLC and the bioassay. The antibiotic eluted from the *R_f* 0.2 area (Fraction 1) inhibited the growth of all bacteria tested except for *Erwinia cartovora* subsp. *carotovora*. Anti-*P. maculans* substance was detected at *R_f* 0.51 (Fraction 2) on the TLC chromatogram (Fig. 1).

The anti-*P. maculans* substance was used to analyze physico-chemical properties, because the purity of this sample was estimated by HPLC. The UV spectrum was recorded on a SHIMADZU UV-2200 spectrophotometer. The purified sample was also subjected to fast atom bombardment mass spectrometry (FAB-MS), ¹H-NMR at 125 MHz and ¹³C-NMR with a 500 MHz spectrometer.

The UV spectrum of the anti-*P. maculans* substance exhibited a characteristic absorption maximum at 259 nm in methanol. In the FAB-MS spectrum of the substance, the molecular ion was observed at *m/z* 820 (Fig. 2). As can be seen in Fig. 3, the ¹H-NMR spectrum (CD₃OD, 125 MHz) of the antibiotic showed proton signals from a methyl group between 0.8-1.0 ppm. Several protons were coupled with each other in 1.0-3.0 ppm. Between 3.0-4.5 ppm, there were protons with oxygenated carbons. Proton patterns in 7.0-7.5 ppm were attributed to several benzene rings. The ¹³C-NMR spectrum (CD₃OD, 500 MHz) showed signals between 20-60 ppm from olefinic carbons (Fig. 4). There were signals of benzene rings between 60-120 ppm. These results of the spectral analysis indicated a molecular weight of 819 for the antibiotic and a complicated structure with several

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Table 1. Antibiotic spectrum of the substances produced by *Bacillus subtilis* AB89

Indicator	Origin ^{a)}	Activity ^{b)}		
		Crude extract	Fraction 1 ^{c)}	Fraction 2 ^{d)}
<i>Phloeospora maculans</i> Ku9109	AKU	+++	—	+++
<i>Pyricularia oryzae</i> TH68-86	TNAES	+++	—	+++
<i>Rhizoctonia solani</i> C505	AMU	+++	—	+++
<i>Rhizoctonia solani</i> C325	IFO	+++	+	+++
<i>Agrobacterium tumefaciens</i> ATCC23308 ^T	ATCC	+++	+++	—
<i>Acidovorax avenae</i> subsp. <i>avenae</i> ATCC19860 ^T	ATCC	++	++	+
<i>Bacillus cereus</i> IAM1029	IAM	+	+	—
<i>Burkholderia gladioli</i> pv. <i>gladioli</i> ATCC10248 ^T	ATCC	+	+	—
<i>Burkholderia glumae</i> 2	KNAES	+	+	—
<i>Clavibacter michiganensis</i> subsp. <i>nebraskensis</i> ATCC27794 ^T	ATCC	++	+	—
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> EH8519	EAES	++	—	+
<i>Ralstonia solanacearum</i> C319	KTES	+++	+++	—
<i>Pseudomonas syringae</i> pv. <i>syringae</i> MAFF301861	MAFF	++	++	—
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> ATCC35933 ^T	ATCC	+++	+++	—

a) Abbreviations for culture collections: AKU, Faculty of Agriculture, Kyushu University, Fukuoka, Japan; TNAES, Tohoku National Agricultural Experiment Station, Japan; AMU, Faculty of Agriculture, Meijyo University; IFO, Institute for Fermentation, Osaka, Japan; ATCC, American Type Culture Collection, USA; IAM, Institute of Applied Microbiology, University of Tokyo; KNAES, Kyushu National Agricultural Experiment Station, Kumamoto, Japan; EAES, Ehime Agricultural Experiment Station, Ehime, Japan; KTES, Kagoshima Tobacco Experiment Station, Kagoshima, Japan; MAFF, Ministry of Agriculture, Forestry and Fisheries, Ibaraki, Japan.

b) Activity index (diameter of inhibition zone): —, Not detected; +, 0–5 mm; ++, 5–10 mm; +++, 10–15 mm

c) Eluate from the spot at *R_f* 0.20 on the TLC plate developed with 1-butanol-ethanol-ammonia solution (1 : 2 : 1, v/v/v)

d) Eluate from the spot at *R_f* 0.51.

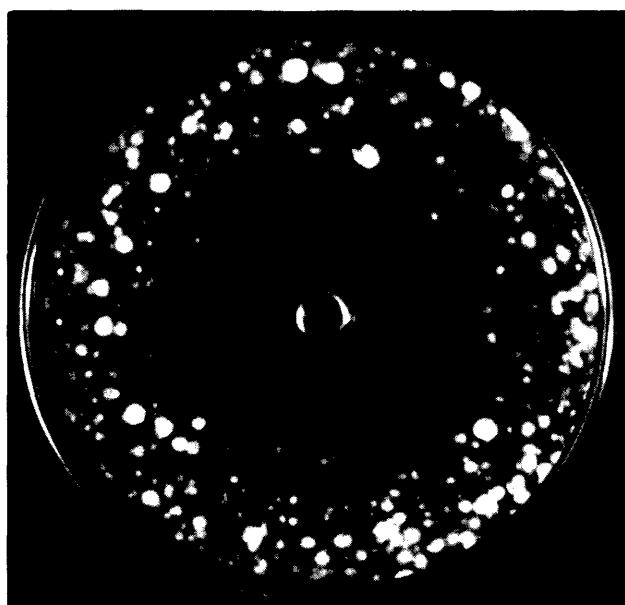


Fig. 1. Inhibition zone formed on lawn of *Phloeospora maculans* by *Bacillus subtilis* AB89. Activity was examined at the original concentration of the culture fluid.

benzene rings for the antibiotic.

Acid hydrolysis and cellulose TLC analyses revealed the absence of simple sugars and uronic acids (data not shown). Data indicated the substance could be a polyene antibiotic. It is well-known that polyene antibiotics, which possess four to seven conjugated double bonds,

are produced by the genus *Streptomyces*⁸⁾. However, conjugated polyene antibiotics such as difficidin, oxydifficidin, proticin and aurantinin have also been reported from members of the genus *Bacillus*^{6,8,10–14)}.

The anti-*P. maculans* substance produced by *B. subtilis* AB89 may be useful in plant disease control. Further analysis of other antibiotics produced by this bacterium is in progress. The chemical structure of present anti-*P. maculans* substance will be determined in a future study.

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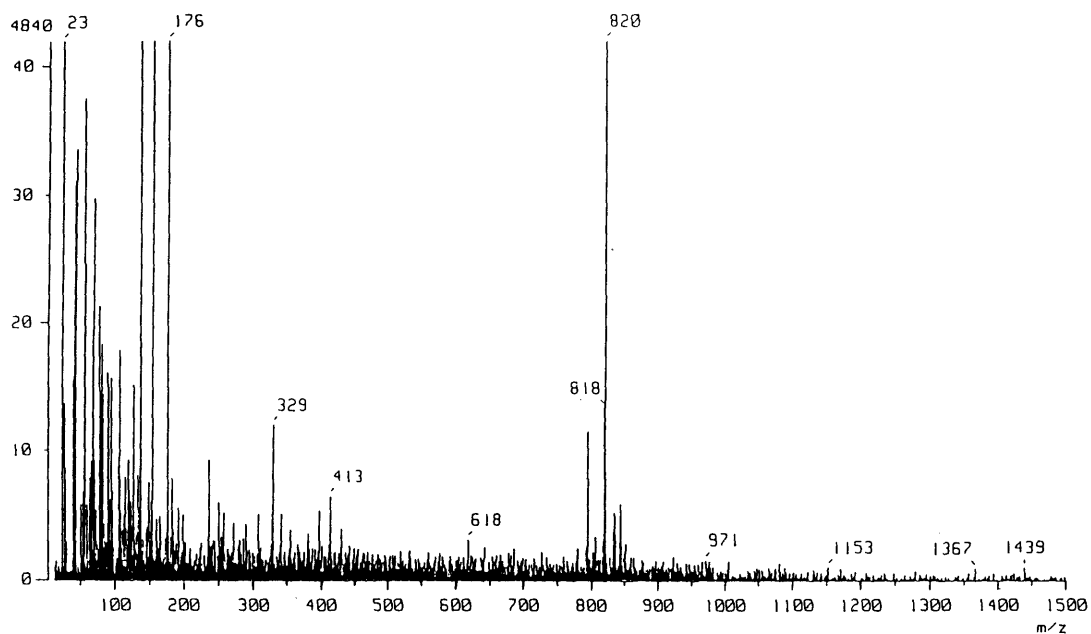


Fig. 2. Mass spectrum of the antibiotic produced by *Bacillus subtilis* AB89. The antibiotic was analyzed by FAB-MS at 2-3 kV (accelerating voltage) with a DMSO-glycerol matrix and gave $[M+H]^+$ ion at m/z 820.

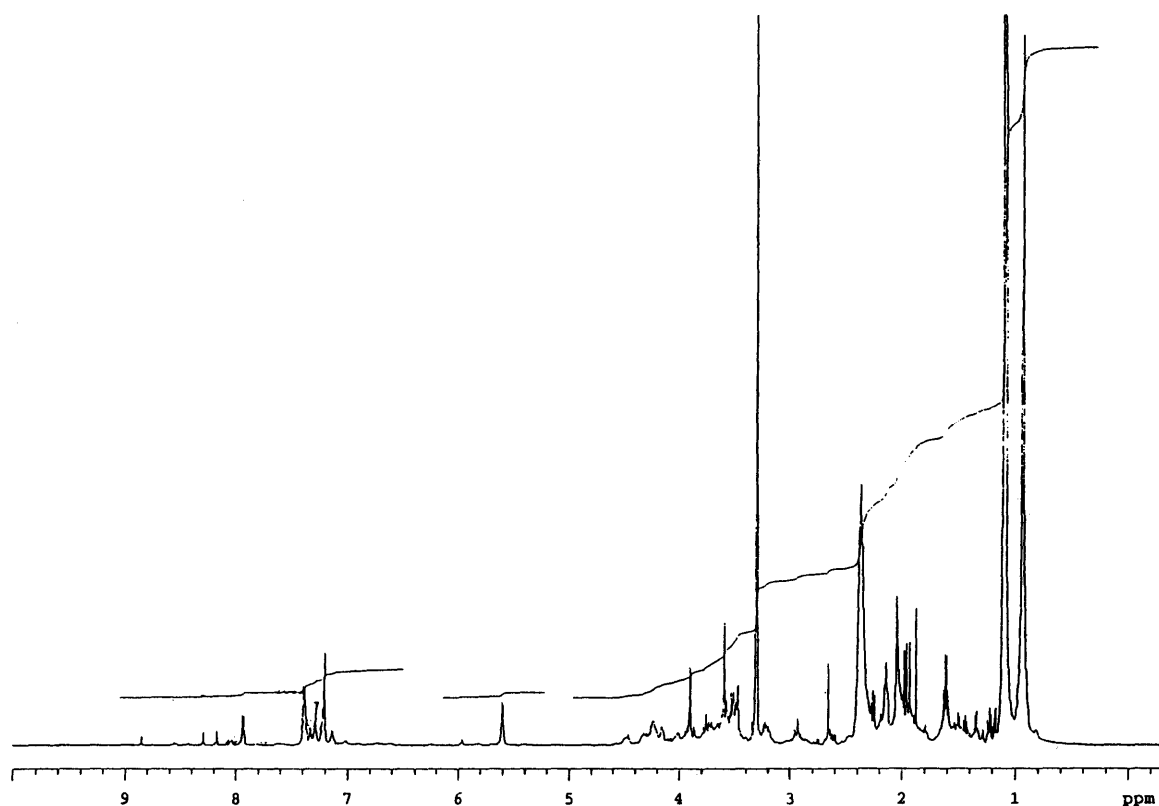


Fig. 3. ^1H -NMR spectrum of the antibiotic produced by *Bacillus subtilis* AB89.

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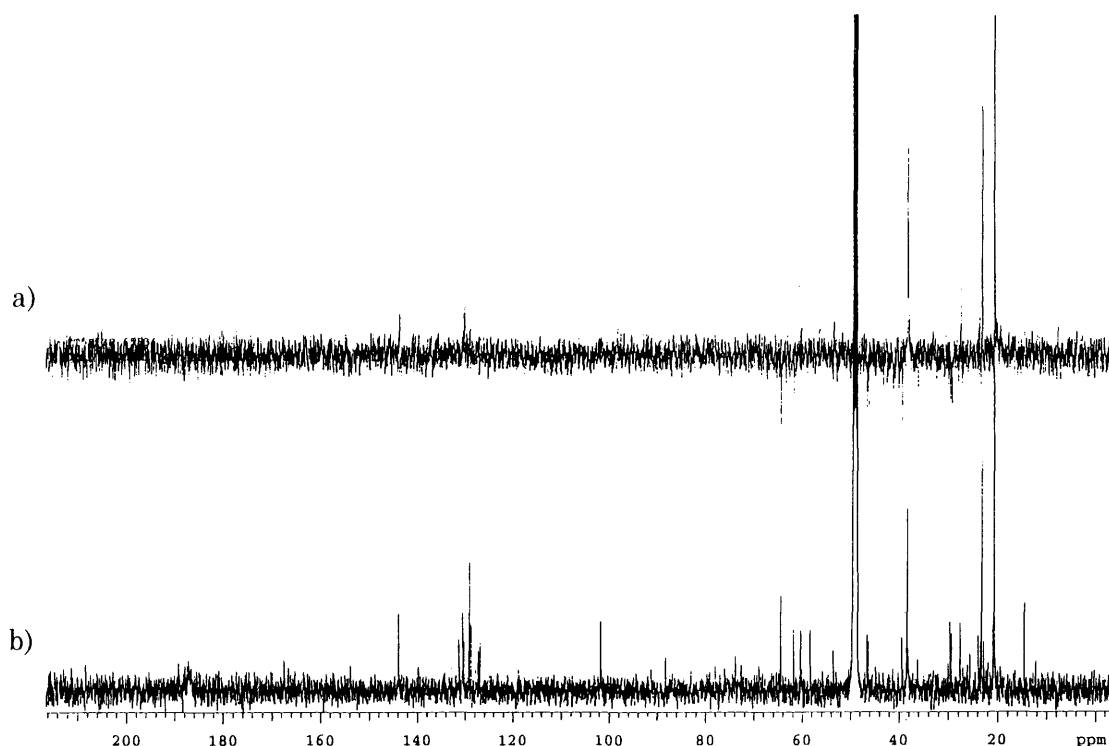


Fig. 4. ^{13}C -NMR spectra of the antibiotic produced by *Bacillus subtilis* AB89. a) INEPT spectrum of the antibiotic. b) Complete decoupled ^{13}C spectrum of the antibiotic.

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和 文 摘 要

川波政和・古屋成人・飯山和弘・金 京姫・森元 聡・松山宣明: *Bacillus subtilis* AB89 株の産生する抗クワ褐斑病菌物質の純化とその特性

抗クワ褐斑病菌物質を *Bacillus subtilis* AB89 株の培養液中に見出し、その純化を試みた。培養液より 1-butanol で粗抽出し、薄層クロマトグラフィー (TLC) ならびに逆相型の高速液体クロマトグラフィー (HPLC) に供試することにより純化標品を得た。本純化物を各種機器分析に供試した結果、分子量が 819 で数個のベンゼン環を有する複雑な構造をもつポリエー系の新規抗菌物質である可能性が示唆された。

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