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Full Paper

Magenta pigment produced by fungus

Sanae Chiba,[1] Naoko Tsuyoshi,[2] Ryosuke Fudou,[2] Makoto Ojika,[3] Yoshimasa Murakami,[1] Yoshiro Ogoma,[1] Masakatu Oguchi,[1] and Shigeru Yamanaka[1,*]

[1] *Faculty of Textile Science and Technology, Shinshu University, Nagano 386–8567, Japan*
[2] *Institute of Life Science, Ajinomoto Co. Inc., Kawasaki 210–0801, Japan*
[3] *Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464–8601, Japan*

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A fungus producing magenta was isolated from cellulosic material by visual observation on Czapek's agar media and the product was conventionally analyzed. The fungal strain that produced magenta pigment was closely related to *Phoma herbarum*. The type of fibers added to Czapek's medium influenced which pigments were produced. Mycelia attached to the surface of nylon-6 and excreted magenta pigment into the fibers. The pigment structure was partially determined. This is the first report of the production of magenta pigment by a microorganism specifically in the presence of nylon-6 fibers, via an unknown mechanism. This phenomenon raises the question of why and how the fungus disperses the pigment inside the fiber and suggests that fabrics can be dyed using microorganisms.

Key Words——dyes; fungus; magenta; *Phoma*; pigment

Introduction

Demand for natural instead of synthetic pigments for coloring fabrics, foods and cosmetics is increasing. Unlike pigments that are synthetic, those from natural sources allow subtle differences in tone because such pigments generally comprise various color components. Microbes have recently received focus as sources of natural pigments (Mapari et al., 2005). Many fungal species produce pigments. *Monascus* fungi (Shin et al., 1998; Tseng et al., 2000) produce red or yellow pigments that are used as food colorants. *Hahella* produces red, prodigiosin (Nakashima et al., 2005), *Ashbya* produces yellow riboflavin (Stahmenn et al., 2001), *Phoma* produces orange aza-anthraquinone (Birch et al., 1964) and *Chromobacterium* produces blue violacein (Rettori and Durán, 1998).

We discovered a fungus that produces magenta pigment. We found that the produced colors were magenta, pink or green depending on the type of fiber added to the culture medium. These findings indicated that various fibers influence the metabolic pathways involved in the production of secondary pigment metabolites.

Materials and Methods

Microorganisms. We isolated a fungus from a wet bacterial cellulose mat produced by acetobacter in our laboratory.

Culture conditions. Identification of fungus: We extracted DNA from mycelia cultivated on PDA (Potato Dextrose Agar; Difco) for 1 week at 25°C.

Pigment production: The fungus was cultivated in the following media: PDA or Czapek's agar (0.3%

* Address reprint requests to: Dr. Shigeru Yamanaka, Department of Functional Polymer Science, Faculty of Textile Science and Technology, Shinshu University, 3–15–1 Tokida, Ueda, Nagano 386–8567, Japan.

Tel and Fax: +81–45–712–5317

E-mail: yamanaka@shinshu-u.ac.jp and syamana@ytv.home.ne.jp

$NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 3% sucrose, 2% agar). The microorganism was inoculated in the above medium and cultivated at various temperatures for 7 days in dark conditions.

Phylogenetic analysis. Mycelial DNA was extracted using DNeasy Plant Mini Kits (QIAGEN, Hilden, Germany). Regions of 18S rDNA fragments were amplified using puRe*Taq* Ready-To-Go PCR beads (Amersham Biosciences, NJ, USA) and the primer pairs, NS1 (5′-GTAGT GCTTG TCTC-3′) and NS8 (5′-TCCGC AGGTT CACCT ACGGA-3′) (White et al., 1990). The amplified PCR products were purified using QIAquick PCR Purification Kits (QIAGEN) and sequenced using an ABI PRISM 3100 (Applied Biosystems, CA, USA) with ABI PRISM BigDye Terminator Kits (Applied Biosystems). The following 8 internal primers were included in the sequencing reactions: NS1–8 (White et al., 1990).

The determined sequences were aligned with those of reference *Dothideomycetes* strains obtained from the GenBank/EMBL/DDBJ databases. The 18S rDNA sequences data of the magenta producing strain have been deposited in DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB252869.

We generated multiple sequence alignments using CLUSTAL (Thompson et al., 1994), and a phylogenetic tree was constructed by the neighbor-joining method. The tree topology was confirmed by bootstrap analysis (Felsenstein, 1985).

Cultivation on medium containing various fibers. The following fabrics were added to Czapek's agar and liquid media: nylon-6, silk and polyester [polyethyleneterephthalate] (all from KURABO Co., Japan), filter paper (ADVANTEC Co. No.2) and hemp (SANKEI Co., Japan). Excepting filter paper, these fabrics were scoured using detergents in the conventional way before use.

The cultures were incubated at 25°C for 7 days in dark conditions. Nylon-6 (2 g) and control cultures were added to 100 ml of liquid Czapek's medium in 500 ml flasks and agitated at 120 rpm on a rotary shaker in dark conditions.

Each fabric specimen was placed on the agar medium and a loopful of fungus was spread on the fabric surface. The inoculum was directly spread onto the agar for control cultures.

Analysis of nylon-6 after cultivation. Pigment distribution in the nylon-6 culture was analyzed using specimens on agar cultures. The surface of the nylon-6 and the fiber core were examined using 3D real surface view electron microscopy (YE-9800, KEYENCE) and by light microscopy to determine the distribution of color produced by the microorganisms.

Dyed nylon-6 was washed with the detergent, Sunmorl WX-24 (NICCA, Japan) to eliminate impurities, and then cut and embedded in epoxy resin to examine the core (Luft, 1961).

Extraction of pigment. Culture broth (4 L) was passed through filter paper to harvest mycelia and fibers. Pigment was extracted from about 15 g (wet weight) of the mixture of mycelia and fibers by 3 washes with 200 ml methanol at 25°C. The crude methanol extract was passed through filter paper, and the filtrate (crude magenta pigment) was dried in vacuo.

Physicochemical properties. We examined the solubility of the crude magenta pigment in methanol, ethanol, acetone, isopropanol, ethyl acetate, chloroform, *n*-hexane and water, and also measured absorption spectra in the ultraviolet and visible regions (V-530 spectrometer; JASCO).

Preliminary isolation and purification of pigment. Crude pigment (0.89 g) was sequentially separated using various proportions of MeOH and EtOAc to precipitate impurities. The supernatant fraction (0.23 g) was separated into six fractions by elution through a silica gel flush column (HI-FLUSH SI-40W-M; 20× 60 mm; 12 g; Yamazen) with EtOAc–80% MeOH (80 : 20→55 : 45; 50 min)–0.5% AcOH. The two fractions (66 mg and 20 mg) containing a pigment that was less hydrophilic than the originally detected magenta were combined and separated into five fractions using the same column and EtOAc–90% MeOH [85 : 15 to 72 : 28 (26 min) to 60 : 40 (12 min) linear gradient]. A fraction (1.4 mg) containing the pigment revealed by TLC as a less hydrophilic magenta spot was further investigated by preparative TLC (20×10 cm; 0.25 mm thick). A main magenta band (0.5 mg) on the TLC plate was analyzed by ^{1}H-NMR (600 MHz; CD_3OD).

Results

Tested microorganisms

We examined 42 microorganisms that produced red (n=10), yellow (n=31) and blue (n=1) pigments on agar media but none of them produced magenta.

However, we isolated a fungus that produced magenta pigment from wet bacterial cellulose mats that were dyed in magenta in our laboratory.

Identification of pigment-producing fungus

The fungus grew relatively quickly on malt extract agar (Difco) at 25°C, and the colonies reached 3–4 cm in diameter within 7 days with sparse aerial mycelia. Spores did not form on any tested mycological media. This sterile fungus formed abundant red pigment on malt extract agar and PDA.

Figure 1 shows a dendrogram constructed using the neighbor-joining method, based on the 18S rDNA sequence of the fungus and its relatives. The fungus was phylogenetically positioned within the order *Pleosporales* and was 99.7% similar to the closest relative, *Phoma herbarum* (AY337712). These results indicated that the fungus was closely related to *Phoma herbarum* in the order *Pleosporales*. The strain was deposited to the Ajinomoto culture collection (AJ-

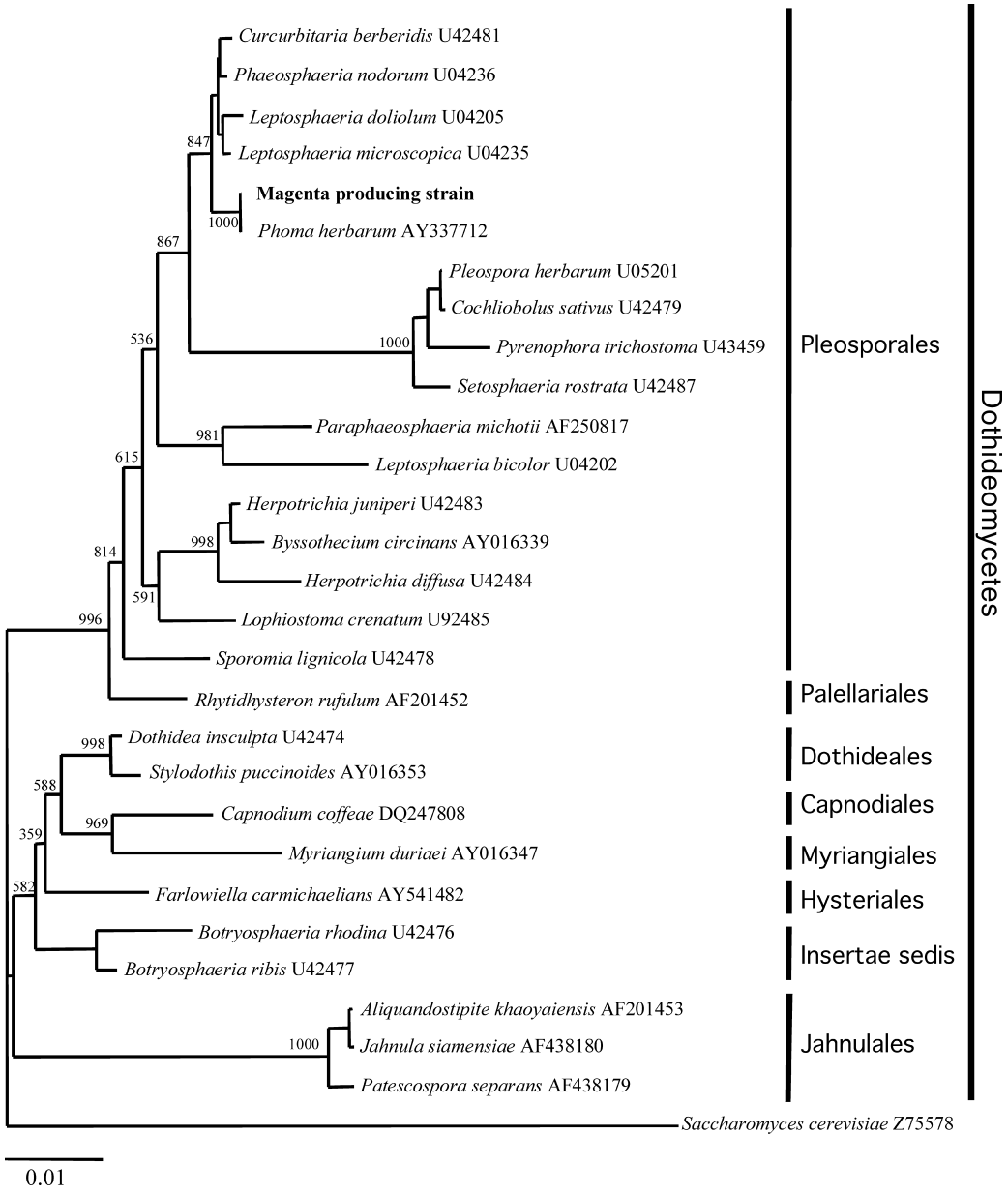

Fig. 1. Phylogenetic tree based on 18S rDNA sequences showing phylogenetic relationship of magenta-producing strain with its close relatives.

Bootstrap values (based on 1,000 replications) are shown at branching points. Bar: one nucleotide substitution per 100 nucleotide sites.

117604).

Culture conditions

The fungus was inoculated onto PDA and Czapek's agar media and incubated at 15, 20, 25 and 30°C for 7 days in dark conditions. Orange-yellow to red pigments were produced in the PDA and Czapek's agar medium. Temperature influenced color production, since the fungus cultured on Czapek's agar medium at 25°C produced more intense color. We therefore adopted these culture conditions for subsequent experiments.

Pigment production on agar medium covered with fabric

Figures 2 and 3 show fungal growth and pigment production on simple Czapek's agar medium and on the same medium covered with filter paper, nylon-6, silk, hemp and polyester, since the fungus was isolated from cellulose stained with magenta. We found that the type of fiber affected the colors produced by the fungal colonies. The control cultures produced orange to reddish-orange pigments (Fig. 2A-1) with faintly reddish-orange mycelia (Fig. 3B-1). On filter paper the pigments were red to pink (Fig. 2A-2) and the fibers were pink (Fig. 3B-2). Nylon-6 and silk yielded magenta colonies (Fig. 2A-3 and 4) and fungus, and both fabrics were stained magenta. Figure 3B-3 shows nylon-6 stained with magenta excreted by the mycelia. In contrast, colonies growing on hemp and polyester produced yellowish-green to deep green pigments (Fig. 2A-5 and 6).

We investigated the effects of nylon-6 added to liquid cultures of the same medium as that of the agar culture. The color tones under these conditions were quite similar to the corresponding agar cultures (data not shown). Control mycelia and broth were more intense brownish red than in agar culture. In liquid cultures containing nylon-6, this fabric and the mycelia were stained magenta and red to pink respectively, but the culture broth was almost colorless. The colors are described according to the Methuen Handbook of Color (A. Kornerup & J. H. Wanscher, EYRE METHUEN-LONDON, 1981).

Analyses of nylon-6 dyeing

We visualized the relationship between the mycelia and nylon-6 by electron microscopy. Mycelia spread on the surface of nylon-6 crossed (Fig. 4), but did not penetrate the fibers. Cross-sections of the nylon-6 show that the cores of the fibers are magenta (Fig. 5). The staining process expanded from the periphery to the center and also along the longitudinal direction of the fiber. These findings indicate that mycelia surrounding the nylon-6 excreted magenta into the fibers. On the other hand, about 0.1 g of nylon-6 suspended in 10 ml of methanol containing 200 mg of crude pigment did not become stained with the pigment after incubation under the same conditions (Czapek's agar).

Silk was also stained magenta and cross-sections showed that the pigment uniformly extended throughout the fibers (data not shown).

Physicochemical properties of crude pigment

The crude magenta pigment was completely soluble in methanol, slightly soluble in ethanol, acetone, isopropanol, ethyl acetate and chloroform, but insoluble in both *n*-hexane and water.

The absorption maxima of the crude pigment were at 530, 360 and 220 nm.

Preliminary isolation and purification of pigment

A crude extract containing magenta pigment was precipitated in organic solvent to remove impurities and the soluble material (0.23 g) was further fractionated by silica gel chromatography twice as described in MATERIALS AND METHODS. Preparative TLC yielded a final 0.5 mg of magenta pigment.

The ^{1}H-NMR spectra revealed signals from the purified pigment and from lipid contaminants and 2D NMR (16.5 h) showed that all signals except those from the lipid essentially disappeared. These findings indicated that the pigment was unstable and became degraded during these processes. The signals that diminished or disappeared in ^{1}H-NMR after 2D NMR were assigned to the partial structures shown in Figs. 6 and 7.

Discussion

We identified a fungus that produced magenta and other pigments on Czapek's agar in the presence of various fabrics. The mycelia, as well as fibers of both nylon-6 and silk on agar, were stained magenta, whereas hemp and polyester resulted in deep green mycelia. Thus, the nature of the fibers affected color production by the fungi. We investigated magenta production on nylon-6 fibers in detail. From a practical viewpoint, our results indicate the possibility of micro-

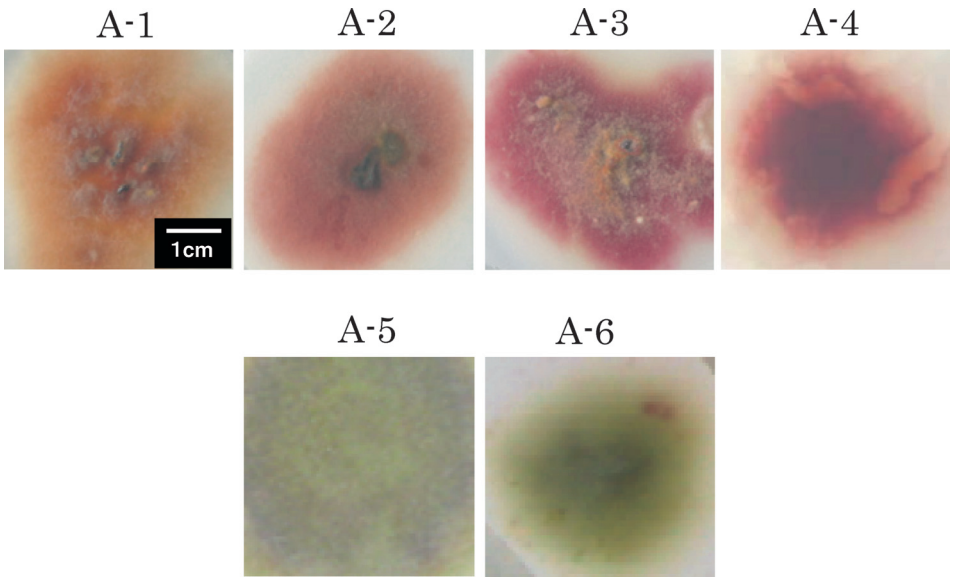

Fig. 2. Fungus incubated on Czapek's agar covered with various types of fabric.
A-1, Czapek's agar only (control); A-2, filter paper; A-3, nylon-6; A-4, silk; A-5, hemp; A-6, polyester.

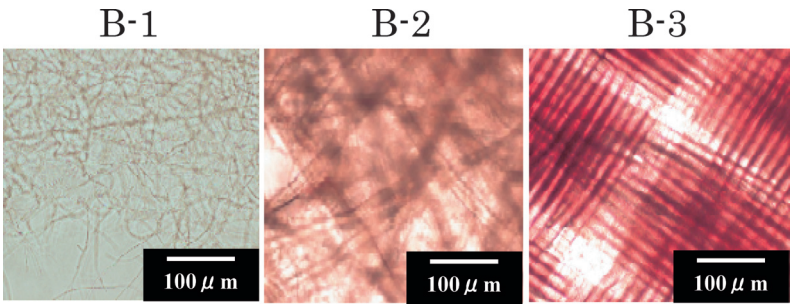

Fig. 3. Optical micrographs of fibers with attached fungus.
B-1, Czapek's agar (control); B-2, filter paper; B-3, nylon-6 fibers.

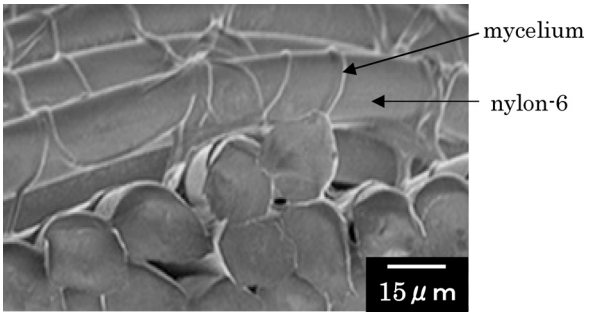

Fig. 4. Electron micrograph showing surface appearance of nylon-6 with attached mycelia.
Mycelia spread on the surface of nylon-6 crossed.

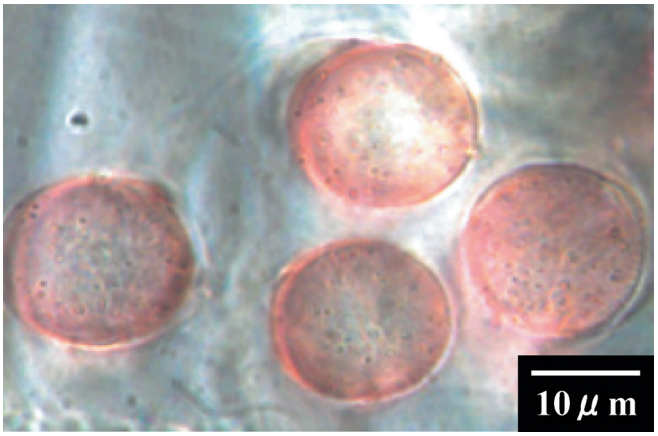

Fig. 5. Optical micrograph showing cross section of nylon-6 stained with magenta.
The staining processes expand from the periphery to the center.

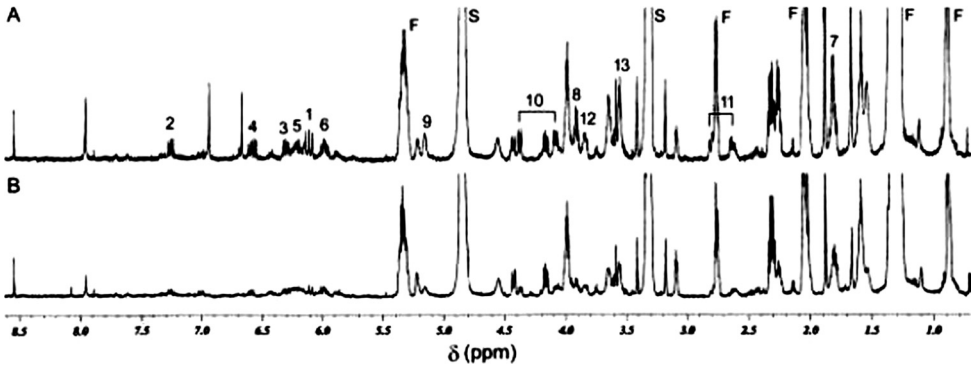

Fig. 6. ^{1}H NMR spectra (600 MHz in CD_3OD) of the partially purified magenta pigment before (A) and after (B) 2D NMR measurement (16.5 h).

The numbers correspond to the position numbers in Fig. 7. S: solvent signals. F: signals of lipids.

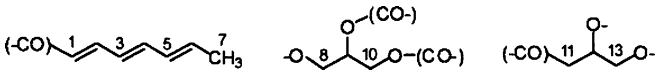

Fig. 7. Partial structure of magenta pigment produced by fungus isolated in this study.

bial dyeing.

The contact-sensing ability of fungi has been reviewed (Perera et al., 1997). For example, *Candida albicans*, a fungus that is pathogenic to humans can orient its growth in response to topographical cues.

Some plant fungi sense host as well as artificial surfaces, and these organisms have specialized infective cells called appressoria that are induced by specific physical and chemical substratum cues (Holger et al., 2000).

We speculate that the mycelia of our fungus responded not only to topographical cues, but also to the chemical structure of the fibers. The common chemical feature of nylon-6 and silk is an amide bond, which might induce a fungal biochemical pathway towards magenta pigment synthesis (Figs. 2 and 3).

Since the pigment dissolved in methanol did not stain fibers after 7 days at 25°C, the mycelia might have been responsible for introducing the pigment into the fiber. Presumably some metabolites produced in contact with the fabric surface promoted color diffusion into the fibers, or pigment precursors diffused within the fiber and later combined to form magenta in the fiber matrix. Determination of how pigments secreted by this fungus penetrate fabrics remains to be investigated.

The determination of entire structure seems to be impossible because the pigment essentially disappeared during analytical processes as mentioned previously.

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