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Two morphological groups derived from *Clonostachys cylindrospora* and their relationship to *trans*-4-hydroxy-(L)-proline productivity

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The *trans*-4-hydroxy-(L)-proline productivity and morphological characteristics of strains isolated from two different conidium-producing structures in *Clonostachys cylindrospora* were compared. *trans*-4-Hydroxy-(L)-proline was found to be produced only by strains originating from conidia on penicillate conidiophores, not by strains originating from conidia on verticillate conidiophores. Strains from each conidium-producing structure were also segregated by morphological properties; i.e., the amount of aerial hypha produced on modified Weitzman's agar, production of water-soluble yellow pigment in potato-dextrose agar, the primarily formed conidium-producing structures, and sclerotium formation. These phenomena indicate that morphological characteristics and metabolite productivity are correlated and destined at the stage of conidial production in *C. cylindrospora*.

Key Words——*Clonostachys cylindrospora*; penicillate conidiophores; pleomorphism; *trans*-4-hydroxy-(L)-proline; verticillate conidiophores.

In pleomorphic conidial fungi, plural state of conidial production are known. With a few exceptions, however, the relationships between biological function and morphological characteristics such as in the production of metabolites have not been studied. Species of *Clonostachys* Corda are known to produce two different conidium-producing structures (Hawksworth and Punithalingam, 1975). Conidia are produced in chains, and adhere obliquely to each other on a penicillate conidiophore (Figs. 1, 2, 5). They are produced in drops on phialides, which are arranged in verticillate order (Figs. 3, 4, 6).

A hyphomycete strain SANK 14591, isolated from leaf litter collected in Tsukuba, Ibaraki, Japan, was found to produce *trans*-4-hydroxy-(L)-proline (*t*-hydroxy-proline) (Serizawa et al., 1995), which is a source compound for the side chain moiety of carbapenems (Shibata et al., 1986). It was identified as *Clonostachys cylindrospora* Arnaud (Serizawa et al., 1995; Tubaki, 1963), considered to be a close relative of a species of *Gliocladium* Corda (Barron, 1968; Matsushima, 1975). Later, the productivity of *t*-hydroxy-proline from this strain was found to be inconsistent. To stabilize the productivity of t-hydroxy-proline, a method of obtaining single-conidium isolates was employed in which special attention was paid to morphological differences between conidiophores. During this work, a correlation was found between productivity of *t*-hydroxy-proline and morphological characteristics related to pleomorphism in single-conidium isolates from each of the two conidium-producing structures.

Materials and Methods

Single-conidium isolation Single-conidium isolation was carried out by using a Skerman's micromanipulator (Skerman, 1968). Penicillate and verticillate conidiophores were distinguished by observation under a dissecting microscope. A small portion of agar with a conidium-producing structure was cut out under a dissecting microscope, with particular attention not to disturb the conidium-producing structure, and transferred to another agar plate. The type of conidium-producing structure was confirmed using a 10× objective lens. Single-conidium isolates from penicillate conidiophores and verticillate conidiophores were each obtained by using the Skerman's micromanipulator. Strains thus isolated were maintained on agar slants.

Observation of morphological characteristics To observe colony morphology and the conidium-producing structures, each strain was inoculated at the center of potato-dextrose agar (PDA; Nissui, Tokyo) and modified Weitzman's agar (oatmeal 10 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, $NaNO_3$ 1 g, agar 20 g and water 1 L) plates, and incubated for 7 days at 23°C.

***t*-Hydroxy-proline estimation** The amount of *t*-hydroxy-proline was measured by using the two-step pre-column labelling method (Cunico et al., 1986; Einarssan et al., 1983) and a Hewlett Packard automatic amino acid analysis system which consisted of AminoQuant HP1090M

Table 1. Characteristics of P- and V-strains of *Clonostachys cylindrospora* SANK 14591.

	On modified Weitzman's agar	On potato-dextrose agar	Sclerotium	Primarily formed conidiophores
P-strains	thick with many aerial hyphae	yellow water-soluble pigment excreted	formed	Penicillate
V-strains	plane with few aerial hyphae	no pigment excreted	not formed	Verticillate

and Fluorometer HP-1046A (Hewlett Packard Co., Illinois), as mentioned by Serizawa et al. (1995).

Results

Single-conidium isolates Each conidium-producing structure tended to be formed separately and was relatively easy to distinguish (Figs. 5, 6). Single-conidium isolates were numbered with the prefix "CL-," as CL-1, 2, 3, etc. From more than 30 strains, 24 strains (7 strains from conidia on the penicillate conidiophores and 17 strains from conidia on the verticillate conidiophores) were selected at random for further study.

Morphological characteristics Although all strains grew at the same rate, strains derived from each conidium-producing structure could be clearly distinguished from each other (Table 1). On PDA, the strains derived from conidia on the penicillate conidiophores (P-strains) formed many aerial hyphae, developing thick floccose white colonies. After 1 week a yellow water-soluble pigment was excreted into the agar (Fig. 9). On modified Weitzman's agar, they showed similar colony morphology, but no water-soluble pigment was observed at 1 week. Sclerotia were formed in the agar by continuous cultivation.

Strains derived from conidia on the verticillate conidiophores (V-strains) grew at the same rate as the P-strains. On PDA, they formed floccose umbonate thick white colonies as the P-strains did (Fig. 8), but V-strains excreted no yellow water-soluble pigments (Fig. 9). On modified Weitzman's agar, a few aerial hyphae developed on the agar, forming thin colonies (Fig. 10). No sclerotia were formed by continuous culture. Both P- and V-strains produced both penicillate and verticillate conidiophores in culture, but they differed in the order in which the two types of conidiophores were produced. V-strains produced verticillate conidiophores after 1 week and the penicillate conidiophores after more than 1 month of continuous cultivation. P-strains formed no conidia after 1 week. The penicillate conidiophores were formed after 3 week, and the verticillate conidiophores after 1 month. Sclerotia (Fig. 7) were produced in P-strains after prolonged incubation, but not in V-strains.

Productivity of *t*-hydroxy-proline *t*-Hydroxy-proline was produced only by P-strains, not by V-strains (Table 2). The amount of *t*-hydroxy-proline produced by P-strains varied from 14 to 25 μg/ml.

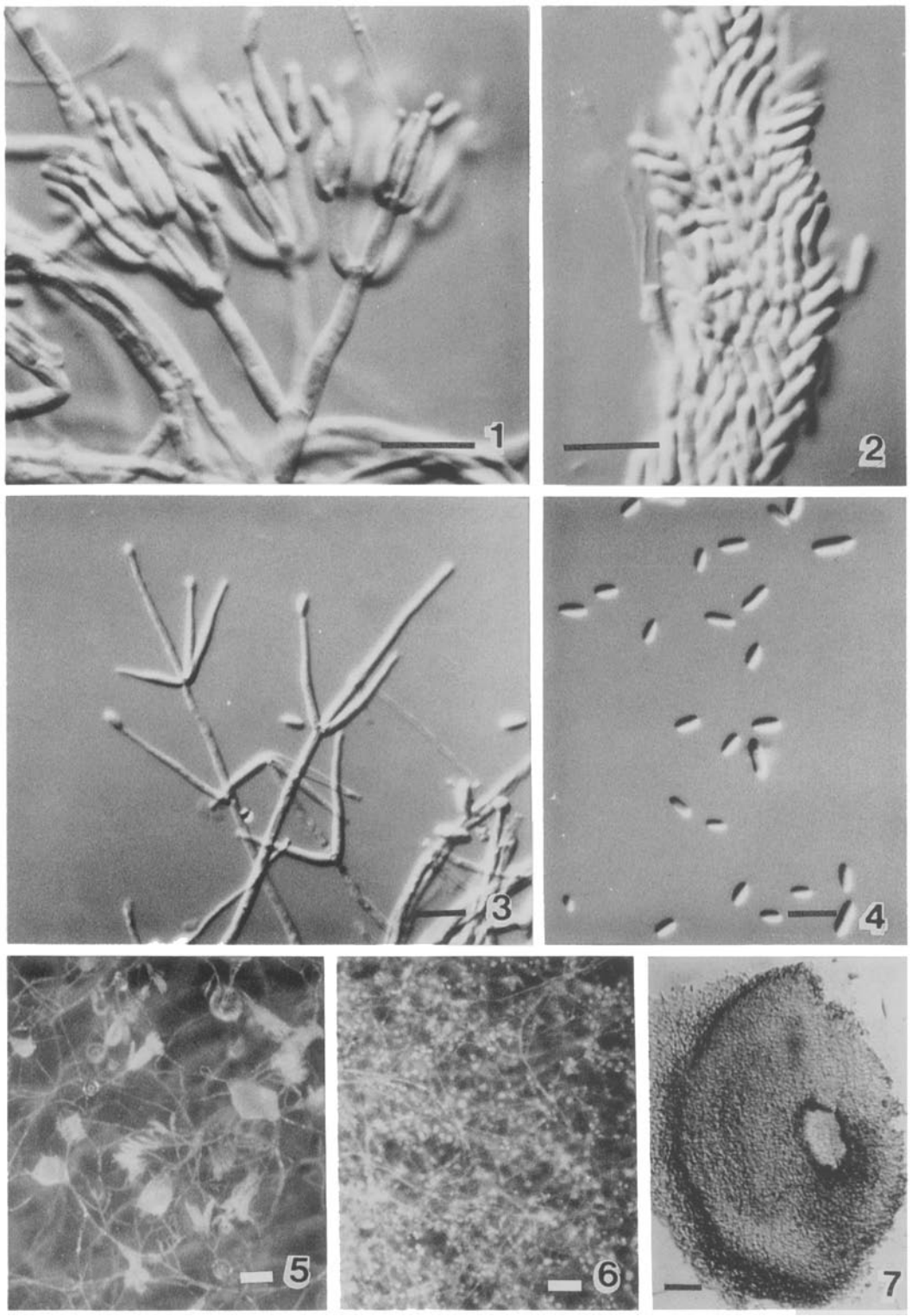
Discussion

Pleomorphism is an intriguing subject in mycology not only because of its practical importance in nomenclature, but also because of its biological significance. The relationship between morphological characteristic and biological function is known in several fungi, such as heterogeneous aflatoxin distribution in conidia or sclerotia in *Aspergillus* species (Wicklow and Shotwell, 1983; Wicklow et al., 1988) and yeast-hypha conversion and wall composition in *Candida albicans* (Deacon, 1980). These changes are reversible and can be shifted to either way depending on cultural conditions. The phenomena reported herein provide an example of the differences in metabolism corresponding to morphological differences. However, they can be segregated from the other examples mentioned above because they were determined at the stage of the conidia from which the strains

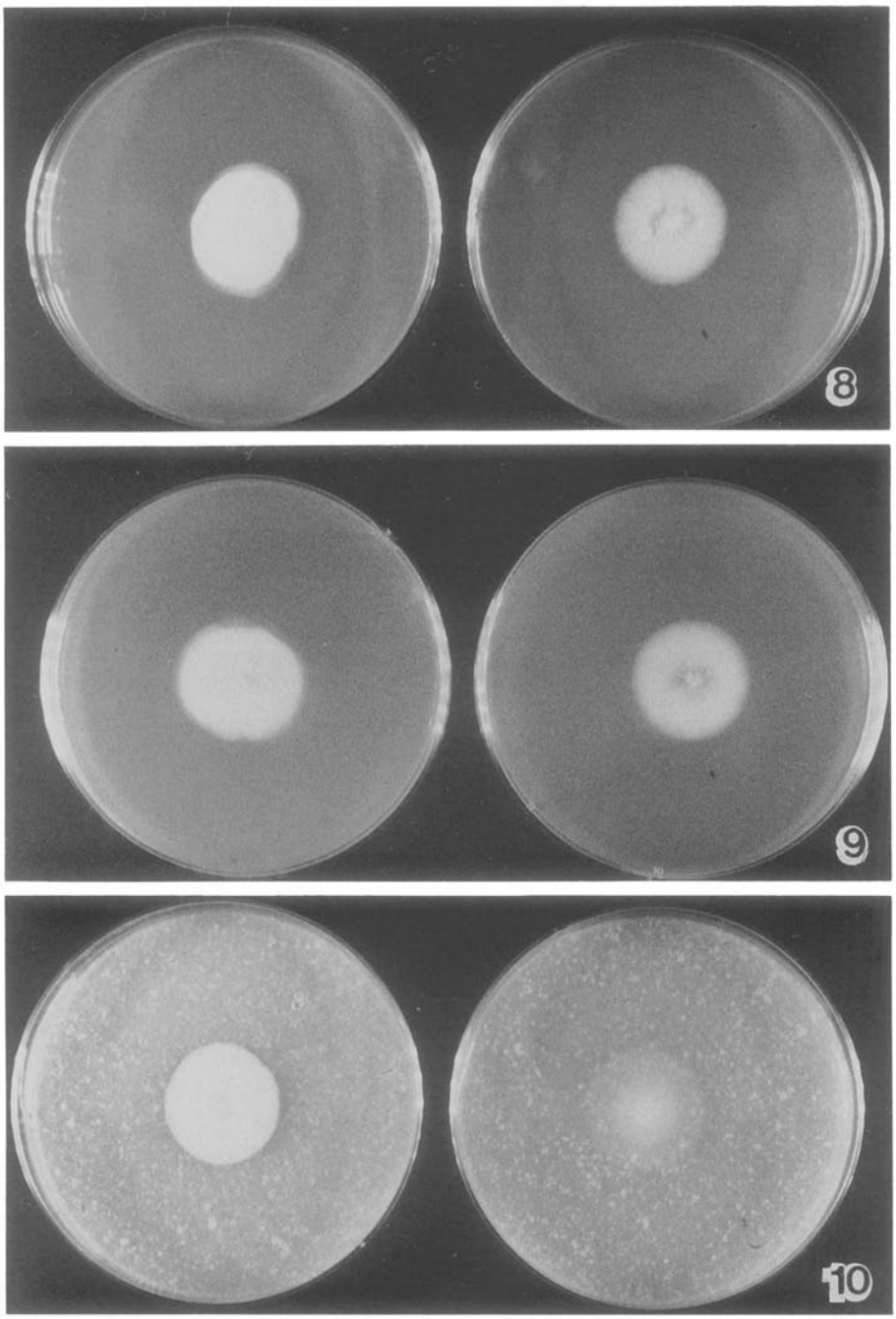
Table 2. *trans*-4-Hydroxy-(L)-proline productivity by single-conidium isolates from *Clonostachys cylindrospora* SANK 14591.

Strain no.	Strain type	*trans*-Hydroxyproline production (μg/ml)	Strain no.	Strain type	Hydroxy-proline production (μg/ml)
CL-1	P-strain	16.03	CL-14	V-strain	ND
CL-2	P-strain	14.18	CL-15	V-strain	ND
CL-4	V-strain	ND*	CL-20	V-strain	ND
CL-5	V-strain	ND	CL-28	V-strain	ND
CL-6	V-strain	ND	CL-36	V-strain	ND
CL-7	V-strain	ND	CL-44	V-strain	ND
CL-8	V-strain	ND	CL-52	V-strain	ND
CL-9	V-strain	ND	CL-65	P-strain	13.97
CL-10	V-strain	ND	CL-72	P-strain	25.12
CL-11	V-strain	ND	CL-79	P-strain	20.04
CL-12	V-strain	ND	CL-86	P-strain	21.98
CL-13	V-strain	ND	CL-93	P-strain	17.51

* ND, not detected.



Figs. 1–7. Micrographs of *Clonostachys cylindrospora* SANK 14591 showing the two conidiophore types. 1, Penicillate conidiophores. 2, Conidial column on the penicillate conidiophore, in which conidia adhere obliquely to each other. 3, Verticillate conidiophores. 4, Conidia from the verticillate conidiophore. 5, Detail of colony where the penicillate conidiophores are abundantly formed, under dissecting microscope. Note the conidial columns formed at the tips of the phialides. 6, Detail of colony where the verticillate conidiophores are abundantly formed, under dissecting microscope. Note the conidial drops formed at the tips of the phialides. 7, Section of the sclerotium formed in P-strains in modified Weitzman's agar. Scale bars: 10 μm in Figs. 1–4; 0.1 mm in Figs. 5, 6; 0.2 mm in Fig. 7.



Figs. 8–10. Comparison of colonies of the P- and V-strains on PDA and modified Weitzman's agar. 8, Colonies of P- (CL-1, left) and V- (CL-4, right) strains formed on PDA after incubation for 1 week at 23°C. Both colonies were morphologically similar and indistinguishable from the surface. 9, The same colonies from the reverse. The reverse of the P-strain (CL-1, left) is paler than the V-strain (CL-4, right) because of yellow water-soluble pigments excreted into the agar. 10, Colonies of P- (CL-1, left) and V- (CL-4, right) strains formed on modified Weitzman's agar after incubation for 1 week at 23°C. Aerial hyphae were sparsely developed in the V-strain, compared with the P-strain.

were derived. It seems that the morphological characteristics and productivity of *t*-hydroxy-proline are destined at the stage of conidial production. The parallelisms of morphological differences and productivity of *t*-hydroxy-proline suggest possible morphological roles of *t*-hydroxy-proline in protein structure. There are two possible explanations for this phenomenon: i) The original strain was heterokaryotic and the nuclei distributed in the penicillate conidiophores are genetically qualitatively different from those in the verticillate conidiophores; ii) the nuclei provided to both conidiophore types are qualitatively equal, but the means of genetic expression differ between one state and the other. The limited production of *t*-hydroxy-proline and sclerotia in the P-strains, not in the V-strains, supports the first possibility. But this needs to be proved by fur-

ther investigation.

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