A New Variety of *Pullularia fermentans* Wynne et Gott

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米山穰*: *Pullularia fermentans* Wynne et Gott の新変種

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An interesting fungus degrading rutin in a peculiar way¹⁾ was encountered and isolated by Dr. S. Hattori and his co-worker in their laboratory of Tokyo and was sent to the author for identification. At first the newly isolated fungus appeared to belong to the group of "Yeast" because of 1) the abundance of free cell formation of the fungus²⁾ and 2) its lack of dark pigmentation. Further investigation, however, seemed to justify its inclusion in the genus *Pullularia*, after comparing this fungus with some strains of this genus^{3), 4), 5).}**

Although dark pigmentation is known up to now as one of the characteristics of *Pullularia*, the new isolate does not produce dark pigment on most media. Also, the appearance of the giant colony of this isolate is peculiar, compared with those of other species of *Pullularia*.

Comparative studies of pigmentation and growth characteristics of all the recognized species of *Pullularia*, including the new isolate, were therefore made under various conditions.

Wynne in 1956⁶⁾ proposed a revision of the genus *Pullularia* based almost entirely on biochemical and physiological considerations rather than on morphology. An attempt was therefore made by the author to classify the new isolate according to this new scheme.

Among the species of *Pullularia* studied, the new isolate resembles the genus *Candida*⁷⁾ most closely. It may thus be given attention by some mycologists because it might fill a phylogenetic gap between the group of "Yeasts" and some black "Fungi Imperfecti".

Materials and Methods

Materials: 1) the fungus in question isolated from the rutin solution, perhaps air-borne, the Univ. of Tokyo, Japan, 2) a culture of so-labelled *Pullularia pullulans*

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** According to Mr. I. Noguchi, Dr. S. Hattori's co-worker, the property of degrading rutin as seen in the new variety is also observed in the Nagao strain of *Pullularia* and *Pullularia fermentans* var. *fermentans* but not in *Saccharomyces cerevisiae* and very poorly in *Aspergillus niger*. The property of degrading rutin may therefore be a distinguishing characteristic of the genus *Pullularia*.

from the Nagao Institute, Japan,* 3) a culture of *Pullularia werneckii* from Prof. Dr. K. Minoura, Osaka Univ., Japan and 4) a culture of *Pullularia fermentans* var. *fermentans* from Dr. Wynne, Texas Univ., Texas (U. S. A.).

Culture media used: malt extract, malt extract agar, Sabouraud dextrose agar (Difco), dextrose-peptone agar,** corn-meal agar (Difco), nutrient agar (Difco), Littman oxgall agar (Difco), microassay culture agar (Difco), Czapek dextrose agar, ammonium sulfate medium,*** potassium nitrate medium.****

Cultures were made on malt extract, nutrient broth (Difco), in test tubes, Sabouraud dextrose agar (Difco), dextrose-peptone agar, corn-meal agar (Difco), nutrient agar (Difco), Littman oxgall agar (Difco), microassay culture agar (Difco), Czapek dextrose agar, ammonium sulfate medium and potassium medium on both slant and plate, respectively. These cultures were incubated aerobically at 25° and observed on alternate days for the presence of pigmentation and growth. Final readings were made at the end of two weeks. Maximum temperature for the growth of fungus was tested on malt extract and malt extract agar at 37°, after two weeks. Method for testing the production of acid from carbohydrates was that of Wynne, except that a Horiba pH meter was used instead of a Beckman Model G pH meter.

Experimental Results

1. Classification of the new isolate in the genus *Pullularia*:

Classification was based both on morphological and on physiological considerations and comparison with known strains of the genus *Pullularia* (Fig. 1). Blastospores $4-7 \times 8-13\mu$, chlamydospores $7 \times 10-13\mu$, arthrospores $6 \times 13-15\mu$, cladosporium forms $7 \times 13\mu$. On Sabouraud dextrose agar, 14 days, at 25°, flat, light colored colony with white surface mycelium, finely fuzzy margin. On malt extract agar, at 25°, at first light pinkish-lilac colored,⁸⁾ later dark pigmentation. No dark pigment produced on almost all media such as nutrient agar, corn-meal agar, Littman oxgall agar, microassay agar, Czapek dextrose agar, ammonium sulfate medium and potassium medium. Light colored rings of pellicle in broths such as nutrient, phenol red base broth containing various sugars. No aerial mycelium observed. No growth at 37°. Ferments dextrose, mannose, sucrose, raffinose and xylose.

2. Pigmentation:

Observations on the pigmentation of some strains of *Pullularia* including the new

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* The author is indebted to the Nagao Institute for supplying the culture.

** The components of this medium were the same as that of Sabouraud dextrose agar (Difco) except that the peptone was purchased in Japan from the Kyokuto-Seiyaku Co.: Bacto-dextrose—10 g., Peptone (Kyokuto-Seiyaku)—40 g., Bacto agar—15 g., per 1 liter dist. water.

*** Dextrose—30 g., $(\text{NH}_4)_2\text{SO}_4$ —5 g., KH_2PO_4 —1 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.5 g., Bacto-agar—15 g., per 1 liter of dist. water.

**** Dextrose—30 g., KNO_3 —30 g., KH_2PO_4 —1 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.5 g., Bacto-agar—15 g., per 1 liter of dist. water.

Fig. 1 Microscopic morphology of the *Pullularia*.

Vertical column

1 st row, the new variety.

2 nd row, *P. fermentans* var. *fermentans*.

3 rd row, Nagao. strain, so-labelled *Pullularia pullulans*.

4 th row, *P. uerneckii*.

Horizontal column

1 st, blastospores, on Sabouraud dextrose agar, 3 days, at 25°.

2 nd, and 3rd., arthrospores and mycelial hyphae.

4 th, chlamydospores

5 th, cladosporium forms.

All are the same enlargement; one scale unit, 2.5 μ .

Fig. 2 Giant colonies of some strains of *Pullularia* on various media.

a, b, and c, malt extract agar with NaCl; a, 5%; b, 10%, c, 12.5%, respectively.

d, Czapek dextrose agar; e, Potassium nitrate medium;

f, Sabouraud dextrose agar with vitamin B₁₂, 100 μ g. per 1 ml. added.

a-e, upper, Nagao strain;

left, new variety;

right, *P. werneckii*;

lower, *P. fermentans* var. *fermentans*.

f, right, *P. fermentans* var. *fermentans*;

left, new variety.

Table 1. Pigmentation of *Pullularia* on various media.*

	Malt extract broth**	Malt extract agar with NaCl added:				Sabouraud dextrose agar	Dextrose-peptone agar	Nutrient broth**	Nutrient agar	Corn meal agar	Littman oxgall agar	Microassay culture agar	Czapek dextrose agar	Ammonium sulfate agar	Potassium nitrate agar	Phenol red broth containing various sugars**
		0%	5%	10%	12.5%											
<i>P. werneckii</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nagao strain of <i>Pullularia</i>	+	+	+	—	—	+	+	+	+	+	+	—	+	+	+	+
<i>P. fermentans</i> var. <i>fermentans</i>	+	+	—	—	—	+	+	+	—	+	S	—	S	+	S	+
New variety	+	+	+	—	—	—	—	—	—	—	—	—	—	S	—	—

* Readings were made on the slant cultures at the end of two weeks at 25°.

** Pellicle pigmentation.

+ dark pigmentation.

S slightly dark pigmentation.

— no dark pigmentation.

variety, are shown in Table 1 and partly Fig. 2. It was found that distinctly dark pigmentation occurred on the slant Czapek agar culture of *P. fermentans* var. *fermentans* when some amount of vitamin B₁₂ was added (20–100 μ g.) (Fig. 3).

3. Giant colony:

A giant colony of the new variety was different from those of other species of *Pullularia* (Fig. 2-d, e, and Fig. 4). In the case of *P. werneckii*, mycelial phase is so predominant that it is called "mold growth"⁶⁾. On the contrary, the new variety produced more blastospores than mycelial cells. Besides, most of the blastospores did not become mycelial cells which is usual in "mold growth" type strain, but new blastospores sprouted again and again. Besides, it was often seen that a blastospore detached from the mother cell as if it sprang out (Fig. 5).

4. The rate of growth in *Pullularia*:

The Nagao strain, the new variety, *P. fermentans* var. *fermentans* and *P. werneckii* were in order not only in rapid growth but also in size of giant colonies up to three weeks. This was the case when these fungi were compared with each other grown in parallel on plate cultures of such media as malt extract agar, dextrose-peptone agar, nutrient agar, corn-meal agar, microassay culture agar, Czapek agar, ammonium sulfate medium, potassium nitrate medium (Fig. 2-d, e, and Fig. 4).

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Fig. 3

Fig. 5

Fig. 4

Fig. 3 Five tube nutritional test of *P. fermentans* var. *fermentans* left to right,

(1)-(3), Czapek agar:

(1), blank,

(2), with vitamin B₁₂, 20 µg. per 1 ml. added.(3), with vitamin B₁₂, 100 µg. per 1 ml. added.

(4)-(5), Sabouraud dextrose agar:

(4), blank,

(5), with vitamin B₁₂, 100 µg per 1 ml. added.Fig. 4 Giant colonies of some strains of *Pullularia*, in which emphases were placed on their appearances as well as their pigmentation, on Sabouraud dextrose agar at 25°, two weeks, with exception of one week of Nagao strain. Upper left, new variety; upper right, Nagao strain; lower left, *P. fermentans* var. *fermentans*; lower right, *P. werneckii*.Fig. 5 Showing the blastospores sprouting from a short mycelial cell of the new variety of *Pullularia*.

Hanging drop culture of malt extract, 25°; upper, original; middle, after 30 minutes; lower, after 40 minutes.

When 100 μg . per ml. of vitamin B₁₂ was added to the Sabouraud dextrose agar plate cultures of the new variety and *P. fermentans* var. *fermentans* the growth of the latter fungus was promoted and the appearances of the two fungi bore a resemblance to each other, with the exception of the pigmentation at the central portion (Fig. 2-f).

5. Salt tolerance:

As seen in Fig. 2, on malt extract agar added with 12.5% NaCl the growth of *P. fermentans* var. *fermentans* and the Nagao strain were extremely inhibited, but the new variety as well as *P. werneckii* was not affected so much.

Discussion

1. Classification of the new isolate:

The new isolate seems to be most closely related to *Pullularia fermentans* var. *fermentans* though the characters of light hyphae and the peculiarity of the giant colony of this fungus would seem to eliminate other varieties of *P. fermentans* described up to now as the variety for this new isolate.

The name of *Pullularia fermentans* var. *candida* is proposed, since it resembles the fungi in the genus *Candida* as stated often in this paper.

***Pullularia fermentans* Wynne et Gott. var. *candida* Yoneyama var. nov.**

Blastosporae 4—7×8—13 μ , chlamydosporae 7×10—13 μ , arthrospora 6×13—15 μ , cladosporium format 7—13 μ . Coloniae in agarico dextroso-peptone planae, pallide roseo-lilacinae, mucosae, area centrali elevata irregulariter tuberculata, margine subtiliter fimbriatae. Pigmentum produtum et centrum in agarico maltoso et dextroso Sabouraudii: Nullum pigmentum in agaro farinae frumanti, in agaro Littmani, in agaro nutriente, in agarum Czapekii. Non crescit cum 37°. Glucosum, mannosum, maltosum, sucrosum, raffinose et xylosum fermentat.

Isolata in culturis ex solutione rutinorm in Tokiensi, Japonia.

2. Pigmentation:

The following schema would help to explain the occurrence of pigment in species belonging to *Pullularia*:

O represents a genic factor which would cause the pigmentation in all species of *Pullularia*, leading to Q which represents pigmentation. P is something which would play a role between O and Q. X is a substance which may be responsible for processing of the course O→P, and Y, another substance for the course P→Q. It seems to be a characteristic of the pigmentation in *Pullularia* that the course O→P→Q is followed.

Dark coloring strains such as *P. pullulans*, *P. werneckii*, and the Nagao strain seem to be capable of completing this course, that is, they have strong genic factors and

are able to produce both X and Y on most media; whereas the new variety seems not to complete this course, that is to say, it has a certain genic defect or failure as regards the course, so that it does not produce X, Y, or both of them^{9), 10), 11), 12)}. That the pigmentation of the new variety does occur on malt media is not easy to explain, but X or Y, or a substance or substances which might induce occurrence of X or Y, in the fungus may be present in malt extract. *P. fermentans* var. *fermentans* seems to have an intermediate position between the two type

3. *Giant colony*:

A giant colony is, as Bornside¹³⁾ pointed out, regarded as a group of cells arranged as a result of fungal development and environmental conditions. So the difference between two giant colonies of the two species of fungi on the same solid media may possibly due to two factors: 1) the way, in which the fungus tends to produce either yeast-like cells or mycelial cells, 2) mode of vegetative reproduction, i.e budding or mycelial elongation plus branching.

Regarding the conversion of the mycelial cells to yeast-like cells, there appeared several reports^{14), 15), 16), 17), 18)}, which ascribed the conversion to the effect of cultural conditions. However, yeast-like cells which appear predominantly and the peculiar mode of their sprouting as seen in the culture of the new isolate may be used as taxonomic criteria¹⁹⁾, when compared with other species of *Pullularia* under a certain condition.

4. *The growth of the species in Pullularia*:

The fact that the growth of *P. fermentans* var. *fermentans* could be promoted on Sabouraud dextrose agar by adding a little quantity of vitamin B₁₂ suggests that a simple chemical substance may be of some relation to the growth of the fungus as is the case with the production of dark color. It is therefore apparent that there exists an intimate affinity between *P. fermentans* var. *fermentans* and the new isolate.

5. *Salt tolerance (Tolerance in hypertonic medium)*:

This property should be sufficient in making a distinction between the new variety and *P. fermentans* var. *fermentans* or the Nagao strain; it is likely to assume its natural habitat is different from those of other species. However, it is regrettable to say that the natural habitat is unknown to us up to now.

The following schema may be finally drawn:

Moving from the right (dark fungi) to the left (the yeast group), there is decrease in pigmentation and rate of the mycelial phase. Fermentation ability is present in *P. fermentans* and the new variety and P-F (center of the schema, which is not an actual species but an imaginative one). Many examples which corresponded to P-F were discovered by Wynne and the Nagao strain seemed to be an example of this type.

Summary

1. A taxonomic study was made of an organism isolated from rutin solution and to which the name of *Pullularia fermentans* var. *candida* was given by the lack of dark pigment of hyphae on most media and by the peculiar appearance of a giant colony from abundance of blastospores and the mode of their sprouting.

2. Comparison of dark pigmentation and giant colonies of the species of *Pullularia* was made, respectively; the lack of dark pigmentation and the peculiarity of a giant colony of the new variety were considered to be worthy of using as taxonomic criteria.

3. The position of the new variety which seems to be an intermediate form between the genus *Pullularia* and the genus *Candida* (Yeast) was discussed.

4. A substance which might be responsible for possible pigmentation or growth of the *Pullularia* was presumed.

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摘 要

東大の服部教授の研究室で、ルチンを分解する菌が分離されたので、その同定を行なった。本菌は酵母に非常によく似ているようだったが、培養条件により *Pullularia* の特徴の片鱗を示したので、長尾研究所からの *Pullularia pullulans* と比較してみて、*Pullularia* に属させるべきものとわかった。

Pullularia は従来 *P. pullulans* と *P. werneckii* の 2 種だけ知られていたが、Wynne (1956) は ホジキン氏 病患者の病巣から *Pullularia* を分離し、それがある炭水化物から酸を生成するので *Pullularia fermentans* という新種をもうけて *Pullularia* 属の定義を修正するよう提唱した。その修正の基礎は *Pullularia* の分類のを規準として炭水化物からの酸形成能を採用することにある。東京からの本菌は、その形態と Wynne の提唱による分類規準とからみて *P. fermentans* var. *fermentans* にもっとも近縁であった。しかしたいていの培地上で *Pullularia* の特徴である暗色色素を形成しないこと、巨大コロニーの様相が特異であることから新変種とし、*Pullularia fermentans* var. *candida* とした。なお本菌は高張培地にもかなりよく耐えるが、それは比較に用いた *P. fermentans* var. *fermentans* や長尾研究所からの菌 so-labelled *P. pullulans* と異なるので、この新変種の天然のすみかは、従来しられている *Pullularia* のすみかとは異なったものと思われる。

この研究では *Pullularia* の四つの菌株について、暗色色素の形成とコロニーの様相が種々の条件下で比較されたが、*Pullularia* の暗色色素形成と生長の速度とが、ある場合には、簡単な化学物質によって支配されることがわかった。それで *Pullularia* のある菌株は microbioassay の研究材料としてもよいのではないかと思われる。