Characterization of the genus *Pseudozyma* by the formation of glycolipid biosurfactants, mannosylerythritol lipids

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Abstract

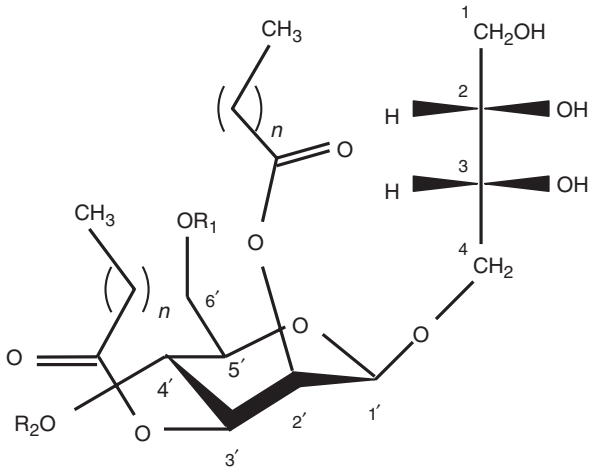
Pseudozyma antarctica is one of the best producers of the glycolipid biosurfactants known as mannosylerythritol lipids (MELs), which show not only excellent surface-active properties but also versatile biochemical actions. In order to obtain a variety of producers, all the species of the genus were examined for their production of MELs from soybean oil. *Pseudozyma fusiformata*, *P. parantarctica* and *P. tsukubaensis* were newly identified to be MEL producers. Of the strains tested, *P. parantarctica* gave the best yield of MELs ($30\,g\,L^{-1}$). The obtained yield corresponded to those of *P. antarctica*, *P. aphidis* and *P. rugulosa*, which are known high-level MEL producers. Interestingly, *P. parantarctica* and *P. fusiformata* produced mainly 4-*O*-[(4′,6′-di-*O*-acetyl-2′,3′-di-*O*-alkanoyl)-β-D-mannopyranosyl]-*meso*-erythritol (MEL-A), whereas *P. tsukubaensis* produced mainly 4-*O*-[(6′-mono-*O*-acetyl-2′,3′-di-*O*-alkanoyl)-β-D-mannopyranosyl]-*meso*-erythritol (MEL-B). Consequently, six of the nine species clearly produced MELs. Based on the MEL production pattern, the nine species seemed to fall into four groups: the first group produces large amounts of MELs; the second produces both MELs and other biosurfactants; the third mainly produces MEL-B; and the fourth is non-MEL-producing. Thus, MEL production may be an important taxonomic index for the *Pseudozyma* yeasts.

Introduction

There is considerable interest in the development of bio-based materials such as biodegradable plastics and biosurfactants from the global environmental point of view. Biosurfactants have attracted considerable interest in recent years, due to their unique properties (e.g. biodegradability, mild production conditions, and a variety of functions; Banat *et al.*, 2000). Biosurfactants are extracellular amphiphilic compounds produced by a variety of microorganisms, and are used in a broad range of industrial applications, including the food, cosmetic and pharmaceutical industries, and also environmental protection and energy-saving technology (Kitamoto *et al.*, 2002). Moreover, biosurfactants are considered to play important roles as immunoregulators and immunomodulators in adhesion and desorption on cellular systems (Cameotra & Makkar, 2004; Singh & Cameotra, 2004). However, biosurfactants have not been widely used in industry because of the relatively low efficiency of production and recovery. Therefore, it is of great interest to discover novel biosurfactant producers, and to improve the large-scale production of biosurfactants.

Mannosylerythritol lipids (MELs) are glycolipids that contain 4-*O*-β-D-mannopyranosyl-*meso*-erythritol as the hydrophilic moiety, and acetyl groups as well as fatty acids as the hydrophobic moiety (Fig. 1). MELs are secreted by several microorganisms, and are some of the most promising biosurfactants known (Kitamoto *et al.*, 2002; Lang, 2002). MELs exhibit not only excellent surface-active properties (Imura *et al.*, 2004a, b) but also versatile cell differentiation activities with respect to human leukemia (Isoda *et al.*, 1997a, b), rat pheochromocytoma (Wakamatsu *et al.*, 2001) and mouse melanoma cells (Zhao *et al.*, 1999). In addition, MEL-A, acetylated at C4 and/or C6, markedly increases the efficiency of gene transfection mediated by cationic liposomes (Inoh *et al.*, 2001, 2004).

Previously, *Pseudozyma antarctica* T-34 (Kitamoto *et al.*, 2002), *P. aphidis* DSM 70725 (Rau *et al.*, 2005), *P. rugulosa* NBRC 10877 (Morita *et al.*, 2006), *Kurtzmanomyces* sp. I-11 (Kakugawa *et al.*, 2002) and *Ustilago maydis* DSM 4500



MEL-A: $R_1 = R_2 =$ Ac
MEL-B: $R_1 =$ Ac, $R_2 = H$
MEL-C: $R_1 = H$, $R_2 =$ Ac
($n = 6 \ldots 10$)

Fig. 1. Chemical structure of mannosylerythritol lipids.

(Spoeckner *et al.*, 1999) were reported to secrete large amounts of MELs from soybean oil. The producers of MELs, however, have remained limited. We thus focused our attention on a search for a novel producer of MELs among the above genera, and examined all the *Pseudozyma* species for MEL productivity.

Here we describe for the first time the production of MELs by *P. fusiformata*, *P. parantarctica* and *P. tsukubaensis*. We also address the classification of the genus *Pseudozyma* based on MEL production.

Materials and methods

Microorganisms

The strains of the genus *Pseudozyma* used in this study are listed in Table 1. Stock cultures were cultivated for 3 days at 25 °C on YM agar medium containing 1% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract, and 1.5% agar. They were stored at 4 °C and renewed every 2 weeks.

Media preparation and culture condition

Seed cultures were prepared by inoculating cells grown on slants into flasks containing a growth medium [4% glucose, 0.3% $NaNO_3$, 0.03% $MgSO_4$, 0.03% KH_2PO_4, 0.1% yeast extract (pH 6.0)] at 30 °C on a rotary shaker (300 r.p.m.) for 2 days.

Table 1. Strains used in this study

Species	Strain	Other designation
Pseudozyma antarctica	JCM 3941	CBS 6678
Pseudozyma aphidis	JCM 10318T	CBS 517.83 = CBS 6821
Pseudozyma flocculosa	JCM 10321T	CBS 167.88 = NBRC 10875
Pseudozyma fusiformata	JCM 3931T	ATCC 38777 = CBS 6951 = NBRC 10225
Pseudozyma parantarctica	JCM 11752T	DMST 15422
Pseudozyma prolifica	JCM 10319	CBS 319.87
Pseudozyma rugulosa	JCM 10323T	CBS 170.88 = NBRC 10877
Pseudozyma thailandica	JCM 11753T	DMST 15423
Pseudozyma tsukubaensis	JCM 10324T	ATCC 24555 = CBS 6389 = NBRC 1940

T, type strain; ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelculues; DMST, National Institute of Health, Department of Medical Sciences, Nonthaburi, Thailand; JCM, Japan Collection of Microorganisms.

Seed cultures (1.0 mL) were transferred to test tubes containing 2 mL of an experimental medium [8% (w/v) soybean oil, 0.3% $NaNO_3$, 0.03% $MgSO_4$, 0.03% KH_2PO_4, 0.1% yeast extract (pH 6.0)], and then incubated as above for 7 days.

Isolation of glycolipids

The MELs produced were extracted from the culture medium with an equal amount of ethyl acetate. The extracts were analyzed by thin-layer chromatography (TLC) on silica plates (Silica gel 60F; Wako) with a solvent system consisting of chloroform:methanol:7M ammonium hydroxide (65 : 15 : 2, v/v). The compounds on the plates were located by charring at 110 °C for 5 min after spraying an anthrone reagent as previously reported (Rau *et al.*, 2005). The purified MEL-A, MEL-B and MEL-C were prepared from soybean oil by *P. antarctica* T-34 as reported previously (Kitamoto *et al.*, 2000), and then used as a standard on TLC and in the following experiments.

Quantification of MELs by HPLC

Quantification of MELs was carried out by HPLC on a silica gel column (Inertsil SIL 100A 5 μm, 4.6 × 250 mm; GL Science Inc., Japan) with a low-temperature evaporative light-scattering detector (ELSD-LT; Shimadzu, Japan) using a gradient solvent program consisting of various proportions of chloroform and methanol (from 100 : 0 to 0 : 100, v/v) at a flow rate of 1 mL min^{-1} (Rau *et al.*, 2005). The quantification of MELs was carried out by HPLC based on the standard curve using the pure MEL fraction that was obtained from *P. antarctica* T-34, as described previously (Kitamoto *et al.*, 1990b). All measurements reported here are calculated values from at least three independent experiments.

Structural analysis

The structure of the purified glycolipid (MEL-A or MEL-B) was characterized by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) with a Varian INOVA 400 (400 MHz) at 30 °C using $CDCl_3$ solution. The fatty acid profiles of the glycolipids were examined mainly with the method described previously (Kitamoto *et al.*, 1998). The methyl ester derivatives of the fatty acids were prepared by mixing the above purified glycolipids (10 mg) with 5% HCl/methanol reagent (1 mL). After the reaction was quenched with water (1 mL) (Tokyo Kasei Kogyo, Tokyo, Japan), the methyl ester derivatives were extracted with *n*-hexane and then analyzed by GC-MS (Hewlett Packard 6890 and 5973N) with a TC-WAX (GL Science Inc., Tokyo), with the temperature programmed from 90 °C (held for 3 min) to 240 °C at 5 °C min^{-1}.

Results

Of the nine species of the genus *Pseudozyma*, *P. antarctica*, *P. aphidis* and *P. rugulosa* were positioned closely together on the molecular phylogenetic tree constructed using ITS1, 5.8S rRNA gene and ITS2 sequences (Fig. 2). These three species have been reported to produce large amounts of MEL from soybean oil (Kitamoto *et al.*, 1990a, b, 2002; Rau *et al.*, 2005; Morita *et al.*, 2006). On the other hand, the MEL production of the other six species is still unknown. In order to search for a novel MEL producer, we then examined all the species of the genus for production of MELs from soybean oil.

The cells were cultured in an experimental medium containing soybean oil as the sole carbon source, and then an equal amount of ethyl acetate was added to the culture medium. The ethyl acetate fractions obtained were spotted onto a TLC plate (Fig. 3). The purified MEL-A, MEL-B and MEL-C were used as a reference, and the glycolipids produced were detected with the anthrone reagent. Three known MEL producers, *P. antarctica*, *P. aphidis* and *P. rugulosa*, showed the typical spots of MEL-A, MEL-B and MEL-C.

Interestingly, another three species, *P. parantarctica*, *P. fusiformata* and *P. tsukubaensis*, were found to show spots corresponding to MELs. More significantly, the efficiency of glycolipid production by *P. parantarctica* seemed to be comparable to that of the known producers. *Pseudozyma fusiformata* showed slight production of glycolipids, similar to *P. parantarctica*. *Pseudozyma tsukubaensis* produced glycolipids showing spots corresponding to MEL-B and MEL-C, but not MEL-A. No MEL spots were detected with the extracts from the other three species, *P. flocculosa*, *P. prolifica* and *P. thailandica*.

To identify the structure of the glycolipids produced by *P. parantarctica*, *P. fusiformata* and *P. tsukubaensis*, the purified major products were further studied by ^{1}H- and ^{13}C-NMR spectroscopy (Table 2). The spectra obtained from the major products from *P. parantarctica* and *P. fusiformata* corresponded well with those of the previously reported MEL-A of *P. antarctica* T-34 (Kitamoto *et al.*, 2000). On the other hand, the spectrum obtained from the major product from *P. tsukubaensis* corresponded well with that of the previously reported MEL-B of *P. antarctica* T-34 (Kitamoto *et al.*, 2000). Consequently, these three strains were confirmed to be MEL producers; the former two strains produced mainly MEL-A (as a main product), MEL-B and MEL-C, and the latter produced MEL-B and MEL-C.

The fatty acid compositions of the major MELs produced from soybean oil by *P. parantarctica*, *P. fusiformata* and *P. tsukubaensis* were also analyzed by the GC-MS method (Table 3). The fatty acid compositions of the MELs produced by *P. parantarctica* and *P. fusiformata* were nearly the same as those of the MELs produced by *P. antarctica* T-34. On the other hand, the fatty acid composition of the MELs produced by *P. tsukubaensis* closely resembled that of the previously reported MEL-B of *Kurtzmanomyces* sp. I-11 (Kakugawa *et al.*, 2002). Accordingly, there seem to be different kinds of fatty acid biosynthetic pathways with regard to MEL-A and MEL-B, as reported by Rau *et al.* (2005) and Morita *et al.* (2006).

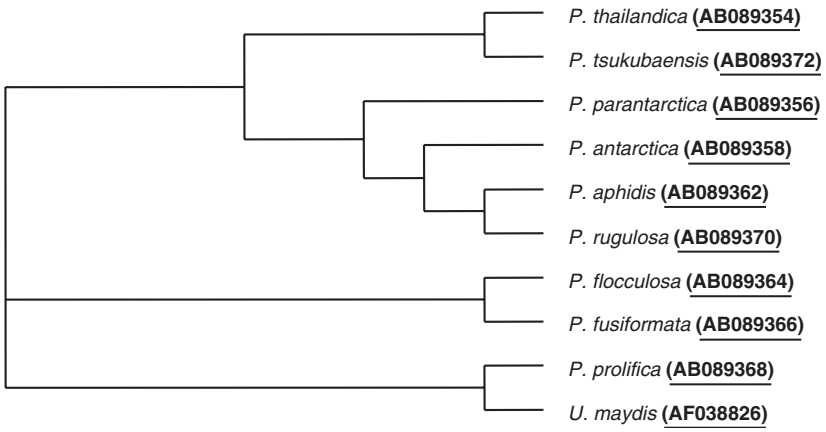


Fig. 2. Molecular phylogenetic tree constructed using ITS1, 5.8S rRNA gene and ITS2 sequences of the genus *Pseudozyma* and *U. maydis*. The DDBJ/GenBank/EMBL accession numbers are indicated in parentheses.

To quantify the amounts of MELs produced by the strains of the genus *Pseudozyma*, the ethyl acetate phases were then subjected to HPLC (Figs 4 and 5). As a typical reference, the purified standard MELs obtained from soybean oil by *P. antarctica* gave three peaks with retention times of *c.* 7.25, 7.72 and 8.17 min, respectively. *Pseudozyma aphidis* and *P. rugulosa* also showed nearly the same pattern of peaks, as previously reported (Rau *et al.*, 2005; Morita *et al.*, 2006). As expected from the results of the TLC and NMR study, the ethyl acetate phases from *P. parantarctica* and *P. fusiformata* gave the main peak corresponding to MEL-A. In contrast, *P. tsukubaensis* gave two peaks corresponding to MEL-B and MEL-C, respectively. Interestingly, *P. parantarctica* gave the best yield of MELs of the strains tested, and the amount of MELs reached over 30 g L^{-1} (Fig. 5). The yield was *c.* 38% on a weight basis to soybean oil supplied, the same level as that of the known producers.

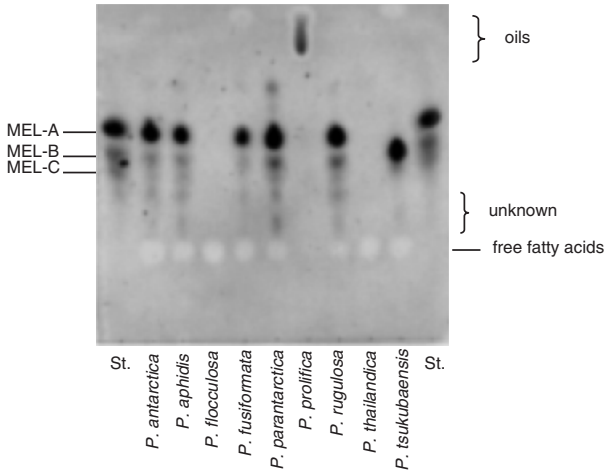


Fig. 3. Secretion of glycolipids by the genus *Pseudozyma*. Samples were extracted from culture medium using equal amounts of ethyl acetate, and the organic solvent fraction was spotted onto a TLC plate. The spots were visualized with the anthrone reagent.

Discussion

We have confirmed three yeast strains, *P. parantarctica*, *P. fusiformata* and *P. tsukubaensis*, to be novel MEL producers. Consequently, six of the nine species of the genus clearly produced MELs.

The strains belonging to the genus *Pseudozyma* are ustilaginomycetous anamorphic yeasts, and are classified in the *Ustilaginales*, including smut fungi and *Ustilago* (Spoeckner *et al.*, 1999; Sugita *et al.*, 2003), which are mainly isolated from plants. Indeed, *U. maydis* is positioned closely to the genus *Pseudozyma* on the molecular phylogenetic tree, as illustrated in Fig. 2, and produces two kinds of glycolipid biosurfactants, namely ustilagic acid [2-(2′,4′-diacetoxy-5′-carboxy-pentanoyl)-octadecyl-cellobioside] and MELs.

Table 2. NMR data of of MEL by *P. parantarctica*, *P. fusiformata* and *P. tsukubaensis*

	P. parantarctica		*P. fusiformata*		*P. tsukubaensis*	
Functional groups	^{1}H-NMR δ (ppm)	^{13}C-NMR δ (ppm)	^{1}H-NMR δ (ppm)	^{13}C-NMR δ (ppm)	^{1}H-NMR δ (ppm)	^{13}C-NMR δ (ppm)
D-Mannose						
H-1′ (C-1′)	4.71 d	99.3	4.70 d	99.3	4.69 d	99.3
H-2′ (C-2′)	5.51 d	68.6	5.50 d	68.6	5.49 d	68.7
H-3′ (C-3′)	5.06 dd	70.7	5.07 dd	71.7	4.91 dd	73.1
H-4′ (C-4′)	5.24 t	65.8	5.22 t	65.8	3.82 m	65.6
H-5′ (C-5′)	3.71 m	72.4	3.69 m	72.5	3.56 m	74.5
H-6′ (C-6′)	4.23 m	62.3	4.23 m	62.5	4.44 m	63.2
meso-Erythritol						
H-1 (C-1)	3.75 m	63.4	3.75 m	63.3	3.76 m	63.5
H-2 (C-2)	3.68 m	71.9	3.67 m	71.9	3.68 m	71.9
H-3 (C-3)	3.74 m	71.1	3.74 m	71.1	3.73 m	71.1
H-4a (C-4a)	3.86 dd	72.2	3.86 dd	72.2	3.87 dd	72.1
H-4b (C-4b)	3.99 dd	72.2	3.99 dd	72.2	3.99 dd	72.1
Acetyl group(s)						
$-CH_3$	2.10, 2.03 s	(20.6, 20.5)	2.11, 2.03 s	(20.6, 20.5)	2.13 s	(20.7)
$-C=O$		(170.9, 169.6)		(170.9, 169.6)		(171.8)
Acyl groups						
$-CH_3$	0.87 t (× 2)	(13.9, 13.8)	0.87 t (× 2)	(13.9, 13.8)	0.88 t (× 2)	(13.9 (× 2)
$-(CH2)_n$-	1.26–1.40 b	(22.4–31.7)	1.26–1.40 b	(22.4–31.7)	1.25–1.40 b	(22.3–31.7)
$-CO-CH_2$- (C-3′ position)	2.22 m	33.8	2.23 m	33.8	2.28 m	33.9
$-CO-CH_2$- (C-2′ position)	2.43 m	34.0	2.43 m	34.0	2.39 m	34.0
$-C=O$		(173.8, 172.9)		(173.8, 173.0)		(173.8, 173.7)

s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; b, broad

Table 3. Fatty acid composition of MEL from soybean oil by *P. antarctica*, *P. parantarctica*, *P. fusiformata*, *P. tsukubaensis* and *Kultzmanomyces* sp. I-11

	Composition (%)				
Fatty acid type	*P. antarctica*	*P. fusiformata*	*P. parantarctica*	*P. tsukubaensis*	*Kurtzmanomyces* sp. I-11*
C6:0	—	2.5	—	1.7	1.4
C8:0	24.6	34.7	26.6	28.1	36.4
C8:1	6.0	3.4	4.5	1.1	1.1
C9:0	2.1	1.3	—	—	—
C10:0	26.3	10.7	22.3	1.3	1.2
C10:1	24.8	10.9	26.9	1.7	4.9
C10:2	7.2	5.8	6.4	1.1	2.0
C12:0	3.7	17.0	5.0	12.0	11.9
C12:1	1.6	1.8	1.9	11.5	5.5
C12:2	—	—	—	0.5	0.6
C13:0	—	1.4	—	0.6	1.3
C14:0	—	3.4	—	1.4	—
C14:1	—	1.1	—	7.8	5.6
C14:2	1.6	3.8	2.3	27.1	25.9
Unknown	2.1	2.2	4.1	4.1	2.2

*Kakugawa *et al*. (2002)

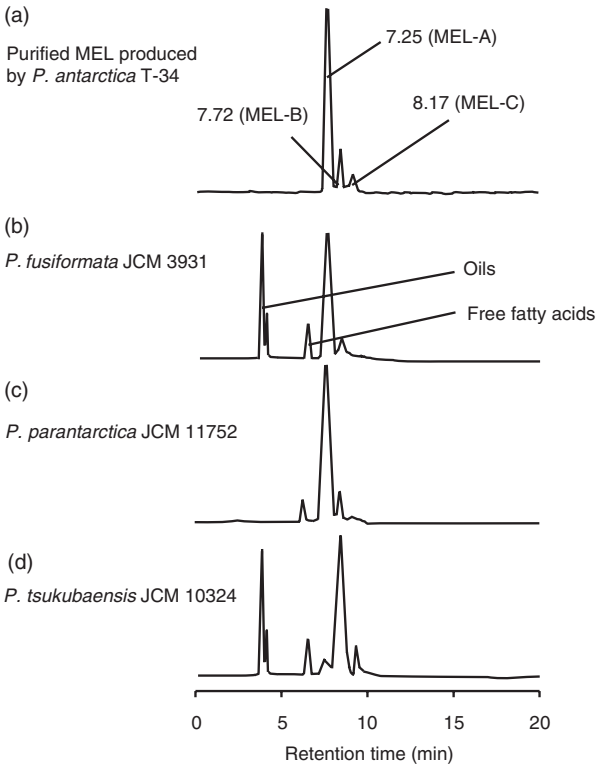


Fig. 4. HPLC analysis of glycolipids produced from soybean oil by (b) *P. parantarctica*, (c) *P. fusiformata* and (d) *P. tsukubaensis*. The glycolipids were extracted and subjected to HPLC analysis. (a) The purified MEL standard produced from soybean oil by *P. antarctica* was used as a reference.

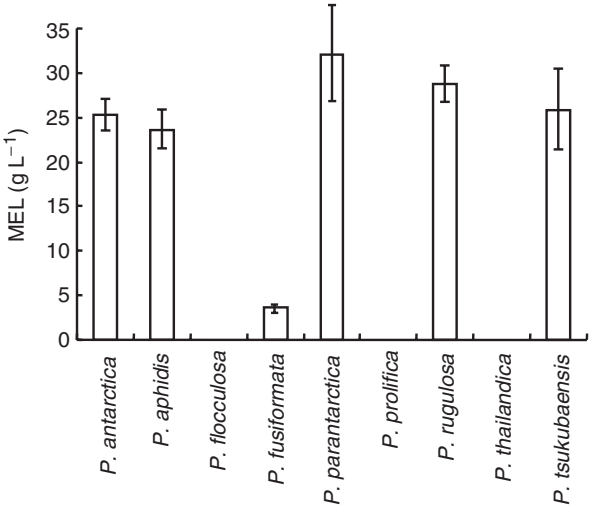


Fig. 5. MEL formation by nine strains of the genus *Pseudozyma* at 30 °C for 7 days. MELs were quantified by HPLC with three independent experiments. Vertical bars show the standard error of the mean based on three independent measurements.

Pseudozyma parantarctica, which was recently isolated from a patient's blood in Thailand, produced large amounts of MELs from soybean oil in this study. The strain is phylogenetically closely related to the three known MEL producers, *P. antarctica*, *P. aphidis* and *P. rugulosa*, according to the rRNA gene sequence tree, as shown in Fig. 2.

These facts indicate that the genes involved in MEL biosynthesis must be highly conserved among the genus *Pseudozyma* and related strains, and that these strains are taxonomically characterized by their MEL productivity. Hence, *P. parantarctica* and the three known producers should be placed in the same group on the basis of their high production of MELs and on the short distance between them on the phylogenetic tree. The details of MEL production by *P. parantarctica* will soon be presented elsewhere.

The efficiency of MEL production of *P. fusiformata* was clearly lower than that of the above four species. On the phylogenetic tree, the strain is distant from the high-level MEL producers. In addition, *P. fusiformata* was reported to secrete ustilagic acid, as well as *U. maydis* (Kulakovskaya *et al*., 2005). Taken together, these findings suggest that this strain should be placed in a different group from *P. antarctica*, *P. aphidis*, *P. rugulosa* and *P. parantarctica*.

Pseudozyma tsukubaensis produced mainly not MEL-A, but MEL-B, which is identical to the MEL produced by *Kurtzmanomyces* sp. I-11 (Kakugawa *et al*., 2002). The fatty acid composition of the MEL produced by this strain was very similar to that of the MEL produced by *Kurtzmanomyces* sp. I-11, and was quite different from that of the MEL produced by *P. antarctica* (Table 3). This clearly means that the steps for acetylation and for fatty acid synthesis differ between the MEL producers. In fact, *P. tsukubaensis* is distant from the high-level MEL producers on the phylogenetic tree, and is thus likely to belong to a different group.

As indicated above, the nine species of the genus *Pseudozyma* can probably be placed into three groups according to their pattern of production of MELs: (1) *P. antarctica*, *P. aphidis* and *P. parantarctica*; (2) *P. fusiformata*; and (3) *P. tsukubaensis*. In addition to these three groups, we suggest a fourth group for *P. flocculosa*, *P. prolifica* and *P. thailandica*, which show no MEL production. *Pseudozyma flocculosa* produces a different glycolipid named flocculosin (Mimee *et al*., 2005), instead of MEL. As large amounts of residual oils were detected in the extract from *P. prolifica* after cultivation, the activity of lipase seems to be lower than that in the other species. *Pseudozyma thailandica* was found to consume soybean oil to some extent, on the basis of the results of TLC analysis, although no MEL production was detected.

Accordingly, the genus *Pseudozyma* consists of biosurfactant producers, and should be divided into four groups according to the pattern of MEL production: the first group produces large amounts of MELs; the second produces both MELs and other biosurfactants; the third mainly produces MEL-B; and the fourth is not a MEL producer. In yeast, the coenzyme Q pattern, composition of cell wall glucan, mannan and chitin, and cellular fatty acids serve chemotaxonomic purposes (Kobayashi *et al*., 1987; Olsen, 1990). The profiles of secondary metabolites support the established taxonomy based on the morphology of the ascomycetous genus *Talaromyces* (Frisvad *et al*., 1990). Cerebroside (monohexosylceramide) components were also tentatively proposed as a chemotaxonomic marker for *Euascomycetes* (Toledo *et al*., 2001). We suggest that MEL production should thus be an important taxonomic index for the *Pseudozyma* yeasts.

In conclusion, we have reported for the first time three species of the genus *Pseudozyma* producing MELs, and tentatively placed them into four groups on the basis of the characteristics of MEL formation and the sequence identities of rRNA genes. Further work on the molecular events, such as the genes and proteins involved in MEL biosynthesis, should be done to shed light on the physiologic differences between the four categories.

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