Creation of *Hansenula polymorpha* fatty acid auxotrophic mutants for genetic study of fatty acid biosynthesis

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ABSTRACT

Three groups of *Hansenula polymorpha* fatty acid auxotrophic mutants were screened by their fatty acid requirement for growth. The mutants are saturated fatty acid auxotrophic mutants (Sfa^-), monounsaturated fatty acid auxotrophic mutants (Mfa^-) and polyunsaturated fatty acid auxotrophic mutants (Pfa^-) which required saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids, respectively. All Sfa^- mutants could grow on the media supplemented with C14:0 but not grow on the media with neither C16:0 nor C18:0. By fatty acid analysis, two of the Sfa^- mutants, S7 and S16, showed a significant difference in the fatty acid composition. S7 clearly defected in the production of C18:2Δ9,12 and C18:3Δ9,12,15, while S16 significantly accumulated medium-chain saturated fatty acids, C12:0 and C14:0. Mfa^- and Pfa^- mutants could grow on the media supplemented with mono-unsaturated fatty acids better than supplemented with polyunsaturated fatty acids. The mutants obtained in this study could be used as tools for studying in genes involved in the fatty acid synthesis and biological effect of fatty acids.

Keywords: fatty acid auxotrophic mutant; *Hansenula polymorpha*; monounsaturated fatty acid (MFA); polyunsaturated fatty acid (PFA); saturated fatty acid (SFA)

INTRODUCTION

There has been much interest in the beneficial impact of *n*-3 and *n*-6 polyunsaturated fatty acids (PFA). These fatty acids have important roles in human health and nutrition. Reliable dietary sources of PFAs are fish oils and some plant oils. Because of insufficiency of the natural resources for the market demand; therefore, the alternative sources for production of PFA-rich oils are required. Many attempts have paid on improvement of producer strains through engineering of metabolic pathways involved in fatty acid biosynthesis. Yeast is generally considered to be the preferred eukaryotic model for studying many cellular physiologies with the benefits of simplicity of the regulatory systems. *Saccharomyces cerevisiae*, the most representative yeast, is unable to synthesize PFAs despite its ability to produce monounsaturated fatty acids (MFAs) (Fujimori *et al.*, 1997). In contrast, the methylotrophic yeast *Hansenula polymorpha* has the capability to produce PFAs such as linoleic acid (18:2-Δ9,12) and linolenic acid (C18:3-Δ9,12,15) in addition to MFAs. Therefore, this yeast has been recently considered to be an appropriate model organism for studying the mechanism underlying fatty acid biosynthesis (Anamnart *et al.*, 1998; Sangwallek *et al.*, 2014). More understanding of fatty acid metabolism offers new insights into the metabolic fluxes governing the production of valuable fatty acids. The concept of obtaining them from microorganisms in sustainable quantities is attractive. It will be express the genes in specific manner and in a lipid background rich in the fatty acid substrates. With the advance in genetic manipulation, fermentation, and ability to synthesize both *n*-3 and *n*-6 PFAs, *Hansenula polymorpha* prompts to be a candidate for improvement of edible microbial oils and single cell oils (Gleeson and Sudbery, 1988; Laoteng *et al.*, 2005). With the advance in multidisciplinary research emerged, the fatty acid metabolism has been studied extensively for full understanding and consequently for effective manipulation of the composition and content of the desired fatty acids. Selection and chemical mutagenesis as well as construction of transgenic organisms would be potential strategies for genetic modifications for oil quality and production.

In this research project, a number of mutants defecting in fatty acid synthesis and having apparently altered phenotypes including growth, fatty acid composition were screened and selected. The obtained

mutants can be further used as tools for searching gene(s)/enzyme(s) involved in fatty acid synthesis.

MATERIALS AND METHODS

Organisms

The auxotrophic strains *ura3-1* (SH 4330) of *H. polymorpha* were derived from CBS 1976 (NCYC 495), a gift from Jan Kiel (University of Groningen, The Netherlands) and Satoshi Harashima (Osaka University, Japan). The *ura3-1* strain was used as parental or wild type strain (WT) for generating and screening mutant strains.

Media and cultivations

The yeast cells were grown either in the complete medium (yeast extract-peptone-dextrose medium; YPD) containing 1% yeast extract, 2% Bacto peptone and 2% glucose or in minimal medium (synthetic dextrose) containing 0.67% yeast nitrogen base without amino acids and 2% glucose. To screen the fatty acid auxotrophic mutants, C14:0, C16:0, C18:0, C16:1Δ9, C18:1Δ9, C18:2Δ9,12 or C18:3Δ9,12,15 emulsified in 1% Triton X-100 were supplemented in the media at the concentration of 0.5 to 1.5 mM. The transformation and genetic manipulation for *Escherichia coli* was described previously (Sambrook *et al.*, 1989).

Mutagenesis and isolation of *H. polymorpha* fatty acid auxotrophic mutants

Mutagenesis of *H. polymorpha* was performed by treatment with ethyl methanesulfonate (EMS) as previously described (Anamnart *et al.*, 1998). Depending on a desired phenotype, mutagenized cells were plated onto either YPD supplemented with a mixture of saturated fatty acids (SFA; 0.5 mM each of C14:0, C16:0 and C18:0) or a mixture of monounsaturated fatty acids (MFA; 0.5 mM each of C16:1Δ9 and C18:1Δ9) or a mixture of polyunsaturated fatty acids (PFA; 0.5 mM each of C18:2Δ9,12 and C18:3Δ9,12,15) and incubated at 20 ^{O}C or 30 ^{O}C until colonies appeared. The master plates were replica-plated onto YPD plates for screening the fatty acid auxotrophic mutants. For grouping, mutants that could not grow on YPD but able to grow on the media that supplemented with SFA, MFA or PFA were called Sfa^{-}, Mfa^{-} or Pfa^{-} mutants, respectively. Based on the requirement of the specific fatty acid for the growth, mutant was further replicated on the media containing the individual fatty acids. The secondary screening of the mutants will be performed using analyses of fatty acid composition.

Lipid extraction and fatty acid analyses

Lipids were extracted from cell homogenates as previously described (Anamnart *et al.*, 1998) or direct-transmethylation (Lepage and Roy, 1984). Cellular fatty acid composition was analyzed by the Agilent 6890N Series GC, using the Hewlett-Packard HP-INOWAX column (30 m x 0.32 mm x 0.25 µm in film thickness) with a temperature gradient (10 °C/min from 150 °C to 180 °C, 5 °C /min from 180 °C to 200 °C, 0.5 °C /min from 200 °C to 205 °C, 5 °C /min from 205 °C to 250 °C and 5 min at 250 °C). Fatty acids were identified by comparison of their retention times with those of commercially available methyl ester standards.

RESULTS AND DISCUSSION

Isolation and characterization of fatty acid auxotrophic mutants

Cells of *H. polymorpha* SH4330 (*ura3-1*) were subjected to EMS mutagenesis. To identify mutants with incapability to synthesize saturated fatty acid (Sfa^{-} mutants), the mutagen-treated cells were spread onto YPD agar supplemented with SFA. Colonies were then replicated onto the YPD medium. Therefore, cells defective in fatty acid synthesis, especially SFA synthesis, would fail to grow on YPD without SFA supplementation. From comparison of >20,000 colonies between the master and replica plates based on the growth ability, 30 presumptive mutants were identified and designated as S1 to S30. After subculturing for ten generations, 24 mutants still required exogenous SFA for their proliferation. In the same manner, Mfa^{-} and Pfa^{-} mutants were identified by their inability to proliferate on YPD agar without the addition of exogenous MFA and PFA, respectively. After subculture for ten generations, two Mfa^{-} and eight Pfa^{-} mutants displayed their stable inability to proliferate on YPD agar without the addition of exogenous MFA and PFA, respectively.

The Sfa^{-} mutants were further characterized on agar plates of YPD supplemented with either individual saturated fatty acids or the mixture of MFA or PFA (Table 1). Most of the Sfa^{-} mutants could grow on YPD supplemented with C14:0 and could not grow on YPD supplemented with C16:0 or C18:0. The mutants S1, S2, S3, S4, S7, S15 and S29 could slightly grow on the media supplemented with C16:0, only S1 slightly grew on YPD with the mixture of MFA or PFA. Like in the *S. cerevisiae* mutant defective in saturated fatty acid biosynthesis, the growth-promoting effect of long chain fatty acids for the fatty acid synthase (Fas^{-}) mutant distinctly

decreases from myristate to stearate (Schweizer and Bolling, 1970).

The Mfa⁻ mutants were characterized on YPD agar plates supplemented with either individual monounsaturated fatty acids or the mixture of SFA or PFA (Table 2). They showed the same phenotype that they could grow on YPD supplemented with C16:1Δ9 as well as the mixture of MFA but could not grow on YPD supplemented with SFA. They had slightly growth on YPD supplemented with C18:1Δ9 or the mixture of PFA. All of the Pfa⁻ mutants could grow on YPD supplemented with either the mixture of MFA, PFA or individual fatty acids (C16:1Δ9, C18:1Δ9, C18:2Δ9,12) (Table 3). It is noteworthy that C18:3Δ9,12,15 inhibited the growth of both wild type and Pfa⁻ mutants. This may be a result of the toxic effects of the products caused by auto-oxidation of polyunsaturated fatty acids in cellular lipids (Bilinski *et al.*, 1989).

Table 1 Phenotypes of Sfa⁻ mutants (at 30 °C for 3 days).

Strains	YPD	YPD agar supplemented with FA					
		SFA	MFA	PFA	C14:0	C16:0	C18:0
S1	-	++	+	+	++	+	-
S2	-	++	-	-	++	±	-
S3	-	++	-	-	++	+	-
S4	-	++	-	-	++	+	-
S5	-	+	-	-	++	-	-
S6	-	++	-	-	++	-	-
S7	-	++	-	-	++	+	-
S9	-	++	-	-	++	-	-
S11	-	++	-	-	++	-	-
S12	-	++	-	-	++	-	-
S14	-	++	±	±	++	-	-
S15	-	++	-	-	++	+	-
S16	-	++	-	-	++	-	-
S17	-	++	-	-	++	-	-
S18	-	++	-	-	++	-	-
S19	-	++	-	-	++	-	-
S20	-	++	-	-	++	-	-
S22	-	++	-	-	++	-	-
S23	-	++	-	-	++	-	-
S24	-	++	-	-	++	-	-
S27	-	++	-	-	++	-	-
S28	-	++	-	-	++	-	-
S29	-	++	-	-	++	±	-
S30	-	+	-	-	++	-	-
SH4330	+++	+++	+++	+++	+++	+++	+++

+++ means fast growth, ++ means growth, + means slow growth, ± means poor growth, - means no growth.

Table 2 Phenotypes of Mfa- mutants (at 30 °C for 3 days).

Strains	YPD	YPD agar supplemented with FA				
		SFA	MFA	PFA	$C16:1^{\Delta 9}$	$C18:1^{\Delta 9}$
M84	-	-	++	+	++	+
M85	-	-	++	+	++	+
SH4330	+++	+++	+++	+++	+++	+++

+++ means fast growth, ++ means growth, + means slow growth, - means no growth

Fatty acid compositions of *H. polymorpha* fatty acid auxotrophic mutants

Fatty acid compositions of the WT and the Sfa⁻ mutant strains cultivated in YPD supplemented with C14:0 or SFA are presented in Table 4. As observed in *H. polymorpha* strains, fatty acids supplied to yeast cells in the growth media were found to be preferentially incorporated into the cells during the cultivation and become a major fraction of the cellular fatty acids (Anamnart *et al.*, 1998; Rakpuang, 2009) in contrast with the cultures supplemented with exogenous C14:0. In this study, the exogenous C14:0 was not highly accumulated in the cells of the both WT and the Sfa⁻ mutants. These may be a cause of the cellular response to maintain proper C14:0 homeostasis that is a critical mechanism of the cells (Orme *et al.*, 1972).

Table 3 Phenotypes of Pfa⁻ mutants (at 20 °C for 7 days).

Strains	YPD	YPD agar supplemented with FA						
		SFA	MFA	PFA	C16:1$^{\Delta9}$	C18:1$^{\Delta9}$	C18:2$^{\Delta9,12}$	C18:3$^{\Delta9,12,15}$
P36	-	-	++	++	++	++	+	-
P37	-	-	++	++	++	++	++	-
P49	-	-	+	++	+	++	+	-
P52	-	-	+	++	+	++	+	-
P55	-	-	++	++	++	++	++	-
P60	-	-	+	+	+	+	+	-
P62	-	-	+	++	+	+	+	-
P63	-	-	+	++	+	+	+	+
SH4330	+++	+++	+++	+++	+++	+++	+++	+

+++ means fast growth, ++ means growth, + means slow growth, + means poor growth, - means no growth.

Table 4 Fatty acid composition (% of total fatty acids) of Sfa⁻ mutants.

Strains[a]	C12:0	C14:0	C16:0	C16:1 Δ9	C16:2 Δ9,12	C17:0	C18:0	C18:1 Δ9	C18:1 Δ11	C18:2 Δ9,12	C18:3 Δ9,12,15
S1	0	1.32	10.68	2.26	2.24	3.06	4.07	18.10	1.97	42.84	8.51
S2	0	1.09	13.96	2.62	3.57	1.54	4.89	9.56	3.50	40.16	11.19
S3	0.16	1.39	7.58	2.34	3.34	1.60	4.78	11.22	2.68	45.93	12.78
S4	0	3.60	14.20	3.05	3.60	1.12	4.89	8.49	2.28	41.87	14.78
S5	0	2.16	12.65	2.14	1.59	0	5.68	15.97	2.75	39.67	14.77
S6	0	1.81	12.39	2.82	3.88	1.11	5.00	8.81	3.83	39.98	12.15
S7	0	1.37	32.04	6.08	0	0	12.97	34.33	0	0	0
S9	0.19	1.35	13.57	3.45	3.86	1.28	4.76	9.96	3.52	40.83	10.79
S11	0	1.57	13.46	3.02	4.32	1.50	6.37	9.23	3.58	40.90	11.59
S12	0	0.68	8.85	1.07	1.93	1.76	6.99	9.96	3.53	41.05	15.10
S16	30.75	8.78	10.20	1.29	2.03	0	3.17	5.16	0	20.90	7.32
S17	0	2.36	15.84	2.57	2.56	1.21	7.94	11.31	1.97	37.26	13.30
S18	0	1.16	11.60	2.39	3.23	1.36	5.02	10.01	3.55	38.92	15.26
S19	0.20	1.52	16.12	1.97	3.26	1.34	9.74	8.28	2.97	31.53	13.98
S20	0	1.33	13.13	1.73	2.61	0	4.71	8.37	2.96	41.85	17.91
S22	0	2.35	14.65	2.77	3.61	0	4.96	8.49	2.45	38.60	15.53
S23	0.19	1.38	13.38	2.49	2.94	1.35	7.41	8.75	3.38	35.87	14.49
S24	0	1.48	12.61	3.26	4.33	0	4.62	7.95	3.85	42.64	17.48
S27	0	6.04	17.45	3.91	4.26	1.29	7.18	8.95	0	35.34	13.29
S28	0	1.20	11.80	2.26	3.36	1.71	6.19	10.57	3.36	40.79	11.55
S29	0	1.95	11.35	3.16	3.83	1.16	3.85	10.60	3.81	44.14	11.03
S30	0.19	2.44	22.31	1.98	2.55	0.91	7.04	14.20	2.88	34.09	9.65
WT	0	2.93	26.04	4.40	0	0	8.09	23.70	0	22.24	5.31

[a]Fatty acid composition in cells grown on YPD+C14:0 (0.5 mM) at 30 °C, 150 rpm for 48 h.

Myristate is essential for eukaryotic cells, not only to provide a concentrated energy source and to serve as an important building block for a membrane structure, but also to mediate post-translation modifications of proteins (Cross, 1987; Towler *et al.*, 1987). A deficit in the myristoyl-CoA pool leads to arrest of cell growth in yeast (Duronio *et al.*, 1991). In contrast, excess intracellular accumulation of the free-fatty acid can be toxic (Oshiro *et al.*, 2003; Tong *et al*. 2006). Prasitchoke *et al.*, (2008) found that C14:0, but not other fatty acids, caused severe growth retardation in *Hpelo1*Δ and *Hpelo2*Δ mutants, defective in elongation of very long-chain fatty acids, of yeast *H. polymorpha*. Thus, it is important to understand how cells regulate the C14:0 pool, and other fatty acids. From the results of fatty acid analysis, two Sfa$^-$ mutants S7 and S16 showed a significant difference in the fatty acid composition. S7 clearly defected in the production of C18:2Δ9,12 and C18:3Δ9,12,15. We have already identified that the S7 strain has a double lesion affecting fatty acid synthesis and Δ12-desaturase (Sooksai *et al.*, 2013). While S16 significantly accumulated medium-chain saturated fatty acids, C12:0 and C14:0. The myristoyl-CoA pool in the S16 mutant could be changed by the dynamic interplay between non-Fas acyl chain elongation systems and degradative pathways.

Fatty acid compositions of the WT and the Mfa- and Pfa- mutant strains cultivated in YPD supplemented with C16:1Δ9 are presented in Table 5. We designed to use C16:1Δ9 as the exogenous unsaturated fatty acid for supplementing growth of the both Mfa$^-$ and Pfa$^-$ mutants for fatty acid analysis because *H. polymorpha* WT usually contains a very small amount of C16:1Δ9 (Anamnart *et al.*, 1998) and both Mfa$^-$ and Pfa$^-$ mutants could grow on the media supplemented with either C16:1Δ9 or C18:1Δ9. The changes in fatty acid profiles occurred when the cultures were exposed to supplemented fatty acids implies that there was a modification of the activity of enzymes involved in fatty acid synthesis, elongation, desaturation and β-oxidation. The data obtained from the analysis of the cell cultures of Mfa$^-$, Pfa$^-$ and WT grown in the presence of C16:1Δ9 showed a high level of 16:1Δ9,12. These data evidently showed that C16:1Δ9 and its derivatives were incorporated into the lipid-containing cell constituents instead of the C18 unsaturated fatty acids.

Table 5 Fatty acid composition (% of total fatty acids) of Mfa$^-$ and Pfa$^-$ mutants.

Strains[a]	C12:0	C14:0	C16:0	C16:1 Δ9	C16:2 Δ9,12	C17:0	C18:0	C18:1 Δ9	C18:1 Δ11	C18:2 Δ9,12	C18:3 Δ9,12,15
M84	0	0.83	30.58	24.77	9.89	0.48	30.38	0	1.13	0	0
M85	0.14	0.93	29.97	22.84	9.63	0.52	31.34	1.92	1.07	0	0
P36	0.12	1.18	25.69	19.52	10.92	0	24.44	4.77	2.56	6.15	0
P37	0.14	1.10	18.18	33.19	11.80	0.64	12.50	5.74	9.14	4.94	0
P52	0.14	0.84	24.56	31.16	13.99	0	20.53	1.38	1.85	1.39	0
P55	0	0.72	15.89	52.48	5.56	1.41	10.87	1.50	10.09	0	0
P60	0	1.02	25.56	31.56	18.39	0	19.74	0	0	0	0
P63	0	0.61	20.57	37.84	15.04	0.53	19.17	0	2.49	0	0
WT	-	0.45	16.38	32.63	3.70	-	7.36	7.93	9.17	7.00	0.85

[a]Fatty acid composition of cells grown in YPD+C16:1Δ9 (0.5 mM) at 30 °C, 150 rpm for 48 h.

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