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Metabolic correlation between polyol and energy-storing carbohydrate under osmotic and oxidative stress condition in *Moniliella megachiliensis*

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***Moniliella megachiliensis*, the osmo-tolerant basidiomycetous yeast was found to accumulate intracellularly energy-storing carbohydrates (trehalose and glycogen) along with polyols (glycerol and erythritol) up to stationary growth phase. In trehalose-loaded cell, osmotic-stress resulted in the rapid generation of glycerol, and oxidative stress with menadione resulted in the rapid generation of erythritol. Under either of these conditions, the levels of the energy-storing carbohydrates were depleted, while little glucose uptake was observed. These results suggested that the intracellular pools of trehalose and glycogen were rapidly converted to glycerol in response to osmotic stress, and to erythritol in response to oxidative stress and altered redox balance. Expression of *tps1* encoding trehalose synthetic enzymes paralleled trehalose accumulation in the cell during the culture in 2% glucose, in contrast, expression of *tpp1* or *tpp2* encoding trehalose-6-phosphate phosphatase was little increased under the same condition. Expression of *tre* (*tre1*/*tre2*) encoding trehalose hydrolase (trehalase) increased with time associated with depletion of trehalose during oxidative stress. From these results, we concluded that glycerol and erythritol, the compatible solutes in *M. megachiliensis* were metabolically interrelated to energy-storing carbohydrates such as trehalose or glycogen during conditions of osmotic or oxidative stress.**

[**Key words:** Erythritol; Glycerol; Trehalose; *Moniliella megachiliensis*; Stress response]

Moniliella megachiliensis SN-124A is a basidiomycetous yeast that was isolated from dry fruit (1). This microbe is able to endure even in 60% glucose because of its extremely high osmo-tolerance (2). We reported previously that *M. megachiliensis* produces high levels of erythritol and low levels of glycerol when cultivated in high-glucose medium (2). Erythritol is currently produced fermentatively, and is used in a wide variety of foods, cosmetics, pharmaceuticals, and chemicals owing to its unique and appealing characteristics such as low energy value, non-carcinogenicity, and potential anti-oxidative function (3–6). Elevation of erythritol yield is one of the most important issues to meet its expanding demand at present. In eukaryotes, erythritol is synthesized via the pentose phosphate pathway (PPP); it is obtained by enzymatic reduction (via $NADP^+$-dependent erythrose reductase (ER)) of the precursor erythrose, itself the product of dephosphorylation of erythrose 4-phosphate (7). In contrast, the bacteria *Oenococcus oeni* reportedly derives erythritol from the precursor erythritol 4-phosphate, which is itself the product of phosphoketolase (8).

Our work also addressed the mechanism of osmotic stress response and concomitant polyol biosynthesis by this strain, demonstrating the following: (i) The Hog1 ortholog of *M. megachiliensis* functions in a HOG osmotic stress-response pathway that is similar to, but much more efficient than, that of *Saccharomyces cerevisiae* (9), (ii) *M. megachiliensis* possesses three erythrose reductases (ER1, ER2, and ER3), with *er3* being the most responsive to osmotic stress as judged by gene expression and by the number and location of stress response element (STRE), and erythritol accumulation together with increasing glucose concentration (10), (iii) *M. megachiliensis* has two transaldolase genes (MmTAL1 and MmTAL2), the MmTAL1 gene is specifically expressed under oxidative stress, whereas, the expression of MmTAL2 is induced under hyperosmotic environment (11).

Further, we noticed incidentally in that work, that both erythritol and glycerol generated in *M. megachiliensis* cells even in the absence of glucose uptake. This observation suggested that carbon sources other than assimilated glucose might serve as substrates for polyol biosynthesis in *M. megachiliensis*.

In general, trehalose and glycogen is known to be energy-storing carbohydrates in many microorganisms (12–14). Trehalose has been known to play an important role in protecting certain organisms from desiccation or freezing (15). It promotes survival of *S. cerevisiae* under heat condition by protecting proteins from denaturation at elevated temperature and suppressing the aggregation of denatured proteins (16). Trehalose is synthesized from UDP-glucose and glucose 6-phosphate via a sequential two-step reaction catalyzed by trehalose 6-phosphate synthase (TPS) and trehalose 6-phosphate phosphatase (TPP or TPS2 in *S. cerevisiae*) (17,18). Conversely, trehalose is hydrolyzed into two molecules of glucose by the action of trehalase (TRE) (19). In *S. cerevisiae*, two kinds of trehalases have been identified: acid trehalase (ATH1) and

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neutral trehalase (NTH1 and NTH2) (20). Glycogen (α-glucosidic polymer) is elongated by glycogen synthase (GSY), an enzyme that transfers the α-1, 4-glucosidic moiety of UDP-glucose to the existing glycogen chain (21).

Glycogen is degraded by glycogen phosphorylase (GPH), which releases glucose 1-phosphate from the reducing end of linear α-1, 4-glucosidic bonds of glycogen together with inorganic phosphate (22). The activities of TRE and GPH are regulated via phosphorylation by cAMP-dependent protein kinase (PKA) (23,24); trehalose 6-phosphate exhibits end-product inhibition of hexokinase, the enzyme that converts glucose to glucose 6-phosphate to provide a starting substrate for glycolysis (25). Thus, the pathways for polyol synthesis are regulated in parallel with the synthesis of energy-storing carbohydrates. However, little information is available about the interrelation of polyol biosynthesis and energy-storing carbohydrates like trehalose or glycogen in *M. megachiliensis*. In the work described here, we investigated metabolic correlations between the polyol and energy-storing carbohydrate pools under conditions of hyper-osmotic and oxidative stresses on this specifically osmo-tolerant yeast. Our results provide insights into the biosynthesis of erythritol and glycerol in glycolytic metabolism, and may lead to improvements in polyol production yield via metabolic engineering.

MATERIALS AND METHODS

Strains and media *M. megachiliensis* SN-124A (deposited as FERM BP-1429, National Food Research Institute, Microbial Gene Bank, Ibaraki, Japan) was used throughout the experiment, and was cultured in YPD medium composed of 1.0% yeast extract, 2.0% peptone and 2.0% glucose at 30°C. This strain was originally described as *Aureobasidium* sp. (1), after then, designated *Trichosporonoides megachiliensis* (26). *Escherichia coli* DH5α (27) used for all cloning applications, was propagated at 37°C in LB medium composed of 2.0% tryptone, 1.0% yeast extract and 2.0% NaCl. GY medium consisted of 2% glucose and 0.5% yeast extract, with the glucose concentration supplemented to 20% or 40% as needed for specific experiments.

Culture methods All *M. megachiliensis* cultures were grown at 30°C with shaking, using one of the three following methods. To evaluate the stress response at intermediate to late stage, *M. megachiliensis* was cultured in GY medium containing 2% glucose as a normal stress-free medium or 20% glucose as an osmotic stressing medium for up to 72 h; portions were harvested at 12-h intervals. To evaluate the stress response of cells that were accumulated (trehalose-loaded cells), *M. megachiliensis* was grown for 24 h (i.e., to stationary phase) by culturing in GY medium with 2% glucose; every hour, a portion of the culture was transferred to fresh GY medium supplemented to 40% glucose or 1 M NaCl as osmotic stressor, 0.1 mM menadione as oxidative stressor, 2% galactose medium containing 0.5% yeast extract as non-fermentable carbon source, and then incubated for an additional 4 h. For RT-PCR analysis, the transferred (glucose or menadione supplemented) cultures were incubated only for 120 min with aliquots harvested at intervals of 30 min, concerning 2% glucose, 40% glucose and 0.1 mM menadione. As oxidative stressor, menadione was used here since it is considered to work not on the surface of the cell but inner cytosol unlike H_2O_2. To evaluate stress response of the cells that were not accumulated trehalose (trehalose non-loaded cells), *M. megachiliensis* was cultured in GY medium with 2% glucose for 12 h (i.e., until log phase); a portion of the culture was transferred to fresh GY medium supplemented to 40% glucose or 0.1 mM menadione and incubated for an additional 2 h. For each method and at each time point, harvested cultures were collected by centrifugation (1000 ×*g*, 10 min, 4°C), washed twice with TE buffer and stored frozen at −80°C pending analysis.

Identification of carbohydrate-related genes of *M. megachiliensis* The draft sequence of the *M. megachiliensis* genome was determined as previously described (28). Following whole-genome shotgun sequencing at Hokkaido System Science (Sapporo, Japan) using the GS FLX Titanium system, assembled sequence contigs were converted to BLAST database format for local BLAST searches using stand-alone BLAST software (ver. 2.2.22) downloaded from the NCBI website (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/). Putative gene/protein homologs were confirmed by individual comparisons to the GenBank database and manual annotation. Functional domains in the corresponding protein sequences were predicted using Conserved Domain Search (NCBI). Nucleotide sequences of the genes were confirmed using Big Dye Terminator v1.1/3.1 (Applied Biosystems, Tokyo, Japan) and an ABI automatic sequencer (Perkin Elmer Japan, Tokyo, Japan). These sequence data were analyzed by using GENETYX-MAC ver. 15 (GENETYX Corporation, Tokyo, Japan).

Determination of intracellular polyol, trehalose and glycogen content Determination of intracellular polyol and trehalose concentrations were performed as previously described (10). Glycogen was analyzed using the previously reported method (29), modified as follows. After neutralization with 1.0 mL of 2 N HCl, glycogen extracted from cells of *M. megachiliensis* was hydrolyzed to glucose using glucoamylase and α-amylase. The resultant glucose concentrations were determined using the ICS-3000 (DIONEX, Osaka, Japan) chromatographic system and a Carbo pac PA-1 column. Amount of glycogen was expressed as glucose moles hydrolyzed.

RT-PCR Isolation of total RNA and synthesis of cDNA were carried out as previously described (10). Gene expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using expression of the GAPDH-encoding locus as an internal control. PCR was performed using 1 μL of diluted cDNA template in a 20-μL reaction. Cycling conditions were optimized for each gene to obtain amplification under the exponential phase of PCR amplification. Oligonucleotide sequences of RT-PCR primers are provided in supplementary data (Table S1). A single primer pair (TRE-for and TRE-rev) was used to amplify transcripts of both *tre1* and *tre2*, taking advantage of the high sequence similarity between these two genes. Transcription of other genes was assayed using primer pairs as follows: TPS1-for and TPS1-rev for *tps1*; TPS2-for and TPS2-rev for *tps2*; TPP1-for and TPP1-rev for *tpp1*, and TPP2-for and TPP2-rev for *tpp2*.

Chemicals All chemicals used were purchased commercially as reagent grade. α-amylase and glucoamylase, were obtained by courtesy from Amano Enzyme Inc. (Nagoya, Japan).

RESULTS

Intracellular accumulation of polyols and carbohydrates with growth time As noted previously (10), erythritol and glycerol accumulate intracellularly in *M. megachiliensis*. We additionally demonstrated the time course of intracellular accumulation of trehalose and glycogen after osmotic or oxidative stress treatments. In a GY medium containing 2% glucose (Fig. 1a) or 20% glucose (Fig. 1b), trehalose and glycogen were detected from 12 h, with maximal values observed between 36 and 48 h. In 2% glucose medium, peak trehalose and glycogen concentrations of approximately 420 and 160 μmol/g wt cell were respectively detected at 36 h. A significant amount of assimilated glucose thus was converted to energy-storing carbohydrates like trehalose and glycogen, as compared to the lower observed amounts of glycerol and erythritol. On the other hand, growth in medium at 20% glucose resulted in much higher amounts of erythritol and glycerol, and slightly lower amounts of trehalose and glycogen than seen in 2% glucose medium. As shown in Fig. 1c, growth curves of the cells in 2% glucose and 20% glucose were almost the same; they reach maximums approximately after 24 h.

Time course of polyol, trehalose and glycogen content Fig. 2 illustrates the change in polyol content in *M. megachiliensis* during osmotic (40% glucose and 1 M NaCl), oxidative (0.1 mM menadione) stress or 2% galactose without glucose as non-fermentable carbon source. The cells used were pre-cultured in 2% glucose medium for 24 h, thereby permitting accumulation of substantial amounts of trehalose and glycogen. Under stress-free conditions (2% glucose), polyol levels in the cell showed little change, while trehalose decreased to approximately one third of initial content, and glycogen levels decreased nearly to zero after 2 h (Fig. 2a). In contrast, cells incubated in medium with 40% glucose, the osmotic stressing medium, exhibited increases in glycerol and erythritol (6- and 4-fold, respectively, after 2 h) and rapid decreases of trehalose and glycogen (Fig. 2b). As well as 40% glucose medium, cells incubated in the medium containing 1 M NaCl, exhibited increases in glycerol and erythritol (6- and 3-fold, respectively, after 2 h) and rapid decreases of trehalose and glycogen (Fig. 2c). Moreover, cells grown in medium containing 0.1 mM menadione exhibited striking increases in the level of erythritol (approximately 10-fold after 1 h) (Fig. 2d). In case of 2% galactose, no increase in polyol was observed, despite of significant decrease of trehalose