Physiology of Methylotrophic Yeasts

Subjects: Microbiology

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Methanol is abundant in the phyllosphere, the surface of the above-ground parts of plants, and its concentration oscillates diurnally. The phyllosphere is one of the major habitats for a group of microorganisms, the so-called methylotrophs, that utilize one-carbon (C1) compounds, such as methanol and methane, as their sole source of carbon and energy. Among phyllospheric microorganisms, methanol-utilizing yeasts can proliferate and survive in the phyllosphere by using unique molecular and cellular mechanisms to adapt to the stressful phyllosphere environment.

methanol methylotrophs phyllosphere diurnal adaptation peroxisome

1. Introduction

In nature, methanol is ubiquitous. Its main origin is considered to be the methyl ester groups of pectin, one of the major components of the plant cell wall [1][2]. Methanol is produced through the hydrolysis of pectin methyl esters by pectin methylesterase. Once released, methanol can be utilized by microorganisms living in the phyllosphere, defined as the aerial parts of plants, or emitted into the atmosphere as a volatile organic compound whose global emission is estimated to be 100 Tg per year [1][3][4]. The atmospheric concentration of methanol has been reported to fluctuate depending on the opening and closing of stomata [5]; however, the amount of methanol in the phyllosphere had not been quantified. Recently, we revealed that the concentration of methanol available for microorganisms on the surface of plant leaves also oscillates during the daily light–dark cycle. Results showed that the methanol concentration in the phyllosphere was higher in the dark period and lower in the light period, which was opposite to atmospheric methanol (Figure 1) [6][7], suggesting that phyllospheric microorganisms utilize the methanol hydrolyzed from the plant pectin in a direct manner, rather than using methanol present in the air.

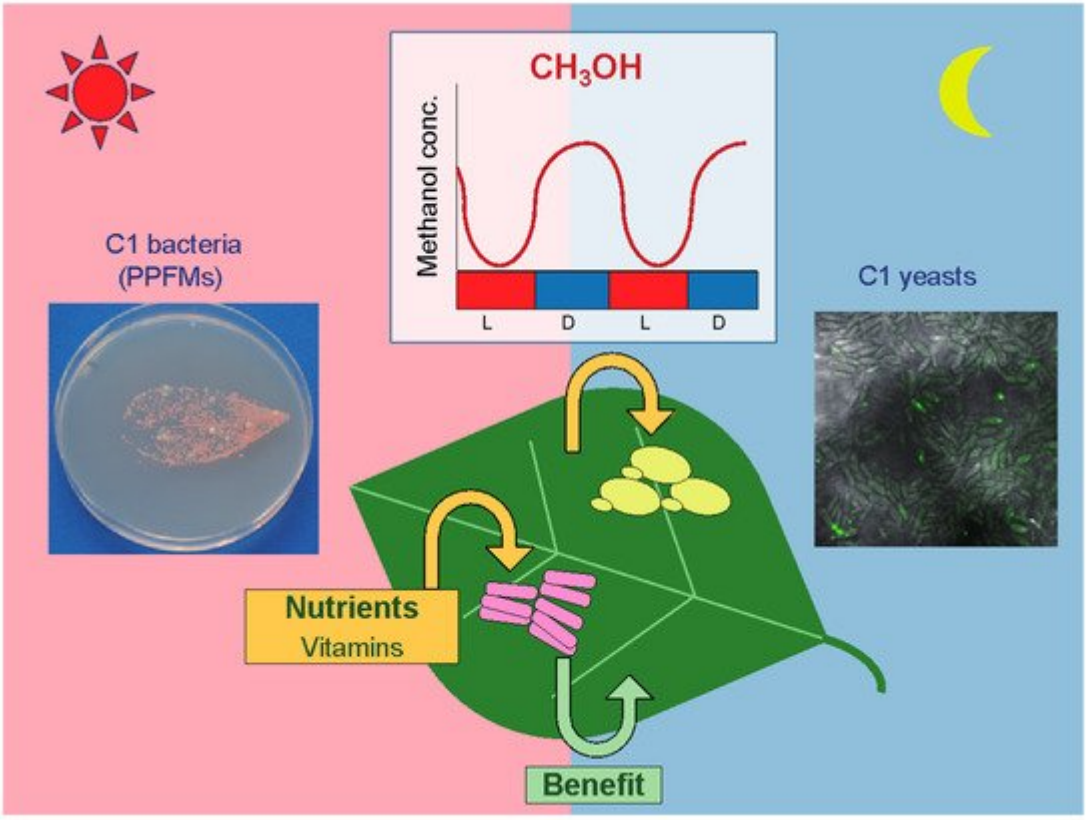

Figure 1. Colonization of methanol-utilizing methylotrophs in the phyllosphere where methanol concentrations oscillate diurnally. Methanol-utilizing bacteria (one-carbon (C1) bacteria, pink-pigmented facultative methylotrophs (PPFMs)) and yeasts (C1 yeasts) colonize the surface of plant leaves and acquire nutrients produced by plants. Concentrations of methanol in the phyllosphere oscillate diurnally, with lower concentrations in the light period (L) and higher concentrations in the dark period (D). After the leaf printing on the agar medium containing methanol as the sole carbon source, pink-pigmented colonies were observed (left panel photo). *Candida boidinii* cells expressing the fluorescent protein Venus proliferate on *Arabidopsis thaliana* leaves (right panel photo).

A wide variety of microorganisms colonize the phyllosphere and the area of soil surrounding plant roots (rhizosphere). Interactions between plants and microbes have been considered to affect not only the growth and proliferation of both organisms, but also the ecosystem and global environment. The total area of the global phyllosphere is estimated to be 10^9 km^2, twice as large as the surface of the earth, and such space could be colonized by bacterial populations of 10^{26}–10^{27} cells, as well as lower numbers of archaea and fungi [8]. While plant–rhizobia and plant–mycorrhizae interactions in the rhizosphere have been thoroughly investigated, studies of plant–microbe interactions in the phyllosphere have been limited to those involving plant pathogens. As such, positive and neutral interactions between phyllospheric microbes and their host plants have been closely researched only in the last decade [9][10].

Among phyllospheric microorganisms, methanol-utilizing bacteria, known as pink-pigmented facultative methylotrophs (PPFMs), are the dominant colonizers of plant leaf surfaces (Figure 1) [11][12]. Methylotrophs are a diverse group of microorganisms that utilize reduced one-carbon (C1) compounds, such as methanol and

methane, as their sole sources of carbon and energy. C1-utilizing microorganisms include bacteria, archaea, and fungi. Most methylotrophic fungi are yeasts. PPFMs are members of the genus *Methylobacterium*, although some have recently been reclassified into *Methylorubrum* [13], and some of these are known to have the ability to promote plant growth [14][15]. Along with PPFMs (C1 bacteria), some methylotrophic yeasts (C1 yeasts), which belong to the genera *Candida* and *Komagataella*, also colonize the phyllosphere (Figure 1) [6]. These yeasts can grow vigorously on methanol-containing media and have been used as hosts for heterologous protein production using strong and regulatable methanol-induced gene promoters [7][16][17][18][19]. Because of their intracellular dynamics, these yeasts have also been used as model organisms to investigate the molecular and cellular mechanisms of the development and degradation peroxisomes, which are essential organelles for methanol metabolism.

Phyllospheric microorganisms are exposed to a variety of environmental factors, such as low nutrients, temperature, drafts, and UV light, which change diurnally [9]. Methylotrophs living in the phyllosphere have therefore evolved physiological adaptations to grow and survive under such stressful conditions.

2. Proliferation of Methylotrophic Yeasts on Plant Leaves Where Methanol Concentrations Fluctuate Diurnally

The ability to utilize methanol as a carbon source is considered to be one of the reasons why methylotrophs are the dominant colonizers of the phyllosphere. This hypothesis is supported by the fact that *Methylorubrum extorquens* AM1 mutant strains defective in methanol metabolism are less competitive than the wild-type strain during colonization on plant leaves [20][21].

While methylotrophic yeasts have often been isolated from various plant resources, it was unknown until recently whether these yeasts can proliferate in the phyllosphere. The methylotrophic yeasts *Candida boidinii* and *Komagataella phaffii* (*Pichia pastoris*) were found to proliferate on the leaf surface of growing *Arabidopsis thaliana* plants [6]. Yeast cells expressing a fluorescent protein were inoculated onto plant leaves and their growth was observed by fluorescence microscopy and quantitative PCR analysis for two weeks. We found that *C. boidinii* cells grew slowly, replicating approximately 3−4 times within 11 days of inoculation. Furthermore, *C. boidinii aod1*Δ and *das1*Δ strains in which genes encoding the peroxisomal methanol-metabolizing enzymes alcohol oxidase (AOD) and dihydroxyacetone synthase (DAS), respectively, were disrupted could not proliferate on leaves, indicating that methanol metabolism is necessary for growth in the phyllosphere.

Another question that had not been answered until recently was how much methanol is present on plant leaves and available to methylotrophs. To examine the methanol concentration in the phyllosphere, we developed a cell-based methanol sensor using the methylotrophic yeast *C. boidinii* expressing the fluorescent protein Venus under the control of the methanol-induced *DAS1* gene promoter [6]. The sensor cells were inoculated on the surface of leaves of *A. thaliana* plants that had been growing for 2–3 weeks after germination in a plant growth chamber with a daily light-dark cycle (14 h light, 10 h dark). After a 4 h incubation, the fluorescence intensity was measured. The estimated methanol concentration was higher in the dark period (25–60 mM) than in the light period (0–5 mM), suggesting that the local methanol concentration in the phyllosphere of growing young leaves oscillates during the

daily light-dark cycle (Figure 1). In addition, transcript levels of the methanol-induced genes *AOD1* and *DAS1* corresponded to the phyllospheric methanol concentration measured by the sensor cells. In contrast to young leaves, the methanol concentration on wilting or dead leaves was estimated to be greater than 250 mM, and did not show diurnal oscillation. Given that the amount of methanol available to phyllospheric microorganisms fluctuates naturally, it is reasonable to propose that methanol-induced gene expression in methylotrophic yeasts was acquired through evolution to adapt to the phyllospheric environment.

We also investigated the nitrogen sources utilized by *C. boidinii* in the phyllosphere [22]. Since *C. boidinii* can utilize nitrate and methylamine as nitrogen sources, we focused on *YNR1*, encoding nitrate reductase, and *AMO1*, encoding amine oxidase, and examined their physiological functions in the phyllosphere. The wild-type and *amo1Δ* strains were able to proliferate on growing young leaves of *A. thaliana* plants, whereas the *ynr1Δ* strain could not. The *YNR1* gene, but not the *AMO1* gene, was expressed in cells inoculated on young leaves, and its expression level fluctuated diurnally, indicating that the nitrate concentration fluctuates diurnally. Further observation, however, found that expression of the *AMO1* gene was induced on wilting leaves. These results suggest that available nitrogen sources for *C. boidinii* change from nitrate on young leaves to methylamine on wilting leaves. Subsequently, we investigated the in vitro fate of *YNR1* after alternating the nitrogen source from nitrate to methylamine, and found that a selective autophagic pathway was involved in the nitrate metabolic change. Together, these results indicate that carbon and nitrogen sources available to methylotrophs in the phyllosphere change not only during the day–night cycle, but also during the life cycle of the plant.

3. Molecular and Cellular Mechanisms of Adaptation to the Phyllosphere Environment in Methylotrophic Yeasts

During growth on methanol, methylotrophic yeasts develop large numbers of peroxisomes that contain AOD, DAS, and other key enzymes for methanol metabolism [17]. When cells are shifted to a glucose or ethanol medium from a methanol medium, peroxisomes are degraded by peroxisome-specific autophagy, which is termed pexophagy. In the phyllosphere environment, where methanol concentrations oscillate diurnally, peroxisome dynamics should be determined by the methanol concentration. We observed that the number of peroxisomes in *C. boidinii* cells on young leaves increased in the dark period and decreased in the light period, corresponding to the methanol concentration [6]. Furthermore, our results demonstrated that *C. boidinii* mutants with disruptions in Pex5 (responsible for peroxisomal protein import), Atg1 (a pivotal kinase for all autophagic pathways), and Atg30 (a receptor molecule on peroxisomes recognized by core Atg proteins) were unable to proliferate on plant leaves, which revealed that regulation of peroxisome dynamics is essential for the proliferation of methylotrophic yeasts in the phyllosphere.

To adapt to the phyllosphere environment and regulate cellular functions in response to the methanol concentration, methylotrophic yeasts must be able to sense a wide range of methanol concentrations in the phyllosphere. We found that the cell-surface proteins Wsc1 andWsc3 in *K. phaffii* are responsible for sensing the environmental concentration of methanol and for regulating methanol-induced gene expression, i.e., genes encoding proteins involved in peroxisome synthesis and methanol metabolism [23]. Moreover, KpWsc1 and its

downstream MAPK (a mitogen-activated kinase) cascade negatively regulate pexophagy in the presence of methanol (higher than 0.15%) through suppression of Atg30 phosphorylation [24]. These results indicate that Wsc1 regulates not only methanol-induced gene expression followed by the development of peroxisomes, but also pexophagy in response to the methanol concentration sensed by the two distinct signaling pathways (Figure 2).

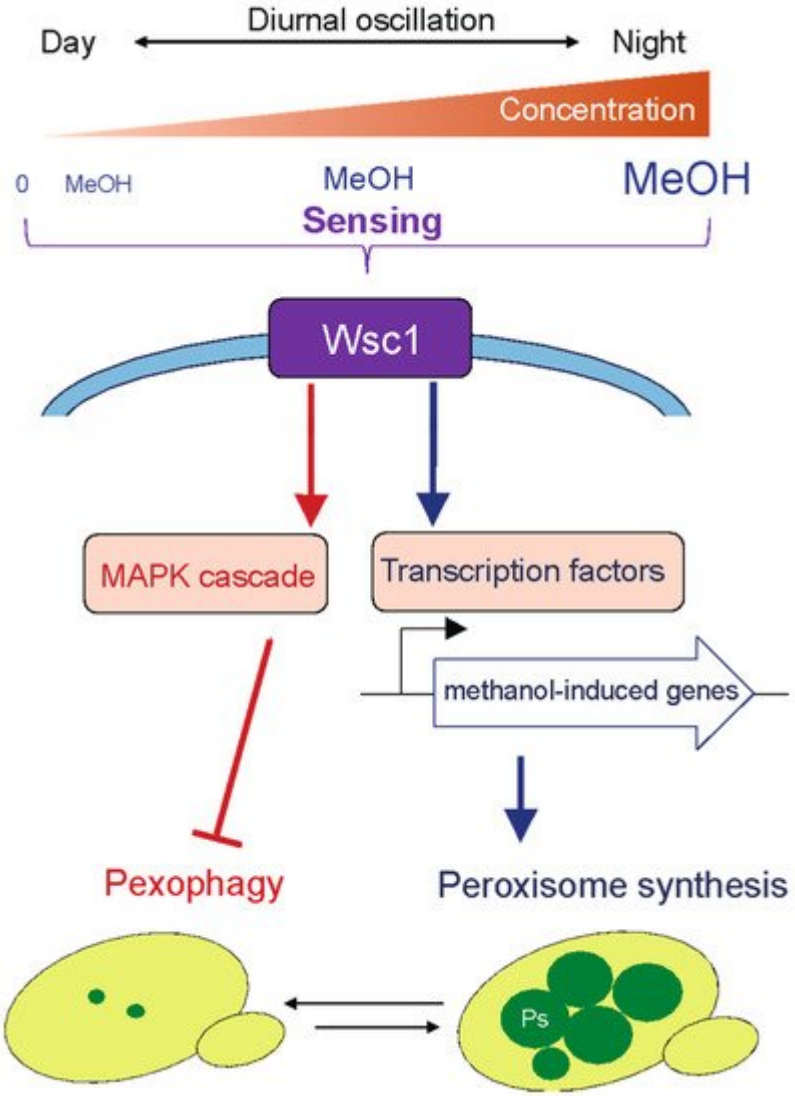

Figure 2. The cell-surface protein Wsc1 senses the methanol concentration in the phyllosphere and regulates peroxisome dynamics in methylotrophic yeast. Wsc1 senses a wide range of methanol concentrations that oscillate diurnally in the phyllosphere. A signal from Wsc1 is transmitted to the transcription factors, activating expression of methanol-induced genes followed by the development of peroxisomes. Under lower methanol concentrations and carbon source-depleted conditions, peroxisomes are degraded by pexophagy. Wsc1 and the downstream MAPK cascade repress pexophagy in the presence of methanol concentrations higher than 0.15%.

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