

Study on the Mechanism of Lipid Droplet Degradation in *Aurantiochytrium limacinum*

呉, 勝

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Name : 呉 勝 (WU SHENG)

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(*Aurantiochytrium limacinum* の油滴分解機構に関する研究)

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Thesis Summary

Thraustochytrids are a group of unicellular marine protists known for their ability to synthesize and accumulate various polyunsaturated fatty acids such as docosahexaenoic acid and eicosapentaenoic acid, which are stored in lipid droplets (LDs). They are widely recognized as important oleaginous microorganisms and are used in lipid research and industrial applications. Like other organisms, thraustochytrids degrade LDs to release fatty acids for energy production under nutrient deprivation. However, the specific mechanisms underlying LD degradation in thraustochytrids remain poorly understood. This study aimed to elucidate the mechanisms and physiological significance of the LD catabolism in thraustochytrids.

In mammals and plants, LDs are primarily degraded through autophagy (lipophagy) and intracellular lipases (lipolysis). Autophagy is an evolutionarily conserved lysosome/vacuole-dependent process responsible for the degradation and recycling of cellular components. However, autophagy in thraustochytrids remains unexplored. To our knowledge, this study is the first to elucidate the autophagic pathway in *Aurantiochytrium limacinum*, a representative genus of thraustochytrids. Autophagy-related (Atg) protein homologs in the draft genome of *A. limacinum* were identified using yeast Atg protein sequences as queries. These proteins exhibit high homology with the Atg proteins in model organisms. A key hallmark of autophagy is the cleavage of the Atg8 C-terminus by Atg4 to expose a glycine residue, followed by its covalent conjugation to phosphatidylethanolamine, facilitated by Atg7 and other Atg proteins, which are essential for Atg8 lipidation and autophagosome formation. Genes associated with this process are highly conserved in *A. limacinum*. Expression of green fluorescent protein (GFP)-tagged Atg8 (GFP-Atg8) in *A. limacinum* showed a cytosolic distribution, and GFP-Atg8 progressively localized to specific vesicles (termed GFP-Atg8 positive vesicles) under glucose depletion. In $\Delta atg4$ and $\Delta atg7$ cells, GFP-Atg8 localization remained unchanged even after glucose exhaustion. However, the expression of a pre-cleaved mCherry-Atg8-Gly construct in $\Delta atg4$ cells partially restored vesicular localization. These findings demonstrate the conservation of autophagic machinery, including the Atg8 lipidation process, in *A. limacinum*.

Following glucose depletion, LDs were observed within GFP-Atg8 positive vesicles in most cells, accompanied by a decrease in triacylglycerol (TAG) content, suggesting potential LD degradation via lipophagy. The characteristics of GFP-Atg8 vesicles were analyzed through co-localization with the endosomal marker Rab5 and labeling with the endocytic tracer FM4-64, revealing that these

vesicles functionally resembled endosomes (termed endosome-like vesicles). Furthermore, acidification of GFP-Atg8 vesicles, resulting from fusion with acidic vacuole-like organelles (VLOs), was confirmed by treatment with chloroquine (CQ), an autophagy inhibitor that prevents the fusion of endosome-like vesicles and VLOs. The spatial association between LDs and FM4-64-labeled membranes further supports the idea that LDs are directly engulfed by endosome-like vesicles via membrane invagination, resembling microlipophagy, where LDs are directly engulfed by lysosomes. Notably, the efficiency of LD internalization into endosome-like vesicles was significantly reduced in $\Delta atg7$ cells, indicating that an intact autophagic pathway is essential for efficient lipophagy in *A. limacinum*.

Interestingly, the intracellular TAG content did not increase upon lipophagy inhibition under glucose starvation conditions in this microorganism. Conversely, TAG levels were significantly decreased in CQ-treated and $\Delta atg7$ cells. Quantification of the total cellular lipase activity demonstrated increased lipase activity in both CQ-treated and $\Delta atg7$ cells. RNA-seq analysis showed the downregulation of lipid synthesis-related genes and upregulation of lipid degradation-related genes when lipophagy was inhibited. Among these genes, the expression of a putative lipase (protein ID: 136024), localized to the LD membrane, was upregulated in both CQ-treated and $\Delta atg7$ cells. Disruption of 136024 led to TAG accumulation and impaired cell growth, whereas its overexpression reduced the TAG content, demonstrating the critical role of 136024 in LD metabolism and cellular growth in *A. limacinum*.

In conclusion, this study revealed a dual regulatory network in which lipophagy orchestrates LD degradation via endosome-like vesicles, while lipophagy inhibition triggers compensatory lipolysis activation to sustain lipid degradation. These findings provide novel insights into the mechanisms underlying lipid degradation and metabolism in thraustochytrids. The insights obtained in this study contribute to the production of valuable fatty acids using thraustochytrids.