Analysis of thermotolerance enhancement mechanism of heat shock metabolite (HSM) produced by actinomycete

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Research objective

We discovered that actinomycetes produce a high-temperature culture-specific substance that is not produced at normal temperature and named this metabolite "Heat Shock Metabolite (HSM)."[1)] This research aimed to analyze what HSM is and how and why it is produced by actinomycetes to discover natural products with novel skeletons and to elucidate their regulatory systems and physiological significance in HSM production. This study aimed to elucidate the thermotolerance-promoting mechanism of maniwamycins, which are HSMs produced by *Streptomyces* sp. JA74. However, the physiological significance of maniwamycins has not yet been reported.

Methods & Results

Effects of maniwamycins on growth at high temperature

First, we attempted to determine why *Streptomyces* sp. JA74 produces maniwamycins in high-temperature culture. We cultured strain JA74 on an agar medium containing maniwamycins, dihydromaniwamycin E, or maniwamycin E, and evaluated the effects of maniwamycins on growth at high temperatures. Normally, the growth of strain JA74 deteriorates at 51°C and becomes inoperable at 52°C. In contrast, maniwamycins tended to promote growth at 51°C and enable growth at 52°C (Figure 1). These results indicated that maniwamycins increased the growth temperature limit of strain JA74 and promoted its growth at high temperatures. Currently, we are evaluating the effects of disruption of the maniwamycin biosynthetic genes on growth at high temperatures. In subsequent analyses, we focused on dihydromaniwamycin E and termed the compound as "DME."

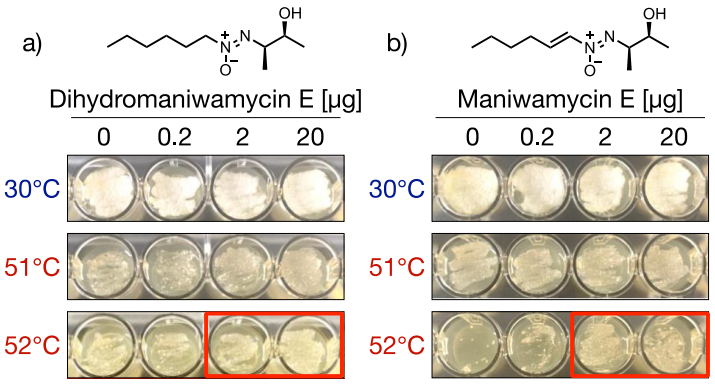

Fig. 1 Effects of dihydromaniwamycin E (a) and maniwamycin E (b) on growth of *Streptomyces* sp. JA74

Analysis of the mechanism of thermotolerance-promoting activity by DME

Although there is some knowledge on the cytotoxicity and antibacterial activity of natural products with azoxy structures, very little information is present on their biological activity. Therefore, there are few clues for understanding their functionality. In order to elucidate the mechanism of the thermotolerance-promoting activity of DME, we attempted to analyze the binding proteins of DME using two methods.

・ Binding protein analysis by "**cell line panel analysis**"

First, we attempted to analyze the binding proteins of DME by "cell line panel analysis," which is performed using the Molecular Profiling Support Activity.[2] Comparative analysis with the sensitivity patterns of various inhibitors to cancer cell lines predicted (**1**) DNA polymerase, (**2**) p38 MAPK, (**3**) BCRP/ABCG2, and (**4**) adenylate cyclase (AC) as candidate binding proteins of DME in human cells. Among these, we decided to focus on (**4**) because the inhibition of (**1**) was expected to cause toxicity, and (**2**) and (**3**) were proteins that are not present in actinomycetes. Adenylate cyclase converts ATP to cAMP, the amount of ATP can be increased by inhibiting DME and using it for growth at high temperatures. Increased energy metabolism is reported to be involved in the high-temperature tolerance of other thermotolerant microorganisms.[3] First, we evaluated the effect of 2′,5′-dideoxyadenosine, a known inhibitor of AC, on the growth of strain JA74. 2′,5′-Dideoxyadenosine showed a tendency to promote growth at high temperature same as with DME (Figure 2a). Furthermore, we tested whether forskolin, known to activate AC, inhibits growth at high temperatures. Forskolin did not exhibit the desired activity (Figure 2b). Because the function of forskolin has been reported in human cells, it is possible that it does not function in actinomycetes. Therefore, we are currently attempting to establish a method for quantifying ATP and cAMP levels during normal- and high-temperature incubations.

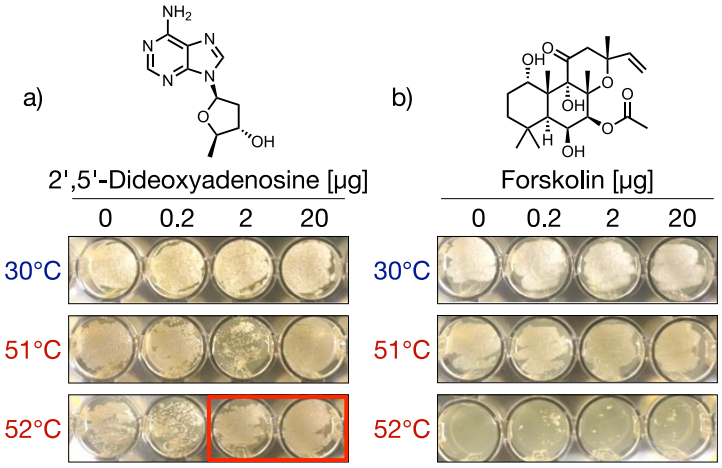

Fig. 2 Effects of Adenylate Cyclase (AC) inhibitor (a) and activator (b) on growth of *Streptomyces* sp. JA74

Binding protein analysis using "**magnetic beads**"

Second, we attempted to identify DME-binding proteins using magnetic beads (TAMAGAWA seiki Co., Ltd, Nagano, Japan). First, we confirmed whether the hydroxyl group of DME is essential for its thermotolerance-promoting activity. We evaluated the activity of the compound in which the hydroxyl group was protected with a benzoyl group and found that it showed growth-promoting activity at high temperatures, similar to DME. Therefore, we immobilized the hydroxyl moiety onto magnetic beads and attempted to identify the binding proteins in the extract of strain JA74. We pulled down the proteins bound to DME using the prepared magnetic beads; however, no protein bands binding to DME were detected (Figure 3). Therefore, we are currently investigating methods for preparing protein solutions and magnetic beads.

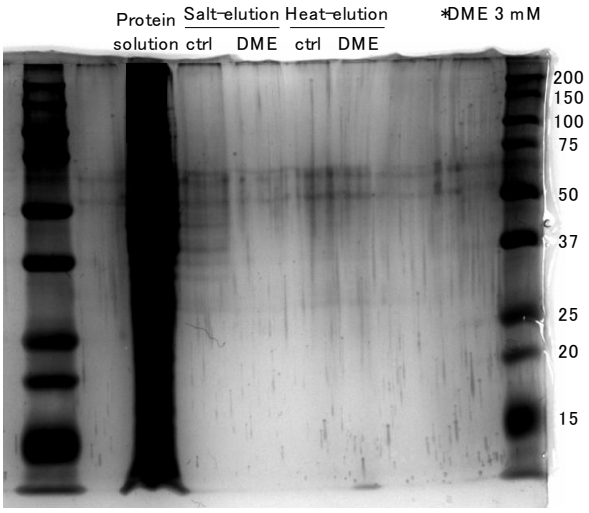

Fig. 3 Analysis of binding proteins

Future plans

In addition to continuing the AC inhibition and magnetic bead analysis of DME, we will focus on the "biological activity" of DME to analyze its thermotolerance-promoting mechanism. There are several examples of other HSMs whose bioactivity in human cells has led to an understanding of thermotolerance-promoting mechanisms. In addition, the biological activity of DME was evaluated and was found to exhibit antiviral and anticancer stem cell activities.[4] As we are currently focusing our analysis on proteins at the cell membrane surface, we would like to link the mechanism of action to our understanding of the thermotolerance-promoting activity of DME.

Conclusion

Maniwamycins, which are HSMs produced by *Streptomyces* sp. JA74, were found to have growth-promoting activity in bacteria that reproduce at high temperature.

References

1) Saito, S., Kato, W., Ikeda, H., Katsuyama, Y., Ohnishi, Y., Imoto, M. (2020) Discovery of "heat shock metabolites" produced by thermotolerant actinomycetes in high-temperature culture. *J. Antibiot.* **73:** 203–210.
2) Molecular Profiling Support Activity (https://www.molpro.jp).
3) Yamada, M., Akada, R., Takasaka, T., Azuma, Y., Hoshida, H., Matsushita, K. (2015) Molecular mechanisms that impart thermostability in thermostable fermentative microorganisms. *Kagakutoseibutsu*. **53:** 763–773.
4) Saito, S. *et al.* (2022) Dihydromaniwamycin E, a heat-shock metabolite from thermotolerant *Streptomyces* sp. JA74, exhibits antiviral activity against influenza and SARS-CoV-2 viruses. *J. Nat. Prod.* **85:** 2583–2591.