Simple Assessing of Calcification Catalyzed by Thermophilic Bacteria

Naoto Yoshida

Department of Biochemistry and Applied Biosciences, University of Miyazaki, 1-1 Gakuen Kibanadai-Nishi, Miyazaki 889-2192, Japan

Abstract:
Several bacterial species have been found to involve the biomineralization phenomenon promoted by them. The formation of calcium carbonate mineral (calcification) is most abundant phenomena as biomineralization by bacteria. Here we introduce the simple assessing method of calcification catalyzed by thermophilic bacteria. To obtain the fresh biomass of thermophilic bacterium, the thermophilic bacteria was cultured on conventional nutrient agar medium at 60°C. Fresh biomass of bacteria is simply placed on calcite promoting hydrogel surface, and incubated at 60°C. After incubation for 24 to 72 h, a number of single crystals can be found in the biomass. This method provides simple assessing for screening of calcification upon thermophilic bacteria.

Keywords: Biomineralization, calcite, thermophilic bacteria, Geobacillus sp., calcite promoting hydrogel

Introduction
Drew has firstly discovered the novel phenomena which bacteria catalyzed the carbonate mineral formation (Drew, 1913). He isolated a denitrifying bacterium able to form calcium carbonate mineral in liquid media and named it “bacterium calcis”. Calcium carbonate mineral includes three type of polymorphs (i.e. calcite, aragonite, and vaterite). Most of the calcium carbonate minerals formed by bacteria are known to be categorized into calcite.

So far, the study on calcite formation by bacterial catalysis provided us the knowledge that there are two types for calcite formation that is 1) urea-type calcite (Stocks-Fischer et al., 1999) and 2) lower fatty acid-type calcite (Boquet et al., 1973; Shaheen et al., 2021). Urea-type calcite is formed using carbon dioxide produced by the hydrolysis of urea with urease. Lower fatty acid-type calcite is formed using formic acid, acetic acid, propionic acid, or butyric acid as raw materials.

The representative bacteria which form urea-type calcite is Bacillus pasteurii (alkaliphilic) (Bachmeier et al., 2002; Dikshit et al., 2022; Hadi et al., 2022), B. megaterium (Sun et al., 2019), B. simplex (Enyedi et al., 2020), B. sphaericus (Adzami et al., 2018). These bacteria express much amount of urease. The formation of lower fatty acid-type calcite is known to involve in the metabolism of Bacillus subtilis (Barabesi et al., 2007, Ferral-Pérez et al. 2020), and halophilic bacteria such as Deleya Halophila (Rothenstein, et al. 2012), Halomonas enribalina (Rivadeneyra et al. 1998), Halobacillus trueperi (Rivadeneyra et al. 2004).

Yoshida et al. has reported first time that thermophilic bacteria catalyze the formation of calcite single crystals (Yoshida et al., 2010). Thermophilic bacterium, Geobacillus thermoglucosidasius NY05 enables to form lower fatty acid-type calcite at 60 degrees using formic acid, acetic acid, propionic acid, pyruvic acid, or butyric acid. Based on studying on formation of
calcite single crystal by thermophilic bacterium, this report presents the simple assessment method for the lower fatty acid-type calcite mineralization by moderately thermophilic bacteria.

Isolation of Thermophilic Bacteria

In this method, the sources of thermophilic bacteria are typically isolated from geothermal soil and high-temperature compost. One examples of medium for bacterial isolation and culture is Soyton Casein digest (SCD) nutrient agar medium (soytone 0.5 g, casein digest 1.5 g, NaCl 5.0 g, agar 1.5% in 1 L of distilled water, pH 7.3). Moderately thermophilic bacteria are easily able to isolated from geothermal soil and compost during incubation at high temperature up to 80˚C. The collected soil is suspended in an appropriate amount in sterile water or a sterile nutrient liquid medium, then the supernatant in soil suspension is applied to the SCD agar medium in Petri dish. Put the Petri dishes in a plastic bag with a zipper to prevent them from drying out and incubate at 60 degrees. If there are bacteria that can grow under the culture conditions at 60˚C, they will form colonies. The colony is lifted and single colony isolation is repeated and purified according to a conventional single colony isolation method.

Preparation of Calcite - Promoting Hydrogel

Distilled deionized water containing 20 mM sodium acetate, 7 mM calcium chloride, and 1.5% (w/v) pure agar was autoclaved and solidified in Petri dish (9 cm in diameter). This was used as calcite-promoting hydrogel.

Assessing for Calcite Mineralization

Moderately thermophilic bacteria, *Geobacillus thermoglucosidasius* NY05 is deposited at type culture in Japan Collection of Microorganisms (JCM) (catalogue No. 31848). This bacterium can be used as representative example in this method. In order to obtain fresh biomass, *G. thermoglucosidasius* was seeded onto soytone-casein digest (SCD) agar medium and cultivated for 18 h at 60°C. Fresh *G. thermoglucosidasius* biomass (10–20 mg as wet weight) was scraped off with a loop and applied to the surface of calcite-promoting hydrogel so that it formed a circle approx. 1 cm in diameter (parent colony) (Fig. 1). This was incubated for 24-72 h at 60°C. Following inoculation of the calcite-promoting hydrogel, visible calcites formation was observed within the parent colony (Fig. 1). Hexagonal-shaped calcites (141.7 ± 13.8 µm) tended to form within the parent colony, whereas spherical or dumbbell-shaped calcites (114.7 ± 7.8 µm) preferentially formed within the parent colony.

![Figure 1. Microscopic observation of calcite formation by *Geobacillus thermoglucosidasius* on calcite forming hydrogel (x 7).](image)

Predicted Way to Form Calcite Using Lower Fatty Acid

The mechanism underlying lower fatty acid-type calcite formation by *G. thermoglucosidasius* NY05 can be explained as follows. Essential factors required for calcite single crystal formation are substances that serve as the nucleus for crystal growth and supply calcium and carbon dioxide. Calcium is supplied from the calcite-promoting hydrogel. Endospore formation is initiated by some of the vegetative cells due to the oligotrophic environment on the calcite-
promoting hydrogel. The oligotrophic environment on the calcite-promoting hydrogel allows vegetative \textit{G. thermoglucosidasius} NY05 cells in the parent colony to initiate endospore formation. Endospore formation progresses while absorbing calcium ions (Yung et al., 2020; Sinnelä et al., 2021); thus, calcium ions are concentrated on the endospore surface. Other vegetative cells can utilize acetate to synthesize glucose via succinate by using the glyoxylate pathway. Newly synthesized glucose is converted into carbon dioxide containing carbon derived from acetate via the tricarboxylic acid (TCA) cycle. Vegetative cells would be a source of carbon dioxide, which is supplied through gluconeogenesis from acetate. Simultaneous sporulation, calcium accumulation, and carbon dioxide generation from vegetative cells induce calcite single crystal growth. Therefore, the state in which endospores coexist with vegetative cells is required to induce calcite single crystal formation. The mechanism driving the self-aggregation of endospores remains poorly understood. Further work is thus required to elucidate the mechanism of \textit{G. thermoglucosidasius} endospore aggregation.

**Conclusion**

The calcite mediated by bacterial metabolism show the significant fluorescent property. The calcite mediated by bacteria without rare earth elements opens the way for the development of calcium carbonate phosphors that may prove to be safe, cost-effective, sustainable, and environmentally friendly.

**Conflict of Interests**

No conflict of interest.

**References**


