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Effect of Doping with Metals, Silicate, And Phosphate Ions on Fluorescence Properties and Morphology of Calcite Single Crystals Synthesized in *Geobacillus thermoglucosidasius* Parent Colonies

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Abstract

Geobacillus thermoglucosidasius cells grown on nutrient agar medium (parent colony) were placed on the surface of a calcite-promoting hydrogel containing acetate and calcium. After incubation at 60°C for 4 days, calcite single crystals of 110-130 μm in diameter were synthesized within the parent colony. The calcite contained 6.6% (atom%) Mg and showed striking fluorescent properties. Changes in the morphology and development of fluorescence intensity of calcite synthesized on calcite-promoting hydrogel doped with different metal ions, silicate ion, and phosphate ion were investigated. Doping with Mg, P, or Sr ions yielded calcite lattices with the respective metal ions substitutionally dissolved into calcium sites. On calcite-promoting hydrogel containing 2 mM Mg ion, the calcite Mg content increased to 22 atom%. Doping of the hydrogel with Al, Si, or P ions yielded calcites with increased fluorescence intensity (190-196% of that of control calcites). Contrary to expectations, doping of the hydrogel with Mn, Sr, Fe, or Co ions yielded calcites with decreased fluorescence intensity (64.4-96.9% of that of control calcites). When the hydrogel was doped with Mg or P ions, the calcite surface became smooth or sheet-like, respectively, in contrast to the rough-surfaced spherical morphology of control calcites. Distinctive crevice structures were observed on the surfaces of calcites synthesized on hydrogel doped with Mn or Al ions. Fluorescence microscopy showed that calcites emitted blue, green, or red fluorescence when illuminated with light at wavelengths of 360-370, 460-500, or 530-560 nm, respectively. Calcites synthesized on hydrogel doped with Al, Si, and P ions emitted stronger fluorescence than control calcites when illuminated with light at 360-370 nm. Our studies have demonstrated that *G. thermoglucosidasius* is useful for the preparation of calcite phosphors in the absence of rare earth elements.

Keywords: Biomineralization; Thermophilic bacterium; *Geobacillus thermoglucosidasius*; Calcite; Fluorescent intensity; Metal ions

Introduction

Biomineralization refers to the processes by which organisms form minerals. Examples of biomineralization include the incorporation of calcium phosphate into teeth and bone [1,2]; deposition in the lithosphere of a silicate ("plant opal") by *Oryza sativa* [3]; and formation in the hydrosphere of shells by mollusks [4,5], otoliths by fish [2], and coccoliths by the alga *Emiliania huxleyi* [6,7]. Notably, these marine organisms combine calcium and bicarbonate ions in seawater to form calcium carbonate crystals.

Bacteria also produce inorganic materials by either intra- or extracellular processes; the resulting crystals are often of nano-scale dimensions and exhibit an exquisite morphology. An example of intracellular biomineralization is the synthesis of magnetite particles by magnetic bacteria; these aligned chains of membrane-enveloped crystals enable the bacteria to migrate along the lines of the earth's magnetic field [8]. An example of extracellular biomineralization is the synthesis of extracellular CdS nanocrystals by the photosynthetic bacterium *Rhodopseudomonas palustris* when grown at room temperature [9]. The formation of these CdS nanocrystals was shown to depend on the activity of a bacterial cysteine desulfhydrase. Another example of extracellular biomineralization is the production of spherical nano (8 nm) ZnS semiconductor particles by immobilized *Rhodobacter sphaeroides* [10]. To date, various species of bacteria have been shown to synthesize phosphorite [11-13], silicates [14], gypsum [15,16], iron goethite [17], and manganese oxide [18,19].

Boquet et al. [20] first reported that formation of calcite by soil bacteria is a general phenomenon. They found that 210 bacterial isolates formed calcite within colonies after 18 days of incubation on solid medium composed of 0.25 g of calcium acetate, 0.4 g of yeast extract, 1.0 g of glucose, 1.5 g of agar, and 100 ml of distilled water (pH 8.0). *Bacillus pasteurii* precipitates calcite during growth in alkaline liquid medium containing urea as the sole energy source [21]. The mechanism for calcite formation by *B. pasteurii* involves a bacterial urease that metabolizes urea to produce CO_2 and ammonia; the resulting increase of pH results in co-precipitation of calcium and carbonate ions to form calcite [22].

In a previous study, we isolated a gram-positive, spore-forming thermophilic bacterium from a high-temperature compost-treatment facility and identified the strain as *Geobacillus thermoglucosidasius* YN05 based on the 16SrRNA gene sequence [23]. Our previous studies indicated that this thermophilic bacterium mediates the formation

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of specific crystals when cultured at 60°C using either solid or liquid medium containing acetate, calcium, and magnesium. Specifically, when a fresh colony of *G. thermoglucosidasius* grown on Trypto-Soya (SCD) nutrient agar was placed on agar hydrogel as a parent colony and then incubated for 4 days at 60°C, single spherical crystals (110-130 μm in diameter) formed extracellularly. Energy-dispersive X-ray (EDX) and X-ray diffraction analyses revealed that the crystals consist of magnesium calcite [23]. Experiments performed using stable carbon isotope–labeled acetate showed that *G. thermoglucosidasius*–mediated formation of calcite depends on carbon derived from acetate. Thus, the formation of calcite by *G. thermoglucosidasius* was predicted to employ a novel mechanism distinct from that used for formation of calcite by *B. pasteurii*. Our previous study also showed that calcite single crystals exhibit specific fluorescence properties, with optimal excitation/emission wavelengths of 369.6 and 446.9 nm, respectively.

Many previous studies have reported industrial applications of fluorescing inorganic crystals consisting of phosphate [24], sulfate [25], or carbonate compounds. Rare earth elements (e.g., Eu or Ce) or rare metals (e.g., Sb or Mn) have been used as luminescent probes. Industrial phosphors are often produced by sintering fluorescent host material with luminescent probes at 800-1200°C. Pan et al. [26] synthesized a Eu-doped calcium carbonate red phosphor by co-precipitation with ammonium bicarbonate at room temperature. Kang et al. [27] reported the synthesis of a Eu-doped calcium carbonate phosphor by simple microwave irradiation at a power of 300 W.

In the present study, we allowed *G. thermoglucosidasius* to biomineralize calcite single crystals on calcite-promoting hydrogels doped with different metal ions or silicate or phosphate ions. We investigated the effect of the doped elemental ions on the fluorescence properties and crystal morphology of the doped calcite and assessed whether the doped elemental ions were substitutionally dissolved into calcium sites in the calcite crystal lattice.

Materials and Methods

Chemicals

Metal salts, silicate, and phosphate were purchased from Wako Chemical Co., Japan. Barium magnesium aluminate (BAM) was provided by Nichia Co. Ltd. Powdered seashell (PSS), which was used as a control, was generated in-house using a mortar and pestle to crush Japanese little neck clam shells to particles of approximately 200 μm in diameter.

Culturing of *G. thermoglucosidasius*

Cultures of *G. thermoglucosidasius* NY05 grown on SCD nutrient agar (Nissui, Japan) were stored at 4°C. Cultures stored in SCD medium were stable for > 1 year under refrigeration (data not shown). Stored *G. thermoglucosidasius* cells were inoculated onto fresh SCD agar and cultured for 14 h at 60°C. The resulting vegetative cells were used as parent colonies for crystal generation.

Formation of calcite single crystals

The standard calcite-promoting hydrogel consisted of a 1.5% (w/v) agar (Nacalai Tesque, Kyoto, Japan) suspension containing 25 mM sodium acetate and 7 mM calcium chloride, which was solidified in 9-cm-diameter Petri dishes. Standard calcite-promoting hydrogel was supplemented with the following salts: $AlCl_3 \bullet 6H_2O$, K_2SiO_4, $MnCl_2 \bullet 4H_2O$, $FeCl_3 \bullet 6H_2O$, $SrCl_2 \bullet 6H_2O$, $Na_2MoO_4 \bullet 2H_2O$, LiCl, $Na_2H_2PO_4$, or $MgSO_4 \bullet 7H_2O$ to 2.0 mM final concentration; $TiCl_4$, $CoCl_2 \bullet 6H_2O$, $NiCl_2 \bullet 6H_2O$, $CuSO_4 \bullet 5H_2O$, or $ZnCl_2$ to 0.01 mM final concentration; or $CdCl_2 \bullet 1/2H_2O$ to 0.005 mM final concentration.

To form a parent colony, an aliquot of 10 mg (wet weight) of fresh *G. thermoglucosidasius* cells was placed in a 1.0-cm-diameter circle on standard or elemental ion–doped calcite-promoting hydrogel and incubated at 60°C for 4 days.

Purification of calcites

As described in a previous report [23], gel solubilization solution (QX1) (Qiagen, Valencia, CA, USA) was used for calcite purification.

Elemental analysis

EDX elemental analyses of purified calcite single crystals were performed using an EMAX-5770 electron microscope equipped with a microanalysis system (Horiba, Tokyo, Japan). The analyses were carried out at an accelerating voltage of 20 kV and a probe current of 0.26 nA.

Morphological observations

Following colloidal gold (50 Å) sputtering, calcite crystals were examined by scanning electron microscopy (SEM) using a Hitachi S-4100 instrument (Tokyo, Japan) operated at an accelerating voltage of 5.0 kV. Light microscopic observations of calcite morphology were carried out as described previously [23].

Evaluation of fluorescence intensity

The fluorescence intensity of calcites synthesized on hydrogels doped with different elemental ions was evaluated using 96-well plates (Nunc, Denmark) and a GENios fluorescent plate reader (TECAN, Durham, NC, USA). A total of 5 μg of purified calcite single crystals was added to each well. To compare the fluorescence intensity of various calcites, equivalent amounts of calcium carbonate (Wako Chemical Co.) and PSS were added to each well. The fluorescence intensity of 20 μg of BAM, a commercially available phosphor, was measured as a positive control. Changes in the fluorescence intensity of calcites were assessed using a plate reader at excitation and emission wavelengths of 360 and 465 nm, respectively.

Fluorescence microscopy observations

Calcites synthesized under *G. thermoglucosidasius* mediation were observed under an Axioskop 2 plus fluorescent microscope (Zeiss, Oberkochen, Germany) and an Eclipse-ME600 polarized microscope (Nikon, Tokyo, Japan). For fluorescence microscopy, the following filter blocks were used: exciter 360-370 nm, 460-500 nm, and 540-550 nm; dichroic mirror 380 nm, 505 nm, and 570 nm; emitter > 399 nm, 510-560 nm, and 573-648 nm.

Results

Elemental analysis

Our previous report demonstrated that calcites are synthesized within parent *G. thermoglucosidasius* colonies [28]. EDX analysis was used to determine the elemental ratio (except for carbon and oxygen atoms) of calcite crystals synthesized on elemental ion–doped calcite-promoting hydrogels. Analysis of the reagent-class calcium carbonate (Wako Chemical Co.) used in this study indicated a 100% elemental ratio for Ca (Figure 1). When calcite-promoting hydrogel was doped with metal ions at a concentration of 2 mM, calcites containing each of the tested metal ions were observed within the parent colony, with the following exception: doping with 2 mM Ti, Co, Cu, Ni, Zn, or

Cd ions resulted in strong inhibition of calcite synthesis. When the doping concentration of Ti, Co, Cu, Ni, or Zn ions was reduced to 0.01 mM, calcites were synthesized within the parent colony. *Geobacillus thermoglucosidasius*–mediated calcite synthesis was strongly inhibited by Cd ion even at 0.01 mM, however, indicating that Cd is the most toxic of the elements (ions) tested in this study. When Cd ion was doped at 0.005 mM, calcites formed within the parent colony.

Elemental analysis of control calcites synthesized on standard calcite-promoting hydrogel revealed a Mg content of 6.6% (atom%) (Figure 1). Thus, calcite single crystals synthesized by the mediation of *G. thermoglucosidasius* consist of a magnesium calcite–type calcium carbonate. Doping of the calcite-promoting hydrogel with elemental ions inhibited the incorporation of Mg ions into the calcite crystal lattice. Doping with Fe, Al, Si, or P ions decreased the Mg content of calcite synthesized within the parent colony to 1.07, 1.01, 0.36, and 0.62% (atom%), respectively. Doping with Cd ion decreased the Mg content of calcite synthesized within the parent colony to 0.61% (atom%). The Mg content was clearly maximized in calcites that had been synthesized on calcite-promoting hydrogel doped with 2 mM Mg, with the Mg content of calcite single crystals increased to 22% (atom%). Doping with Zn ion did not result in the coordination of Zn into the calcite lattice but did increase the calcite Mg content by 8.08% (atom%).

As observed with Mg, doping with P, Mn, or Sr ions resulted in coordination of these ions into the calcite lattice (Figure 1). Coordination of the remaining elements into the calcite lattice was not observed. The P and Sr content in calcite synthesized within the parent colony was 38.8 and 30.1% (atom%), respectively. In contrast, the content of Mn was low, at 2.3% (atom%), in calcite synthesized in parent colonies grown on hydrogel doped with Mn ion.

Morphological observations

The morphology of the calcites synthesized within parent colonies as examined by SEM are shown in Figure 2. A variety of calcite single crystal morphologies were observed, the most common being a spherical form. The diameter of these calcite spheres was usually between 50 and 150 μm, although the diameter of some crystals was as large as 200 μm or as small as 20 μm. The surface of the calcites appeared to be covered with a scale-like layer of more or less porous material.

Figure 2 shows the morphology of control calcite single crystals synthesized on standard calcite-promoting hydrogel containing only sodium acetate and calcium chloride: semi-spherical, diameter of 110-130 μm, with a rough surface. Doping with Mg or Sr ions yielded crystals with distinct morphologies. When calcite-promoting hydrogel was doped with Mg ion, *G. thermoglucosidasius* formed smooth-faced spherical crystals with diameters of 50-70 μm. Several crystals appeared to be twins or aggregates of the spherical structure. When hydrogel was doped with Sr ion, crystals formed as smooth-surfaced dumbbell or spherical shapes. In hydrogel doped with Mn ion, pseudo-polyhedral calcites with bubble-like structures larger than 70 μm also were observed. Our microscopic observations of calcite single crystals demonstrated that doping with Mg, Sr, or Mn ions yields calcites with generally spherical forms. The calcite single crystals synthesized on hydrogel doped with Al ion were much more heterogeneous in morphology, involving angular crystals with scale-like surfaces. Hydrogel doped with Si ion yielded angular crystals with cleavage planes; scale-like structures also appeared to grow from the surface of the cleavage planes. Doping of the hydrogel with P ion resulted in calcites with tabular forms. *Geobacillus thermoglucosidasius*–mediated synthesis of calcite crystals yielded a range of morphologies, including spherical, tabular, and angular crystals in the size range of 20-200 μm. The shape of a crystal must reflect the nature of the environment in which crystal grows [29].

Evaluation of fluorescence intensity

As shown in Figure 3, standard reagent-class calcium carbonate (Wako Chemical Co.) and PSS yielded fluorescence intensities of 1,866 and 2,256, respectively. Calcite single crystals synthesized within the parent colony on standard calcite-promoting hydrogel (containing only sodium acetate and calcium chloride) showed a fluorescence intensity of 14,277. Thus, the fluorescence intensity of calcites synthesized in *G. thermoglucosidasius* parent colonies was 6.3-7.6 times higher than that of standard reagent-class calcium carbonate and PSS. The doping of standard hydrogel with Mg, Al, Si, or P ions resulted in a notable

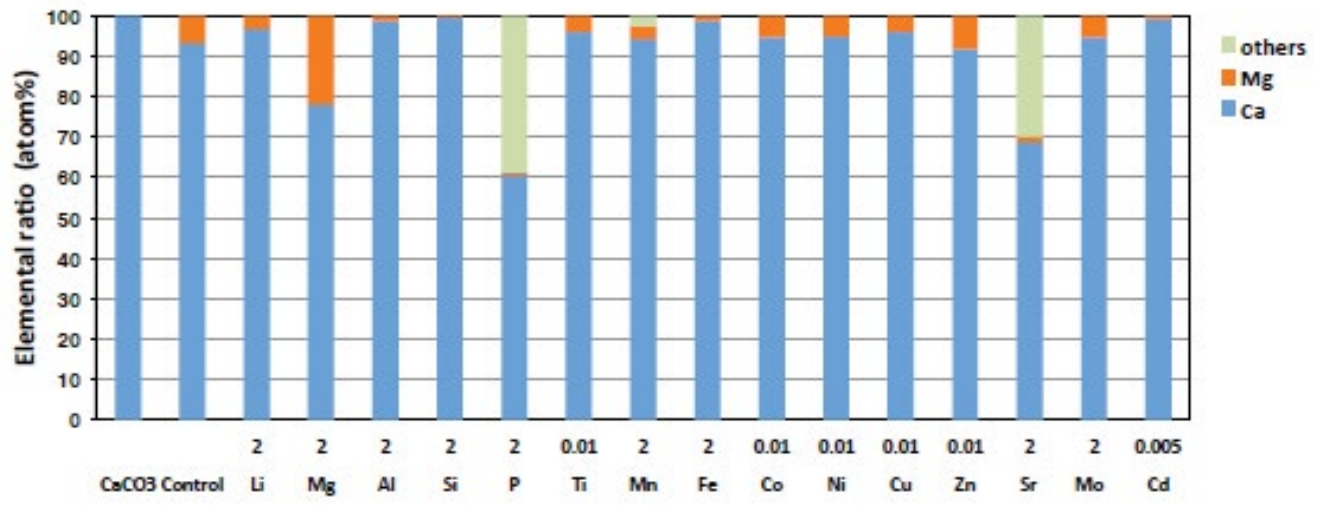

Figure 1: Elemental analysis of calcites synthesized on calcite-promoting hydrogels doped with different metal ions. The labels "$CaCO_3$" and "control" indicate reagent-class pure calcium carbonate and calcite synthesized on standard calcite-promoting hydrogel, respectively. Numerals indicate the concentration (mM) at which each ion was doped in the respective standard calcite-promoting hydrogel.

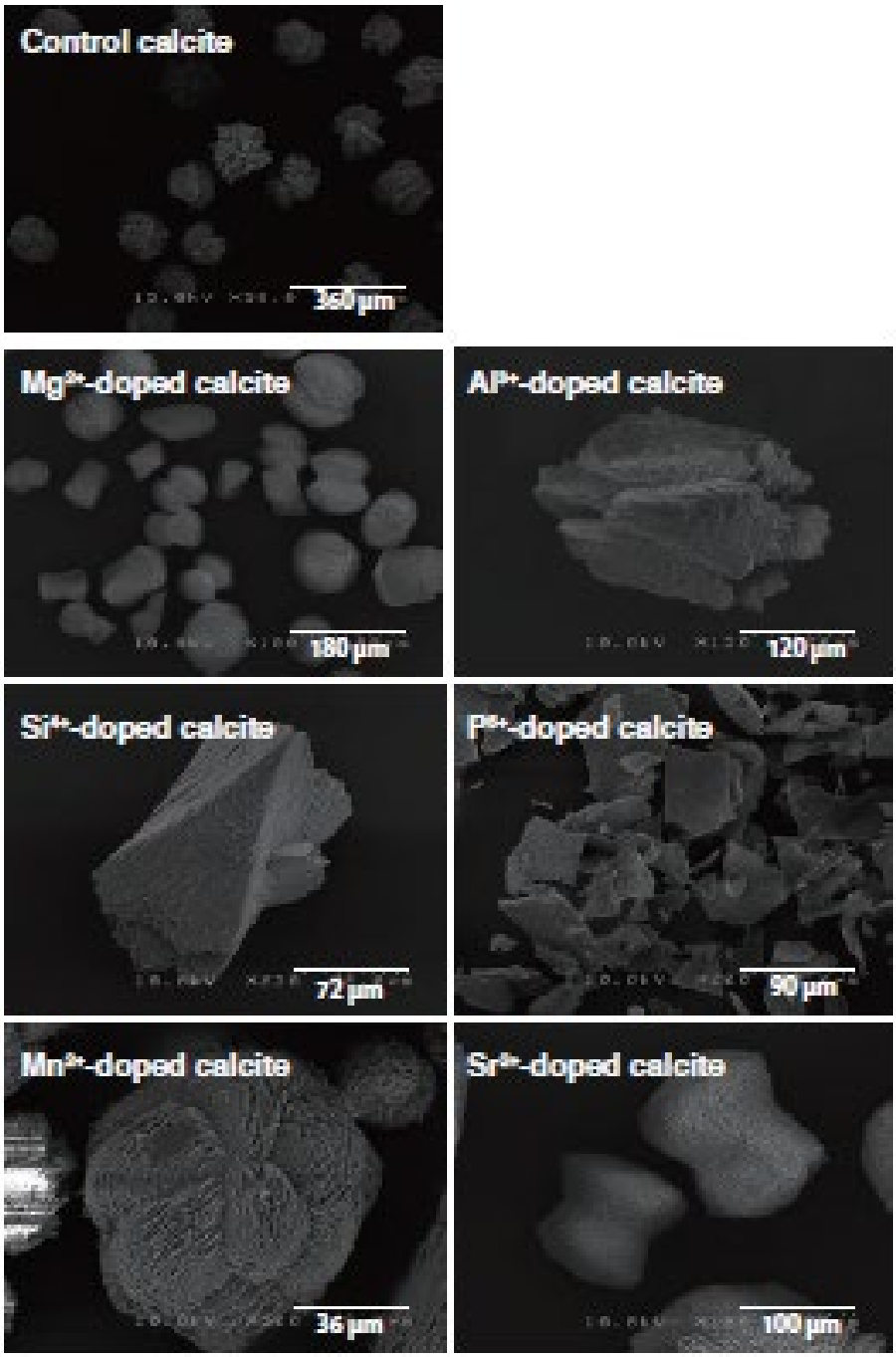

Figure 2: Scanning electron microscopy observations of calcite crystals synthesized within parent colonies. Calcite-promoting hydrogel was doped with different metal ions, as indicated in the upper left of each photograph. Control calcite was synthesized using standard calcite-promoting hydrogel.

increase in the fluorescence intensity of calcites synthesized within the parent colony (23,572; 27,895; 27,900; and 27,187, respectively). In contrast, the fluorescence intensity of calcites synthesized on hydrogel doped with Co, Cu, Fe, or Mo ions decreased, with values that were 85.9-97.8% of the control calcite intensity. Doping of calcite-promoting hydrogel with Li, Ni, Zn, or Cd ions resulted in increased fluorescence intensity of calcites synthesized within the parent colony (19,895; 15,919; 18,207; and 21,585; respectively). In contrast, doping with Sr or Mn ions, which were coordinated into the calcite crystal lattice, resulted in decreased fluorescence intensity, with values falling to 96.9 and 64.4%, respectively, of the fluorescence intensity of the control calcite. Doping with Al, Si, or P ions resulted in fluorescence intensities that were 190-196% of the control calcite fluorescence intensity. The fluorescence intensity of calcite synthesized within the parent colony on Cd-doped hydrogel was 151% of the control calcite fluorescence intensity. Statistical significance was accepted at the $P < 0.05$ level. As a

phosphor control, we assayed BAM ($[Ba, Eu^{2+}]MgAl_{10}O_{17}$), a compound that is widely used as a blue-emitting phosphor for plasma display panels [30]. Manufactured BAM (20 μg) exhibited a fluorescence intensity of 24,831.

Fluorescence microscopy observations

The fluorescence properties of the calcites synthesized on hydrogels doped with elemental ions were characterized using fluorescence microscopy. Light and fluorescence microscopic images of calcite single crystals synthesized within *G. thermoglucosidasius* parent colonies are shown in Figure 4. Control calcite emitted blue, green, and red light when excited at 365 ± 5 nm, 480 ± 20 nm, and 545 ± 15 nm, respectively. Calcite crystals formed by *G. thermoglucosidasius* mediation, on the other hand, were excited by illumination with light in the wavelength interval 260 to 400 nm; the emission wavelength ranged from 350 to 600 nm [23]. Such a wide emission wavelength interval is a novel fluorescence property of *G. thermoglucosidasius*–mediated calcite crystal synthesis. As shown in Figure 3, doping of calcite-promoting hydrogel with Mg, Al, Si, or P ions enhanced the fluorescence intensity of calcite crystals. Fluorescence microscopy also showed that these calcite crystals emitted marked fluorescence. Similar to control calcite, calcites synthesized on hydrogel doped with Mg, Al, Si, or P ions emitted blue, green, and red light when excited at 365 ± 5 nm, 480 ± 20 nm, and 545 ± 15 nm, respectively. Notably, calcite single crystals synthesized on hydrogel doped with P, Si, or Al ions emitted stronger fluorescence than control calcite.

The BAM phosphor used in the experiment took the form of a fine white powder. The wavelength for excitation of BAM is known to be limited to a narrow ultraviolet region. Consistent with previously reported results [30], we observed that BAM fluoresces blue when excited by light in the wavelength range 360-370 nm but does not fluoresce when exposed to light of in the wavelength ranges 460-500 nm or 530-560 nm.

Discussion

In the present study, acetate and calcium were included in the basic calcite-promoting hydrogel. Although Mg was not included as a component of the calcite-promoting hydrogel, calcite crystals formed on this substrate did include Mg at an elemental concentration of 6.6%. We hypothesized that this Mg was derived from the cytoplasm of *G. thermoglucosidasius* cells. We demonstrated that 1.0 mg (dry weight) of *B. subtilis* vegetative cells contained 0.2 μg of Mg; an equivalent dry weight of *G. thermoglucosidasius* cells contained 2.2 μg of Mg (data not shown). It is therefore possible that *G. thermoglucosidasius* contains markedly higher levels of Mg compared with other gram-positive bacteria. Our observations support the above hypothesis. Doping of calcite-promoting hydrogel with metals other than Mg inhibited incorporation of Mg into the calcite lattice. The highest inhibitory effects were seen when doping with Al, P, Fe, Sr, or Cd ions (Figure 2). These ions were assumed to strongly interact with Mg ions, thus inhibiting the incorporation of Mg into the crystal lattice. It is assumed that Sr, an element homologous to Mg and Ca, is incorporated into the calcite lattice by substitution for Mg and Ca.

Our results demonstrated that Mg, P, Mn, or Sr are substituted for Ca in calcites synthesized under the mediation of *G. thermoglucosidasius*. Previous reports have also demonstrated that these elements can substitute for Ca in calcite [13]. Novel calcites synthesized by doping with P, Mn, or Sr might serve as promising materials for material science and bioremediation applications. Recently, Sánchez-Román et al. [13] reported that any of 19 species of moderately halophilic bacteria are capable of precipitating calcite crystals when grown on nutrient medium supplemented with 7.5% NaCl. The resulting calcites were found to contain small amounts of P when assessed by EDX. The effects of growth kinetics, temperature, and solution composition on Mn incorporation into calcite have also been investigated [31]. Mn-bearing overgrowths on calcite seeds were precipitated at 10, 25, and 50°C from solutions with various calcium molar ratios over a range of precipitation rates. The substitution of Sr with Ca in calcite crystals was investigated by Fujita et al. [32].

In the present work, the Mg content of calcites synthesized on calcite-promoting hydrogel containing Al, Fe, or Cd was reduced markedly, falling to levels of 1.07% or less. Calcites biomineralized on calcite-promoting hydrogel doped with Al or Cd ions displayed enhanced fluorescence intensity, but we did not observe a correlation between fluorescence intensity and calcite Mg content. We demonstrated that P, Mn, and Sr are incorporated into the crystal lattice of calcites synthesized on hydrogels containing ions of the respective element. Doping with P ion enhanced the fluorescence intensity,

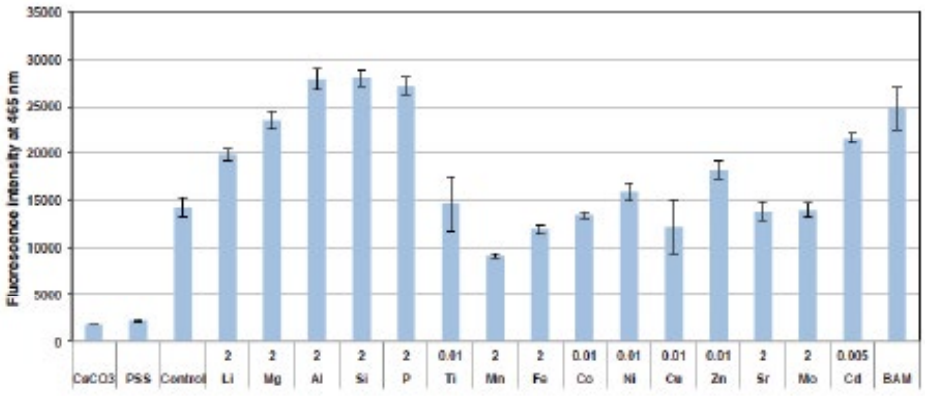

Figure 3: Fluorescence intensity at 465 nm of calcite synthesized on calcite-promoting hydrogels doped with different metal ions. The labels "$CaCO_3$", "PSS", and "control" indicate reagent-class pure calcium carbonate, powdered seashell, and calcite synthesized on standard calcite-promoting hydrogel, respectively. Numerals indicate the concentration (mM) at which each ion was doped in standard calcite-promoting hydrogel. The label "BAM" indicates barium magnesium aluminate. Values represent the mean ± SD fluorescence intensity for three independent experiments.

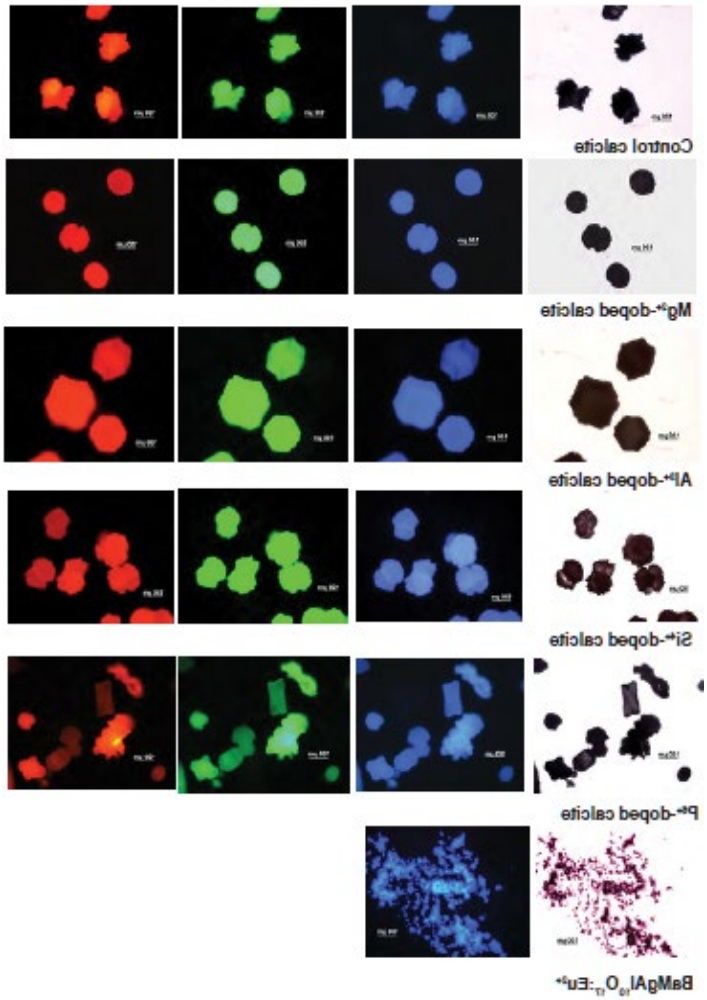

Figure 4: Fluorescence microscopy observations of calcite synthesized within parent colonies. Control calcite was synthesized using standard calcite-promoting hydrogel. Elemental ion-doped calcites were synthesized using calcite-promoting hydrogels doped with the indicated elemental ions at a concentration of 2 mM. Blue, green, and red emissions were observed at excitation wavelengths of 360-370 nm, 460-500 nm, and 530-560 nm, respectively.

whereas doping with Mn or Sr ions reduced the fluorescence intensity of the synthesized calcite. Al and Si enhanced the fluorescence intensity, but these elements were not themselves incorporated into the crystal lattice. Consequently, only P behaved as an activator, enhancing the fluorescence intensity of calcite by substituting for Ca. Incorporation of activator ions into the host lattice was affected by the coordination number of the activator and the crystal structure of the host material. During orientation of the developing crystal lattice, Al, Si, P, or Cd ions may affect interactions between Ca and CO_2, causing Ca-C bonds to form in the crystal, resulting in an unstable lattice. We postulate that these interactions enhance the fluorescence intensity of the resulting calcites.

Due to their high fluorescence and superior chemical stability, a recent study examined the preparation and characteristics of carbonate calcium fluorescent materials doped with rare earth. Red phosphors based on Eu ion are potential activators that provide strong red emission; efforts to develop applications for these phosphors have focused primarily on their use with calcium carbonate [27]. Red phosphor Eu ion was doped into calcite using a co-precipitation method, indicating that this material produces an intense red emission due to substitution of Eu ion for Ca in the calcite crystals [26].

Conclusions

Our studies have demonstrated that the thermophilic bacterium *G. thermoglucosidasius* is useful for the preparation of calcite phosphors in the absence of solid solutions of rare earth elements [23]. Biological methods for the synthesis of Al, Si, or P ion–doped calcite mediated by *G. thermoglucosidasius* 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. We may expect new industrial applications for calcite phosphors synthesized by *G. thermoglucosidasius* mediation, in which the fluorescence intensity is enhanced by synthesis on calcite-promoting hydrogel doped with Al, P, or Si ions due to their fluorescence stability without loss of intensity.

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