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The Up-regulation of Carbonic Anhydrase Genes of Bacillus mucilaginosus under Soluble Ca\(^{2+}\) Deficiency and the Heterologously Expressed Enzyme Promotes Calcite Dissolution

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Molecular mechanisms and gene regulation are of interest in the area of geomicrobiology in which the interaction between microbes and minerals is studied. This paper focuses on the regulation of the expression of carbonic anhydrase (CA) genes in Bacillus mucilaginosus and the effects of the expression product of the B. mucilaginosus CA gene in Escherichia coli on calcite weathering. Real-time fluorescent quantitative PCR (RT-qPCR) was used to explore the relationship between CA gene expression in B. mucilaginosus and promotion of calcite dissolution under condition of Ca\(^{2+}\) deficiency. The results showed that adding calcite to the medium, which lacks Ca\(^{2+}\), can up-regulate the expression of the bacterial CA genes to accelerate calcite dissolution for bacterial growth. CA genes from B. mucilaginosus were transferred into E. coli by cloning. We then employed crude enzyme extract from the resultant E. coli strain in calcite dissolution experiments. The enzyme extract promoted calcite dissolution. These findings provide direct evidence for the role of microbial CA on mineral weathering and mineral nutrition release.

Keywords: Bacillus mucilaginosus, Ca nutrition acquisition, carbonic anhydrase, geomicrobiology, heterologous expression, RT-qPCR

Introduction

Mineral weathering can cause the migration of mineral elements (Alekseev et al. 2003; Meunier et al. 2007) and affect geochemical processes (Goddersis et al. 2010; Hazen et al. 2008). Physical weathering, chemical weathering and biological weathering are the three main forms of mineral weathering (Warscheid and Braams 2000; Wilson 2004). However, biological weathering involves biochemical and physical processes, such as the secretion of organic acids (McMaster 2012; Neaman et al. 2005; Valix et al. 2001), complexation (Koretsky 2000; Lian et al. 2008b), oxidation-reduction (Brown et al. 2003; Shelobolina et al. 2012; Shock 2009) and bio-mechanical action (Bonneville et al. 2011; Chen et al. 2000). Some materials secreted by organisms has made it easy to identify associations between biological enzymes and mineral weathering (Fru 2011; Xiao et al. 2012a) and to determine whether organisms can affect the weathering rate through enzyme secretion.

Carbonic anhydrase (CA) is widely distributed in animal cells (Jackson et al. 2007; Supuran and Scozzafava 2007), plant cells (Bradfield 1947; Moroney et al. 2001; Sunderhaus et al. 2006) and microorganisms (Bahn et al. 2005; Hewett-Emmett and Tashian 1996; Smith and Ferry 2000). It is capable of catalyzing the hydration reaction of atmospheric and self-generated CO\(_2\) and the reverse reaction \(\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-\) (Meldrum and Roughton 1933; Smith and Ferry 2000; Tripp et al. 2001; Xu et al. 2008). H\(^+\) can promote the dissolution of carbonate rocks, which, in turn, affects the CO\(_2\) cycle. CA from bovine erythrocytes can affect the dissolution of carbonate minerals in phosphate ores under acidic conditions (Papamichael et al. 2002). The dissolution rate for limestone increases about 10-fold at high \(p_{\text{CO}_2}\) values after addition of bovine carbonic anhydrase (BCA) (Liu et al. 2005). With dolomite, the dissolution rate with BCA increases about 3-fold at low \(p_{\text{CO}_2}\) values. These results suggest that the degree of acceleration of carbonate dissolution by CA is different between limestone and dolomite. Demir et al. (2012) also showed that BCA is able to promote
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dolomite dissolution. However, this evidence is insufficient to conclude that CA plays an important role in biogeochemical processes on the Earth’s surface in general.

The weathering in which microorganisms participate is partly related to their need for mineral elements (Mailloux et al. 2009). Because of the great variety, wide distribution and very active metabolisms of these organisms, microorganism-induced weathering of minerals and rocks has attracted a great deal of attention (Jacobson and Wu 2009; Lepleux et al. 2012; Luttege and Conrad 2004; Shirokova et al. 2012; Stockmann et al. 2012; Uroz et al. 2007). CA genes are present in almost all organisms (Vanta et al. 1983), so the study of the function of CA on mineral weathering appears to be very important. Xiao et al. (2012b) showed that a higher expression level of the Aspergillus fumigatus CA gene favored weathering of potassium-bearing rocks. They speculated that CA might promote generation of H2CO3, promoting weathering of silicate minerals and facilitating the release of K+. CA can be detected in different soils in the Karst region (Li et al. 2005b). This suggests that CA is widely distributed in the soil of Karst areas. Li et al. (2005a) detected microbial CA activity in column-built leached soil-limestone Karst systems. They surmised that CA secreted from microbes might be a major factor influencing the release of Ca2+ in natural systems. In addition, microbial extracellular CA could accelerate the release of Ca2+ from limestone (Li et al. 2007; 2009). These results implied that microbial CA might have a significant influence on the bio-karst process. In Li’s experiments, a crude enzyme solution containing CA was made from cell secretions. The composition of the solution was very complex. Other substances, such as organic acids, may have affected limestone dissolution. This made it difficult to determine the contribution of each component of the microbial secretions to limestone dissolution. Zhang et al. (2011) showed that CA activity increased as the amount of limestone in the medium increased. This increasing trend was limited only within a certain range, and enzyme activity did not directly reflect the relationship between the expression of CA and mineral weathering. Although the basis for mineral weathering by microbial CA was investigated in earlier studies, some aspects need clarification. Genomes of mineral weathering microbes generally contain several CA genes; it is unclear whether these genes are different from each other, whether all of them are relevant to mineral weathering and, if so, whether their respective gene products exhibit CA activity. To resolve these issues, molecular biology must be used to study the relationship between the expression and regulation of microbial CA genes and mineral weathering. Molecular biology can also be used to analyze the activity of the product of any single CA gene.

Real-time fluorescence quantitative PCR (RT-qPCR) can be used to detect gene expression at specific times (Iskandar et al. 2004; Nolan et al. 2006). An E. coli expression system, which is the most common type of expression system, was used in the present study (Banerjee et al. 2009; Small et al. 2010; Sorensen and Mortensen 2005). The E. coli expression system is preferred because of its low cost, high productivity and easy operation. An in-depth study of the function of almost any gene can be performed by combining RT-qPCR and E. coli expression.

In our research group, we often use Bacillus mucilaginosus to study the interactions between microbes and minerals (Mo and Lian 2011; Xiao et al. 2012a). Zhang et al. (2011) showed B. mucilaginosus can capture atmospheric CO2 by carbonic anhydrase. Alternatively, this strain is widely used in China and surrounding countries as a plant growth-promoting rhizobacterium (PGPR) (Singh et al. 2010) and can be used as fertilizer. The B. mucilaginosus K02 genome (GenBank database accession number: HM579819) was sequenced and five CA related genes (Gene ID were 12734710, 12739330, 12735171, 12735237 and CP003422 region: 5453463-5454707) were annotated. RT-qPCR was used to explore the relationship between gene expression and mineral weathering. Five gene amplification products were transferred into E. coli, forming recombinant strains. The enzymatic activity of heterologously expressed proteins was determined, and the protein showing the highest activity was selected for calcite dissolution experimentation. The results showed that CA genes expression responds to soluble calcium deficiency and that heterologously expressed protein plays a role in calcite dissolution. The CA genes also provide a new means of investigating the role and regulatory mechanism of microbial CA in biogeochemical processes.

Materials and Methods

Real-time Fluorescent Quantitative PCR

Preparation of calcite powder
Calcite was selected as a typical representative mineral of carbonate rocks. The calcite, from a Karst region of Guizhou province of China, was crushed and a greater than 200-mesh fraction was collected by sieving. The calcite powder was washed by suspension in sterilized ultrapure water (SUW) to eliminate the fine dust from the grinding procedure. The powder was then rinsed three times with ethyl alcohol. The cleaned powder was dried in a convection oven. X-ray diffraction analyses were carried out to identify the mineralogical composition (not shown here). The results showed it was almost pure calcite.

RT-qPCR

RT-qPCR was used to determine whether the calcite could promote the expression of all five CA genes of the B. mucilaginosus strain. Control cultures of B. mucilaginosus were grown in nitrogen-containing medium having the following composition per liter: sucrose 10.0 g, (NH4)2SO4 1.0 g, CaCl2 0.44 g, MgSO4 0.5122 g, KCl 0.1 g, and Na2HPO4·12H2O 2.507 g. Experimental cultures were grown in the same medium, except that the 0.44 g of CaCl2 were replaced by 0.2 g powdered calcite. After 2 d, 4 d or 6 d of culture, the bacteria were centrifuged (11500×g, 4°C, 1 min).

The supernatant was discarded, and the collected cell was frozen in liquid nitrogen. Total RNA was extracted (Invitrogen, following the manufacturer’s instruction) and reverse transcribed into cDNA. The RT-qPCR reaction system and
reaction conditions followed the manufacturer’s instructions of TaKaRa. As an internal reference, 16S rRNA was used (Table S1). The Ct value was recorded for subsequent analysis. After optimizing the primers by testing different primers, a single melting temperature was determined for each of the 6 pairs of primers (not shown here). The mean of ΔΔCt was set as zero when the bacteria were cultured using the only calcium source, CaCl₂, on the second day. The relative expression level (REL) was calculated using the following formula: 

\[ REL = 2^{-\Delta\Delta Ct} \]

Construction of Transformants

To verify that heterologously expressed CA can promote calcite dissolution, five clones of all of the CA genes were constructed. A modified SDS-CTAB method (Niu et al. 2008) was utilized to extract genomic DNA because of the secreted extracellular polysaccharide. PCR was performed as described in the molecular cloning section (Sambrook and Russell 2002). The forward and reverse primers (ps1, ps2, ps3, ps4, and ps5) were designed with Primer Premier 5.0. They are listed in Table S2. The amplified fragments were named TCA1, TCA2, TCA3, TCA4, and TCA5. For in vitro amplification, PCR was performed in accordance with the manufacturer’s instructions for DNA polymerase (Sangon Biotech (Shanghai) Co., Ltd.). The identities of the PCR products were verified using agarose gel electrophoresis. Plasmid pET30a (+), which contained the kanamycin resistance gene (Kan R), was used as the vector. The purified PCR products were digested at the Kpn I and Hind III sites introduced by the primers and then inserted into Kpn I and Hind III-restricted pET30a (+) to create pXLL01, pXLL02, pXLL03, pXLL04 and pXLL05. Recombined plasmids were transferred into competent cells to form transformants. Five kinds of plasmids were digested with restriction enzymes to confirm whether the recombinant plasmids were correct. Finally, the recombinant plasmids were sequenced.

Protein Expression, Verification and Determination of Protein Activity

The pET30a (+) and five types of recombined plasmids were transferred into E. coli BL21 (DE3) competent cells. After isopropyl β-thiogalactopyranoside (IPTG) induction, cultivation was continued for an additional 6 h. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify protein expression. One mL of culture solution was centrifuged (16,200 × g, 4˚C, 5 min). The supernatant was discarded and the cell precipitate was re-suspended in 300 μL lysis buffer (1 mM EDTA and 20 mM Tris-HCl, pH 8.0). The cell wall was broken by ultrasonication in 3 × 10 s at 10 s intervals. One hundred microliters of suspension was transferred to a clean centrifuge tube and the remainder was centrifuged as described above. The loading buffer (2×: LD: 1 M Tris-HCl [pH 6.8] 100 mM DTT 3 g, 10% SDS 400 mL, Bromophenol blue 2 g, glycerol, 240 mL) was added to the suspension and the supernatant in a volume ration of 1:1, respectively.

All proteins and the marker were treated at 95˚C for 5 min. SDS-PAGE was performed using a modified version of the SDS-PAGE Laemmli protocol. Proteins were stained using Coomassie brilliant blue R-250. Decolorization was performed until the band appeared clear. For Western blot analysis, after the samples were electrophoresed, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes for immunoblotting. The membrane was blocked, and the blots were probed with double antibodies. The membrane was immersed in TMB substrate and kept in the dark for 10 min. Finally, protein bands were visualized on PVDF membranes.

Enzyme activity was measured by a method reported in a previous study (Pocker and Stone 1967). Phosphate buffer was prepared at different pH values (6.5, 7.0, 8.0, 9.0 and 9.5). The preparation process for crude enzyme solution (CES) from 90 mL of bacterial suspension followed the description mentioned above for protein preparation. Lysis buffer was replaced by SUW and 2 mL cell resuspension solution was broken by ultrasonication in 6 × 10 s at 10 s intervals. The enzyme activity was calculated by the optical density (OD) value of the color of the product of the enzyme reaction. When OD was determined, the OD value of the reaction solution containing CES, which was from the E. coli containing pET30a, was set as zero as the control. Then the enzyme activities of CESs from five recombinants were determined.

Calcite Dissolution

First, 0.0500-0.0505 g of powdered calcite was added to the 100 mL volume flask in three replicates. CES was obtained from the ultrasonication of E. coli containing pET30a (+) and pXLL04, named CA0 and CA4, respectively. CA0 and CA4 were obtained using the method described above (protein activity section). It is clear that CA0 only contain CA from expression of E. coli genome rather than heterologous expression. Fifty milliliters of SUW containing 200 μL of BCA (2.5 × 10⁻⁶ g/mL), CA0 or CA4 were added to the flasks named as the BCA group, CA0 group and CA4 group, respectively. Finally, the flasks were placed in a rotary shaker (140 rpm, 35˚C).

After 0 min, 10 min, 20 min, 30 min, 60 min, 120 min and 240 min, three flasks containing calcite particle suspension with CA0 and three others containing calcite particles with CA4 were sacrificed and their content filtered by use of separate 0.45 μm filter membranes. The remaining reaction solution was discarded after sampling. The Ca²⁺ in the 20 mL filtered reaction solution from the controls and experimental setups was titrated using ethylenediaminetetraacetic acid disodium salt (EDTA-Na₂) referring to published literature (Nielsen 2010). Because the calcite was 99% pure, containing just a small amount of quartz, and because the Mg²⁺ in the system did not interfere with the analysis, the titer of EDTA-Na₂ was taken to indicate the concentration of Ca²⁺ in a sample. The relative dissolving-out quantity of Ca²⁺ was set as zero at 0 min. At 5 min and 180 min, calcite particles, in the reaction system with CA4 (CA4
group), were taken out and rapidly dried for microscopic examination.

**Results**

**Relationship between the Expression of B. mucilaginosus CA Genes and Calcite Weathering**

The experiment was designed to explore whether calcite can induce the expression of CA genes by comparing the gene expression quantity of *B. mucilaginosus* cultured in a medium containing calcite (insoluble calcium) with a gene expression quantity of the same strain cultured in a medium containing soluble calcium. The mRNA relative expression quantities of different CA genes are shown in Figure 1.

Overall, variations in expression trends were similar for all five CA genes. In the experimental group (containing calcite without CaCl$_2$), REL gradually improved from the 2nd to the 6th day. However, the REL values for the controls (containing CaCl$_2$ without calcite) remained in the single digit range and did not differ significantly. The REL between experimental and control was as great as two orders of magnitude because of the difference in the source of calcium.

**Cloning of CA Genes, Protein Expression and the Determination of CA Activity**

The *B. mucilaginosus* K02 genome was extracted. All five types of CA genes were amplified and the bands of their PCR products were identified in 1% agarose gels (Figure S1A). The positions of the bands representing the lengths of the genes were consistent with the calculated values. The recombinant plasmids were constructed by linking the PCR products and digested plasmid pET30a and then they were transfected into competent cells. The recombinant plasmids were extracted and verified by double-enzyme digestion. The sizes of the two bands were consistent with the calculated values (Figure S1B). The sequences of the inserted DNA were determined. The results showed that the target sequences were inserted into the vector plasmids without even one mutated base (data not shown).

The expression systems were constructed by transferring recombinant plasmids into *E. coli* BL21. In brief, in order to facilitate description, proteins were expressed in hosts containing pXLL01, pXLL02, pXLL03, pXLL04 and pXLL05. These proteins are here called PCA1, PCA2, PCA3, PCA4 and PCA5, respectively. The total proteins and supernatant proteins were visualized using SDS-PAGE (Figure 2A). The results showed that the molecular weight of the proteins ranged from 25 kDa to 55 kDa. This was consistent with calculated values, so it can be used as preliminary evidence that these proteins were target proteins. In the five recombinants, PCA3 was most plentiful and PCA5 was least plentiful. The solubilities of these five types of proteins were significantly different. PCA4 showed the most solubility and PCA5 showed the least. Although many inducing conditions (temperature: 25°C and 30°C, final IPTG concentration: 0.1, 0.7 and 1 mM) were explored, an expression gap still remained, indicating the different levels of expression of these five types of CA genes. Codon preference of *E. coli* is likely the reason why there exists the expression gap.

Under the control of the T7 promoter, recombinants were found to produce His-tagged proteins, which are capable of combining with antibodies. Western blots were used to further confirm whether these proteins were target proteins. Poorly soluble proteins were more likely to produce false positives; therefore, these proteins were used in Western blot analysis (Figure 2B). Although PCA3 showed the highest expression levels, its poor solubility was obvious (Figure 2A). PCA3 and PCA5 were selected for further experiments. The levels of these proteins were determined. Their size was found to be consistent with the results of SDS-PAGE (Figure 2A). These proteins were again shown to be the target proteins. The appearance of several untargeted bands may be attributed to expressed His-tagged proteins native to *E. coli*.

A standard curve for the CA activity assay was constructed. The equation of the standard curve was $y = 9.426x - 0.0134$, and the coefficient of correlation was 0.99546. The activity of expressed proteins from the recombinants was determined. All five kinds of proteins showed CA activity (Figure 3) indicating that they were not evolutionary junk. PCA5 demonstrated only limited activity, possibly because it formed only small amounts and these had low solubility. CA4 showed high amounts of enzymatic activity in either acidic or alkaline environments. The maximum activity of CA4, 109.2 U/mL, was observed at pH 9.0. Variations in enzymatic activity were observed in repeated experiments, but CA4 consistently demonstrated the highest enzymatic activity. The difference in enzyme activity was found to be statistically significant (p < 0.05).

**Calcite Dissolution Kinetics**

Overall, the release of Ca$^{2+}$ per unit time was greatest during the first 10 min. Thereafter, the reaction slowed gradually until equilibrium was achieved. The standard deviation (<5%) for replicate determinations was very small indicating that relatively high accuracy during the entire experimental process. At any time, the order of the dissolution rate from largest to smallest was BCA, CA4 and CA0. The curves in Figure 4 show that calcite dissolution reached equilibrium in 240 min. Figure 5A shows that initially the particle surfaces were essentially free of organic material. Figure 5B shows that, subsequently, the particle surfaces became covered with organic matter.

**Discussion**

We tried to prepare growth curves with *B. mucilaginosus* in the presence and absence of calcite. However, the large amount of exopolysaccharide formed by this strain (Deng et al. 2003; Lian et al. 2008a) prevented precise determinations of growth by this organism. At first, there were only a few bacterial cells. Upon continued incubation of the culture,
the cell population gradually increased. Thereafter, the increased extracellular polysaccharide had trapped calcite particles to such an extent as to slow down the rate of calcite dissolution. As a result, calcium ions needed for growth became less available to the bacteria. In addition, because of local degradation of extracellular polymer of aerobic heterotrophic microorganisms, the alkalinity of the (micro)-environment may be elevated to accelerate the precipitation

Fig. 1. mRNA relative expression quantities of different CA genes. (A), (B), (C), (D), and (E) show the expressions of gene1, gene2, gene3, gene4 and gene5, respectively. C and E represent the control and experimental groups. The calcium resource in the C and E group is CaCl2 and calcite, respectively.
of CaCO₃ (Dupraz and Visscher 2005). The stress of limitation in calcium availability caused the bacteria to synthesize more CA to facilitate calcite dissolution. In the control cultures, sufficient dissolved calcium was still available, so that growth was not limited. The REL of the control, which was almost constant over time, was significantly lower than that of the experimental cultures.

This experiment provides direct evidence that calcite can promote the expression of CA genes in *B. mucilaginosus*. It implies a causal connection linking the expression of CA genes and the process of calcium utilization (Figure 6). Translated protein accelerated CO₂ hydration and produced an acidic environment that favored the dissolution of calcite. Although previous studies (Li et al. 2005a; Li et al. 2007) have shown that CA from microorganisms can play a role in mineral dissolution, some of the isozymes, which may not participate in mineral weathering, have a central role in bacterial metabolism. We have shown, in the present study, that all five CA genes significantly up-regulated the production of corresponding enzyme proteins for playing a supplementary role in the promotion of calcite weathering in response to Ca²⁺ limitation.

To further determine whether CA has a role in promoting CaCO₃ dissolution, the gene for each of the CAs was cloned. The heterologously expressed proteins were generated and their activity determined. All the different CA proteins were active. This showed that our cloning method is applicable to the study of single CA gene expression. The ability of *B. mucilaginosus* to produce isozymes allows it to respond to some unfavorable environmental changes without negative effects on its metabolism. This phenomenon merits in-depth study to explain why so many CA genes co-exist in one species and what a central function each kind of CA plays. In

Fig. 2. Protein expression. (A) SDS-PAGE analysis of CA genes expression in *E. coli* Lanes: 1, molecular size marker; 2, negative control pET30a(+) in *E. coli* BL21 after IPTG induction; 3, total protein expressed in BL21 containing pXLL01; 4, the supernatant protein of lane 3; 5, total protein expressed in BL21 containing pXLL02; 6, supernatant protein of lane 5; 7, total protein expressed in BL21 containing pXLL03; 8, supernatant protein of lane 7; 9, total protein expressed in BL21 containing pXLL04; 10, supernatant protein of lane 9; 11, total protein expressed in BL21 containing pXLL05; 12, supernatant protein of lane 11. (B) Western blotting analysis CA genes expression in *E. coli*. Lanes: 1, molecular size marker; 2, negative control pET30a(+); 3, the total protein expressed in BL21 containing pXLL03; 4, the supernatant protein of lane 3; 5, the total protein expressed in BL21 containing pXLL05; 6, the supernatant protein of lane 5. The protein bands indicated by arrows show non-specific binding.

Fig. 3. CA activity of different crude enzyme solutions: relative enzymatic activity of CA at different pH values.

Fig. 4. Calcium ion concentrations at different times after the addition of different materials. BCA, CA0 and CA4 groups represent the reaction system adding BCA, CA0, and CA4, respectively.
addition, the determination of enzymatic activity may serve as the basis for follow-up calcite dissolution experiments.

CA catalyzes the hydration of CO2 leading to the formation of carbonic acid, regardless of whether the CO2 is of atmospheric or metabolic origin. Dissociation of the carbonic acid yields H\(^+\), which favors dissolution of CaCO\(_3\). Calcite dissolution is determined in term of the overall reaction (CaCO\(_3\) + CO\(_2\) + H\(_2\)O \to Ca\(^{2+}\) + 2HCO\(_3\)^\text{−}\)). Stoichiometry requires that one molecule of CO2 be converted to H\(^+\) and HCO\(_3\)^\text{−} for each molecule of Ca\(^{2+}\) released. During the calcite dissolution, the following relation holds: \( V \frac{d[CO_2]}{dt} = AR \) (Buhmann and Dreybrodt 1985; Dreybrodt et al. 1996). Here [CO\(_2\)], V and A represent the concentrations of CO\(_2\), the volume of the solution and the area of the mineral surface, respectively, and R is the flux of Ca\(^{2+}\) from the calcite surface and depends upon the chemical composition of the reaction system.

The release of Ca\(^{2+}\) per unit time was greatest during the first 10 min and the rate of dissolution in the CA4 group slowed down after the first 10 min compared with the BCA group (Figure 4). Figure 5 shows that, initially, the calcite particles had no organic deposit on them but did have such deposits 180 min after the start of the dissolution experiment. Coating the calcite particles in certain organic substances caused decreases in the dissolution rate over time (Luttge and Conrad 2004). At relatively small V/A ratios, the rate of dissolution was mainly affected by the conversion of CO2 (Dreybrodt et al. 1996). During the first 10 min, the amount of Ca\(^{2+}\) released was higher in the CA4 group than in the CA0 group, and the dissolution rate was almost the same with BCA (Figure 4). This showed that CA protein from B. mucilaginosus CA gene transcription in vitro promoted calcite dissolution.

A pseudo–second-order kinetic model (Ho and Ofomaja 2006) was constructed for description of the dissolution behavior of calcite particles after 10 min:

\[ \frac{t}{q_t} = \frac{1}{kq_e} + \frac{1}{q_e}t \]

Here, t is time, \( q_t \) is the quantity of Ca\(^{2+}\) released at a given time, k is the rate of Ca\(^{2+}\) release, and \( q_e \) is the equilibrium concentration of Ca\(^{2+}\). A pseudo–second-order kinetic model of calcite dissolution is shown in Table 1. Although the surfaces of the calcite particles became covered in biofilm-like organic matter (Figure 5B), which inhibited dissolution to some extent, CA continued to play a certain role in weathering. The K value of the CA4 group was greater than that of the CA0 group. When equilibrium is reached, the \( q_e \) of the CA4 group was roughly equal to that of the CA0 group. This is consistent with the theory that enzymes do not change the

<table>
<thead>
<tr>
<th>Group</th>
<th>Model</th>
<th>Equation</th>
<th>( q_e ) (mg*L(^{-1}))</th>
<th>k (mg*L(^{-1})min(^{-1}))</th>
<th>( R^2 )</th>
</tr>
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<tbody>
<tr>
<td>BCA</td>
<td></td>
<td>( \frac{t}{q_t} = 5.060 + 0.0425t )</td>
<td>19.42</td>
<td>152.6</td>
<td>0.996</td>
</tr>
<tr>
<td>CA0</td>
<td></td>
<td>( \frac{t}{q_t} = \frac{1}{kq_e} + \frac{1}{q_e}t )</td>
<td>12.60</td>
<td>25.90</td>
<td>0.981</td>
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<tr>
<td>CA4</td>
<td></td>
<td>( \frac{t}{q_t} = 2.979 + 0.0760t )</td>
<td>13.16</td>
<td>58.12</td>
<td>0.990</td>
</tr>
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equilibrium point but only increase the rate of the reaction. The \( q_e \) of CA4 and CA0 was lower than that of the BCA group. Two reasons for this may be that: (I) some particles may be isolated when they become coated in organic matter, and (II) organic matter may be suitable for the regeneration of CaCO\(_3\) at the heterologous nucleation sites, which leads to the formation of a secondary precipitate consuming Ca\(^{2+}\) in solution. The reaction may be described as follows:

\[
\text{Ca}^{2+} + 2\text{HCO}_3^- \rightarrow \text{CaCO}_3 \downarrow + \text{CO}_2 + \text{H}_2\text{O} \quad \text{or} \\
\text{Ca}^{2+} + \text{CO}_3^{2-} \rightarrow \text{CaCO}_3 \downarrow
\]

This shows that heterologously expressed CA proteins can promote calcite dissolution, which suggests that it may be feasible to solve geochemical problems using molecular biology. The current project provides a new perspective on mineral weathering and a foundation for further research into the role of microbial CA in the biogeochemical cycle. CA can promote mineral dissolution by increasing the reaction rate. Microorganisms can enhance their viability in the mineral-nutrient-poor environments by obtaining mineral elements in a timely manner through the auxiliary function of CA.

However, many studies (Kump et al. 2000; Ludwig et al. 1999) have not taken the role of enzyme catalysis in the mineral weathering process into account. This has caused the role of microorganisms in mineral weathering to be underestimated. In this way, our experimental results pose two questions worth studying in-depth: (I) whether all the CAs of the microbes play a supplemental role in mineral weathering; and (II) whether microbial mineral weathering from secreted enzymes will accelerate the carbon cycle as global concentrations of CO\(_2\) increase.

**Conclusions**

RT-qPCR and heterologous expression analysis were used to study the relations of gene expression, calcite dissolution and the acquisition of Ca nutrition. RT-qPCR showed that calcite up-regulated the expression of CA genes and fostered the synthesis of CAs, which, in turn, promoted calcite dissolution. Heterologous expression of five types of CA products in *E. coli*, determined by *B. mucilaginosus* CA gene sequences, were demonstrated to possess CA activity, and one type was shown to have the function of promoting calcite dissolution. These findings provide direct evidence that CAs from *B. mucilaginosus* play an auxiliary role in calcite weathering and Ca nutrition release under the condition of Ca\(^{2+}\) deficiency, which is conducive to the survival of bacteria.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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