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Effect of carbonic anhydrase on silicate weathering and carbonate formation at present day CO₂ concentrations compared to primordial values

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It is widely recognized that carbonic anhydrase (CA) participates in silicate weathering and carbonate formation. Nevertheless, it is still not known if the magnitude of the effect produced by CA on surface rock evolution changes or not. In this work, CA gene expression from *Bacillus mucilaginosus* and the effects of recombination protein on wollastonite dissolution and carbonate formation under different conditions are explored. Real-time fluorescent quantitative PCR was used to explore the correlation between CA gene expression and sufficiency or deficiency in calcium and CO₂ concentration. The results show that the expression of CA genes is negatively correlated with both CO₂ concentration and ease of obtaining soluble calcium. A pure form of the protein of interest (CA) is obtained by cloning, heterologous expression, and purification. The results from tests of the recombination protein on wollastonite dissolution and carbonate formation at different levels of CO₂ concentration show that the magnitudes of the effects of CA and CO₂ concentration are negatively correlated. These results suggest that the effects of microbial CA in relation to silicate weathering and carbonate formation may have increased importance at the modern atmospheric CO₂ concentration compared to 3 billion years ago.

The evolution of the Earth has been a complex, long-term process¹. The overall trend in the composition of its surface minerals has involved a constant decrease in silicate and an increase in carbonate minerals. Physical^{2,3} and chemical⁴ weathering processes are the main forces driving silicate weathering. In recent decades, the fact that living creatures, especially microorganisms, are involved in mineral weathering has been recognized by a growing number of researchers^{5–7}. Microbial weathering results from a combination of many factors⁸ including: bio-mechanical action, the secretion of organic acids, chelation effects, redox reactions, and others. Participation of some active substances during biological weathering makes mineral weathering and enzymatic action more closely linked. Thus, it is worth exploring whether organisms can secrete enzymes to accelerate the weathering of silicate minerals or not and, if they do, how big a role the biological enzymes play in different habitats.

The free metal ions that arise from silicate weathering are involved in the precipitation of carbonates, and this process is accompanied by the fixing of atmospheric CO₂^{9–11}. An important constraint on the formation of carbonates is the concentration of carbonate (CO₃^{2–}) in the metallogenic environment¹². The acceleration of carbonate formation due to the action of biological enzymes is thus attributed to the increased formation of HCO₃[–] and CO₃^{2–} in the carbonate deposition process. Carbonic anhydrase (CA) was first found in human erythrocytes¹³ and is widely present in animals, plants, and microorganisms. CA shows appreciable CO₂ hydrase activity (catalytic constants k_{cat} lie in the range 3.9–8.0 × 10⁵ s^{–1} and kinetic efficiencies k_{cat}/K_m are in the range 4.3–9.7 × 10⁷ M^{–1}s^{–1})¹⁴. Thus, CA is capable of catalyzing the reversible hydration reaction, CO₂ + H₂O ↔ H⁺ + HCO₃[–], of atmospheric and self-generated CO₂^{15,16}.

It has been found that when the environmental CO₂ concentration changes, organisms may be able to regulate the expression level of the CA gene to adapt to those changes^{17,18}. For example, the CA gene expression level in mature leaves of young legumes changes following the diversification of CO₂ concentration: the CA expression level is reduced if the CO₂ concentration is elevated¹⁹. *Chlamydomonas reinhardtii* will also increase its CA



expression level to take full advantage of CO₂ when the CO₂ concentration decreases from 5 to 0.04%²⁰. The expression level of their CA genes is increased at lower CO₂ concentrations. The above research shows that CA may not work at higher CO₂ concentrations or, perhaps, that it has a more important role in the face of a CO₂ deficiency. Consequently, it seems more meaningful to express this kind of gene to capture CO₂ when available levels are low.

When microorganisms grow in environments that have no limits on the availability of elements, many metabolic pathways become very slow (or even stop) to avoid unnecessary material and energy use. A more efficient, economical way is always chosen if they grow in relatively harsh conditions. Expression levels of one, or several, genes will be different according to the difficulty in obtaining nutrition. An anaplerotic role for CA has been proposed which, for example, accounts for the unusual behaviour observed in terrestrial cyanobacteria such as *Nostoc flagelliforme* during hydration–dehydration cycles²¹. The present authors recently showed that the level of CA gene expression in *Aspergillus fumigatus*²² and *Aspergillus niger*²³ is enhanced if the only potassium source available is potassium feldspar (to allow the organisms to obtain potassium more effectively). As it accelerates CO₂ hydration, CA can promote the generation of H₂CO₃, thus promoting weathering of silicate minerals and facilitating the release of K⁺. Moreover, the increased expression of CA by *Bacillus mucilaginosus* favours its survival when the growth environment lacks Ca²⁺ but is rich in calcite²⁴. Therefore, an enhanced expression level of the CA gene has a positive impact on microbial growth in environments in which soluble mineral elements are lacking but mineral particles are abundant. The microorganisms not only acquire mineral nutrition but also, at the same time, accelerate the weathering of silicate or calcite. Thus, biological adaptation, with the aid of CA, makes carbon, calcium, and silicon circulation more active.

Carbonate formation is not only an important part of the evolution of surface minerals but also a significant method of fixing atmospheric CO₂^{11,25}. Microbial lithification may be the by-product of metabolism^{26,27}. Some organisms can actively capture CO₂ and convert it into solid carbonate through CA catalysis²⁸. When the CO₂ concentration is reduced by several orders of magnitude, biomineralization behaviour (in which CA takes part) may affect the growth and even survival of the organism. Previous studies have confirmed that many organisms, such as microbes^{29,30}, coral^{31,32} and animals^{33,34}, can take advantage of CA's role in CaCO₃ formation at atmospheric levels of CO₂. Miyamoto *et al.*, for example, showed that CA from the nacreous layer in oyster pearls is conducive to the formation of CaCO₃ crystals³⁵. Moreover, CA accelerates deposition of minerals and shows greater activity at low CO₂ concentrations³⁶. It has also been reported that CA can contribute to carbonate precipitation at high concentrations³⁷. Thus, there is no definitive conclusion as to whether the role of CA is more obvious with a reduction of CO₂ concentration during CaCO₃ deposition, or not.

In the work presented here, we use real-time quantitative PCR (RT-qPCR) to study the effect of sufficiency or deficiency in calcium and CO₂ concentrations on CA gene expression. Inversely, the function of CA in wollastonite dissolution and CaCO₃ formation, at different CO₂ concentrations, was investigated using heterologous expression and protein purification. The object of the study is to explore whether the magnitude of the silicate weathering and carbonate formation produced by CA is different at the modern atmospheric CO₂ concentration compared to that 3 billion years ago.

Results

The involvement of CA in wollastonite weathering at the atmospheric CO₂ level. The results from Experiment 1 are shown in Fig. 1 (see the Methods section for details on the different experiments performed). The trends in the pH variation for the two treatments (i.e. with and without CaCl₂) are similar (Fig. 1a). There was a sharp

initial decrease in pH from day 0 (the primary culture) to day 2. In the days which followed, the pH rose slightly. A significant difference was that a moderate reduction in pH occurred with wollastonite as the only calcium source (compared with that containing CaCl₂) from day 4 to day 6.

The soluble silicon content (SSiC) of the group with added CaCl₂ was significantly greater than that in the other group on day 2 (Fig. 1b). However, the differences were not statistically significant ($p = 0.09$ and 0.37 , respectively) when the two conditions were compared on days 4 and 6 (Fig. 1b). From day 4 to 6, the SSiC did not increase appreciably ($p = 0.066$) when the medium contained CaCl₂. However, there was a statistical difference ($p = 0.033$) when the medium only contained wollastonite.

As far as the effect of sufficiency or deficiency in calcium on CA gene expression is concerned (see Experiment 2 in the Methods), none of the CA genes showed markedly different expressions in the two conditions on days 2 and 4 (Fig. 2). The low expression of CA genes and no difference between expressions in the two culture conditions in the early- and mid-growth stages, demonstrates that CA function may not be essential at these points. All five CA genes showed much higher expression levels on day 6 when only wollastonite was present compared to when wollastonite and CaCl₂ were used (Fig. 2). Furthermore, there was a sharp increase in expression of all genes from day 4 to day 6 when the culture was deficient in calcium. These results indicate that the participation of CA was urgently needed to accelerate wollastonite dissolution in order to provide Ca²⁺ under such conditions.

If *B. mucilaginosus* sensed calcium deficiency, significantly increased expression levels of the five CA genes were observed (see Fig. 2). Nevertheless, whether or not a single CA can show any significant effect on the dissolution of wollastonite at the current atmospheric CO₂ concentration remains unanswered. The current authors tried to find an answer to this by testing the effects of recombinant protein (PCA4) from CA4 gene by heterologous expression on wollastonite dissolution (see Experiments 4 and 5 in the Methods section). The effects of PCA4 on wollastonite dissolution can be seen in Fig. 3. The size of the target protein is consistent with the actual calculated value (28.62 kDa), and no contaminating proteins remained after dialysis (Fig. 3a). The ratio of the dialysis and soluble proteins was about 1 : 1.225 (gray value), so only a small amount of protein was lost. As can be seen from the dissolution curve (Fig. 3b), the Ca²⁺ concentration, after adding PCA4, was higher everywhere compared to that without it. As the reaction continued, the wollastonite dissolution reached equilibrium. There were only trace amounts of Ca²⁺ released after 8 h. According to the change in the amounts of Ca²⁺ released over time, a pseudo-second-order kinetics model³⁸ was constructed to describe the dissolution behaviour of the wollastonite under both conditions (see Table 1). As can be seen from the kinetics equations (Table 1), the value of the dissolution rate k after adding PCA4 was $1.402 \times 10^{-3} \text{ mg g}^{-1} \text{ min}^{-1}$, and $9.24 \times 10^{-4} \text{ mg g}^{-1} \text{ min}^{-1}$ without CA.

The effect of CO₂ on CA gene expression and the decreased importance of CA for wollastonite dissolution at high CO₂ concentration. The relative expression levels of the CA genes (displayed in Fig. 4) were significantly different at different sampling times and CO₂ concentrations (see Experiment 3 in the Methods). The expression of CA3, CA4, and CA5 genes showed no obvious differences on days 2 and 4. This indicates that CO₂ does not affect the expression of these three genes at this growth stage. However, the expression of CA1, CA3, CA4, and CA5 genes on day 6 were related to CO₂ concentration. Furthermore, CO₂ concentration and CA gene expression are negatively correlated. The relative level of expression decreased three- to five-fold when the CO₂ concentration increased by two orders of magnitude. Additionally, the difference in expression levels obtained by

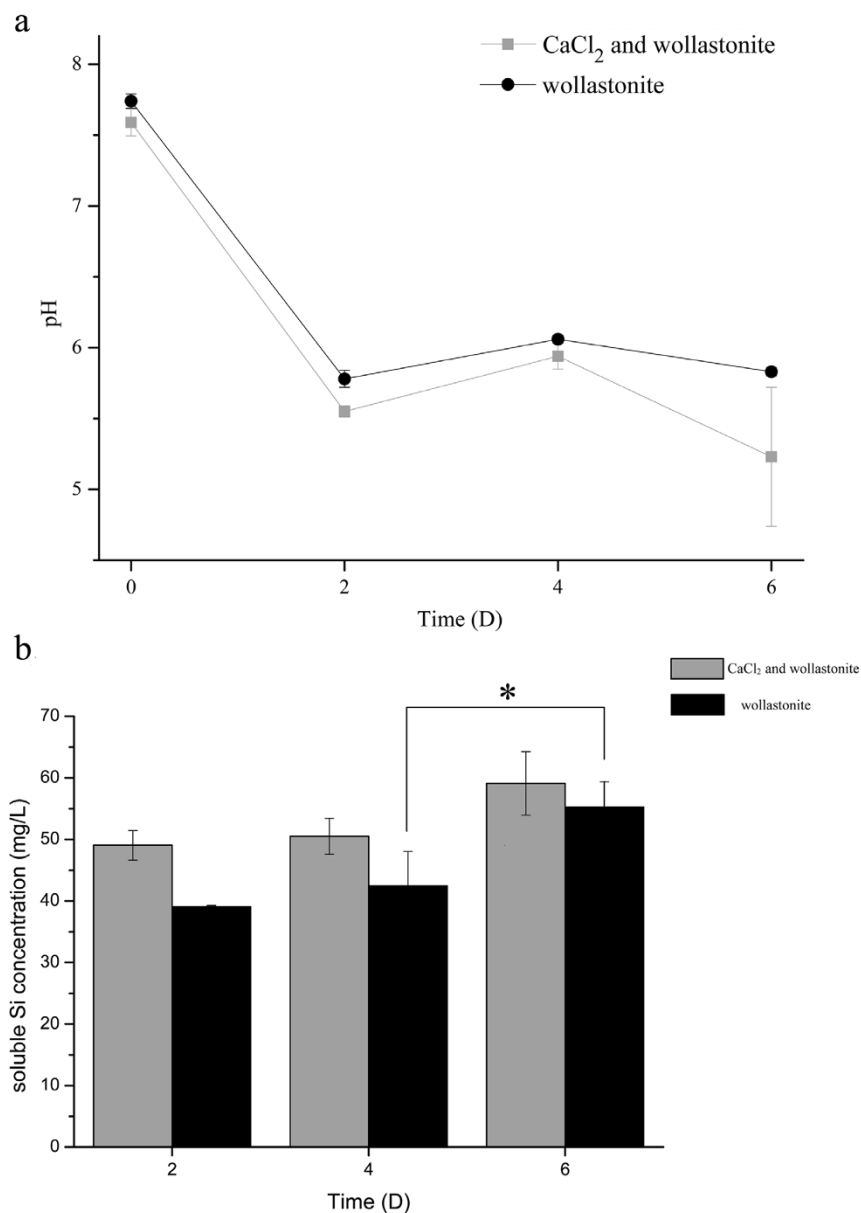


Figure 1 | Variation of the pH and soluble silicon concentration of the bacterial cultures at different sampling points (Experiment 1): (a) the pH value at three sampling points; and (b) the concentration of soluble silicon at three sampling points. Bacteria were cultured in a medium with CaCl₂ and wollastonite as calcium sources (gray bar) or with wollastonite only (black bar). *The results from the two treatments are significantly different ($p = 0.033$).

comparing days 6 and 2 reached two orders of magnitude at 0.039% CO₂. The CA1 gene demonstrated differential expression on day 4. This suggests that the stress of Ca²⁺ deficiency was felt by bacteria at that time. In this case, CA1 was preferred to accelerate the dissolution of wollastonite. This selectivity allows the bacteria to not only adapt to vertiginous environments in a timely manner but also prevents a waste of materials and energy due to superfluous gene expression.

To further confirm that the role played by CA in wollastonite dissolution is decreased at higher CO₂ concentrations, the effect of PCA4 on wollastonite demineralization was determined at both CO₂ concentrations (see Experiment 5). It can be seen from the observed trends in the amount of dissolved Ca²⁺ (see Fig. 5a) that the dissolution of the wollastonite gradually equilibrated at 0.039% CO₂ concentration even though PCA4 was added to the reaction system as well. In contrast, Ca²⁺ was released continuously under high CO₂ conditions. The difference in Ca²⁺ concentration emerged as early as the tenth minute. As the reaction proceeded, the difference increased.

Thus, after 8 h, the Ca²⁺ concentration at 3.9% CO₂ exceeded twice that present at atmospheric CO₂ levels. These results suggest that CA plays a greater role at lower CO₂ concentrations than at higher CO₂ concentrations.

The impact of CO₂ on the value of CA in carbonate formation. The results on the impact of CO₂ on the role of CA in carbonate formation are shown in Fig. 5b. Regardless of whether the reaction system contains PCA4 or not, the CO₂ concentration is positively correlated with CaCO₃ production. At any CO₂ concentration, the CaCO₃ content (w/w) is significantly different due to the participation of PCA4 ($p = 5 \times 10^{-6}$, 1.2×10^{-5} , and 3.3×10^{-5} at day 2, 4, and 6, respectively). At 10% CO₂ concentration, the mass of CaCO₃ was approximately 0.065 g without PCA4 and more than 0.070 g with PCA4. The proportion of CaCO₃ that formed due to the participation of PCA4 was about 15%. At low concentrations of CO₂ (0.4%), the masses were approximately 0.005 g without PCA4 and

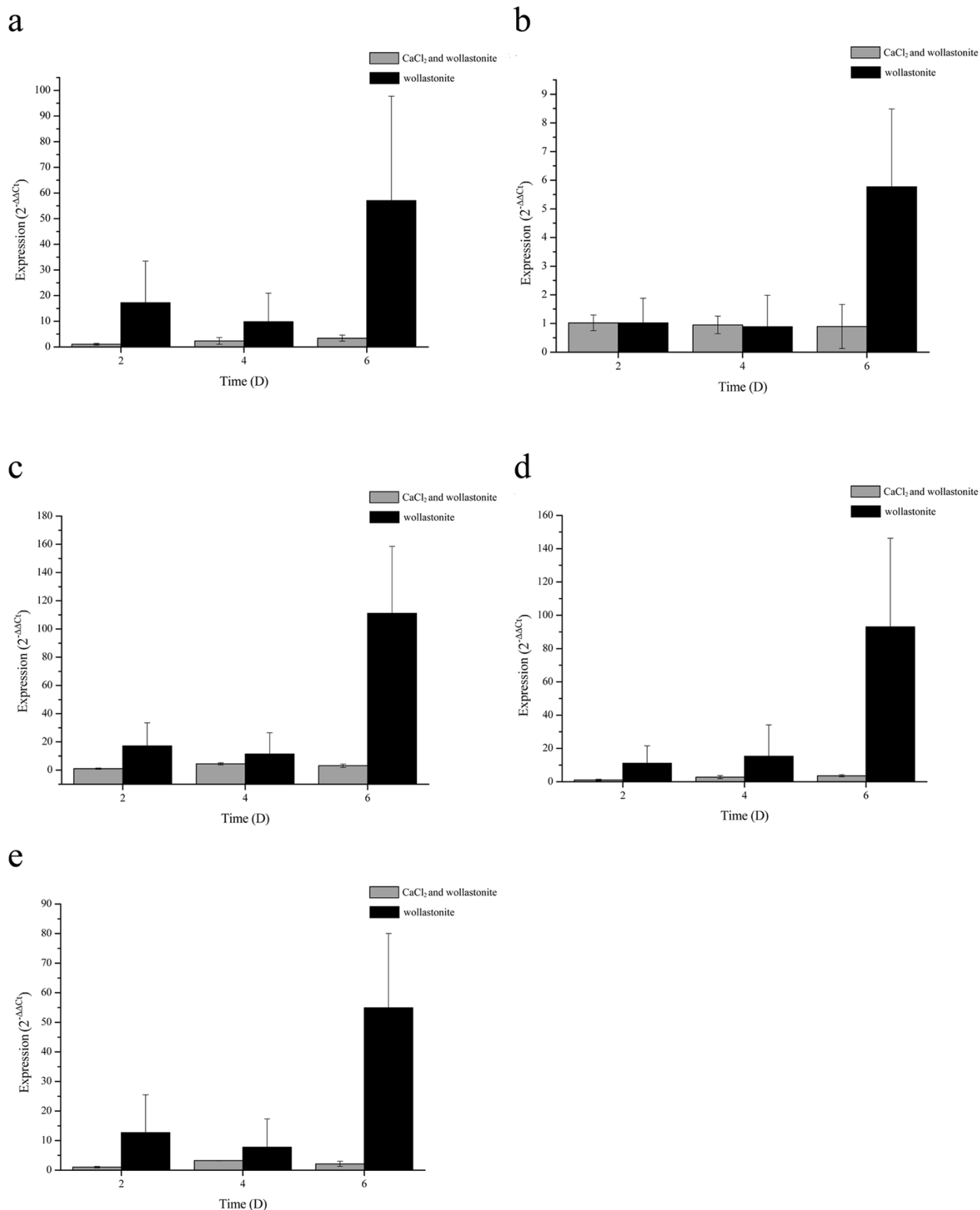


Figure 2 | mRNA relative expression levels of five CA genes (Experiment 2). (a), (b), (c), (d), and (e) show the expression of CA1, CA2, CA3, CA4, and CA5, respectively. Gray bars and ‘CaCl₂ and wollastonite’, denote that the calcium sources were CaCl₂ and wollastonite. Similarly, black bars and ‘wollastonite’ denote that the calcium source was wollastonite only. The bacteria were cultured using a concentration of 0.039% CO₂.

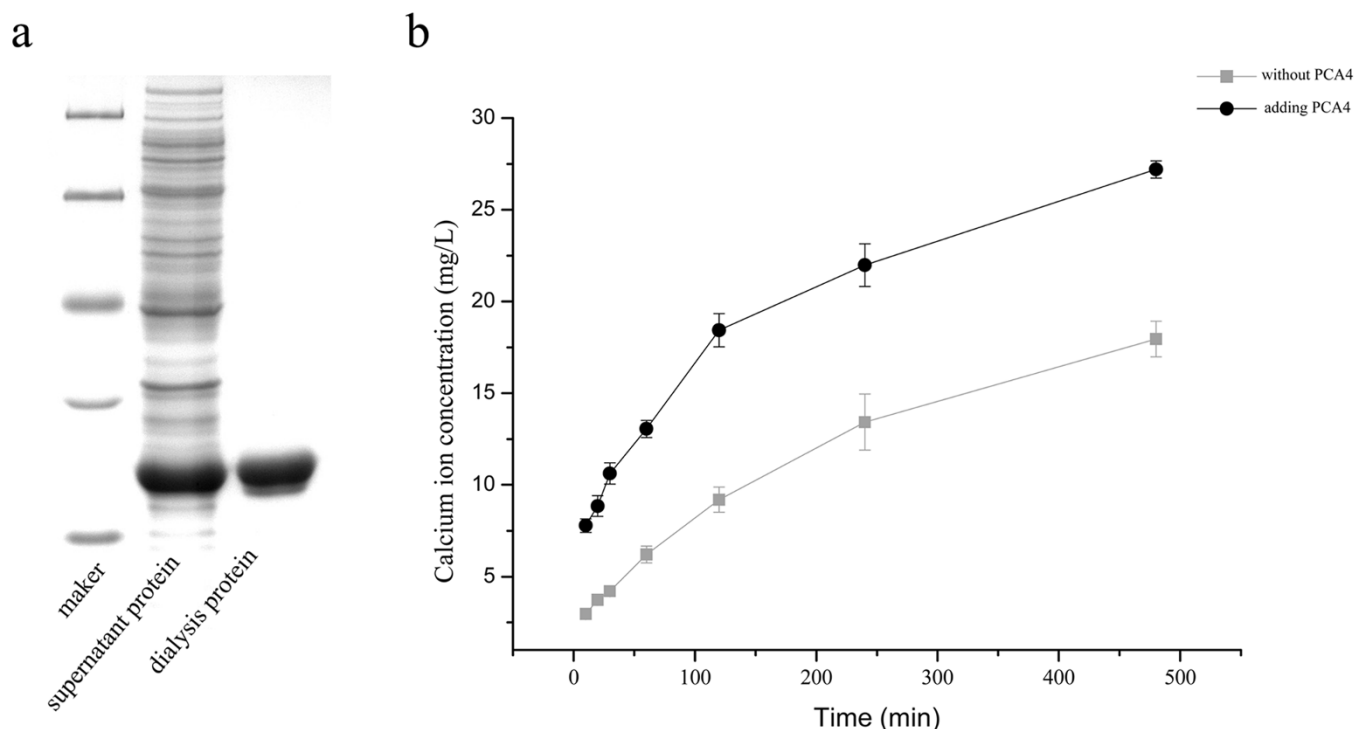


Figure 3 | The effect of purified PCA4 on wollastonite dissolution (Experiments 4 and 5). (a) SDS-PAGE analysis of recombinant protein (PCA4). The sizes of the protein markers are 116.0, 66.2, 45.0, 35.0, and 25.0 kDa, respectively. (b) Ca^{2+} concentrations at different sampling times, with or without PCA4 in the reaction system, using a concentration of 0.039% CO_2 .

more than 0.020 g with PCA4. The proportion of the CaCO_3 formed as a result of the recombinant protein was up by 419%, largely due to the behaviour of the CA. Thus, PCA4 causes a much greater difference in the amount of CaCO_3 at lower CO_2 concentrations. The fact that the effect of CA is more remarkable at low CO_2 concentrations, rather than the opposite, is notable.

Discussion

Organisms growing on the surfaces of rocks, and thereby causing weathering to occur, are largely there to obtain nutrition^{39–41}. Some of the most important inorganic nutrients required for proper cell function are obtained from rocks⁴². In the experiments testing whether wollastonite can induce the expression of CA and whether the weathering behaviour caused by the participation of CA from *B. mucilaginosus* contributes to a certain proportion of the overall mineral weathering effect at atmospheric CO_2 levels or not, wollastonite was the only available calcium resource when the *B. mucilaginosus* was cultured in media lacking soluble calcium but containing wollastonite. As one group had artificially added CaCl_2 , it is illogical to describe wollastonite dissolution using Ca^{2+} concentration. In view of this, SSiC was used to represent wollastonite dissolution. The number of bacteria on day 2 were about $(2.78 \pm 0.48) \times 10^7 \text{ ml}^{-1}$ and $(2.70 \pm 0.69) \times 10^7 \text{ ml}^{-1}$ with and without CaCl_2 , respectively. We observed a sharp decrease in pH, which may be due to organic acids being secreted by *B. mucilaginosus* in both treatments. Liu *et al.* showed that *B. mucilaginosus* produces organic acids to decompose

silicate minerals during its growth, e.g. oxalic acid and citric acid⁴³. The overall effect of the bacteria on wollastonite weathering with added CaCl_2 was stronger, and soon afterwards more soluble silicon was released. Despite the weaker effect without added CaCl_2 , enough Ca^{2+} was released to meet the amount needed for bacterial growth on day 2. Consequently, CA protein may not play an obvious role in wollastonite dissolution and the CA gene expression levels showed no significant differences. As culturing continued, the consumption of organic acids may result in a slight increase in pH. In the culture condition with wollastonite as the only calcium resource, the pressure of calcium deficiency may have been felt on day 6. The wollastonite-only group had a relatively larger bacterial population, $(1.21 \pm 0.11) \times 10^9 \text{ ml}^{-1}$, and less Ca^{2+} than the group containing CaCl_2 ($117.35 \pm 10.62 \text{ mg/L}$). A single unit of Ca^{2+} , which bacteria were able to gain, was much lower in the group without CaCl_2 . In this case (in the medium without CaCl_2), the demand of *B. mucilaginosus* for soluble calcium may be stronger. The RT-qPCR results show that the expression of all the CA genes were up-regulated by two orders of magnitude from day 4 to day 6 when wollastonite was the only calcium resource. The consistency between this increase in CA gene expression and wollastonite dissolution (Fig. 1b, from day 4 to 6) suggests that CA plays a role in the dissolution of wollastonite at atmospheric CO_2 concentrations. The dissolution of wollastonite consumed part of the H^+ produced by CO_2 hydration and generated a certain amount of HCO_3^- :

Table 1 | The fits of the wollastonite dissolution data to a pseudo-second-order kinetics model

Group	Model	Equation	q_e (mg g^{-1})	k ($\text{mg g}^{-1} \text{ min}^{-1}$)	R^2
Without PCA4	$\frac{t}{q_t} = \frac{1}{kq_e^2} + \frac{1}{q_e}t$	$\frac{t}{q_t} = 12.71 + 0.108t$	9.225	9.24×10^{-4}	0.959
With PCA4		$\frac{t}{q_t} = 4.439 + 0.078t$	12.67	1.402×10^{-3}	0.986

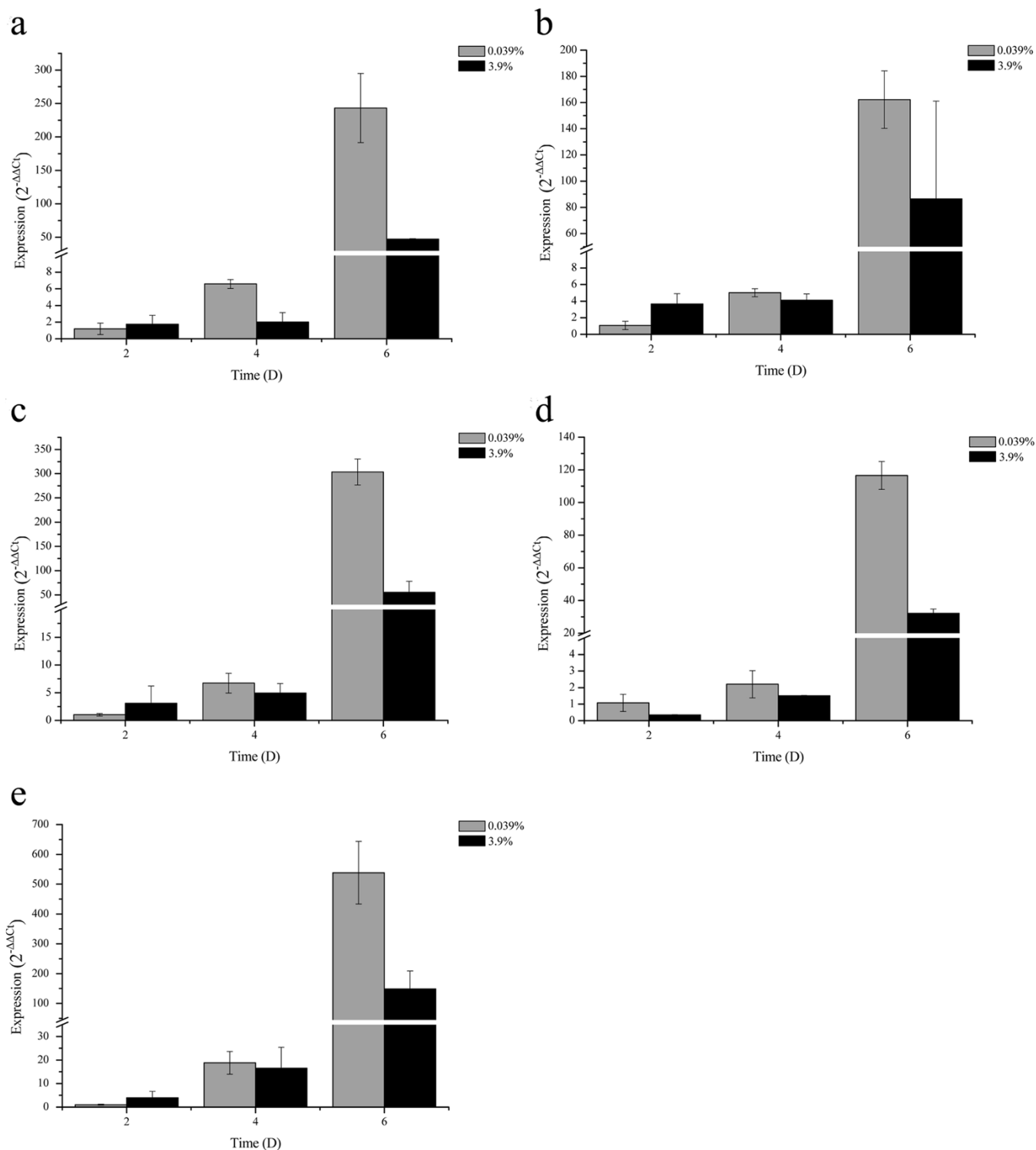
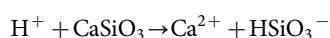
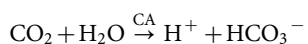
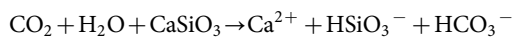


Figure 4 | mRNA relative expression levels of five CA genes of *B. mucilaginosus* cultured with wollastonite as the calcium resource using 0.039% and 3.9% CO₂ concentrations (Experiment 3). (a), (b), (c), (d), and (e) show the expression levels of CA1, CA2, CA3, CA4, and CA5, respectively.



The overall change can be written:



- (1) Therefore, the extent of the pH decrease was lower than that with CaCl₂ and wollastonite as calcium resources (Fig. 1a). This is due to the production of HCO₃⁻ and consumption of CO₂ in the medium.
- (2) There are two aspects to the facilitation of wollastonite dissolution by increasing the expression level of CA: (i) The amount of H⁺ is an important factor if the bacteria is to obtain adequate Ca²⁺ from wollastonite dissolution; (ii) CO₂ hydration can produce HCO₃⁻, which is an important substrate for many fundamental biological pathways such as: gluconeogenesis, lipogenesis, ureagenesis, pyrimi-
- (3)

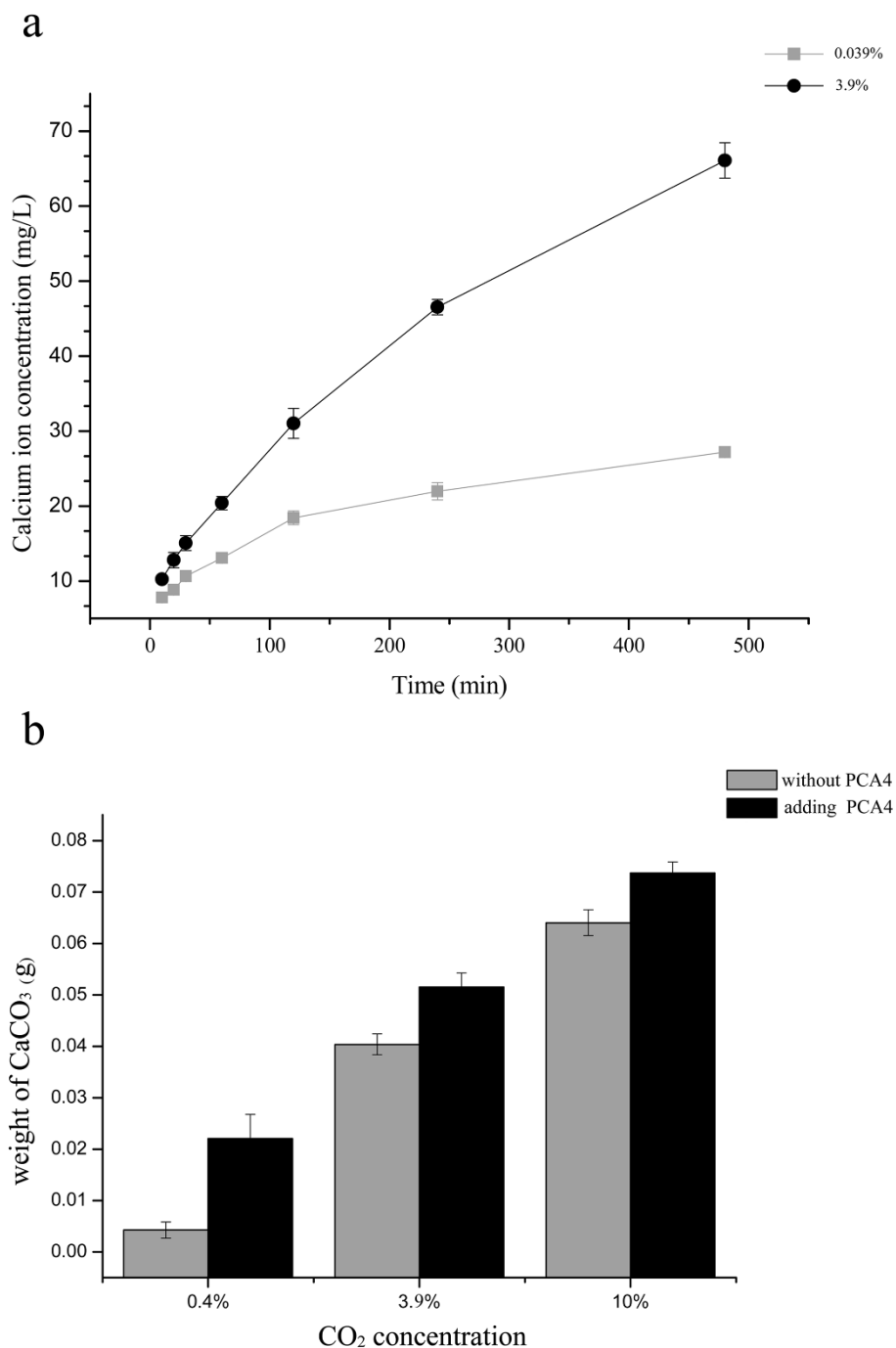
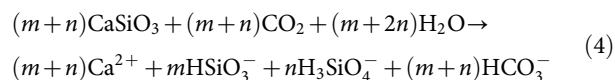


Figure 5 | The effect of PCA4 on wollastonite dissolution and CaCO₃ formation using different CO₂ concentrations. (a) Ca²⁺ concentration at different sampling times with PCA4 added to the reaction system at atmospheric and 3.9% CO₂ concentration levels. (b) mineralization in the reaction system without PCA4 (gray bar) or with (black bar) using different CO₂ concentrations.

dine synthesis, and synthesis of several amino acids⁴⁴. CA can participate in the formation of malonyl-CoA, which is catalyzed by acetyl-CoA carboxylase, with bicarbonate and acetyl-CoA as the substrate⁴⁵. Therefore, CA is an important regulator of fatty acid metabolism. Synthesis of fatty acids helps to improve membrane fluidity, which has a certain effect on the efficiency of nutrient acquisition²³.

Wollastonite dissolution proceeds according to the reaction:



It can be seen from the stoichiometry of this equation that each mole of Ca²⁺ released consumes one mole of CO₂, which means the rela-

tionship that holds between the quantity of Ca²⁺ released and CO₂ consumed during the process of wollastonite dissolution is:

$$V \frac{d[\text{CO}_2]}{dt} = AR^{46,47} \quad (5)$$

Here V , $[\text{CO}_2]$, and A represent the solution volume, CO₂ concentration, and the area of the mineral surface, respectively; R is the flux of Ca²⁺ from the wollastonite surface. Thus, for a given volume of solution and mineral surface area, release of Ca²⁺ is proportional to the consumption of CO₂. From the results of fitting the data to a pseudo-second-order kinetic equation, it is apparent that the k values, after adding PCA4, were higher than those without PCA4.



This further confirms that CA has a significant role in promoting dissolution of wollastonite at 0.039% CO₂ concentration. Therefore, enhancement of the CA expression level is an effective way to promote weathering of minerals in order to get the desired inorganic nutrients when the microorganism grows where the CO₂ concentration is low. This behaviour of the bacteria, to a certain extent, also accelerates the weathering of silicate minerals. The involvement of CA in the demineralization of silicate minerals has also become a recognized part of global biogeochemical cycles.

An increase in CA gene expression level is advantageous to the microbe's survival chances in soluble-calcium deficient environments. However, does CA have a significant accelerating effect on mineral dissolution at high CO₂ concentrations? The proportion of CO₂ in the Earth's primordial atmosphere was up to 10%⁴⁸. Previous studies have shown that bacteria can grow in the presence of a CO₂ concentration of 5%^{49,50} and even 10%⁵¹. In our experiment, the wollastonite underwent dissolution to varying extents at two different levels of CO₂ concentration (Fig. 5a). The low saturation level resulted in a reduction in dissolution rate and the process gradually reached dissolution equilibrium at 0.039% CO₂ concentration. In contrast, at 3.9% CO₂ concentration, the solution had a relatively high saturation level and the dissolution rate remained essentially unchanged as CO₂ continuously dissolved in the reaction. Therefore, the CO₂ primarily affected wollastonite dissolution and the function of the CA was not obvious in a sustained high-CO₂ partial pressure environment. During the early appearance of life (3 billion years ago), silicate weathering mainly occurred due to physical and chemical effects — the contribution of CA to silicate weathering at this time may have been minimal. Atmospheric CO₂ concentrations gradually decreased (by more than two orders of magnitude) during the process of terrestrial evolution. This implies that the current expression level of biological CA is far higher now than it was in the period during which life emerged. Consequently, the participation of CA in silicate weathering may be much higher now than it was three billion years ago. This means that CA played an increasingly important role in the evolution of the Earth.

Whether it is physical, chemical, or biological weathering that affects the silicate minerals, the process is always accompanied by a release of metal ions. Some can react with HCO₃⁻ or CO₃²⁻ in aqueous solution to revert to a solid form²⁵. This is also the basic process governing both silicate weathering and carbonate formation. Mineralization experiments have shown that the highest amount of CaCO₃ occurs under 10% CO₂ and yet the required HCO₃⁻ or CO₃²⁻ during this mineralization mainly arises from spontaneous CO₂ hydration. The role played by CA in the formation of CaCO₃ crystals is only responsible for a small proportion of them. Although bacterial CA may have been helpful in promoting the formation of carbonate more than 3 billion years ago²⁵, the role might have been negligible because of the small amount of biomass and high CO₂ concentration. As the atmospheric CO₂ concentration decreased, non-enzymatic CO₂ hydration reactions would have become relatively weak. However, the involvement of CA, to some extent, compensated for this reduced rate. When applying CA to capture atmospheric CO₂, the enzyme efficiency required to accelerate CO₂ capture increases as the partial pressure of the CO₂ decreases⁵². The least amount of total mineralization was found at the minimum concentration of CO₂, but the difference in the amount of CaCO₃ was maximized at that point. This suggests that CA is more significant at lower CO₂ concentrations. To some degree, the participation of CA mitigates the reduced rate of carbon fixation and carbonate formation due to the decrease in CO₂ concentration.

In summary, CA gene expression is negatively correlated with the ease of obtaining soluble calcium and CO₂ concentration. Moreover, considering the importance of the effect of purified CA on wollastonite dissolution and CaCO₃ precipitation, the magnitude of the effect of CA is significantly weakened at higher CO₂ concentrations. In

view of the value of the effect of CA at the current atmospheric CO₂ concentration and that 3 billion years ago, the results suggest that the role of microbial CA may have become increasingly more apparent and important as terrestrial surface rocks have evolved.

Methods

Minerals. The wollastonite, Ca₃(Si₃O₉), used in the present study was provided by the State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences (Guiyang, China). The mineral was crushed and washed according to the method described by Daval *et al.*⁵³. Briefly, the crushed wollastonite powder was washed using absolute ethanol and sterilized ultrapure water (18.2 MΩ cm⁻¹) to eliminate the fine dust resulting from the grinding procedure. Analysis using X-ray diffraction showed that the wollastonite powder contained only trace amounts of calcite and quartz.

Experiment 1 – Effects of *B. mucilaginosus* on wollastonite dissolution. *B. mucilaginosus* was cultured in a nitrogen-containing medium with different calcium resources to test the effect of *B. mucilaginosus* on wollastonite dissolution (see Table 2). The composition of one kind of medium per litre was as follows: sucrose 10.0 g, (NH₄)₂SO₄ 1.0 g, CaCl₂ 0.44 g, wollastonite 1.16 g, MgSO₄ 0.5122 g, KCl 0.1 g, and Na₂HPO₄·12H₂O 2.507 g. The other medium consisted of the same components except for omission of the CaCl₂. As far as possible, to avoid adsorbed metals and cell exudates being introduced into the medium during inoculation, a seed solution was used according to the description given by Fein *et al.*⁵⁴ (with a little modification). Briefly, the bacteria were cleaned using sterilized ultrapure water (SUW), sterilized HNO₃ (1 M), SUW, sterilized EDTA (0.001 M), and SUW, respectively. Finally, the precipitate was suspended using 20 ml of SUW and used as seed liquid for inoculation. This operation removes as much inherent calcium as possible. Meanwhile, control media were inoculated with deactivated bacteria to eliminate interference from abiotic factors or mineral dissolution only in the medium. The ratio of seed solution to medium was about 1 : 10 (v/v). The culture conditions were set at 30 °C and 130 rpm at the current atmospheric CO₂ concentration (0.039%). The pH value of the culture solution was tested at set sampling times (2, 4, and 6 days) using a pH-meter (METTLER-TOLEDO SevenEasy S20). The number of bacteria was counted using a microscope (Zeiss Axio Imager A1, Zeiss, Germany). Moreover, some of the culture solution (15 ml) was then centrifuged (10397 g, 4 °C, 30 min) using a centrifuge (Sigma 3 k30) and 5 ml of the supernatant were collected. The remaining liquid was discarded. The precipitate was re-suspended using 10 ml of 1 M ammonium acetate, broken (a minute at a time for a total of three times) using an ultrasonic cell disrupter (Sanyo Soniprep150), and cleaned ultrasonically for 30 min. Ammonium acetate solution was added again to make the total volume up to 15 ml. The solution was mixed and centrifuged (10397 g, 4 °C, 30 min) and the supernatant collected. The two kinds of supernatant were mixed in equal volumes and the concentrations of Ca²⁺ and SiC were detected using ICP-AES (Thermo IRIS Intrepid II XSP). A two-tailed *t*-test was performed using STATISTICA 6.0 software. The data met the assumptions of the test. The mean and its standard deviation were calculated based on three independent experiments.

Experiment 2 – Effects of calcium resources on CA gene expression. Five CA-related genes [Gene IDs: 12734710 (CA1), 12739330 (CA2), 12735171 (CA3), 12735237 (CA4), and CP003422 region: 5453463–5454707 (CA5)] were annotated in the *B. mucilaginosus* K02 genome. To test the degree of difficulty (or ease) of acquiring Ca²⁺ on CA expression, the same culture conditions were used as in Experiment 1. After 2, 4, or 6 d culturing time, the bacteria were centrifuged (11500 g, 4 °C, 1 min). The supernatant was discarded, and the collected cells frozen in liquid nitrogen. Total RNA was then extracted (using an Invitrogen kit in accordance with the manufacturer's instructions) and reverse transcribed into cDNA. The correct RT-qPCR reaction conditions were adopted in accordance with the manufacturer's instructions (SYBR® Premix Ex Taq™ (TliRNaseH Plus), TaKaRa). As an internal reference, 16S rRNA was used (see Table S1). After optimization by testing different primers, a single melting temperature was determined for each of the six pairs of primers, 85.8, 88.2, 87.8, 85.7, 84.8, and 85.5 °C, respectively. The *Ct* value was recorded for subsequent analysis (when the fluorescent signal of each reaction tube reached a set threshold, the number of reaction cycles involved gives the *Ct* value). The mean of $\Delta\Delta Ct$ was set to zero on the second day when the bacteria was cultured using wollastonite and CaCl₂. The relative expression level (REL) was then calculated using the following formula:

$$REL = 2^{-\Delta\Delta Ct} \quad (6)$$

Experiment 3 – Effects of CO₂ concentration on CA gene expression. The CO₂ concentration was set to either 0.039% or the higher CO₂ concentration (3.9%) to determine the effect of CO₂ concentration on CA gene expression (see Table 2). In this experiment, the calcium resource in the medium was only wollastonite. The culture conditions were the same as in Experiment 1. Bacteria collection, RNA extraction, reverse transcription, and the RT-qPCR experiment were carried out as in Experiment 2. The mean of $\Delta\Delta Ct$ was set to zero on the second day at 3.9% concentration. REL was then calculated using Eq. (6).



Table 2 | The experimental design used in this study

Experiment	purpose	experimental strain and enzyme	calcium resources	CO ₂ concentration (%)
1	effects of <i>B. muciliginosus</i> on wollastonite dissolution	<i>B. muciliginosus</i>	wollastonite/wollastonite and CaCl ₂	0.039
2	effects of sufficiency or deficiency in calcium on CA gene expression	<i>B. muciliginosus</i>	wollastonite/wollastonite and CaCl ₂	0.039
3	effects of CO ₂ concentration on CA gene expression	<i>B. muciliginosus</i>	wollastonite	0.039/3.9
4	construction of the heterologous expression vector, induction expression and purification of recombinant protein	<i>E. coli</i> and enzyme	—	0.039
5	effects of PCA4 on wollastonite dissolution	enzyme	wollastonite	0.039/3.9
6	effects of PCA4 and CO ₂ concentrations on CaCO ₃ precipitation	enzyme	CaCl ₂	0.4/3.9/10

Experiment 4 – Construction of the heterologous expression vector and induction expression and purification of recombinant protein. The construction of engineered *E. coli* in which five kinds of CA can be expressed was reported previously²⁴. The engineered *E. coli*, which over-expresses CA protein from transcription and translation of the CA4 gene referred to as PCA4, was used in the present study. Our recently published research showed that PCA4 had the best solubility and activity compared to four other proteins²⁴, and so it was selected for use in this study. Briefly, the CA4 gene was amplified using PCR and then two kinds of restriction endonuclease (Kpn I and Hind III) were introduced using the relevant primers. PCR products and plasmid pET30a were both digested using Kpn I and Hind III and then linked to construct the expression vector. Recombinant plasmids were introduced into *E. coli* BL21 to form recombinant bacteria. Protein was produced by the induction of a final concentration of 1 mM IPTG. After induction, over-expressed PCA4 was obtained using ultrasonication. As there is impure protein mixed with the PCA4, the mixed proteins were purified using Ni-NTA agarose (QIAGEN) in accordance with published research³⁷. Shortly after, the mixed proteins were loaded into the Ni-NTA agarose. Then, washing with a buffer (50 mM NaH₂PO₄, 300 mM NaCl, 40 mM imidazole, pH 8.0) and elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0) was carried out to remove impure proteins and collect the targeted proteins (PCA4). The eluent containing target proteins was dialyzed twice in dialysate (100 mM tris-sulfate 100, pH 8.0) for 16 h in total. The complete process of protein purification and dialysis was carried out at 4 °C. SDS-PAGE (12.5% polyacrylamide) was used to analyze the target protein as described by Laemmli⁵⁵ with a little modification. Proteins were stained using Coomassie brilliant blue R-250 and decolouration was performed until the band appeared clear. The “gray value” of the proteins were calculated using Photoshop software and used to represent its content.

Experiment 5 – The effect of CA on wollastonite dissolution. Ultrapure water (49 ml) was added to an Erlenmeyer flask containing 0.116 g of wollastonite; three replicates were tested. Then, 1 ml of ultrapure water and the same amount of PCA4 were rapidly added to the flasks at 35 °C and 130 rpm. Samples were collected at 0, 10, 20, 30, 60, 120, 240, and 480 min (the remaining samples were discarded after each sampling time). The liquid was filtered using a 0.45 μm filter membrane. The concentration of Ca²⁺ was determined by titration using ethylenediaminetetraacetic acid disodium salt (EDTA–Na₂). To explore whether the importance of the role of CA in wollastonite dissolution at high CO₂ concentration was similar, the same operation was carried out at 3.9% CO₂ concentration. To analyze the data, a two-tailed *t*-test was used. The data presented is the mean (along with the standard deviation) of three independent experiments.

Experiment 6 – Mineralization reaction under different CO₂ concentrations. A 10 ml (0.2 M) portion of Tirs–HCl (pH 9.0) was mixed with an equal volume of CaCl₂ (0.2 M) in a clean, sterile Petri dish. Then, 1 ml of ultrapure water and an equal volume of PCA4 were added to the reaction system at 35 °C and rotated at 80 rpm under three different CO₂ concentrations (0.4%, 3.9%, and 10%). There were three independent replications of each treatment. After 20 min, the supernatant was discarded and the sample dried overnight at 65 °C. The residual crystalline CaCO₃ was weighed. The dry weight of 1 ml of PCA4 solution is only a few micrograms, so it is negligible in relation to the weight of the CaCO₃ formed. The statistical approach used to analyze the data was the same as that described above.

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Author contributions

B.L. and L.X. wrote the main manuscript text; B.L., C.L., L.X. and S.W. designed the experiments; J.H. and L.X. carried out the experiments, and L.X. prepared figures 1–5. All authors reviewed the manuscript.

Additional information

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