



RESEARCH ARTICLE OPEN ACCESS

Metabolic Insights Into Microbially Induced Calcite Formation by Bacillaceae for Application in Bio-Based Construction Materials

Michael Seidel¹ | Charlotte Hamley-Bennett² | Bianca J. Reeksting² | Manpreet Bagga³ | Lukas Hellmann⁴ | Timothy D. Hoffmann² | Christiane Kraemer⁴ | Irina Dana Ofițeru³ | Kevin Paine⁵ | Susanne Gebhard^{1,2}

¹Institut für Molekulare Physiologie, Johannes-Gutenberg-Universität Mainz, Mainz, Germany | ²Department of Life Sciences, Milner Centre for Evolution, University of Bath, Bath, UK | ³School of Engineering, Newcastle University, Newcastle upon Tyne, UK | ⁴Nucleic Acid Core Facility, Johannes-Gutenberg-Universität Mainz, Mainz, Germany | ⁵Department of Architecture and Civil Engineering, University of Bath, Bath, UK

Correspondence: Susanne Gebhard (sugebhar@uni-mainz.de)

Received: 14 November 2024 | **Revised:** 26 February 2025 | **Accepted:** 11 March 2025

Funding: This work was supported in part by funding from the Engineering and Physical Sciences Research Council (EPSRC, UK) through the Resilient Materials for Life (RM4L) (EP/P02081X/1) project and the Engineering Microbial-Induced Carbonate Precipitation via Meso-Scale Simulations (eMICP) (EP/S013997/1; EP/S013997) project.

Keywords: acetate metabolism | calcium detoxification | MICP | self-healing concrete

ABSTRACT

Microbially induced calcite precipitation (MICP) offers promising solutions for sustainable, low-cement infrastructure materials. While it is known how urea catabolism leads to biomineralisation, the non-ureolytic pathways of MICP are less clear. This limits the use of the latter in biotechnology, despite its clear benefit of avoiding toxic ammonia release. To address this knowledge gap, the present study explored the interdependence between carbon source utilisation and non-ureolytic MICP. We show that acetate can serve as the carbon source driving calcite formation in several environmental Bacillaceae isolates. This effect was particularly clear in a *Solibacillus silvestris* strain, which could precipitate almost all provided calcium when provided with a 2:1 acetate-to-calcium molar ratio, and we show that this process was independent of active cell growth. Genome sequencing and gene expression analyses revealed an apparent link between acetate catabolism and calcite precipitation in this species, suggesting MICP may be a calcium stress response. Development of a simple genetic system for *S. silvestris* led to the deletion of a proposed calcium binding protein, although this showed minimal effects on MICP. Taken together, this study provides insights into the physiological processes leading to non-ureolytic MICP, paving the way for targeted optimisation of biomineralisation for sustainable materials development.

1 | Introduction

The construction industry is, after transport, the second largest contributor to our current carbon emissions, with cement production alone contributing approximately 8% of anthropogenic CO₂ release (Farfan et al. 2019). Alternative means of either replacing cement in construction materials or expanding the

useful lifespan of cement-based infrastructure are therefore highly desirable from both an environmental and a sustainability viewpoint. The ability of many bacteria to precipitate calcium carbonate in the form of calcite has been gaining interest over recent years for its potential use in construction materials (Castro-Alonso et al. 2019; Justo-Reinoso et al. 2021; Seifan and Berenjian 2018). Calcite is the main component mineral of

Michael Seidel and Charlotte Hamley-Bennett contributed equally to this work.

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2025 The Author(s). *Environmental Microbiology* published by John Wiley & Sons Ltd.

natural limestone; hence its production by bacteria may offer a green route to low-cement or cement-free materials.

One application of microbially induced calcite precipitation (MICP) that has seen considerable progress over the past decade is so-called 'self-healing' concrete. While concrete is extremely strong under compression and very durable under ideal conditions, many environmental stresses or bending strains can lead to the formation of microcracks. These cracks increase water permeability, leading to corrosion of steel reinforcements and reducing the service life of structures (Richardson et al. 2020; Seifan and Berenjian 2018). In self-healing concrete, bacteria are encapsulated, usually in spore form, and added directly to the concrete during casting, together with nutrients and often calcium salts (Alazhari et al. 2018; Seifan and Berenjian 2018; Richardson et al. 2020; Justo-Reinoso et al. 2021; Bagga et al. 2023). Once cracks occur, the encapsulating material of the bacteria breaks to release the cells, while the ingress of water dissolves the nutrients and triggers spore germination. The formation of calcite by the activated bacteria eventually seals the crack and restores the water-tightness of the material to prevent further damage (Alazhari et al. 2018; Seifan and Berenjian 2018; Richardson et al. 2020; Justo-Reinoso et al. 2021).

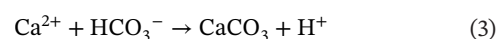
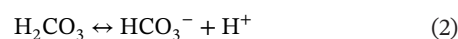
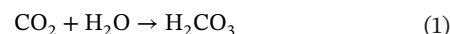
Other potential applications of MICP include consolidation of soils, bioremediation of environmental heavy metal or radionuclide contaminations, or the development of Engineered Living Materials (Srubar 2021; Fu et al. 2023; Dikshit et al. 2022; Kumar et al. 2023). The diversity of technologies that are seeking to exploit the ability of bacteria to form water-insoluble minerals highlights the potential of MICP for the development of sustainable future materials. Despite the intensity of biotechnological research and development of MICP-based applications, we have a surprisingly limited understanding of the molecular mechanism that facilitates biomineralisation. Such knowledge will, however, be required to select the best-suited bacterial species and optimise the applications for maximal mineral formation.

The majority of applications using MICP to date have been focussed on ureolytic bacteria. Here, the enzyme urease catalyses the hydrolysis of urea to ammonia and carbonic acid. The ammonia causes a strong increase in pH, which shifts the carbonate equilibrium from carbonic acid to carbonate ions. In the presence of Ca^{2+} ions, the availability of carbonate and the high pH values lead to precipitation of insoluble calcium carbonate (Hoffmann et al. 2021). Mineral precipitation resulting from ureolysis therefore has been described as a 'passive' process, based on induced chemical changes in the macroenvironment surrounding the bacteria (Castanier et al. 1999). While ureolysis leads to fast and strong biomineralisation, the production of two moles of ammonia per mole of urea can lead to excessive nitrogen loading in the environment and toxic effects on animals, particularly in aquatic ecosystems (European Environment Agency 2019).

An alternative MICP mechanism that has seen promising developments, particularly in self-healing concrete, does not require the presence of urea and is usually simply described as 'non-ureolytic', reflecting our limited understanding of the drivers of biomineralisation. While this form of MICP occurs more slowly than the ureolytic process, non-ureolytic bacteria have been

used successfully in self-healing concrete (Jonkers et al. 2010; Justo-Reinoso et al. 2021; Bagga et al. 2023). Moreover, we could show that several non-ureolytic bacteria isolated from limestone environments were capable of the same high yields of MICP as their ureolytic counterparts (Reeksting et al. 2020). When tested for self-healing of cracked cement mortars, they gave a comparable, if not better, performance, showing their feasibility for industrial application (Reeksting et al. 2020).

Broadly, MICP in non-ureolytic bacteria is thought to rely on carbon dioxide evolved from bacterial metabolism of organic carbon compounds. In aqueous conditions, the resulting presence of dissolved inorganic carbon (DIC), that is, the sum of carbonic acid, bicarbonate and carbonate ions, can cause the formation of calcium carbonate when high concentrations of calcium ions are provided (Equations 1–3) (Hoffmann et al. 2021; Schreiberová et al. 2019; Mondal and Ghosh 2019).



It has also been proposed that active maintenance of a physiological calcium balance in the bacteria could play a role (Hammes and Verstraete 2002). Intracellular calcium concentrations are estimated to be in the range of 0.1–0.3 μM , which is 1000-fold less than typically found outside the cell, especially in MICP conditions (Norris et al. 1996; Dominguez 2004). In such high calcium environments, passive influx will lead to an accumulation of intracellular calcium. This is potentially damaging to the bacteria, which likely counteract the influx by using active exporters, for example, Ca^{2+} -ATPases, to reduce intracellular calcium concentrations to physiological levels. It has been theorised that such active export could lead to a localised accumulation of calcium ions near the cell surface, where they could react with the DIC from the bacterium's carbon metabolism to form calcium carbonate minerals (Hammes and Verstraete 2002).

Taking these contributing components of bacterial metabolism together shows that non-ureolytic MICP is a much more complex process than ureolytic MICP. To understand how biomineralisation occurs and to design effective applications, it is essential to gain detailed mechanistic insights. This involves studying the metabolic properties of non-ureolytic MICP bacteria. Additionally, it is important to understand how carbon and calcium metabolism are linked to mineral formation, both on a physiological and genetic level.

In this study, we set out to address this question by investigating the links between carbon metabolism and MICP in a subset of our previously isolated environmental, non-ureolytic MICP strains (Reeksting et al. 2020). Development of a defined medium based on acetate as the main carbon source allowed us to show that, in some species, carbon catabolism indeed was directly correlated with calcium carbonate formation. Whole genome sequencing of five isolates was used to identify candidate genes that may be involved in a physiological link between carbon metabolism, calcium detoxification and MICP. For the most MICP-active isolate, *Solibacillus silvestris* CGN12, we

could show that calcium addition caused increased expression of genes for both acetate metabolism and calcium detoxification. To investigate this further, an initial system for the genetic modification of *S. silvestris* CGN12 was developed to delete a putative calcium binding protein and assess its impact on MICP. Overall, our data provide insights into the mechanisms of non-ureolytic MICP and experimental evidence that carbon catabolism and potentially calcium detoxification directly influence biomineralisation.

2 | Results

2.1 | Calcium Carbonate Precipitation Using Acetate as a Carbon Source

From a metabolic perspective, ureolytic MICP is straightforward to understand, being largely linked to the hydrolysis of urea to carbonate and ammonia through the activity of the enzyme urease (Stocks-Fischer et al. 1999). For non-ureolytic MICP, the metabolic mechanisms leading to biomineralisation are far less clear. Past studies have mainly used yeast extract-based media for assessing the ability for MICP and the quantity of calcite minerals formed during the growth of non-ureolytic bacteria, for example, the model MICP species *Alkalihalobacillus pseudofirmus* (formerly *Bacillus pseudofirmus*) and *Sutcliffiella cohnii* (formerly *Bacillus cohnii*) (Justo-Reinoso et al. 2021). While growth in such complex media leads to reliable production of biomass and, in the presence of calcium ions, calcite formation, it does not provide insights into specific metabolic pathways used by the bacteria, for example, linking carbon metabolism to calcite formation.

To address this, we set out to develop a more defined growth medium that would allow meaningful comparisons between the performance of model species and uncharacterised environmental strains during growth on a specific carbon source. We focussed on the non-ureolytic environmental species from a previously assembled strain collection, most of which belonged to the genus *Bacillus* or close relatives (Reeksting et al. 2020). Furthermore, we based the growth medium on the composition of CSE medium, a chemically defined medium commonly used to grow *B. subtilis* (Commichau et al. 2008), but with modifications as detailed in the Methods section. In brief, the medium contained a low amount of yeast extract to serve as a source for essential amino acids, vitamins and trace elements. As the carbon source, we selected acetate because this, as well as lactate, are the only two defined carbon sources routinely used in the application of non-ureolytic MICP in cementitious materials and would therefore ensure material compatibility (Justo-Reinoso et al. 2021). Initial genomic analysis suggested that not all of our isolates would be able to use lactate as a carbon source, leading us to select acetate. We named the final medium YA (for yeast extract and acetate) or YAC (yeast extract, acetate, calcium) medium. For routine assays, sodium acetate was provided at a concentration of 100 mM, with additional provision of 100 mM calcium nitrate where MICP was to be tested.

In preliminary tests, this medium was able to support robust growth of many of the initially selected isolates. Of these, we

TABLE 1 | Overview of environmental isolates used for MICP analysis.

Strain	Species ^a	Temperature preference	Reference
BA32	<i>Metabacillus dongyingensis</i>	Mesophilic	(Reeksting et al. 2020)
BA41	<i>Brevibacillus choshinensis</i>		
BA42	<i>Brevibacillus frigiditolerans</i>		
BA43	<i>Bacillus idriensis</i>		
CG7-2	<i>Lysinibacillus varians</i>		
CGN12	<i>Solibacillus silvestris</i>		
CGN14	<i>Jeotgalibacillus</i> sp.		
PD1-1	<i>Bacillus licheniformis</i>		
UBN2	<i>Bacillus frigiditolerans</i>		
UBN3	<i>Solibacillus isronensis</i>		
Psy5	<i>Peribacillus simplex</i>	Psychrotrophic	This study

^aIdentified via 16S rDNA sequencing or available full genome sequences.

chose 10 for further analysis (Table 1). These bacteria all showed mesophilic growth at 30°C. For some applications of MICP outside the laboratory, it is, however, likely that bacteria with a lower temperature growth optimum are required. To this end, we repeated the isolation procedure described previously (Reeksting et al. 2020), but incubated the cultures at 7.5°C. One of the isolates obtained from this (strain Psy5), which could grow well in YA medium and showed robust psychrotrophic growth between 7.5°C and 20°C, was selected for further analysis and included in our list of study species (Table 1). Finally, we included the two model species for non-ureolytic MICP, *A. pseudofirmus* and *S. cohnii*, for comparison and to provide a performance baseline.

For this selection of 13 strains, we assessed which species were capable of MICP using 100 mM acetate as a carbon source. The mesophilic isolates as well as *A. pseudofirmus* and *S. cohnii* were grown at 30°C for 6 days, while the psychrotrophic bacterium Psy5 was grown at 10°C for 12 days due to its slower growth rate. After this time, the amount of precipitated calcium carbonate was quantified. This showed that all tested species could precipitate calcium carbonate under the chosen growth conditions (Figure 1A). The amount of calcium carbonate formed ranged from 20 mM in isolate CGN14 to 60 mM in isolate Psy5, with the two model species falling approximately in the middle of this range. Comparing these values to the theoretical maximal formation of calcium carbonate (100 mM), based on the amount of calcium salt provided in the

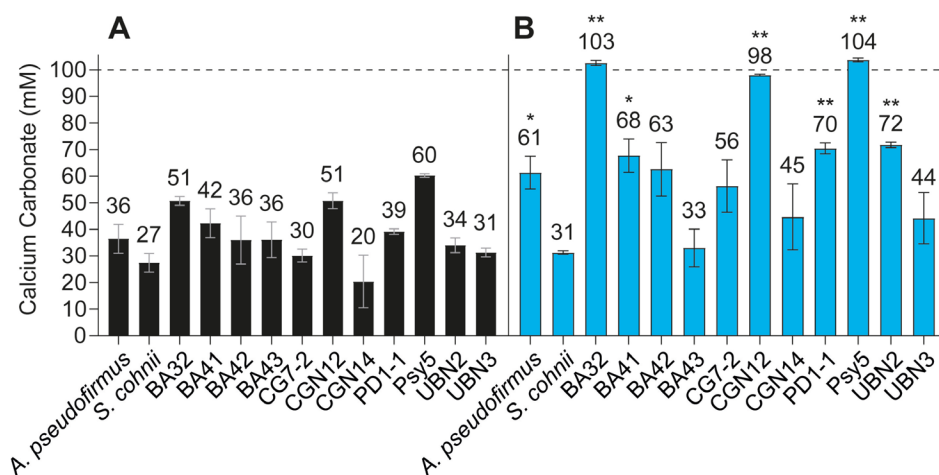


FIGURE 1 | Comparison of calcium carbonate precipitation in YAC medium. *A. pseudofirmus*, *S. cohnii* and 11 environmental isolates were grown in YAC medium containing 100 mM calcium nitrate and either (A) 100 mM or (B) 200 mM sodium acetate. All cultures were grown for 6 days at 30°C except Psy5, which was grown at 10°C for 12 days, at which time the calcium carbonate precipitate was harvested and quantified. Numbers above the columns show the mean of calcium carbonate in mM harvested from 2 to 3 biological repeats. Error bars are the standard deviation of the mean. Results of statistical comparison of data in panel B to data in panel A for each strain by Student's *t*-test are shown as * for $p < 0.05$ and ** for $p < 0.005$. The horizontal line visualises the theoretical maximum of calcium carbonate precipitation based on 100 mM available calcium nitrate. Results are shown as the mean and standard deviation from three biological repeats.

growth medium, showed that only about half of the calcium became biomineralised in these experiments. This suggested that MICP activity was limited by an unknown factor under the chosen conditions.

A likely candidate for the limiting factor was the provision of the carbon source, because metabolic activity under aerobic conditions should convert acetate to CO₂, resulting in an increased concentration of DIC, one of the requirements for calcium carbonate formation (Talaiekhazan et al. 2013). To test if this was the case, the concentration of acetate was doubled, and the experiment was repeated under otherwise identical conditions (Figure 1B). Upon doubling the acetate concentration in the medium, the environmental strains BA32, CGN12 and Psy5 showed a strong and significant increase in calcium carbonate production, precipitating all of the available calcium. The strains PD1-1 and UBN2 also showed a moderate increase in calcium carbonate production, while the remaining strains did not show a significant increase.

Apart from demonstrating that the MICP efficiency in the initial experiment for some strains indeed had been limited by the provision of the carbon source, these results showed that increasing the acetate concentration had different effects across the bacteria tested. In CGN12, BA32 and Psy5, doubling the acetate concentration doubled the MICP yield, suggesting a directly proportional relationship between carbon source and biomineralisation. At the other extreme, MICP by BA43 and *S. cohnii* was not affected by changing the acetate concentration. In these species, it appears that MICP was not immediately linked to the breakdown of this carbon source, even though in all tested cultures no acetate was detectable by the end of the experiment, showing that the organisms were able to consume the provided carbon source. Elucidating the exact reasons for this was beyond the scope of this study, but it is plausible that the precipitation activity of each species

might be improved by optimising the provision of nutrients depending on specific needs.

2.2 | Acetate Metabolism as a Means of Controlling Calcite Precipitation

To further explore the relationship between acetate concentration and calcium carbonate precipitation, we decided to focus on the three strongest precipitators, CGN12, BA32 and Psy5, as well as the better-understood model species *A. pseudofirmus* and *S. cohnii*. HPLC analysis of culture supernatants from day 6 post-inoculation (12 days for Psy5), as in the experiments shown in Figure 1, had not shown any detectable acetate remaining, indicating the carbon source had been consumed completely at some point during the incubation time. Hence, we reduced the growth time to 3 days (6 days for Psy5) so that the rate of acetate utilisation could be determined.

Precipitation profiles were produced at different calcium nitrate concentrations (either 50 or 100 mM) while keeping the acetate concentration fixed at 100 mM. As a general trend, changing only the calcium nitrate concentration appeared to have little effect on the precipitation of calcium carbonate (compare black hatched to black solid bars in Figure 2A). To test if acetate was again the limiting factor, we doubled the amount of acetate (200 mM; Figure 2A, blue bars). Increasing the acetate concentration in the presence of 50 mM calcium salt had no effect (compare blue hatched to black hatched bars), showing that here MICP was limited by the provision of calcium ions. For *A. pseudofirmus*, *S. cohnii* and Psy5 grown in the presence of 100 mM calcium nitrate, there were no obvious changes in precipitation patterns compared to the lower acetate concentration (Figure 2A, blue vs. black bars). However, for BA32 and CGN12, doubling the acetate concentration led to increased precipitation at higher calcium concentrations. This effect was

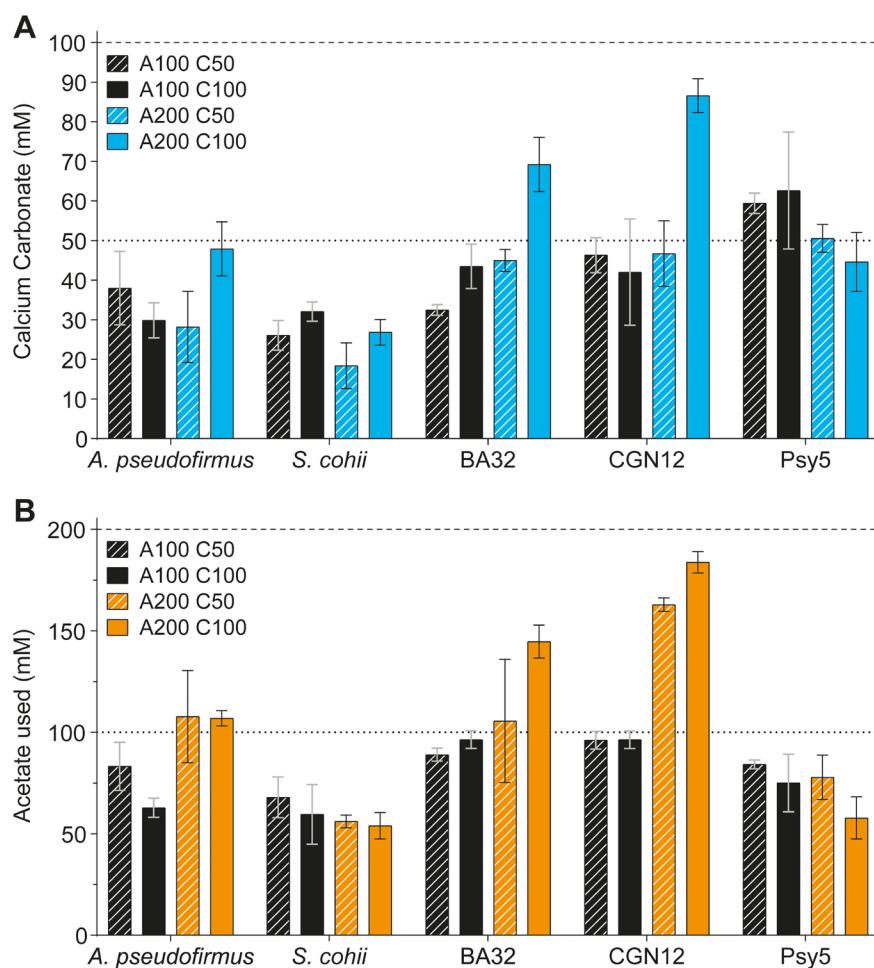


FIGURE 2 | Comparison of calcium carbonate precipitation and acetate consumption between strains. Strains were grown in YAC medium with varied concentrations of acetate and calcium for 3 days at 30°C for all strains except Psy5, which was grown for 6 days at 10°C. A100 = 100 mM, A200 = 200 mM sodium acetate; C50 = 50 mM, C100 = 100 mM calcium nitrate. (A) Quantification of calcium carbonate precipitation at the end of the experiment. Horizontal lines indicate the theoretical maximal calcium carbonate precipitation at 50 mM (thin dashed line) and 100 mM (thick dashed line) calcium nitrate. (B) Acetate consumption (in mM) by the end of the experiment determined via HPLC analysis. Horizontal lines indicate the theoretical maximum amount of acetate utilisation at 100 mM (thin dashed line) or 200 mM (thick dashed line) initial sodium acetate provision. Results are shown as the mean and standard deviation from three biological repeats.

most pronounced for CGN12, where the amount of precipitated calcium carbonate almost doubled with the increase in acetate (Figure 2A, blue hatched vs. blue solid bars). These data confirmed that there was a direct connection between acetate provision, calcium concentration and the amount of calcium carbonate biomineralisation in CGN12 and BA32.

To directly link the increases in precipitation with acetate consumption, acetate usage by each species under the chosen conditions was determined (Figure 2B). *S. coihii* showed similar levels of acetate utilisation at 100 and 200 mM, not increasing usage in response to higher acetate levels, and also calcium concentration had no impact on acetate consumption. Acetate utilisation in *A. pseudofirmus* increased slightly, but not proportionally, when more acetate was available (Figure 2B, orange vs. black bars). At lower acetate concentrations, calcium showed a slight inhibitory effect on acetate consumption by this bacterium (83 vs. 63 mM used); however, this effect was not present at the higher acetate concentration (107 vs. 108 mM used). When provided with 100 mM acetate, CGN12 consumed almost

all of the available acetate (96 mM used), which was unaffected by different calcium concentrations. By doubling the available acetate, acetate usage was nearly doubled (163 mM used), showing that this species' metabolism was exclusively limited by carbon source provision in the YA medium. Increasing the calcium concentration led to a further small increase in acetate consumption by CGN12 (184 mM used). BA32 showed a similar pattern of usage to CGN12, degrading almost all of the available acetate (89–96 mM used) when 100 mM was provided. Similar to CGN12, at the higher acetate concentration, doubling the calcium concentration also stimulated acetate consumption by BA32 (106 vs. 145 mM used).

These data supported our earlier observation that calcium precipitation by BA32 and CGN12 was directly driven by acetate consumption, while in the other species further aspects must be involved that are not easily explained with C- and Ca²⁺-source availability. Particularly in CGN12, it appeared that optimal biomineralisation required a two-fold molar excess of acetate over calcium. A possible explanation for this seemingly fixed ratio

could be the provision of DIC from acetate metabolism, which can then react with available calcium ions to form calcium carbonate minerals. This point is discussed further below.

Interestingly, Psy5, which had shown a clear acetate-dependency of biomineralisation when tested over the original 12-day period (Figure 1), did not demonstrate such an effect over the shorter incubation period used here. When provided with 100 mM calcium salt, it only produced about half the amount of theoretically possible mineral, regardless of how much acetate was provided (Figure 2A). Consistent with this, quantification of acetate consumption also showed no change between the four growth conditions (Figure 2B). It is likely that the lower incubation temperature and much slower growth of this species impacted its metabolism and biomineralisation in a way that halving the incubation period did not allow the completion of either acetate consumption or calcium carbonate formation.

To further investigate the links between acetate metabolism and calcium carbonate biomineralisation, we focused on the environmental isolate CGN12 and the model species *A. pseudofirmus* for the rest of this study. The rationale for this selection was that CGN12 showed the strongest link between acetate consumption and MICP, while *A. pseudofirmus* at least showed a small increase in biomineralisation at the elevated acetate concentration.

2.3 | Differences in Precipitation Kinetics Between Species

To gain a better understanding of the link between acetate metabolism and biomineralisation on a temporal scale, we next determined the precipitation kinetics for CGN12 and *A. pseudofirmus*. Both species were inoculated into YAC medium containing 100 mM calcium nitrate and either 100 or 200 mM sodium acetate, and cell growth as colony forming units per mL⁻¹ (CFU/mL), remaining acetate concentration and amount of precipitated calcium carbonate were determined after 1, 2, 3 or 6 days of incubation (Figure 3).

In agreement with our previous observations, CGN12 precipitated calcium carbonate depending on the available acetate, reaching maximal levels when all acetate was consumed (Figure 3, orange symbols). This was after 2 days in the cultures with 100 mM acetate, after which precipitate amounts no longer increased. At the higher acetate concentration, the substrate was only fully consumed by Day 6, and accordingly, more mineral precipitate was formed until that time point. For *A. pseudofirmus*, acetate consumption was generally slower than for CGN12, requiring 3 days to consume the 100 mM concentration, and residual amounts were still detectable after 6 days when 200 mM were initially provided (Figure 3, grey symbols). As with CGN12, mineral production reached the highest levels at the time point where acetate was completely consumed, although overall lower levels of biomineralisation were observed (approx. 40% of the theoretical maximum) with no increase observed at higher starting acetate concentrations, consistent with the earlier assays.

Because acetate serves as a general carbon source in the medium, we needed to ensure that the apparent beneficial effect of acetate on calcium precipitation by CGN12 was not simply due to a general improvement of growth when more carbon source was available. To this end, we determined the Monod constant for CGN12 growth on acetate, that is, the concentration that supported the half-maximal exponential growth rate, akin to Michaelis–Menten kinetics of enzymes (Pirt 1975). This resulted in values of $K_s = 69$ mM (Monod-constant) and $\mu_{MAX} = 1.04$ h⁻¹ (maximal theoretical growth rate) (Figure S1), showing that both 100 and 200 mM acetate were considerably higher than the Monod constant, and only a marginal increase in growth should be expected from this change in concentration.

Interestingly, in both species, cell growth (detected as viable cell counts) plateaued already after Day 1 of incubation, at a time when acetate was still available and still being consumed. Cell growth was therefore limited by something other than the carbon source, while biomineralisation in CGN12 appeared only to be determined by the availability of sufficient acetate. Consistent with this observation, in both species, biomineralisation was

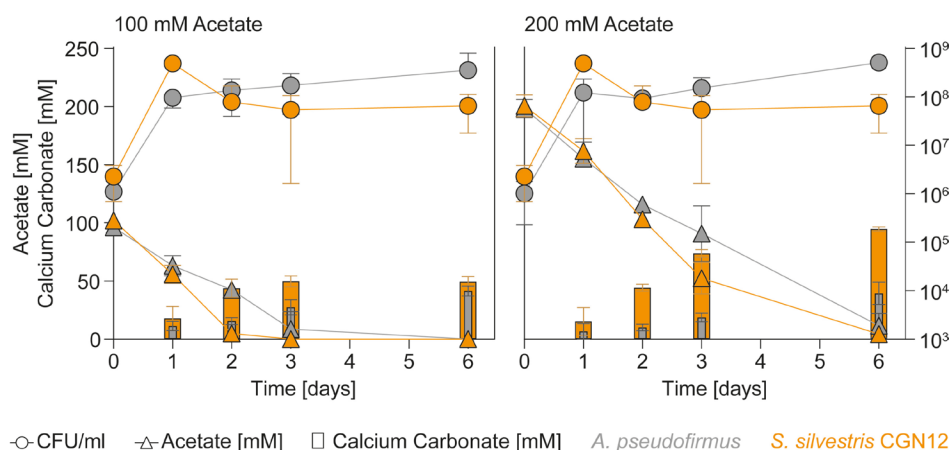


FIGURE 3 | Precipitation kinetics of *A. pseudofirmus* and CGN12. Viable cell counts (CFU/mL), calcium carbonate precipitation and acetate consumption for *A. pseudofirmus* and CGN12 grown in YAC medium at different concentrations of sodium acetate were monitored over 6 days at 30°C with shaking (140 rpm). The concentration of calcium nitrate was fixed at 100 mM. Data are shown as mean and standard deviation of the mean for three biological replicates.

minimal after 1 day and continued to increase with longer incubation. This showed that mineral formation was linked to acetate catabolic activity, but not active cell growth, which is addressed in more detail next.

2.4 | Acetate Drives MICP in Stationary Phase Cells of *S. silvestris* CGN12

The observation that the majority of calcium carbonate formation occurred during the stationary growth phase of CGN12 next prompted us to investigate biomineralisation specifically in this growth phase. This setup would allow us to track only acetate consumption and mineral formation, while cell counts and therefore biomass were held approximately constant. To this end, we inoculated five parallel small flask cultures with CGN12, each containing 50 mL of YA medium (180 mM sodium acetate), and allowed the cultures to reach the stationary phase after 24 h of incubation. At this point, 100 mM calcium nitrate was added to each flask and incubation continued for another 24 h. For each time point, one entire culture was harvested to quantify precipitated calcium carbonate, viable cell counts (CFU/mL) and the remaining soluble calcium and acetate concentrations (Figure 4).

As seen before in the precipitation kinetics tests, at entry into the stationary phase (24 h time point), the majority of the provided acetate remained, and acetate consumption proceeded at a steady rate over the following 24 h, while cell counts remained stable (Figure 4, orange and black symbols). Interestingly, the concentration of soluble calcium showed a similarly steady decrease over the incubation period as for acetate. At the same time, we observed a gradual increase in insoluble CaCO_3 mineral (Figure 4, blue open and closed symbols). The appearance of the mineral accurately

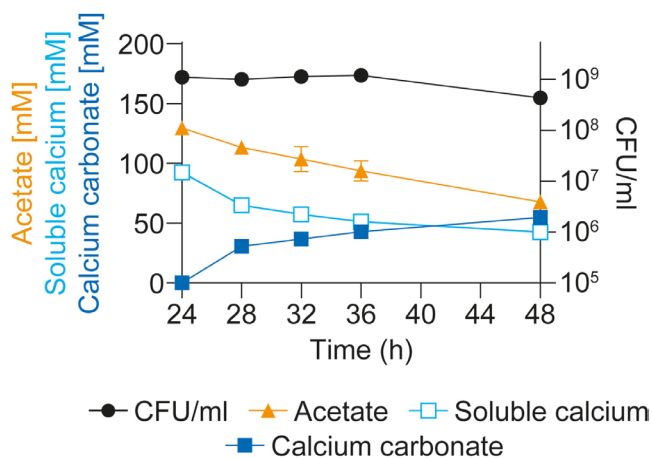


FIGURE 4 | Acetate-driven biomineralization in stationary phase cells of *S. silvestris* CGN12. Cells were grown in five parallel flasks in YA medium with 180 mM acetate for 24 h to reach stationary phase. At this point, 100 mM calcium nitrate was added aseptically, and the cultures were incubated for a further 24 h. At 4, 8, 12 and 24 h after the addition of calcium nitrate, one entire flask was harvested and precipitated calcium carbonate, viable cells (CFU/mL), and the concentrations of soluble calcium and acetate were quantified. Results are shown as mean and standard deviation from two biological repeats.

accounted for the amount of soluble calcium that had disappeared from the medium, with between 92% and 97% of total calcium recovered at each time point. As a negative control, an uninoculated control flask was also tested for precipitation after the addition of calcium nitrate. The slightly alkaline pH of the medium resulted in the production of 1.6 mM (± 0.1) calcium carbonate over the same 24 h period, which was negligible compared to the biological formation of mineral in the bacterial cultures.

The consistent rate of reduction in both acetate and soluble calcium concentrations during the time-course of this experiment suggested that the bulk of the provided acetate was needed during the stationary phase and that at least some of the carbon from acetate degradation likely went towards calcium carbonate production via the formation of DIC.

2.5 | The Yeast Extract Component of YAC Medium Does Not Significantly Contribute to Biomineralisation

So far, we had assumed that the yeast extract in the growth medium did not significantly contribute to DIC provision. To confirm this, the individual contributions of acetate or yeast extract to the precipitation of calcium carbonate were assessed in resting cell assays with CGN12. This format was necessary because CGN12 was unable to grow in the defined medium when yeast extract was omitted, but it could maintain viability if cells pre-grown in YA medium were resuspended to high densities ($\text{OD}_{600}=4$) in a solution that corresponded to YAC medium (50 mM calcium nitrate) but missing the yeast extract component. Following incubation for 3 days, cell viability was determined as CFU/mL, acetate consumption quantified by HPLC, and the precipitate harvested and quantified (Table 2).

When the cells were provided with calcium nitrate and yeast extract but no acetate, no precipitation was observed after 3 days, demonstrating that neither the small amount of yeast extract present in the YAC medium nor carbon dioxide from the atmosphere was contributing relevant amounts of DIC for calcium carbonate precipitation. Hence, under the conditions tested, CGN12 appears to only use the carbon from acetate metabolism for calcium carbonate precipitation.

Where acetate was present, CGN12 was able to consume the entire provided carbon source during the 3 days of incubation. This acetate consumption did not result in further cell growth because viable counts were slightly lower than where no acetate was provided (Table 2). Instead, acetate again appeared to be the limiting factor for the precipitation of the provided calcium nitrate, closely recapitulating the earlier results from cells actively grown in YAC medium. As before, a twofold molar excess of acetate over calcium was required for complete precipitation because at 50 mM acetate, only approximately half the calcium was recovered as CaCO_3 , whereas it was nearly completely precipitated at 100 mM acetate (Table 2). Further comparison of CaCO_3 production to acetate consumption showed that under both conditions the ratio of carbonate to acetate was almost identical at 0.48 (Table 2). Given that acetate is a C_2 -compound, this implies that approximately 25% of the carbon from acetate seemed to go consistently towards mineral formation, regardless of the initial amount of acetate provided.

TABLE 2 | Resting cell precipitation assay of CGN12 under different concentrations of acetate.

Acetate (mM) ^a	Average cell count at end of assay (CFU/mL) ^c	CaCO ₃ produced (mM)	Acetate utilised (mM)	Stoichiometry (CaCO ₃ : acetate)
0 ^b	4.50E+08	0.0 (±0.0)	0.0 (±0.0)	—
50	1.30E+08	23.8 (±1.5)	49.1 (±3.0)	0.48 (±0.01)
100	3.00E+07	47.5 (±0.5)	102.4 (±5.1)	0.47 (±0.02)

^aMedium composition corresponded to YAC medium with 50 mM calcium nitrate but without the yeast extract component.

^bYA base medium with 0.2% yeast extract provided as the sole carbon source.

^cCells were resuspended to a starting OD₆₀₀ = 4, corresponding to viable cell counts of approximately 5×10^8 to 2×10^9 cfu/mL on Day 0. The end-point of the assay was Day 3.

2.6 | Acetate as the Main Carbon Source in Self-Healing Concrete

As MICP in CGN12 can be driven by the provision of acetate in laboratory culture, we next wanted to test if acetate could be used as the main carbon source in an applied setting. To this end, the performance of CGN12 and the non-ureolytic model MICP bacterium *A. pseudofirmus* were assessed in self-healing cement mortars using the previously established standard procedure (Tan et al. 2023). In brief, spores were produced for each strain and then encapsulated in lightweight aggregate as described in materials and methods. Encapsulated spores were cast into mortars with sodium acetate, calcium nitrate and trace amounts of yeast extract as nutrients dissolved in the mixing water, with concentrations corresponding to YAC medium composition. Mortars containing the same nutrients but no added spores ('Control') and plain mortars containing neither nutrients nor bacteria ('Reference') were produced for comparison. Once hardened, mortars were cured and then cracked under 3-point bending to obtain a target crack width of ~0.5 mm. Following this, they were semi-submerged in tap water at room temperature and the self-healing process monitored over 8 weeks (Figure 5A).

Autogenous healing in the reference mortars containing standard components only was minimal, with 1%–3% crack area reduction over the 56-day period. Some self-healing (23%–25%) was observed in the control mortars, where nutrients but no bacteria were added. This is a common observation in these experiments, as self-healing is not tested under sterile conditions, and environmental bacteria are likely to facilitate low levels of healing using the nutrients and additional calcium ions provided (Tan et al. 2020). Mortars containing *A. pseudofirmus* spores showed a lower percentage of crack healing than the control mortars (13% after 56 days), consistent with the low MICP activity of this strain when grown on acetate as the main carbon source. In contrast, CGN12 displayed greater crack healing than the control after 7 days (39%), although this subsequently reduced to 23% after 56 days (Figure 5A,B).

While quantification of the decrease in crack area between samples is a useful tool for monitoring the early stages of crack healing, it can only give visual information of the crack surface but cannot account for healing product formed at the depth of the crack (He et al. 2023). The main aim of using biomineralisation in self-healing concrete is to re-establish the water tightness of the structure, which is lost when microcracks form, in turn leading to corrosion of internal steel reinforcements and eventually

structural failure. To assess self-healing performance, water flow assays can test the restoration of watertightness in cracked mortars, which can be mediated by healing product at both crack surface and deeper in the fissure. When we tested our mortars after 30 and 56 days of healing, reference mortars (which contained no nutrients, calcium or spores) showed almost no recovery of watertightness of 1%–3% (Figure 5C), consistent with the crack area reduction data. Control mortars, which contained nutrients and calcium but no spores, showed up to 27% recovery of watertightness after 56 days, likely due to the activity of environmental bacteria, as mentioned above. While mortars containing *A. pseudofirmus* spores showed a high degree of variability between duplicates and only barely surpassed the control specimens, samples containing CGN12 achieved the greatest recovery of water tightness, averaging 69% after 30 days and 70% after 56 days (Figure 5C).

Taken together, these results show that CGN12 can provide self-healing properties and restoration of water tightness when cast into cement mortars and provided with acetate as the main carbon source.

2.7 | The Genetic Basis for Variation in Calcite Precipitation Between Species

The experimental evidence presented so far suggested that non-ureolytic MICP, at least in some bacteria, is driven by cellular metabolism of carbon sources such as acetate. To explore if the differences between species observed in the precipitation assays could be explained by differences in the metabolic enzymes each species possessed, a targeted genomic comparison was performed. For this, we primarily focused on genes that were annotated as being involved in acetate metabolism. Additionally, we were interested in genes encoding putative calcium binding domains that could be exposed on the cell surface and might influence MICP, positively or negatively, via either creating a local calcium enrichment or sequestration of calcium ions.

To this end, we returned to the five strains from our initial set that showed a positive effect on MICP via increasing the acetate concentration: CGN12, BA32, PD1-1, UBN2 and Psy5 (Figure 1). Genomic sequences for these strains were obtained through a combination of Illumina short-read sequencing and Oxford Nanopore long-read sequencing. The high-fidelity short-read sequences were assembled using the long-read sequences as scaffolds, leading to nearly closed (2–12 contigs) draft genomes for

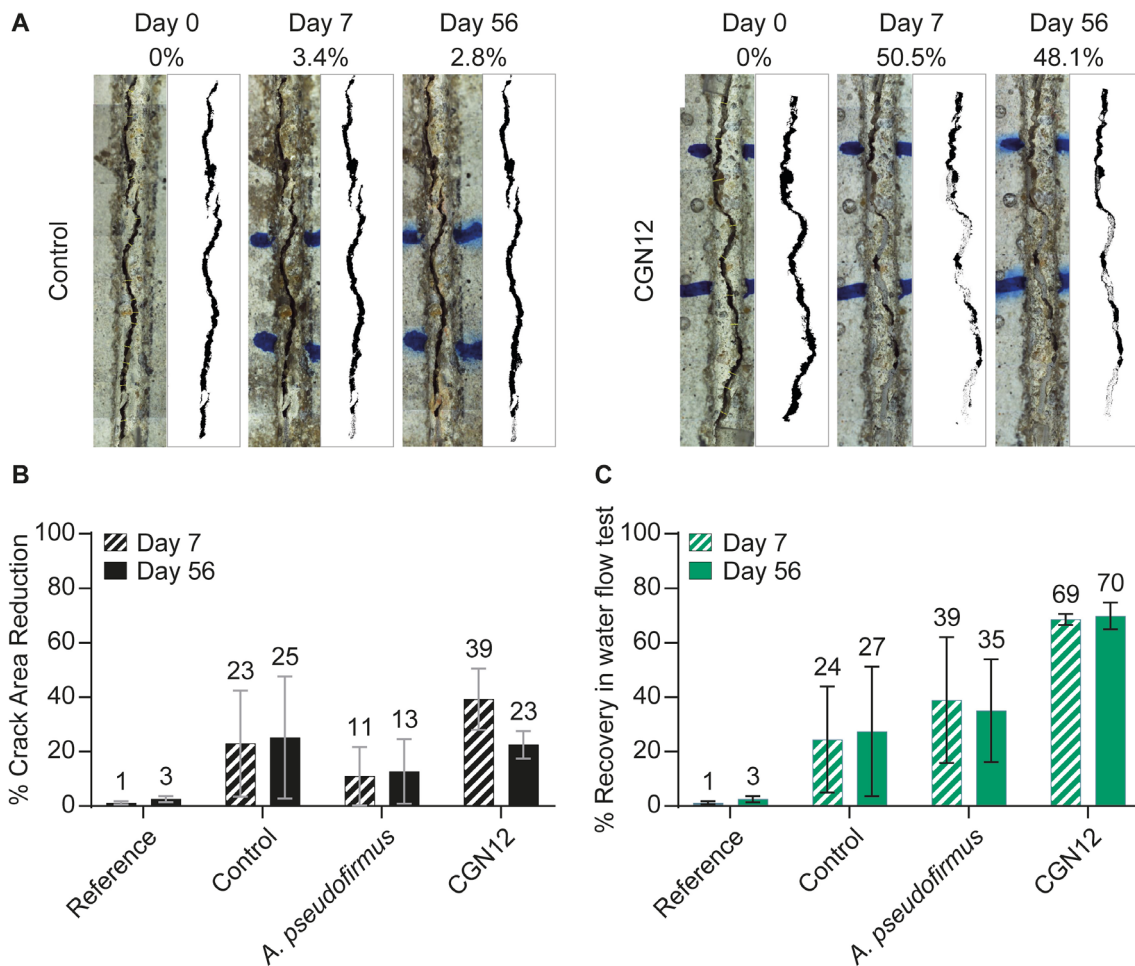


FIGURE 5 | CGN12 efficiently seals cracks in mortar prisms. (A) Crack closure in control and CGN12-containing mortars with acetate provided as the main carbon source. Pictures were taken as a top-down view onto the surface of the specimen. A representative mortar sample is shown immediately after cracking and then after 7 and 56 days of healing. Digital rendering of the crack areas is shown to the right of each sample with the calculated percentage in crack area reduction shown above. Cracks were marked with blue pen to allow the same region to be monitored over time. Due to the limited imaging area of the microscope, images were manually assembled from multiple photographs of overlapping sections of the specimen. (B) Crack healing in mortars shown by average % area reduction of cracks from digitally rendered images of duplicate samples. (C) Crack healing in mortars shown by average % recovery of watertightness determined by water flow tests in duplicate samples. Each dataset in panels B and C contains reference samples (no nutrients, calcium or spores), control samples (nutrients and calcium, but no spores) and samples containing nutrients, calcium and either *A. pseudofirmus* or CGN12 spores.

these new isolates (Table S3). Published sequences for *A. pseudofirmus* and *S. cohnii* were obtained from NCBI (<https://www.ncbi.nlm.nih.gov/>) for comparison.

To analyse the gene content for acetate metabolism, we focused on genes encoding the enzymes of the tricarboxylic acid (TCA) cycle, as well as the enzymes needed to activate acetate for entry into the TCA cycle (Figure 6A). For each of the seven species analysed, we identified the presence or absence of the gene using the KEGG database and determined the copy number for each gene (Table 3, Table S4). In all analysed genomes, genes for the core metabolic pathway for acetate degradation via the TCA cycle were consistently present. All genomes included 2–3 copies of a gene encoding Acetyl-CoA-Synthase (ACS, EC 6.2.1.1), needed to feed acetate into the TCA cycle for degradation. Interestingly, however, the genome of CGN12 also contained a gene for an ADP-forming ACS (EC 6.2.1.13), which is normally only found in archaea. At the same time, its genome was the only one to lack

genes for the enzymes phosphate transacetylase (PTA) and acetate kinase (ACK), encoding the enzymes used by most bacteria to produce acetate as a metabolic end product. These differences may suggest the presence of a unique acetate metabolism in this strain.

Another observation in the acetate metabolic genes was the presence of a gene encoding a putative succinyl-CoA:acetate CoA transferase (SAT, EC 2.8.3.18) in several of the analysed species (Figure 6A, Table 3, Table S4). This enzyme is involved in heterotrophic growth on acetate using a modified TCA cycle where conversion of succinyl-CoA to succinate is coupled to activation of acetate to acetyl-CoA in a single enzymatic reaction and with the expenditure of only a single ATP-equivalent (Pettinato et al. 2022). *S. cohnii*, Psy5 and UBN2 both possess one copy of such a gene, while CGN12 harbours two, highlighting yet another unique feature of its acetate metabolic genes.

In addition to acetate metabolism, we were also interested in the differences in calcium binding proteins between our strains. Table 4 and Table S5 show the copy number and locus tags, respectively, of genes for selected calcium binding proteins: Excalibur domain proteins, which have a calcium binding motif but an unknown function (Rigden et al. 2003), as well as an F-type and a P-type calcium binding ATPase thought to be involved in calcium homeostasis through export (Norris et al. 1996). All analysed species possess a single copy of each gene encoding the two calcium ATPases. Genes coding for an Excalibur domain protein are present in BA32, CGN12, PD1_1 and UBN2, but such a gene is absent from *A. pseudofirmus*, *S. cohnii* and Psy5. Thus, in addition to differences in acetate metabolism, we also detected notable differences in calcium binding proteins that are likely to be surface exposed. Given that acetate metabolism provides DIC, and calcium binding proteins may affect the local concentration of calcium ions near the cell surface, it is conceivable that these genomic differences contribute to the differing MICP properties of the analysed strains.

2.8 | The Effect of Calcium on the Gene Expression of Metabolic Enzymes and Calcium Binding Proteins

To better understand the role of the identified acetate metabolism and calcium binding domain encoding genes for MICP in CGN12, we determined the expression levels of some of these genes under MICP-conditions using quantitative reverse transcriptase PCR (qRT-PCR). As the earlier experiments had shown that acetate-driven MICP by CGN12 occurs mostly in stationary phase, we here created the relevant condition by exposing stationary-phase cells to a calcium stimulus (100mM calcium nitrate) for 30 min. Untreated cells served as the control. The genes of interest were those encoding the two distinct types of ACS enzymes (gene locus AB1K09_14095 for EC 6.2.1.1 and locus AB1K09_11545 for EC 6.2.1.13), one of the genes encoding an SAT (gene locus AB1K09_18240), the P-type Calcium binding ATPase (gene locus AB1K09_00890) and one of the Excalibur domain proteins. For the latter, the gene with locus tag AB1K09_06620 was chosen, which contains only an Excalibur

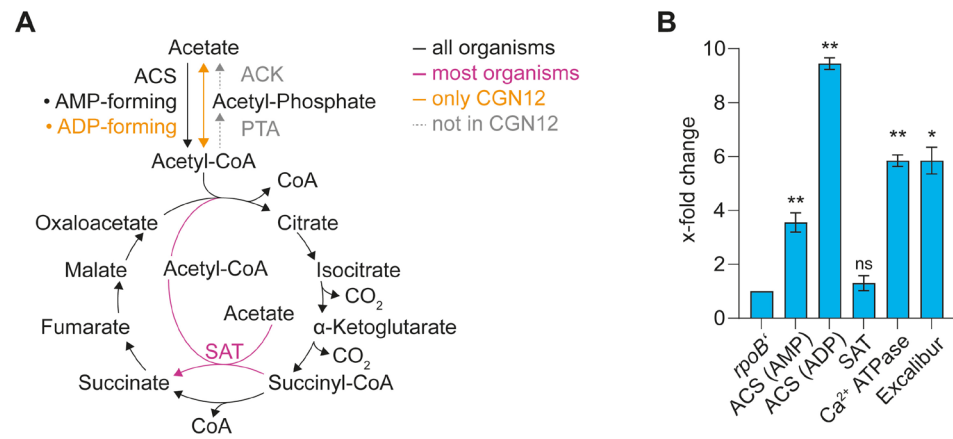


FIGURE 6 | Genomic comparisons of isolated species and changes in gene expression in CGN12. (A) Similarities and differences in how acetate can feed into the TCA cycle in selected species. ACS, Acetyl-CoA-Synthetase, showing two types of the enzyme, one forming AMP and the other forming ADP during the reaction; ACK, Acetate kinase; PTA, Phospho-transacetylase; SAT, Succinyl-CoA:Acetate CoA transferase. (B) Changes of gene expression in CGN12 upon calcium challenge, determined by RT-qPCR. X-fold changes were calculated using the Pfaffl equation from biological duplicates and using the *rpoB'* gene as internal standard. Samples were taken before and 30 min after the addition of 100mM calcium nitrate to stationary phase cells to mimic MICP conditions. *, $p < 0.01$; **, $p < 0.0001$; ns, not significant in two-sided Student's *t*-test.

TABLE 3 | Genomic comparison of the number of acetate and TCA cycle-related genes in the selected species.^a

Organism	Acetyl-CoA synthetase (AMP-forming)	Acetyl-CoA synthetase (ADP-forming)	Acetate kinase	Phosphate acetyltransferase	Succinyl-CoA: acetate CoA transferase
<i>A. pseudofirmus</i>	3	—	1	1	—
<i>S. cohnii</i>	3	—	1	1	1
BA32	2	—	1	1	—
CGN12	2	1	—	—	2
PD1_1	3	—	1	1	—
Psy5	2	—	1	1	1
UBN2	2	—	1	1	1

^aFor details of the locus tag for each identified gene, please refer to Table S4.

TABLE 4 | Genomic comparison of the number of selected calcium homeostasis-related genes in the selected species.^a

Organism	Excalibur domain proteins	F-type Ca ²⁺ ATPase	P-type Ca ²⁺ ATPase
<i>A. pseudofirmus</i>	—	1	1
<i>S. cohnii</i>	—	1	1
BA32	2	1	1
CGN12	2	1	1
PD1_1	1	1	1
Psy5	—	1	1
UBN2	1	1	1

^aFor details of the locus tag for each identified gene, please refer to Table S5.

domain, whereas the second gene (AB1K09_02315) encodes a protein with an Excalibur, S-layer homology and metallo-beta-lactamase domains, which makes it more difficult to predict its potential function. As a constitutively expressed reference gene, the housekeeping gene *rpoB'* encoding the β' subunit of RNA polymerase was used.

As expected, addition of calcium led to an increase in expression of the genes putatively involved in calcium export or binding, with the level of the P-type calcium ATPase gene increasing 5.9-fold and the Excalibur domain gene 5.8-fold compared to the untreated sample (Figure 6B). The upregulation of the ATP-dependent calcium transporter upon calcium addition potentially reflects a need of the cell to remove excess calcium ions that passively entered the cytoplasm through active export. The role of the Excalibur domain protein is less clear and is discussed in more detail below.

Interestingly, both of the ACS-encoding genes showed an increase in expression, 3.5 and 9.5 times higher, respectively, in the treated condition compared to the untreated sample (Figure 6B). The SAT, presumably involved in a modified TCA cycle, did not show any significant increase in expression in the presence of calcium and may therefore either play a minor or constitutive housekeeping role in acetate degradation. These findings suggest an increase in acetate catabolic activity in response to calcium, which may be required to meet the increased energy demand from the upregulation of the ATPase. Such an upregulation of acetate degradation could potentially explain the slightly higher degree of acetate consumption we had observed for CGN12 when grown in high-calcium conditions (Figure 2B).

2.9 | Deletion of the Putative Calcium-Binding Excalibur Domain Protein in *S. silvestris* CGN12

Our RT-qPCR data showed a clear upregulation of a gene encoding a putative calcium-binding Excalibur domain protein (locus tag AB1K09_06620) upon a calcium stimulus. Structure predictions using AlphaFold 3 (Abramson et al. 2024) show that the protein almost exclusively consists of a calcium binding domain, in which all residues of the typical binding motif of DxDxDGxxCE

are present and suitably oriented to bind the predicted Ca²⁺ ion ligand (Figure S2; Rigden et al. 2003). The protein sequence also contains the two fully conserved cysteine residues that characterise Excalibur-domains and likely act as structural constraints through disulphide bond formation (Rigden et al. 2003). The N-terminal end of the protein is predicted to be unstructured and to contain a signal peptide, which is another common feature of Excalibur-domain proteins (Figure S2C; Rigden et al. 2003). The molecular function of this protein or indeed Excalibur domains in general is not yet known, other than that this domain typically occurs in surface-exposed proteins of bacteria and has similarity to the typical calmodulin-like EF-hand Ca²⁺ binding domains (Rigden et al. 2003). The structure prediction of the *S. silvestris* CGN12 Excalibur-domain protein therefore is consistent with its annotation as an extracellular calcium binding protein.

To further investigate this gene's putative role in MICP or calcium homeostasis, we generated an unmarked deletion of the excalibur gene (Δexc) in CGN12 via double homologous recombination. To this end, the well-established vector pMAD, possessing a temperature-sensitive origin of replication and used for the construction of unmarked gene deletions in *B. subtilis* (Arnaud et al. 2004), was modified with an origin of transfer (*oriT*) from plasmid pG2k-oriT-gfp. The resulting plasmid, pMAD-oriT, could replicate in CGN12, and the *oriT* addition facilitated conjugation from *Escherichia coli* donor strains to CGN12. Despite the lower routine growth temperature of CGN12 (30°C) compared to *B. subtilis* (37°C), the temperature shifts required to control vector replication and thus the two homologous recombination events leading to gene deletion could be applied with minor modifications of the established protocols as described in the methods section.

Once the Δexc strain had been obtained, we compared its calcium carbonate precipitation to that of the parental wild type. Overall, we did not observe a marked alteration in precipitation yields between the two strains at the previously used calcium and acetate concentrations (Figure 7). We did observe a small but reproducible shift in the CaCO₃-to-acetate stoichiometry from 0.47 ± 0.01 in WT to 0.54 ± 0.01 in the Δexc strain, indicating that the latter might form slightly more calcium carbonate per acetate consumed than the wild type. Given how consistent the stoichiometry was for CGN12 between repeat experiments and different growth conditions, this 15% shift may be biologically meaningful and imply a role of the Excalibur-domain protein in calcium binding, potentially sequestering the ions and making them unavailable for crystal formation. Further investigations of this protein will be required to determine if it really contributes, positively or negatively, to non-ureolytic MICP in CGN12 or other bacteria with such genes.

3 | Discussion

MICP serves as an exciting approach for more sustainable infrastructure materials. While ureolytic MICP is well understood, there is only little knowledge on the non-ureolytic pathway(s) leading to biomineralisation. A recent review highlighted the improved sustainability and economic feasibility

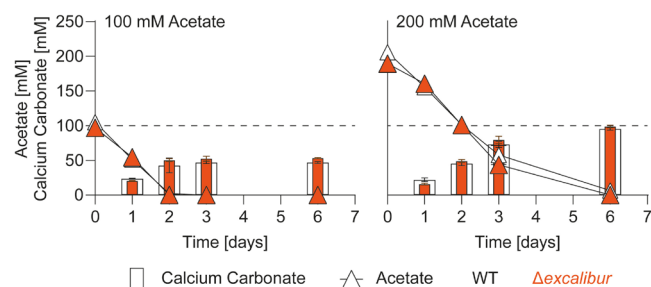


FIGURE 7 | Cells lacking the Excalibur domain protein are not affected in MICP or acetate metabolism. Calcium carbonate precipitation and acetate consumption were determined for wild-type CGN12 and the Dexc deletion strain grown at different concentrations of sodium acetate. The initial concentration of calcium nitrate was 100 mM in standard YAC medium. Cells were grown over 6 days at 30°C with shaking (140 rpm), and one entire flask culture was sacrificed for each time course for quantification of precipitate formed and acetate remaining. Dashed horizontal lines indicate the theoretical maximum calcium carbonate precipitation. Data are shown as mean and standard deviation of the mean for two biological replicates.

non-ureolytic pathways may be able to offer (Fahimzadeh et al. 2022), further underscoring their potential for biotechnological and materials science application. This study therefore aimed to give insights into the metabolic processes of non-ureolytic MICP by investigating several environmental isolates. We could show that in certain species, the provision of acetate as a carbon source can be a major driver of mineral formation. Additionally, full genome sequencing, gene expression analysis, and the development of basic molecular techniques were used to shed some light on the underpinning processes and genetic features that contribute to non-ureolytic MICP.

Specifically, in the isolate *S. silvestris* CGN12, acetate consumption was directly linked to MICP, even though only a small proportion of the provided acetate was used by the bacteria for cell growth. In fact, the majority of acetate consumption occurred during the stationary growth phase, which was also the phase in which detectable MICP took place. Moreover, we observed a consistent carbonate-to-acetate ratio of 0.47–0.48 in the precipitation assays, suggesting that a quarter of the acetate-derived carbon was likely bound into CaCO_3 , allowing *S. silvestris* CGN12 to precipitate almost all of the supplied calcium, as long as enough acetate was available. It should be noted that without a detailed ^{13}C carbon flux analysis, we cannot yet determine if the carbon in the mineral directly derives from acetate, or whether a more indirect mechanism is responsible for the fixed stoichiometry we observed. Nevertheless, the consistent observation of the same carbonate-to-acetate ratio regardless of the growth conditions for *S. silvestris* CGN12 strongly suggests that this factor is an intrinsic property of this bacterium's metabolism. As discussed in more detail below, the only condition where we could observe a small increase in the amount of carbonate formed per acetate consumed was upon deletion of the gene encoding an Excalibur-domain protein. While we do not yet understand the molecular reasons for this observation, it may indicate that deliberately improving this ratio, either through targeted manipulation of existing strains or through specific

selection of isolates with a high carbonate yield per acetate (or other carbon source) use, may be an effective means of optimising MICP performance for application and industrial use.

Overall, our findings on the link between acetate catabolism and calcium carbonate formation are in accordance with previous reports that suggested that non-ureolytic MICP is governed by DIC and the available calcium ions, as well as the presence of nucleation sites (Hammes and Verstraete 2002; Hoffmann et al. 2021). In our experimental setup, we reduced the possibility of indirect CaCO_3 precipitation due to alkaline pH by using a buffered medium. Together, these observations indicate that non-ureolytic MICP can occur independently of active cell growth, with acetate catabolism leading to DIC formation to fuel the MICP process. This adds a new aspect to how heterotrophic pathways can lead to non-ureolytic MICP.

Comparing the different environmental isolates and laboratory strains showed that the metabolic link between acetate consumption and MICP was not universal among all non-ureolytic MICP bacteria. The observed variations suggest that different species may utilise alternative metabolic pathways or carbon sources to drive DIC production, leading to MICP. Of note, we here focussed exclusively on acetate as a potential carbon source. Past studies of using non-ureolytic bacteria for application in self-healing concrete have either used solely yeast extract to provide growth nutrients to the bacteria or supplied acetate or lactate salts as additional carbon sources (Justo-Reinoso et al. 2021). For later use of the bacteria in a concrete-based application, the carbon source must be compatible with cement chemistry; hence, no carbon sources with limited evidence of material compatibility were tested here. Our choice of acetate over lactate was based on preliminary searches of available genome sequences of strains related to our isolates, which indicated that not all strains possessed lactate dehydrogenase encoding genes and would thus not be able to use lactate as a sole carbon source. Indeed, genome sequencing of *S. silvestris* CGN12 later showed that this strain also lacked such a gene. However, for the application of other bacteria in MICP-based technologies, exploration of a potential means of driving MICP with alternative carbon sources should be a valuable future research direction. Understanding such metabolic nuances will be crucial for the application of MICP technologies in the future because it allows a precise design of nutrient provision to maximise mineral formation by a given bacterial strain.

Other pathways that have been shown to lead to non-ureolytic MICP include sulphate reduction, methane oxidation, denitrification and photosynthesis-driven MICP (Hoffmann et al. 2021). However, many of these lead to byproducts that are either toxic (e.g., hydrogen sulphide or ammonia) or cause metal corrosion, rendering them unsuitable for application in construction materials, especially steel-reinforced concrete (Fahimzadeh et al. 2022). The pathways considered suitable for application are limited to denitrification, photosynthesis and oxidation of organic compounds. Denitrification, for example, by *Pseudomonas* sp., has the advantage of functioning in low- O_2 environments, and considerable progress has been made to understand the nutritional requirements for optimal application of such bacteria, for example, in soil consolidation or crack healing in concrete (Erşan et al. 2015a, 2015b; Fahimzadeh et al. 2022). A potential

drawback of denitrification-dependent MICP could be the production of toxic N_2O under some specific application conditions (Fahimzadeh et al. 2022). Photosynthetic MICP requires light and is therefore more suitable for applications like carbon capture or restoration of concrete surfaces than material-embedded processes, for example, soil consolidation or healing of deeper concrete cracks (Zhu et al. 2015; Fahimzadeh et al. 2022). Acetate catabolism by *S. silvestris* CGN12 exemplifies a highly efficient MICP pathway via oxidation of organic compounds. It does not produce unwanted byproducts and is primarily governed by the availability of calcium ions and acetate. It requires the presence of oxygen, but in aerobic environments, the metabolic characteristics described in this study make this bacterium a well-suited organism for application, for example, in self-healing concrete as shown here, or in the future soil stabilisation or even production of engineered living materials.

When testing *S. silvestris* CGN12 in an application-relevant context, our data showed that acetate can serve as an effective nutrient for crack healing in cement mortars. While the variability between replicate mortar specimens was quite high, particularly in the tests for water flow after the healing process, this is a common observation in the assessment of self-healing activity. Variability can arise, for example, from slight differences in initial crack width, irregularities in crack surface or morphology or deviations from perpendicularity of the cracks, which can affect water flow (Tziviloglou et al. 2016). Of note, the variability was highest in control mortars and those containing *A. pseudofirmus* cells, neither of which showed effective healing with acetate as a carbon source. Mortars with *S. silvestris* CGN12 gave consistent values across the triplicates tested (Figure 5), demonstrating its robust healing performance. Another relevant consideration is the difference between crack area reduction (Figure 5B) and restoration of water tightness (Figure 5C). The former can only observe the immediate crack surface, while water tightness assesses the properties also deep inside the crack. As seen for mortars containing *S. silvestris* CGN12, crack surface area reduction may even decline again over prolonged healing time, while the water tightness is maintained over the whole test period. Apparent discrepancies between crack surface reduction and actual healing performance have been observed before. It has been proposed that the healing product may be distributed randomly within the crack volume, depending on the distribution of healing agents, which may mean that less healing material is at the crack surface, while partial crack closure further down can still give increased durability (He et al. 2023). In our experimental setup, it is also possible that the water flow tests on Day 7 dislodged some healing product from the crack surface, potentially allowing for improved healing to occur beneath the surface between 7 and 56 days. Importantly, however, the observed loss of healing at the surface between Days 7 and 56 did not compromise water resistance, which suggests that healing within the crack by *S. silvestris* CGN12 remained intact.

The successful use of acetate as a nutrient in self-healing concrete is a significant step towards reducing the cost of self-healing concrete using non-ureolytic bacteria by replacing the majority of expensive yeast extract with the cheaper sodium acetate. The use of acetate also avoids the drawbacks associated with more complex media, such as retarding effects on cement hydration and potential adverse impacts on the final

material (Schreiberová et al. 2019). The sodium salt of acetate is cost-effective and has been demonstrated to enhance the rigidity of concrete by improving interfacial bonds between hydration products and aggregates (Al-Kheetan et al. 2020). Our results further show that physiological experiments in the laboratory can provide useful insights to optimise the application conditions for MICP-based technologies. Specific supply of nutrients is, however, only one of the many facets that need to be considered in improving the sustainability of our concrete infrastructure. A recent life cycle analysis showed that, using current technology, self-healing concrete with non-ureolytic bacteria fed with yeast extract actually has a significant environmental impact, and this was largely driven by the use of calcium nitrate (Justo-Reinoso et al. 2023). A key requirement to lower a construct's whole-life carbon footprint, and also to improve scalability and economic viability, will be to only use self-healing material in the precise locations where it is needed, that is, the cover zone (Justo-Reinoso et al. 2023; He et al. 2024). Given our results on using a defined acetate:calcium ratio for crack healing by *S. silvestris* CGN12, there might be further scope for reducing costs and environmental impact by replacing not only yeast extract but also calcium nitrate with the single component calcium acetate. Such information on nutritional requirements for MICP was previously identified as a knowledge gap in improving sustainability and economic feasibility of biological self-healing concrete (Fahimzadeh et al. 2022). A comprehensive life cycle analysis considering nutrient provision for the bacteria via calcium acetate, combined with alterations of the materials and construction design process, would be a valuable future undertaking to fully assess the environmental and economic impact of such a self-healing technology.

Genomic comparison between our isolates to better understand the differences in acetate metabolism and its link to MICP revealed an intriguing difference in the pathway feeding acetate into the TCA cycle in the genome of CGN12. In bacteria, acetate activation to acetyl-CoA usually occurs via the enzyme Acetyl-CoA-Synthase (EC 6.2.1.1), which uses the energy from ATP hydrolysis to AMP and pyrophosphate to produce acetyl-CoA. This reaction is considered irreversible under physiological conditions, and bacteria producing acetate as a metabolic end-product or during overflow metabolism generally employ the two sequential reactions of phosphate transacetylase (PTA), converting acetyl-CoA to acetyl phosphate, and then acetate kinase (ACK), converting acetyl phosphate to acetate, with concomitant generation of ATP. Curiously, CGN12 does not possess genes encoding these two enzymes, but instead harbours a gene encoding an ADP-forming ACS (EC 6.2.1.13). This enzyme is almost exclusively found in archaea such as *Thermococcus* and in some protists, but only seldom in bacteria (Schönheit and Schäfer 1995; Reeves et al. 1977; Parizzi et al. 2012; Awano et al. 2014). It catalyses the reversible conversion between acetate and acetyl-CoA and can therefore be used both in acetate production and consumption, although in Archaea it is mostly linked to the former (Schönheit and Schäfer 1995). The strong induction of the ADP-forming enzyme we observed in CGN12 under calcium exposure suggests that its role under these conditions is likely catabolic, that is, activation of acetate to acetyl-CoA, in contrast to reports from other bacteria that may be using the enzyme predominantly in the reverse direction (Parizzi et al. 2012). The

unusual presence of two different ACS, but no ACK or PTA in CGN12, as well as the presence of two succinyl-CoA:acetate CoA transferase (SAT) encoding genes, may indicate that acetate metabolism generally plays a unique role in the physiology of this bacterium, possibly beginning to shed some light on its efficient MICP ability using acetate as the carbon source. It will be interesting to monitor genome sequence information of other high-performing MICP strains as they become available to see if an unusual complement of acetate metabolic genes is more widely correlated with high biomineralisation activity in the presence of acetate.

Intriguingly, in *S. silvestris* CGN12, exposure to high calcium levels led to the upregulation not only of genes related to calcium detoxification but also of those involved in acetate metabolism. Together with our observation that under high calcium conditions, CGN12 appears to consume slightly more acetate, this may indicate a direct link between calcium stress and acetate metabolism and may suggest that MICP serves as an active calcium detoxification mechanism in this species. Such a mechanism might be biologically plausible, given the natural habitat of the bacterium, that is, calcium-rich limestone rock, from which it was originally isolated (Reeksting et al. 2020).

Further support for this notion may be found in previous studies in micro-algae, where it was found that extracellular precipitated calcium could originate from within a microorganism, leading the authors to propose that carbonatogenesis was an active process involving ionic exchanges through the cell membrane (McConnaughey and Whelan 1997; Castanier et al. 1999; Yates and Robbins 1999). Consistent with this, we could show that a gene encoding a calcium-binding P-type ATPase in *S. silvestris* CGN12 was significantly upregulated upon calcium exposure. These findings support a model in which excess intracellular calcium is actively exported from the cell and, using CO₂ from acetate metabolism, is incorporated into extracellular calcium carbonate on the cell surface (Figure 8), consistent with the hypothesis formulated by Hammes and Verstraete (2002). In how far extracellular calcium binding sites are involved in MICP, and whether they promote or hinder biomineralisation remains to be fully explored. CGN12 possesses two proteins with a predicted Excalibur calcium binding domain. Our original hypothesis had been that presentation of calcium ions on the cell surface by such proteins might aid in crystal nucleation. However, the MICP yield of a strain lacking one of these proteins was almost identical to the parent strain, with the main difference being a

small but reproducible increase in the carbonate-to-acetate ratio. This apparent increase in the amount of carbonate formed per acetate consumed went against our initial expectations, that is, that loss of a calcium binding protein from the cell surface may lead to reduced biomineralisation activity. This was based on an assumption that immobilisation of calcium ions near the cell surface may somehow contribute positively to crystal nucleation or growth. Our observation of the opposite effect may indicate that in the wild type the protein might rather sequester the calcium, lowering ion availability and thus also lowering the MICP efficiency. More detailed mechanistic studies will be needed to answer these questions, including experimental validation that the protein does indeed bind calcium on or near the cell surface, which we currently only base on predictions of protein domain architecture and presence of a typical extracellular calcium binding EF-hand-like domain with the conserved binding motif DxDxDGxxCE (Figure S2; Rigden et al. 2003). If successful, a molecular understanding of the links between surface calcium binding and biomineralisation could offer interesting routes to modulating MICP kinetics.

To summarise, the direct link between carbon metabolism and MICP in *S. silvestris* CGN12 reported here provides valuable insights into the physiological and molecular mechanisms of non-ureolytic biocalcification. While not a universal principle across all non-ureolytic MICP strains, knowledge of this relationship offers a means to control MICP through acetate provision, making it a powerful tool in biotechnological applications. Moreover, we provide initial indications of potential genetic determinants of MICP activity, paving the way towards gaining a full understanding of non-ureolytic biomineralisation.

4 | Materials and Methods

4.1 | Strains and Growth Conditions

All bacterial strains and plasmids used in this study are listed in Table 1 and Table S1. Environmental strains were previously isolated from limestone rock scrapings (Reeksting et al. 2020). The psychrotrophic strain Psy5 was isolated as described but incubated at 7.5°C.

For routine maintenance, cells were grown in LB pH 8.2, adjusted with a final concentration of 20 mM Tris-HCl pH 9. *E. coli*

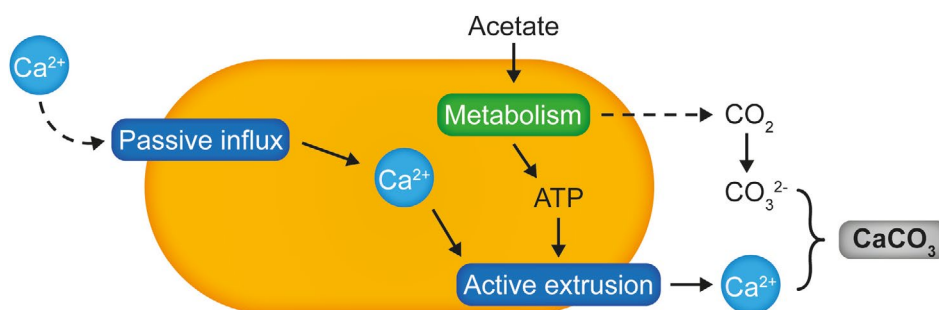


FIGURE 8 | Model of non-ureolytic MICP in *S. silvestris* CGN12. Calcium enters the cell passively and needs to be actively exported. The energy for this process is provided by acetate catabolism, which also produces CO₂ as a byproduct. The CO₂ in solution can react with calcium ions to form CaCO₃, using the bacterial cell surface as a nucleation site.

strains were routinely grown in regular LB at 37°C with shaking (140rpm).

For most experimental cultures, bacteria were grown in yeast extract acetate (YA) medium containing 50 mM Tris-HCl pH 7.8, 0.2% wt/vol yeast extract, 0.5 mM MgSO₄, 0.01 mM MnSO₄ and unless otherwise stated, 100 mM sodium acetate. The design of YA medium was based on the following rationale, using the *B. subtilis* medium CSE as the starting point (Commichau et al. 2008): Metabolic predictions based on species-level identifications by 16S rDNA sequencing obtained in our previous study (Reeksting et al. 2020) suggested that most of the strains would likely display some form of amino acid auxotrophy, so we decided to include a small amount of yeast extract (0.2%) to provide a source of not only nitrogen but also essential amino acids, vitamins and trace elements. The provision of some yeast extract also counteracted the potential drawback of a fully defined medium composed of many individual ingredients, which could make it impractical for industrial-scale applications in the future. For a suitable carbon source for non-ureolytic MICP, we considered that lactate or acetate is already commonly used in applications and therefore should be chemically compatible with cement-based technologies (Justo-Reinoso et al. 2021). As not all bacteria in our collection were likely able to use lactate as a carbon source, again based on initial genomic analyses, acetate was selected for the present study. For routine assays, sodium acetate was provided at a concentration of 100 mM, with an additional provision of 100 mM calcium nitrate where MICP was to be tested.

4.2 | Endospore Production

Endospores of CGN12 and *A. pseudofirmus* were prepared in Difco sporulation medium (DSM); 8 g L⁻¹ nutrient broth, 13.41 mM KCl and 0.49 mM MgSO₄, adjusted to pH 7.6 with NaOH. Prior to use, 1 mM Ca(NO₃)₂, 0.01 mM MnCl₂ and 1 μM FeSO₄ were added from a filter-sterilised stock solution (Sonenshein et al. 1974). Cultures grown in LB for 24 h at 30°C with shaking (140rpm) were used to inoculate 750 mL of sporulation medium to an OD₆₀₀ of 0.02. Cultures were grown for at least 48 h before sporulation was assessed using phase-contrast microscopy. When most cells contained phase-bright endospores, cells were pelleted by centrifugation (4000g, 10 min at RT) and washed three times in 10 mM Tris-HCl pH 9. Cells were treated with chlorhexidine digluconate (0.3 mg mL⁻¹) for 30 min to kill vegetative cells, and then the wash steps were repeated. Spore pellets were frozen at -80°C and freeze-dried under vacuum overnight.

4.3 | Precipitation Assays

Cultures grown for 24 h in YA medium were used to inoculate 20 mL YAC medium, containing 50–100 mM calcium nitrate, to an OD₆₀₀ of 0.02. Precipitation cultures were incubated with shaking (140 rpm) at 30°C. At the indicated time points, precipitate was harvested by centrifugation (500g, 5 min at RT). The supernatant containing the cells was discarded and the precipitate washed in distilled water until all remaining cells were removed. The precipitate was dried at 50°C for 3 days until the

weight remained constant. The amount of precipitate was determined as the difference in weight of each empty tube before harvesting and the post-drying weight. Separate flasks per time point were set up so that at each time point (Days 0, 1, 2, 3 and 6 post inoculation) the calcium carbonate from a complete flask could be harvested and quantified.

4.4 | Determination of Acetate Concentration by HPLC

HPLC was performed on an Agilent 1260 Infinity at 65°C with a Rezex ROA Organic Acid H⁺ 8% column (Phenomenex). The mobile phase was 5 mM H₂SO₄, with a flow rate of 0.6 mL/min over 25 min. The primary detection wavelength was 215 nm, and the UV spectrum of each sample was collected. Samples were passed through a 0.22 μm filter and diluted 1:10 before 10 μL was injected onto the column. Acetate for each sample was quantified by comparing against a standard curve of samples with known acetate concentrations.

4.5 | Determination of Soluble Calcium Concentration

Samples were centrifuged at 4000g for 10 min to remove cells. The supernatant was passed through a 0.22 μm filter before 0.5 mL was combined with 2 mL of Patton-Reeder's solution (0.105 mM calconcarboxylic acid, VWR, in 0.73 M NaOH) and vortexed briefly to mix. The sample was titrated with 25 mM EDTA until the solution turned a permanent bright blue, indicating that all calcium had been complexed. The volume of EDTA required to reach the colour change was recorded and used to calculate the concentration of calcium in the sample, based on EDTA complexing calcium in a 1:1 M ratio.

4.6 | Whole Genome Sequencing

To extract genomic DNA, strains were grown for 24 h in LB pH 8.2. Cells were pelleted and genomic DNA extracted using the Monarch Genomic DNA Purification Kit (New England Biolabs). To obtain high molecular weight gDNA, after incubation with RNase A, sodium acetate was added to a final concentration of 300 mM and the entire solution was mixed with equal parts isopropanol. The precipitated DNA was then spooled on a glass rod and transferred into 100 μL Tissue Lysis Buffer mixed with 113 μL 10 mM Tris-HCl pH 8, before continuing with DNA binding to the column and elution, as described by the manufacturer.

Short read genomic sequences were obtained by Illumina sequencing (Microbial Genome Sequencing Centre, Pittsburgh, PA, USA). Paired-end reads were assembled into contigs using Shovill (<https://github.com/tseemann/shovill>). Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Sequencing libraries for long read sequencing of the strains CGN12, BA32, PD1-1, UBN2 and Psy5 were generated from

400 ng high-molecular weight gDNA per strain using the Oxford Nanopore Technologies Native Barcoding Kit 24 (SQK-NBD112.24) according to the manufacturers' protocol. Quality control was performed using the Invitrogen Qubit 4 FLuorometer. Next, 11 fmol of sequencing library with an estimated fragment size of 7.5 kb was loaded onto an R10.4 MinION flow cell (FLO-MIN112) and sequenced on a GridION X5 Mk1 for 72 h using the 'barcode balancing' option. Subsequent base calling was performed using Guppy v6.3.9 with super high accuracy (SUP) mode, resulting in 125–233 k reads per strain.

Genome assembly was performed two-fold, with Unicycler v0.5.0 (Wick et al. 2017) for short- and long-read hybrid assembly and epi2me wf-bacterial-genomes v0.4.0 (Pedersen and Quinlan 2018; Kolmogorov et al. 2019) for long reads only. Quality and completeness of assemblies were evaluated using common assembly metrics (number and size of scaffolds, N50, N90) and BUSCO v5.0.0 in genome mode with Bacillales database (Manni et al. 2021) and the best assembly for each strain selected. Gene annotation was performed using Prokka v1.14.6 (Seemann 2014). Completeness of the annotation was assessed by extracting all protein sequences of annotated genes and comparing them with BUSCO in protein mode.

4.7 | RT-qPCR

Stationary-phase cells were treated either with or without 100 mM calcium nitrate for 30 min. RNA was isolated using a Total RNA Extraction kit (NEB #T2010). For reverse transcription, 2 µg of the isolated RNA was used to synthesise cDNA from random primers using a High Capacity cDNA RT Kit (Applied Biosystems). LuminoCt SYBR Green qPCR Readymix (Sigma Aldrich) was used for all qPCRs. Each reaction contained 10 µL of Master Mix, 0.2 µL of 100× ROX internal reference dye, 0.3 µM of each primer, 4 µL of diluted cDNA template and ultrapure water to a final volume of 20 µL. qPCR was performed on StepOnePlus Instrument (Applied Biosystems). The qPCR cycle consisted of an initial enzyme activation step at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing and extension at 60°C for 30 s. A melt curve analysis was performed by raising the temperature at the end of each run by 0.3°C from 60°C to 95°C. Standard curves were created for each primer set with qPCR, using a dilution series of pooled cDNA from individually synthesised samples as a template. Fold changes were calculated using the Pfaffl equation (Pfaffl 2001).

4.8 | Deletion of the Excalibur Protein-Encoding Gene in *S. silvestris* CGN12

The temperature-sensitive *oriT* was amplified from pG2k-*oriT*-gfp using primers SG1073 and SG1073 (Table S2) and cloned into pMAD using the BamHI restriction sites (Arnaud et al. 2004). Genomic DNA was extracted from CGN12 (Genejet genomic DNA extraction Kit, Thermo, USA). Flanking regions 1 kb upstream and downstream of the Excalibur domain-encoding gene (gene locus AB1K09_06620) were amplified using primers SG1229 and SG1230 (upstream) and SG1231 and SG1232 (downstream). Fragments were joined by Gibson Assembly (NEBuilder) and ligated into pMAD-*OriT* using the NcoI

restriction sites. For the conjugation to CGN12, *E. coli* S17 containing the plasmid was grown into stationary phase in LB medium containing 100 µg/mL ampicillin. CGN12 was also grown into stationary phase in LB pH 8.2 medium. S17 and CGN12 cells were washed and combined in a 1:1 ratio in 20 mL LB supplemented with 20 mM MgCl₂. Cells were incubated for 1 h at 30°C with shaking and then pelleted by centrifugation and spotted onto LB agar plates containing 10 mM MgCl₂. Plates were incubated at 30°C for 24 h. Cells were scraped off the agar plates, resuspended in 1 mL LB pH 8.2 and serially diluted in 1:10 steps. Dilutions were spotted in a volume of 20 µL per spot onto LB pH 8.2 agar containing MLS selection (0.5 µg/mL Erythromycin and 12.5 µg/mL Lincomycin). Plates were incubated for 72 h at 30°C and colonies patched onto fresh selection plates to confirm growth. Presence of the plasmid in CGN12 was confirmed by colony PCR with primers SG1072 and SG1073. Next, an overnight culture was prepared in LB medium with MLS selection at 30°C. The following morning, 10 mL LB with MLS selection was inoculated to a starting OD₆₀₀ of 0.1 and incubated at 30°C for 2 h. The temperature was then increased to 42°C for 5 h. Serial dilutions were then plated onto LB agar with MLS selection and incubated at 42°C for 24 h. Colonies were selected and inoculated into LB pH 8.2 without antibiotics and incubated for 6 h at 30°C. The temperature was then increased to 42°C for 3 h. Dilutions were plated onto LB pH 8.2 agar without selection and incubated for 24 h at 42°C. Colonies were replica-patched onto MLS and non-MLS plates. MLS-sensitive colonies were checked for the presence of the gene deletion by PCR using primers SG1229 and SG1232. Positive clones were confirmed by sequencing of the PCR product with primer SG1409.

4.9 | Strain and Genome Sequence Deposition

The five strains chosen for full genome sequencing were deposited into the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection (Germany) under the following accession numbers: DSM 118343 (*M. dongyingensis* BA32), DSM 118343 (*S. silvestris* CGN12), DSM 118343 (*P. simplex* Psy5) and DSM 118343 (*P. frigorigerans* UBN2). *B. licheniformis* PD1_1 was already deposited as DSM 110495 (Reeksting et al. 2020).

The hybrid genome sequences were deposited into GenBank with the following accession numbers: JBFEAO000000000 (*M. dongyingensis* BA32), JBFEAN000000000 (*S. silvestris* CGN12), JBFEAM000000000 (*B. licheniformis* PD1_1), JBFEAL000000000 (*P. simplex* Psy5) and JBFEAK000000000 (*P. frigorigerans* UBN2).

4.10 | Preparation of Mortar Samples

Mortar prisms were prepared according to Reeksting et al. (2020). Mortar prisms (65 mm by 40 mm by 40 mm) were cast in duplicate, in two separate layers. The first layer contained (per 2 prisms), 177.8 g sand conforming to standard BS EN 196–1, 61.3 g Portland limestone cement (CEM II/A-L 32.5R), 30.7 g water, 0.7 g yeast extract, 3.0 g calcium nitrate and 2.4 g aerated concrete granules. Spores (1.0×10^{10} CFU) were resuspended in 2.83 g distilled water and soaked into the aerated concrete granules before drying and sealing with polyvinyl acetate (30% wt/

wt). After 3 h, the second, top layer was cast. The second layer contained standard cement mortar (per 2 prisms: 184 g standard sand, 61.32 g cement and 30.7 g water). Reference specimens were cast in two layers but contained only standard cement mortar in both layers. Specimens remained at room temperature for 24–48 h before demoulding and subsequent curing for 28 days submerged in tap water. After curing, specimens were oven dried at 50°C for 24 h. The top third of the prism was wrapped with carbon fibre-reinforced polymer strips to enable generation of a crack of controlled width. A notch (1.5 mm deep) was sawn at midspan to serve as a crack initiation point. Specimens were cracked by 3-point bending using a 30-kN Instron static testing frame. A crack mouth opening displacement (CMOD) gauge was used to measure crack width. Load was applied to maintain a crack growth of 25 µm per minute, and loading was stopped when the crack width was predicted to be 500 µm wide after load removal. A marker pen was used to indicate specific crack sections to enable monitoring of the crack at the same site. Following cracking, prisms were placed in tanks that were open to the atmosphere and filled with tap water to 20 mm below the top of the mortars and then incubated at room temperature for 2 months. Visualisation of crack healing was monitored using a Leica M205C light microscope, and images were taken of freshly cracked mortars and after 1, 4 and 8 weeks of healing.

Author Contributions

Michael Seidel: conceptualization, investigation, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, data curation. **Charlotte Hamley-Bennett:** conceptualization, investigation, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, data curation. **Bianca J. Reeksting:** investigation, writing – original draft, validation, formal analysis, visualization, data curation. **Manpreet Bagga:** investigation, validation, formal analysis, data curation. **Lukas Hellmann:** investigation, methodology, writing – review and editing, formal analysis, data curation, supervision. **Timothy D. Hoffmann:** investigation, methodology, writing – review and editing. **Christiane Kraemer:** methodology, writing – review and editing, formal analysis, supervision, project administration. **Irina Dana Ofițeru:** conceptualization, writing – review and editing, project administration, supervision, resources, funding acquisition. **Kevin Paine:** conceptualization, funding acquisition, writing – review and editing, project administration, supervision, resources. **Susanne Gebhard:** conceptualization, funding acquisition, writing – original draft, writing – review and editing, formal analysis, project administration, supervision, resources.

Acknowledgements

This work was supported in part by funding from the Engineering and Physical Sciences Research Council (EPSRC, UK) through the Resilient Materials for Life (RM4L) (EP/P02081X/1) project and the Engineering Microbial-Induced Carbonate Precipitation via Meso-Scale Simulations (eMICP) (EP/S013997/1; EP/S013997) project. The authors gratefully acknowledge the Technical Staff within the Life Sciences Department and the Department of Architecture and Civil Engineering at the University of Bath for technical support and assistance in this work. We also thank Yannic Zettelmeyer at Johannes Gutenberg University Mainz for genome assembly from the long and short read data as part of his student research project. Open Access funding enabled and organized by Projekt DEAL.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All numerical data underpinning the graphs shown in the figures are available in Table S6.

References

- Abramson, J., J. Adler, J. Dunger, et al. 2024. “Accurate Structure Prediction of Biomolecular Interactions With AlphaFold 3.” *Nature* 630, no. 8016: 493–500. <https://doi.org/10.1038/s41586-024-07487-w>.
- Al-Kheetan, M. J., S. H. Ghaffar, O. A. Madyan, and M. M. Rahman. 2020. “Development of Low Absorption and High-Resistant Sodium Acetate Concrete for Severe Environmental Conditions.” *Construction and Building Materials* 230: 117057. <https://doi.org/10.1016/j.conbuildmat.2019.117057>.
- Alazhari, M., T. Sharma, A. Heath, R. Cooper, and K. Paine. 2018. “Application of Expanded Perlite Encapsulated Bacteria and Growth Media for Self-Healing Concrete.” *Construction and Building Materials* 160: 610–619. <https://doi.org/10.1016/j.conbuildmat.2017.11.086>.
- Arnaud, M., A. Chastanet, and M. Débarbouillé. 2004. “New Vector for Efficient Allelic Replacement in Naturally Nontransformable, Low-GC-Content, Gram-Positive Bacteria.” *Applied and Environmental Microbiology* 70, no. 11: 6887–6891. <https://doi.org/10.1128/AEM.70.11.6887-6891.2004>.
- Awano, T., A. Wilming, H. Tomita, et al. 2014. “Characterization of Two Members among the Five ADP-Forming Acyl Coenzyme A (Acyl-CoA) Synthetases Reveals the Presence of a 2-(Imidazol-4-yl)Acetyl-CoA Synthetase in *Thermococcus Kodakarensis*.” *Journal of Bacteriology* 196, no. 1: 140–147. <https://doi.org/10.1128/JB.00877-13>.
- Bagga, M., I. Justo-Reinoso, C. Hamley-Bennett, et al. 2023. “Assessing the Potential Application of Bacteria-Based Self-Healing Cementitious Materials for Enhancing Durability of Wastewater Treatment Infrastructure.” *Cement and Concrete Composites* 143: 105259. <https://doi.org/10.1016/j.cemconcomp.2023.105259>.
- Castanier, S., G. Le Métayer-vrel, and J.-P. Perthuisot. 1999. “Ca-Carbonates Precipitation and Limestone Genesis—The Microbiogeologist Point of View.” *Sedimentary Geology* 126, no. 1-4: 9–23. [https://doi.org/10.1016/S0037-0738\(99\)00028-7](https://doi.org/10.1016/S0037-0738(99)00028-7).
- Castro-Alonso, M. J., L. E. Montañez-Hernandez, M. A. Sanchez-Muñoz, M. R. Macias Franco, R. Narayanasamy, and N. Balagurusamy. 2019. “Microbially Induced Calcium Carbonate Precipitation (MICP) and Its Potential in Bioconcrete: Microbiological and Molecular Concepts.” *Frontiers in Materials* 6: 126. <https://doi.org/10.3389/fmats.2019.00126>.
- Commichau, F. M., K. Gunka, J. J. Landmann, and J. Stülke. 2008. “Glutamate Metabolism in *Bacillus Subtilis*: Gene Expression and Enzyme Activities Evolved to Avoid Futile Cycles and to Allow Rapid Responses to Perturbations of the System.” *Journal of Bacteriology* 190, no. 10: 3557–3564. <https://doi.org/10.1128/JB.00099-08>.
- Dikshit, R., N. Gupta, A. Dey, K. Viswanathan, and A. Kumar. 2022. “Microbial Induced Calcite Precipitation Can Consolidate Martian and Lunar Regolith Simulants.” *PLoS One* 17, no. 4: e0266415. <https://doi.org/10.1371/journal.pone.0266415>.
- Dominguez, D. C. 2004. “Calcium Signalling in Bacteria.” *Molecular Microbiology* 54, no. 2: 291–297. <https://doi.org/10.1111/j.1365-2958.2004.04276.x>.
- Erşan, Y. Ç., E. Gruyaert, G. Louis, C. Lours, N. Belie, and N. de Boon. 2015a. “Self-Protected Nitrate Reducing Culture for Intrinsic Repair of Concrete Cracks.” *Frontiers in Microbiology* 6: 1228. <https://doi.org/10.3389/fmicb.2015.01228>.
- Erşan, Y. Ç., N. de Belie, and N. Boon. 2015b. “Microbially Induced CaCO₃ Precipitation Through Denitrification: An Optimization Study in Minimal Nutrient Environment.” *Biochemical Engineering Journal* 101: 108–118. <https://doi.org/10.1016/j.bej.2015.05.006>.

- European Environment Agency. 2019. "Ammonia Emissions From Agriculture Continue to Pose Problems for Europe." <https://www.eea.europa.eu/highlights/ammonia-emissions-from-agriculture-continue>.
- Fahimzadeh, M., P. Pasbakhsh, L. S. Mae, J. B. L. Tan, and R. S. K. Raman. 2022. "Multifunctional, Sustainable, and Biological Non-Ureolytic Self-Healing Systems for Cement-Based Materials." *Engineering* 13: 217–237. <https://doi.org/10.1016/j.eng.2021.11.016>.
- Farfan, J., M. Fasihi, and C. Breyer. 2019. "Trends in the Global Cement Industry and Opportunities for Long-Term Sustainable CCU Potential for Power-To-X." *Journal of Cleaner Production* 217: 821–835. <https://doi.org/10.1016/j.jclepro.2019.01.226>.
- Fu, T., A. C. Saracho, and S. K. Haigh. 2023. "Microbially Induced Carbonate Precipitation (MICP) for Soil Strengthening: A Comprehensive Review." *Biogeotechnics* 1, no. 1: 100002. <https://doi.org/10.1016/j.bgtech.2023.100002>.
- Hammes, F., and W. Verstraete. 2002. "Key Roles of pH and Calcium Metabolism in Microbial Carbonate Precipitation." *Reviews in Environmental Science and Biotechnology* 1, no. 1: 3–7. <https://doi.org/10.1023/A:1015135629155>.
- He, S., M. Nuri, H. M. Jonkers, M. Luković, and E. Schlangen. 2024. "Structural Behaviour of Reinforced Concrete Beams With Self-Healing Cover Zone as Lost Formwork." *Developments in the Built Environment* 18: 100458. <https://doi.org/10.1016/j.dibe.2024.100458>.
- He, S., Z. Wan, Y. Chen, H. M. Jonkers, and E. Schlangen. 2023. "Microstructural Characterization of Crack-Healing Enabled by Bacteria-Embedded Polylactic Acid (PLA) Capsules." *Cement and Concrete Composites* 143: 105271. <https://doi.org/10.1016/j.cemconcomp.2023.105271>.
- Hoffmann, T. D., B. J. Reeksting, and S. Gebhard. 2021. "Bacteria-Induced Mineral Precipitation: A Mechanistic Review." *Microbiology (Reading, England)* 167, no. 4: 001049. <https://doi.org/10.1099/mic.0.001049>.
- Jonkers, H. M., A. Thijssen, G. Muyzer, O. Copuroglu, and E. Schlangen. 2010. "Application of Bacteria as Self-Healing Agent for the Development of Sustainable Concrete." *Ecological Engineering* 36, no. 2: 230–235. <https://doi.org/10.1016/j.ecoleng.2008.12.036>.
- Justo-Reinoso, I., A. Heath, S. Gebhard, and K. Paine. 2021. "Aerobic Non-Ureolytic Bacteria-Based Self-Healing Cementitious Composites: A Comprehensive Review." *Journal of Building Engineering* 42: 102834. <https://doi.org/10.1016/j.jobbe.2021.102834>.
- Justo-Reinoso, I., N. Arena, B. J. Reeksting, S. Gebhard, and K. Paine. 2023. "Bacteria-Based Self-Healing Concrete—A Life Cycle Assessment Perspective." *Developments in the Built Environment* 16: 100244. <https://doi.org/10.1016/j.dibe.2023.100244>.
- Kolmogorov, M., J. Yuan, Y. Lin, and P. A. Pevzner. 2019. "Assembly of Long, Error-Prone Reads Using Repeat Graphs." *Nature Biotechnology* 37, no. 5: 540–546. <https://doi.org/10.1038/s41587-019-0072-8>.
- Kumar, A., H.-W. Song, S. Mishra, et al. 2023. "Application of Microbial-Induced Carbonate Precipitation (MICP) Techniques to Remove Heavy Metal in the Natural Environment: A Critical Review." *Chemosphere* 318: 137894. <https://doi.org/10.1016/j.chemosphere.2023.137894>.
- Manni, M., M. R. Berkeley, M. Seppey, and E. M. Zdobnov. 2021. "BUSCO: Assessing Genomic Data Quality and Beyond." *Current Protocols* 1, no. 12: e323. <https://doi.org/10.1002/cpz1.323>.
- McConaughy, T. A., and J. F. Whelan. 1997. "Calcification Generates Protons for Nutrient and Bicarbonate Uptake." *Earth-Science Reviews* 42, no. 1–2: 95–117. [https://doi.org/10.1016/S0012-8252\(96\)00036-0](https://doi.org/10.1016/S0012-8252(96)00036-0).
- Mondal, S., and A. Ghosh. 2019. "Review on Microbial Induced Calcite Precipitation Mechanisms Leading to Bacterial Selection for Microbial Concrete." *Construction and Building Materials* 225: 67–75. <https://doi.org/10.1016/j.conbuildmat.2019.07.122>.
- Norris, V., S. Grant, P. Freestone, et al. 1996. "Calcium Signalling in Bacteria." *Journal of Bacteriology* 178, no. 13: 3677–3682. <https://doi.org/10.1128/jb.178.13.3677-3682.1996>.
- Parizzi, L. P., M. C. B. Grassi, L. A. Llerena, et al. 2012. "The Genome Sequence of *Propionibacterium Acidipropionici* Provides Insights into its Biotechnological and Industrial Potential." *BMC Genomics* 13: 562. <https://doi.org/10.1186/1471-2164-13-562>.
- Pedersen, B. S., and A. R. Quinlan. 2018. "Mosdepth: Quick Coverage Calculation for Genomes and Exomes." *Bioinformatics (Oxford, England)* 34, no. 5: 867–868. <https://doi.org/10.1093/bioinformatics/btx699>.
- Pettinato, E., P. Böhnert, and I. A. Berg. 2022. "Succinyl-CoA:Acetate CoA-Transferase Functioning in the Oxidative Tricarboxylic Acid Cycle in *Desulfurella Acetivorans*." *Frontiers in Microbiology* 13: 1080142. <https://doi.org/10.3389/fmicb.2022.1080142>.
- Pfaffl, M. W. 2001. "A New Mathematical Model for Relative Quantification in Real-Time RT-PCR." *Nucleic Acids Research* 29, no. 9: e45. <https://doi.org/10.1093/nar/29.9.e45>.
- Pirt, S. J. 1975. *Principles of Microbe and Cell Cultivation*. Blackwell Scientific.
- Reeksting, B. J., T. D. Hoffmann, L. Tan, K. Paine, and S. Gebhard. 2020. "In-Depth Profiling of Calcite Precipitation by Environmental Bacteria Reveals Fundamental Mechanistic Differences With Relevance to Application." *Applied and Environmental Microbiology* 86, no. 7: e02739-19. <https://doi.org/10.1128/AEM.02739-19>.
- Reeves, R. E., L. G. Warren, B. Susskind, and H. S. Lo. 1977. "An Energy-Conserving Pyruvate-to-Acetate Pathway in *Entamoeba Histolytica*. Pyruvate Synthase and a New Acetate Thiokinase." *Journal of Biological Chemistry* 252, no. 2: 726–731. [https://doi.org/10.1016/S0021-9258\(17\)32778-3](https://doi.org/10.1016/S0021-9258(17)32778-3).
- Richardson, A. E., B. Nagaratnam, K. A. Coventry, D. Brandy, and L. Amess. 2020. "Crack Healing in Cementitious Materials Including Tests Methods." *Journal of Green Building* 15, no. 1: 37–54. <https://doi.org/10.3992/1943-4618.15.1.37>.
- Rigden, D. J., M. J. Jedrzejewski, and M. Y. Galperin. 2003. "An Extracellular Calcium-Binding Domain in Bacteria With a Distant Relationship to EF-Hands." *FEMS Microbiology Letters* 221, no. 1: 103–110. [https://doi.org/10.1016/S0378-1097\(03\)00160-5](https://doi.org/10.1016/S0378-1097(03)00160-5).
- Schönheit, P., and T. Schäfer. 1995. "Metabolism of Hyperthermophiles." *World Journal of Microbiology & Biotechnology* 11, no. 1: 26–57. <https://doi.org/10.1007/BF00339135>.
- Schreiberová, H., P. Bílý, J. Fládr, K. Šeps, R. Chylik, and T. Trtik. 2019. "Impact of the Self-Healing Agent Composition on Material Characteristics of Bio-Based Self-Healing Concrete." *Case Studies in Construction Materials* 11: e00250. <https://doi.org/10.1016/j.cscm.2019.e00250>.
- Seemann, T. 2014. "Prokka: Rapid Prokaryotic Genome Annotation." *Bioinformatics Oxford, England* 30, no. 14: 2068–2069. <https://doi.org/10.1093/bioinformatics/btu153>.
- Seifan, M., and A. Berenjian. 2018. "Application of Microbially Induced Calcium Carbonate Precipitation in Designing Bio Self-Healing Concrete." *World Journal of Microbiology and Biotechnology* 34, no. 11: 168. <https://doi.org/10.1007/s11274-018-2552-2>.
- Sonenshein, A. L., B. Cami, J. Brevet, and R. Cote. 1974. "Isolation and Characterization of Rifampin-Resistant and Streptolydigin-Resistant Mutants of *Bacillus Subtilis* With Altered Sporulation Properties." *Journal of Bacteriology* 120, no. 1: 253–265. <https://doi.org/10.1128/jb.120.1.253-265.1974>.
- Srubar, W. V. 2021. "Engineered Living Materials: Taxonomies and Emerging Trends." *Trends in Biotechnology* 39, no. 6: 574–583. <https://doi.org/10.1016/j.tibtech.2020.10.009>.

Stocks-Fischer, S., J. K. Galinat, and S. S. Bang. 1999. "Microbiological Precipitation of CaCO_3 ," *Soil Biology and Biochemistry* 31, no. 11: 1563–1571. [https://doi.org/10.1016/S0038-0717\(99\)00082-6](https://doi.org/10.1016/S0038-0717(99)00082-6).

Talaiekhozan, A., A. Keyvanfar, A. Shafaghat, et al. 2013. "A Review of Self-Healing Concrete Research Development." *Journal of Environmental Treatment Techniques* 2, no. 1: 1–11.

Tan, L., B. Reeksting, I. Justo-Reinoso, et al. 2023. "The Effect of Oxygen and Water on the Provision of Crack Closure in Bacteria-Based Self-Healing Cementitious Composites." *Cement and Concrete Composites* 142: 105201. <https://doi.org/10.1016/j.cemconcomp.2023.105201>.

Tan, L., B. Reeksting, V. Ferrandiz-Mas, A. Heath, S. Gebhard, and K. Paine. 2020. "Effect of Carbonation on Bacteria-Based Self-Healing of Cementitious Composites." *Construction and Building Materials* 257: 119501. <https://doi.org/10.1016/j.conbuildmat.2020.119501>.

Tziviloglou, E., V. Wiktor, J. Wang, et al. 2016. "Evaluation of Experimental Methodology to Assess the Sealing Efficiency of Bacteria-Based Self-Healing Mortar: Round Robin Test." In *Proceedings of the RILEM Conference on Microorganisms-Cementitious Materials Interactions*, edited by V. Wiktor, H. Jonkers, and A. Bertron. RILEM Publications SARL.

Wick, R. R., L. M. Judd, C. L. Gorrie, and K. E. Holt. 2017. "Unicycler: Resolving Bacterial Genome Assemblies From Short and Long Sequencing Reads." *PLoS Computational Biology* 13, no. 6: e1005595. <https://doi.org/10.1371/journal.pcbi.1005595>.

Yates, K. K., and L. L. Robbins. 1999. "Radioisotope Tracer Studies of Inorganic Carbon and Ca in Microbially Derived CaCO_3 ," *Geochimica et Cosmochimica Acta* 63, no. 1: 129–136. [https://doi.org/10.1016/S0016-7037\(98\)00297-X](https://doi.org/10.1016/S0016-7037(98)00297-X).

Zhu, T., C. Paulo, M. L. Merroun, and M. Dittrich. 2015. "Potential Application of Biomineralization by *Synechococcus* PCC8806 for Concrete Restoration." *Ecological Engineering* 82: 459–468. <https://doi.org/10.1016/j.ecoleng.2015.05.017>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.