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Construction of two ureolytic model organisms for the study of microbially induced calcium carbonate precipitation

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ABSTRACT

Two bacterial strains, *Pseudomonas aeruginosa* MJK1 and *Escherichia coli* MJK2, were constructed that both express green fluorescent protein (GFP) and carry out ureolysis. These two novel model organisms are useful for studying bacterial carbonate mineral precipitation processes and specifically ureolysis-driven microbially induced calcium carbonate precipitation (MICP). The strains were constructed by adding plasmid-borne urease genes (*ureABC*, *ureD* and *ureFG*) to the strains *P. aeruginosa* AH298 and *E. coli* AF504*gfp*, both of which already carried unstable GFP derivatives. The ureolytic activities of the two new strains were compared to the common, non-GFP expressing, model organism *Sporosarcina pasteurii* in planktonic culture under standard laboratory growth conditions. It was found that the engineered strains exhibited a lower ureolysis rate per cell but were able to grow faster and to a higher population density under the conditions of this study. Both engineered strains were successfully grown as biofilms in capillary flow cell reactors and ureolysis-induced calcium carbonate mineral precipitation was observed microscopically. The undisturbed spatiotemporal distribution of biomass and calcium carbonate minerals were successfully resolved in 3D using confocal laser scanning microscopy. Observations of this nature were not possible previously because no obligate urease producer that expresses GFP had been available. Future observations using these organisms will allow researchers to further improve engineered application of MICP as well as study natural mineralization processes in model systems.

1. Introduction

Microbially induced calcium carbonate precipitation (MICP) is an important process in many engineered and natural systems including: geologic carbon sequestration, radionuclide remediation, soil stabilization and permeability manipulation (Cunningham et al., 2013; De Muynck et al., 2010; Ferris et al., 1996; Fujita et al., 2000; Lauchnor et al., 2013; Phillips et al., 2013; Whiffin et al., 2007). Natural systems, including the earth's global carbon cycle and ocean chemistry, have been affected by bacterial precipitation of calcium carbonate (Riding and Liang, 2005). Microbial carbonate sediments, including those produced by biofilms, are present throughout the geologic record and are widely distributed, making them important for understanding global carbon cycling (Riding, 2000; Vasconcelos et al., 1995). The medical community is also interested in MICP in the context of kidney stones, and mineral formation in ureteral stents and catheters (Morris et al., 1999). Even with the extensive body of literature and interest in MICP, specific mechanisms and implications of how microbes induce mineral formation spatially and temporally are still not clear. For example, the degree to which micron-scale biomass and mineral formation alter flow paths that translate to centimeter and larger scale processes is not fully understood (Armstrong and Ajo-Franklin, 2011; Graf von der Schulenburg et al., 2009).

Perhaps the most highly studied microbial process that can induce calcium carbonate precipitation is ureolysis. Organisms that produce large amounts of urease, such as *Sporosarcina pasteurii* (formerly known as *Bacillus pasteurii* (Yoon et al., 2001)), can be extremely efficient at raising the pH of their environment through hydrolyzing urea and subsequently precipitating calcium carbonate as shown in Eqs. (1)–(4) (Mitchell and Ferris, 2006).

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Urea Hydrolysis:

$$CO(NH_2)_2 + 2\,H_2O \rightarrow 2\,NH_3 + H_2CO_3 \quad (1)$$

Ammonia Protonation and pH Increase:

$$2\,NH_3 + 2\,H_2O \leftrightarrow 2\,NH_4^+ + 2\,OH^- \quad (2)$$

Shift of Carbonate Equilibrium:

$$H_2CO_3 + 2\,OH^- \leftrightarrow HCO_3^- + H_2O + OH^- \leftrightarrow CO_3^{2-} + 2\,H_2O \quad (3)$$

Calcium Carbonate Precipitation:

$$CO_3^{2-} + Ca^{2+} \leftrightarrow CaCO_3(s) \quad (4)$$

The urease enzyme hydrolyzes urea ($CO(NH_2)_2$) to produce two ammonia molecules and one carbonic acid molecule (Eq. (1)). The overall effect of urea hydrolysis is a pH increase and a shift of the carbonic acid equilibrium towards carbonate (Eqs. (2) and (3)). In the presence of calcium, or other divalent cations, precipitation of solid carbonate species takes place once a critical saturation state has been reached (Eq. (4)). In engineered systems, calcium is typically supplied to the system so calcium carbonate is most often the primary precipitate. Other organic and inorganic compounds are often incorporated into the precipitates, potentially changing their characteristics from those expected from pure forms of calcium carbonate (Bosak and Newman, 2005).

Microscopic observations have been used extensively to study MICP processes but real-time imaging that can differentiate biomass from mineral precipitates has been a significant challenge. Electron microscopy only provides an end-point view of the system with unavoidable sample preparation artifacts (Dohnalkova et al., 2011). In contrast, light microscopy allows for the direct observation of microorganisms in a fully hydrated environment. Currently, staining is the only way to use light microscopy to definitively differentiate between biomass and the minerals in a hydrated environment (Schultz et al., 2011). However, staining affects biological processes; so long term studies over the course of days using fluorescent stains are not ideal.

The genetic modification of microbes to incorporate a green or other fluorescent protein is a suitable alternative to direct staining (Larrainzar et al., 2005). Organisms that express a fluorescent protein can be imaged over the course of hours and days without the need to introduce potentially inhibitory or toxic stains. Also, expression of the fluorescent protein can potentially be linked to the transcription of a protein of interest thus providing insight into the specific activity of an enzyme.

The goal of this work was to develop bacterial strains that constitutively express green fluorescent protein (GFP) and active urease. This was accomplished through the insertion of plasmid-borne urease genes into bacteria containing a chromosomal *gfp* insert. In addition to the construction of these organisms, their potential to be used in MICP studies was evaluated based on their ability to hydrolyze urea in batch kinetic studies. The growth and ureolysis kinetics of the newly constructed model organisms were compared to *S. pasteurii*, a model organism commonly used for MICP studies. Finally, the model organisms' suitability for studies in microscopic flow cells was demonstrated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Appropriate growth medium (100 mL, Table 1) was inoculated with 1.0 μL per mL of frozen stock culture. The baffled 250 mL Erlenmeyer flasks were incubated at the appropriate temperature (Table 1) on horizontal shakers at 200 rpm. Cells from overnight cultures were washed twice by centrifugation at 4200 ×*g* and subsequently resuspended in sterile phosphate buffered saline solution (PBS) to remove spent media. PBS had final concentrations of 8.5 g/L NaCl, 0.61 g/L KH_2PO_4, 0.96 g/L K_2HPO_4 (all Fisher Scientific, NJ, USA) and was adjusted to a pH of 7 with concentrated HCl. The final inoculum for all experiments was diluted to an OD of 1.54 (See Section 2.7.1) and inoculated at a volumetric ratio of 1:1000. Experiments were conducted at 37 °C for *Escherichia coli* and *Pseudomonas aeruginosa* strains and 30 °C for *S. pasteurii*.

2.2. Plasmid and model organism construction

To construct urease-producing strains, *P. aeruginosa* AH298 (Werner et al., 2004) and *E. coli* AF504*gfp* (Folkesson et al., 2008) were both transformed with plasmid pMK001 carrying the urease operon from *E. coli* DH5α(pURE14.8) (Collins and Falkow, 1990). The pUC19-based plasmid pURE14.8 carries the urease operon that includes structural genes *ureABC* and putative accessory genes *ureD* and *ureFG* used to acquire nickel (Kim et al., 2006). The full-length sequence of this operon was not previously known (Fig. 2).

Plasmid pMK001 was constructed as follows. To subclone the urease genes, flanking forward and reverse polymerase chain reaction (PCR) primers (Eurofins MWG Operon) were designed with restriction sites *PstI* and *SpeI* added to the respective 5′ end. Primer sequences can be found in the Supplemental Information. The PCR-amplified fragment was digested with the appropriate restriction enzymes and ligated into an equally-digested pJN105 vector. This plasmid contains an L-arabinose-inducible promoter and encodes for gentamycin resistance (Newman and Fuqua, 1999).

The resulting plasmid construct was used to transform chemically competent *E. coli* cells. Gentamicin-resistant transformants were screened by gel electrophoresis of restriction-digested plasmids. Functional tests for ureolysis were performed in Fluka urea broth (Sigma-Aldrich, MO, USA) containing 100 μg/ml gentamicin and 50 mM L-arabinose (Sigma-Aldrich). Antibiotic pressure was maintained in all subsequent screening and kinetic experiments to ensure plasmid retention.

Plasmid pMK001 was finally transformed into the strains AH298 and AF504*gfp*, resulting in strains MJK1 and MJK2, respectively. Both of these strains already contained a mutant, chromosomal *gfp* variant (Folkesson et al., 2008; Werner et al., 2004). Both GFP variants contain an amino acid sequence at the C-terminal end that is recognized for degradation by proteases within the cell (Andersen et al., 1998).

Table 1
Bacterial strains, media and batch growth conditions used in this work.

Strain	Relevant properties	Growth medium	Reference
Pseudomonas aeruginosa AH298	GFP on chromosome	LB [a]	Werner et al. (2004)
Pseudomonas aeruginosa MJK1	AH298 with urease operon added on pJN105 plasmid	LB plus 100 μg/mL gentamicin	This work
Escherichia coli DH5α(pURE14.8)	Urease operon on pUC19 plasmid, source of *Ure* genes for transformations.	LB plus 50 μg/mL ampicillin	Collins and Falkow (1990)
Escherichia coli AF504gfp	GFP on chromosome	LB plus 100 μg/mL ampicillin	Folkesson et al. (2008)
Escherichia coli MJK2	AF504*gfp* with urease operon added on pJN105 plasmid	LB plus 10 μg/mL gentamicin	This work
Sporosarcina pasteurii ATCC 11859	Common urease-positive model organism for MICP studies	BHI [b] plus 20 g/L urea or CMM − [c]	Larson and Kallio (1954)

[a] 25 g/L Luria–Bertani Medium.
[b] 37 g/L Brain Heart Infusion Medium.
[c] Modified Calcite Mineralizing Medium (Ferris et al., 1996) without calcium chloride or sodium bicarbonate and modified to 10 g/L urea rather than 20 g/L.