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# Microbial Growth under Supercritical $CO_2$

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**Growth of microorganisms in environments containing $CO_2$ above its critical point is unexpected due to a combination of deleterious effects, including cytoplasmic acidification and membrane destabilization. Thus, supercritical $CO_2$ ($scCO_2$) is generally regarded as a sterilizing agent. We report isolation of bacteria from three sites targeted for geologic carbon dioxide sequestration (GCS) that are capable of growth in pressurized bioreactors containing $scCO_2$. Analysis of 16S rRNA genes from $scCO_2$ enrichment cultures revealed microbial assemblages of varied complexity, including representatives of the genus *Bacillus*. Propagation of enrichment cultures under $scCO_2$ headspace led to isolation of six strains corresponding to *Bacillus cereus*, *Bacillus subterraneus*, *Bacillus amyloliquefaciens*, *Bacillus safensis*, and *Bacillus megaterium*. Isolates are spore-forming, facultative anaerobes and capable of germination and growth under an $scCO_2$ headspace. In addition to these isolates, several *Bacillus* type strains grew under $scCO_2$, suggesting that this may be a shared feature of spore-forming *Bacillus* spp. Our results provide direct evidence of microbial activity at the interface between $scCO_2$ and an aqueous phase. Since microbial activity can influence the key mechanisms for permanent storage of sequestered $CO_2$ (i.e., structural, residual, solubility, and mineral trapping), our work suggests that during GCS microorganisms may grow and catalyze biological reactions that influence the fate and transport of $CO_2$ in the deep subsurface.**

Geologic carbon dioxide sequestration (GCS) is an emerging strategy to abate $CO_2$ emissions associated with the burning of fossil fuels by capture, compression, and subsurface injection of generated $CO_2$ (1, 2). Although many subsurface geologic formations targeted for storage of compressed $CO_2$ are known to be biologically active environments (3–6), the extent to which biological processes may play a role in the fate and transport of $CO_2$ remains unknown (7). $CO_2$ exists as a supercritical fluid ($scCO_2$) at the temperature and pressures of the vast majority of reservoirs targeted for sequestration (i.e., >31°C and 72.9 atm). $scCO_2$ has generally been regarded as a microbial sterilizing agent due to a combination of factors, including cytoplasm acidification, increased $CO_2$ anion concentration, osmotic stress, membrane permeabilization and leakage via $CO_2$ extraction, and physical cell rupture (8–14).

While there has been no direct evidence that microorganisms can sustain metabolic activity and grow in environments containing $scCO_2$, previous work indicates this possibility. Survival of spores and biofilms after short-term $scCO_2$ exposure (i.e., minutes to hours) (8, 9, 15, 16) is well documented, and recent studies show that mineral matrices may enhance microbial survival of $scCO_2$ exposure by providing substrates for biofilm formation and/or by creating buffered microenvironments (10, 17). Biogeochemical models also suggest that diverse forms of microbial metabolism are thermodynamically favorable under reservoir conditions post-$CO_2$ injection (3, 18), where an aqueous phase in direct contact with $scCO_2$ may have dissolved $CO_2$ concentrations exceeding 2.5 M (3). Furthermore, high-pressure incubations to simulate reservoir conditions with elevated (but not supercritical) $CO_2$ have demonstrated activity of acetoclastic methanogens (under 49.3-atm pressure with 86.4 mM $CO_2$) (19) and homoacetogens (under 395-atm total pressure with 126 mM $CO_2$) (20). Recently, field studies at the Ketzin $CO_2$ sequestration site in Germany and the Otway Basin site in Australia provided evidence that changes in microbial community composition occur following $CO_2$ injection, suggesting that a combination of processes, including differential survival and possibly growth, occurs in the subsurface after exposure to near- and supercritical levels of $CO_2$ (21, 22).

Whether microorganisms survive and remain active post-$CO_2$ injection is relevant for predicting the fate and stability of the injected $CO_2$. Microbial activity can influence the various trapping mechanisms that are crucial to permanent storage of sequestered $CO_2$. Prior work has documented that microbial biofilms can be employed to plug pore spaces and impede the flow of $scCO_2$ through sandstone cores, providing a means of "structural trapping" for a mobile $CO_2$ phase (15, 23). Trapping of $CO_2$ residuals in pore spaces by capillary forces (residual trapping) may be affected by biosurfactant effects on wetting (24). Bacterial surfaces may provide sites for carbonate mineral nucleation, while bacterial activity can increase the rate of mineral weathering and therefore liberate the metal cations necessary for incorporation of $CO_2$ into carbonate minerals (mineral trapping) (25–27). Finally, increased dissolution of $CO_2$ into an aqueous phase (solubility trap-

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ping) has been demonstrated by pH increases induced by bacterial ureolysis under high partial $CO_2$ pressure ($pCO_2$) (28).

In this study, we tested whether environmental microbes could be isolated with the ability to survive and exhibit microbial activity (growth) during exposure to $scCO_2$. We performed a series of experimental enrichment cultures inoculated with subsurface fluid filtrate or well core samples from three subsurface environments targeted for $CO_2$ sequestration: the Frio-2 site near Liberty, TX (29); the Otway Basin site in southeastern Australia (22, 30, 31); and the King Island site near Stockton, CA (32). These three sites are geologically attractive as prospective $CO_2$ injection zones because they consist of high-porosity/permeability sandstone formations overlaid by low-permeability sealing layers capable of structurally trapping buoyant $scCO_2$ in the underlying zone. Enrichment cultivation was followed by isolation and characterization of strains able to grow under $scCO_2$. Microbial growth under $scCO_2$ is surprising given its inhibitory properties and indicates the possibility that microbial activities will influence $CO_2$ trapping during geologic carbon dioxide sequestration.

## MATERIALS AND METHODS

**Subsurface sample collection and storage.** Samples from GCS sites were utilized as inocula for microbial enrichment cultures using $scCO_2$ as the selective agent. Samples from the Frio-2 site were collected as part of the Frio-2 project and shared courtesy of Tommy Phelps (Oak Ridge National Laboratory). For sample collection, 10 to 20 liters of formation fluids was collected by U-tube from the Frio-2 $CO_2$ sequestration site near Liberty, TX, before, during, and after $CO_2$ injection (29, 33). Frio-2 formation fluids from 1,528- to 1,534-m depth were filtered through borosilicate glass filters (nominal pore size, 0.8 μm) and frozen on site. Three samples screened for this study correspond to samples collected prior to $CO_2$ injection, 7.5 h after injection, and 372 days postinjection. Otway Basin samples consisted of rock cores from 929- to 1,530-m depth from the Pemble, Paaratte, and Skull Creek formations (30). Otway cores were collected as part of the CO2CRC project in Southeast Australia (www.co2crc.com.au). The King Island core sample was obtained from the Mokelumne River formation at ~1,447-m depth during drilling of the Citizen Green #1 deep-characterization well by the West Coast Regional Sequestration Partnership (WESTCARB), San Joaquin County, CA. Rock cores were kept refrigerated at 4°C prior to analysis.

**Enrichment cultivation.** Inocula for enrichment cultures were prepared in an anaerobic glove bag with an $O_2$ and $H_2$ monitor (95% $CO_2$-5% $H_2$) and added to 4- or 10-ml pressure vessels containing a 50% volume of growth medium (below). For Frio-2 samples, inocula consisted of 10 μl of hydrocarbon and particulate residue from the surface of glass fiber filters, collected with a sterile scalpel. For Otway Basin cores, drilling fluid tracer penetration data were used to guide collection from the core interior where contamination was least likely. The selected regions of Otway cores were pulverized with a stainless steel mortar and pestle, and 1 g of crushed rock was used as inoculum. For the King Island formation core, no tracer-free interior could be identified, as the sediment was highly permeable and unconsolidated. Thus, a representative sample from the center of the King Island core was used as inoculum and processed in the same manner as Otway cores.

Medium for enrichment cultivation of Frio-2 samples was modified GYP sodium acetate mineral salts broth (GYP) consisting of (in g/liter) 2.0 glucose, 1.0 yeast extract, 1.0 tryptic peptone, 1.0 sodium acetate, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 NaCl, 0.01 $MnSO_4 \cdot 4H_2O$, and 0.01 $FeSO_4 \cdot 7H_2O$. Both GYP and MS media were used for enrichment cultivation from the Otway Basin and King Island cores with supplements targeting different microbial functional groups added to MS medium (34). MS medium consisted of (in g/liter) 0.5 yeast extract, 0.5 tryptic peptone, 10.0 NaCl, 1.0 $NH_4Cl$, 1.0 $MgCl_2 \cdot 6H_2O$, 0.4 $K_2HPO_4$, 0.4 $CaCl_2$, 0.0025 EDTA, 0.00025 $CoCl_2 \cdot 6H_2O$, 0.0005 $MnCl_2 \cdot 4H_2O$, 0.0005 $FeSO_4 \cdot 7H_2O$, 0.0005 $ZnCl_2$, 0.0002 $AlCl_3 \cdot 6H_2O$, 0.00015 $Na_2WoO_4 \cdot 2H_2O$, 0.0001 $NiSO_4 \cdot 6H_2O$, 0.00005 $H_2SeO_3$, 0.00005 $H_3BO_3$, and 0.00005 $NaMoO_4 \cdot 2H_2O$. MS medium supplements consisted of 0.5 g/liter glucose for fermenters; 1.3 g/liter $MnO_2$, 2.14 g/liter $Fe(OH)_3$, and 1.64 g/liter sodium acetate for metal reducers; 0.87 g/liter $K_2SO_4$, 0.83 g/liter $FeSO_4$, and 0.82 g/liter sodium acetate for sulfate reducers; or 1.3 ml trimethylamine and 0.82 g/liter sodium acetate to target methanogens (34). Culture media were added to serum bottles and degassed with a stream of 100% $CO_2$ or 100% $N_2$ gas for 30 min prior to pressurization. $Na_2S$ (at 0.25 g/liter), a reducing agent to maintain anaerobicity, and resazurin (at 0.001 g/liter), a visual redox indicator, were added to culture media. Following inoculation, vessels were pressurized and incubated for 2 to 4 weeks at 37°C for Frio-2 samples and 37 and 60°C for Otway Basin and King Island samples, respectively. In addition to the above media, Luria-Bertani broth (LB) (Difco) and LB agar were used for strain cultivation at 37°C under ambient aerobic conditions.

**TABLE 1** Observed and predicted pH and predicted $CO_2$ concentration as a function of GYP culture medium composition,[a] headspace gas, and pressure at 37°C

| Headspace | Pressure (atm) | Predicted pH[b] | Observed pH[c] | Predicted $[CO_2]$ (M)[b] |
|---|---|---|---|---|
| $CO_2$ | 1 | 5.3 | 5.1 | 0.026 |
| | 100 | 3.6 | 3.9–4.5 | 2.7 |
| $N_2$ | 1 | 7.0 | 7.0 | 0 |
| | 100 | 7.0 | ND[d] | 0 |

[a] Growth medium components (g/kg): 0.72 acetate, 0.431 $Na^+$, 0.607 $Cl^-$, 0.002 $Fe^{2+}$, 0.0025 $Mn^{2+}$, 0.0196 $Mg^{2+}$, 0.08575 $SO_4^{2-}$, 0.1028 $S^{2-}$. Buffering of pH by the acetate system in GYP maintained the final pH above the value predicted for deionized water alone.
[b] Geochemical modeling performed in PhreeqC calling the LLNL database.
[c] pH measured by Orion model 520A pH meter ($P = 1$ atm) or visualized ($P = 120$ atm) via indicator strip (EMD Chemicals).
[d] ND, not determined.

The pH of ambient and $CO_2$-saturated medium was measured at 1 atm and 21°C using an Orion model 520A pH meter. The pH of medium under an $scCO_2$ headspace was measured by visualization of a pH indicator strip (EMD Chemicals) through the sapphire window of a 25-ml view cell (Thar Technologies; 05422-2). In addition, PhreeqC version 2 was used to predict the equilibrium pH and potential precipitation of chemical species in the growth medium (Table 1) under a $CO_2$ or $N_2$ atmosphere and as a function of temperature and pressure. Thermodynamic data were obtained from the Lawrence Livermore National Library (LLNL) database.

**High-pressure incubation.** Vessels for high-pressure growth (see Fig. S10 in the supplemental material) were 316 stainless steel high-performance liquid chromatography (HPLC) column bodies or 316 stainless steel tubing (4- and 10-ml capacity). Vessels were fitted with ball valves (Supelco) or quarter-turn plug valves (Swagelok or Hylok). Vessels were filled to one-half capacity (2 or 5 ml) with cultivation medium, and following inoculation, the headspace of the stainless steel culture vessels, representing 50% of the total vessel volume, was pressurized at a rate of 2 to 3 atm $min^{-1}$ to 100 to 136 atm with industrial-grade $N_2$ gas (Airgas) or with extraction-grade $CO_2$ gas (Airgas) with a helium (He) head pressure such that the final gas mixture was 97 to 99% $CO_2$. Pressurized vessels were incubated in a 37°C warm room, with shaking at 100 rpm to increase mixing of media and subsurface inocula. Following incubation, the vessels were connected with 316 stainless steel tubing and fittings to Swagelok pressure gauges to measure the final vessel pressure before samples were depressurized at a rate of 3 to 5 atm $min^{-1}$ over approximately 30 min. Generally, culture vessels with initial headspace pressures of >100 atm lost between 5 and 25 atm of pressure, due to slow leakage through fittings,

**TABLE 2** $ScCO_2$ enrichment cultivation summary

| Sample origin (incubation time [days] for initial enrichment and passages 1–3, respectively) | Growth medium[a] | Biomass (cells/ml) observed for enrichment/passage at temp (°C)[c] | | | | | | | | Isolate[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Initial | | 1 | | 2 | | 3 | | |
| | | 37 | 60 | 37 | 60 | 37 | 60 | 37 | 60 | |
| Frio-2; formation water particles; 1,500 m (15, 14, 15, 15) | GYP | +++ | ND[d] | +++ | | +++ | | +++ | | MIT0214 |
| Otway core 2; 1,164.12–1,164.33 m; silty claystone (21, 33, 61, 41) | MS + fermenter | − | + | | + | | | | | |
| | MS + metal reducer | − | + | | − | | | | | |
| | MS + sulfate reducer | − | + | | + | | | | | |
| | MS + methanogen | − | + | | + | | + | | | |
| | GYP | − | − | | | | | | | |
| Otway core 3; 1,193.59–1,193.69 m; sandstone (14, 28, 81, 40) | MS + fermenter | + | + | + | + | − | − | | | |
| | MS + metal reducer | + | − | + | | +++ | | +++ | | MITOT1 |
| | MS + sulfate reducer | + | − | − | | | | | | |
| | MS + methanogen | − | − | | | | | | | |
| | GYP | − | − | | | | | | | |
| Otway core 4; 1,239.31–1,239.48 m; silty claystone (28, 81, 41, ND) | MS + fermenter | + | + | + | + | − | − | | | |
| | MS + metal reducer | + | + | − | + | | − | | | |
| | MS + sulfate reducer | + | + | − | − | | | | | |
| | MS + methanogen | − | + | | − | | | | | |
| | GYP | − | − | | | | | | | |
| Otway core 5; 1,273.86–1,273.95 m; sandstone (29, 42, 49, ND) | MS + fermenter | + | − | + | | − | | | | |
| | MS + metal reducer | − | − | | | | | | | |
| | MS + sulfate reducer | − | − | | | | | | | |
| | MS + methanogen | − | − | | | | | | | |
| | GYP | − | − | | | | | | | |
| King Island core; 1,447 m; unconsolidated sandstone (29, 38, 49, 37) | MS + fermenter | + | + | + | − | ++ | | ++ | | |
| | MS + metal reducer | + | − | + | | +++ | | +++ | | WMR1 |
| | MS + sulfate reducer | − | − | | | | | | | |
| | MS + methanogen | + | − | + | | +++ | | +++ | | WMe1, WMe2 |
| | GYP | + | − | + | | +++ | | +++ | | WG1 |

[a] Growth medium consisted of GYP or basal MS medium with supplementation targeting various microbial metabolic groups (see Materials and Methods).
[b] Strain identification by 16S rRNA gene BLASTN: MIT0214, *B. cereus*; MITOT1, *B. subterraneus*; WMR1, *B. safensis*; WMe1, *B. safensis*; WMe2, *B. megaterium*; WG1, *B. amyloliquefaciens*.
[c] +, ++, and +++, biomass observed at $<10^4$, $10^4$ to $10^5$, and $>10^5$ cells/ml, respectively. −, biomass not observed.
[d] ND, not determined.

over the course of a multiple-week incubation, with greater losses associated with longer incubations. Unless specifically noted, all vessel incubation data reported here maintained $scCO_2$ headspace pressures of >72.9 atm, the critical pressure for $CO_2$ mixed with ≤3% inert helium at 37°C (35), or for incubations at lower pressures, final pressure ($P_{final}$) was ≥70% of $P_{initial}$. All pressures were measured at room temperature (21°C). Based on the ideal gas law, we can estimate a maximum pressure increase of 6% associated with incubation of reactors at 37°C, although since $scCO_2$ is a nonideal gas, this may be an overestimate. Following depressurization, cultures were transferred to an anaerobic chamber (Coy Lab Products) containing a 95% $CO_2$-5% $H_2$ atmosphere for subsampling and passaging. All pressure vessels and valves were cleaned and autoclaved between uses, and high-pressure tubing was flushed before use with 10% bleach for 30 min, rinsed with MilliQ $H_2O$, rinsed with 100% ethanol, and dried with $CO_2$ gas.

Enrichment cultures from the Frio-2, Otway, and King Island sites were serially passaged by diluting 10% (vol/vol) of the previous culture in fresh growth medium under a 95% $CO_2$-5% $H_2$ atmosphere, with pressurization to 120 atm with $CO_2$, followed by incubation at 37°C. The contents of enrichment vessels were analyzed for cell abundance at the end of passages and in the inocula prior to incubations. Frio-2 passages 1 to 3 were incubated for 15 days each, while passage 4 was incubated for 60 days; subsequent passages were incubated for 9 to 15 days (Table 2). Otway and King Island passages were incubated for longer time periods (i.e., 1 to 2 months) to increase the likelihood of cellular growth based on earlier observations. Samples from each passage were subjected to microscopic enumeration and archived as glycerol stocks at −80°C.

**Enumeration of cell density.** To quantify biomass, we used a combination of methods, including direct cell counts, viable cell counts, and optical density. Cell density at the beginning and end of incubations was determined by microscopic epifluorescent cell staining using 4′,6-diamidino-2-phenylindole (DAPI; Sigma) or SYTO9 (Invitrogen) with shaking for 10 min in the dark. Five hundred microliters to 1 ml of sample was then filtered onto 25-mm, 0.2-μm-pore-size, black polycarbonate filters (Nuclepore), followed by 2 washes with 1 ml of phosphate-buffered saline (PBS). PBS was incubated on the filtered sample for 1 min to help wash off excess dye. Filters were laid on slides under microscope immersion oil with a coverslip (Thermo Scientific) and were stored at 4°C in the dark until counting. The cell density (in cells/ml) was calculated by multiplying the mean cell counts (in one 10-by-10 microscope grid) by the dilution factor and then by $3.46 \times 10^4$ (as one 10-by-10 grid at ×1,000 magnification corresponds to $1/3.46 \times 10^4$ of a 25-mm filter). Samples

were visualized on a Zeiss Axioplan fluorescence microscope. Images were captured on a Nikon D100 camera using the NKRemote live imaging software. Viability counts, i.e., CFU plating, were performed using Luria broth agar. Viable spore counts were carried out by heating aliquots to 80°C for 10 min to kill vegetative cells (36) prior to plating on Luria broth agar. Culture optical density (600 nm [$OD_{600}$]) was measured on a Bausch and Lomb spectrophotometer (1-cm path length) or via a 96-well microplate reader (BioTek Synergy 2) (200 μl per well). Optical density was not measured for incubations using metal reducer medium due to confounded readings from solid particulate content.

For MIT0214, growth was defined by increased cell density and evidence of vegetative cell morphologies by microscopy. Growth-positive cultures had at least 45-fold-increased direct cell counts relative to initial inocula of less than $1 \times 10^6$ spores/ml, or at least a 5-fold increase in direct cell counts for cultures with inocula greater than $1 \times 10^6$ spores/ml, as MIT0214 final cell densities generally varied between $1 \times 10^7$ and $1 \times 10^8$ cells/ml. For MITOT1, growth was defined by observation of culture turbidity accompanied by at least a 4-fold increase in viable cell counts (CFU/ml) above the initial spore density (on average, increases were >50-fold) and at least 25-fold-higher viable counts compared to replicates without observed turbidity since samples without evident growth all showed a decline in viable counts relative to the initial viable counts of the inoculated cultures, presumably due to a loss of spore viability during the incubation period.

**Extraction of DNA and analysis of 16S rRNA gene diversity in enrichment cultures.** DNA extraction from Frio-2 sample enrichment passages was performed using a protocol modified for Gram-positive bacteria (37). DNA extraction from Otway Basin project passages was performed using two methods, the Qiagen Blood and Tissue DNA extraction kit protocol for Gram-positive cells (Qiagen) or the MoBio soil DNA extraction kit (MoBio). Amplification of 16S rRNA genes from extracted DNA was performed using universal bacterial primers 515F 5′-GTG CCA GCM GCC GCG GTA A-3′ and 1406R 5′-ACG GGC GGT GWG TRC AA-3′ (Frio-2 passages 1, 2, and 7) and 27F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492R 5′-TAC GGY TAC CTT GTT ACG ACT T-3′ (Frio-2 passage 9, Otway passage 3, and colony-purified isolates). PCR mixtures (20 μl per reaction mixture) contained 25 to 75 ng of genomic DNA, 1× Phusion polymerase buffer, 0.4 μM (each) primer (IDT), 0.4 μM deoxynucleotide mixture, and 1 U Phusion polymerase (New England BioLabs). Thermal cycling conditions consisted of an initial 3 min at 95°C followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 90 s, followed by a final extension time of 5 min. Every PCR included negative and positive controls.

Amplified 16S rRNA gene fragments from Frio-2 samples were gel purified (Qiagen gel extraction kit) and ligated into the pJET1.2 vector (Fermentas) according to the manufacturer's protocol. Ligation products were transformed into *Escherichia coli* DH5α or *E. coli* Top10 cells, and clones were selected for sequencing (using LacZ/isopropyl-β-D-thiogalactopyranoside [IPTG] plating). For the Frio-2 enrichment, sequencing reaction mixtures were prepared using BigDye Terminator 3.1 according to the manufacturer's instructions and sequencing was performed on an ABI 3130 platform. Otway project 16S rRNA gene fragments were gel purified (Qiagen gel extraction kit) and sequenced commercially (Genewiz, Cambridge, MA). Removal of vector and primer sequences and manual editing and clustering of operational taxonomic units (OTUs) at 99% nucleotide identity were performed using Sequencher 4.5 (Gene Codes Corp.). Chimeric sequences were identified by Chimera Check 2.7 (RDP II Database) software and removed from analysis. 16S rRNA gene sequences obtained from passages of the $scCO_2$ enrichments from the Frio-2 and Otway Basin sites and from all colony-purified isolates were uploaded to the Ribosomal Database Project (RDP) server (38) for multiple-sequence alignment using their weighted neighbor-joining tree-building algorithm. The stability of the groupings was estimated by bootstrapping on 100 trees, and phylogenetic tree files were downloaded and visualized with MABL (39).

**Isolation of strains and preparation of spores.** Samples from passages 2, 5, and 3 of the enrichment cultures from Frio-2 (sample 9-26-1039-20L), Otway Basin (core 3), and the King Island core, respectively, were plated on LB agar and incubated aerobically at ambient pressure at 37°C to obtain colonies, followed by colony purification by restreaking on LB agar and identification using DNA extraction and 16S rRNA gene sequencing. Since isolates had 16S rRNA sequence types matching cloned sequences corresponding to endospore-forming bacteria, spores were prepared as described in reference 40 to serve as inocula for subsequent characterization. For spore preparation, overnight stationary-phase cultures grown under aerobic ambient conditions in LB medium were diluted 1:50 into modified G medium which consists of the following (in g/liter): 2.0 yeast extract, 0.025 $CaCl_2 \cdot 2H_2O$, 0.5 $K_2HPO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.05 $MnSO_4 \cdot 4H_2O$, 0.005 $ZnSO_4 \cdot 7H_2O$, 0.005 $CuSO_4 \cdot 5H_2O$, 0.0005 $FeSO_4 \cdot 7H_2O$, 2.0 $(NH_4)_2SO_4$, adjusted to pH 7.1 after autoclaving. Cells were incubated with shaking for 72 h to induce sporulation and then centrifuged for 15 min at 4,000 × *g*. Samples were resuspended and centrifuged 5 times in autoclaved wash buffer containing 0.058 g/liter $NaH_2PO_4 \cdot H_2O$ and 0.155 g/liter $Na_2HPO_4 \cdot 7H_2O$ with 0.01% (vol/vol) Tween 20 to prevent aggregation of spores. Spore preparations were heat treated at 80°C for 10 min to kill residual vegetative cells. Spores for each isolated strain were stored in wash buffer at 4°C until further use.

**Physiological characterization.** Physiological tests for isolates displayed in Table 3 were conducted in triplicate. LB medium was used for assaying temperature, pH, and salinity ranges, with positive growth scored by an optical density at 600 nm ($OD_{600}$) of greater than 0.05. Medium pH was adjusted with NaOH or HCl followed by incubation at 37°C. Salinity was adjusted with NaCl in LB medium followed by incubation at 37°C. LB agar was used for colony morphology determination after 20 h of incubation at 37°C. Spore formation was determined by confirming viability after heat-killing cultures grown in modified G sporulation medium and by microscopic evaluation after staining for spores (41).

**Measuring growth and survival in anaerobic $CO_2$ and $N_2$ atmospheres at variable pressure.** The dynamics of growth and survival of strains isolated from $CO_2$ sequestration sites and the type strains *Bacillus subtilis* PY79, *Bacillus mojavensis* JF-2 (ATCC 39307), and *Bacillus cereus* (ATCC 14579) were investigated under variable headspace gas composition and pressures. Inocula consisted of either cells or spores; vegetative cell cultures were grown overnight to stationary phase under aerobic conditions and diluted approximately 100-fold to $10^7$ cells/ml in fresh growth medium while spores maintained in wash buffer were diluted to approximately $10^5$ spores/ml unless otherwise specified. Media for MIT0214 and MITOT1 were GYP and MS media with the metal reducer supplement, respectively. Incubations at pressures from 3 to 136 atm were conducted in 316 stainless steel pressure vessels at 37°C with shaking at 100 rpm. Pressurization was achieved by regulating backpressure from the $CO_2$-He tank. Determination of growth dynamics under 1 atm $N_2$ or 1 atm 95% $CO_2$-5% $H_2$ was conducted in 100-ml serum bottles at 37°C with shaking at 100 rpm.

To test whether *Bacillus* spores could tolerate indirect or direct exposure to $scCO_2$, spores of *B. cereus* MIT0214 (Frio-2 isolate), *B. subtilis* PY79, and *B. mojavensis* JF-2 were aliquoted into Durham vials in equal volumes of spore storage buffer corresponding to $5 \times 10^7$, $7 \times 10^5$, and $1 \times 10^8$ spores, respectively, and dried for 2 to 3 days at 70°C to achieve a desiccated state. Dried spores were either covered in 2 ml of fresh spore storage buffer or left desiccated and then incubated under $scCO_2$ (100 atm, 37°C) in a 1-liter High Pressure Equipment Company (HIP) vessel for 2 weeks. After depressurization, samples were plated for viable counts on LB medium. Desiccated samples were resuspended in 2 ml of buffer before plating.

Antibiotic-amended control experiments were used to confirm growth under $scCO_2$. We amended reactors containing spores with both kanamycin and chloramphenicol antibiotics at 100 and 10 μg/ml, respectively, in parallel with reactors without antibiotics. Additionally, we included no-cell controls for all experiments. Presence or absence of growth was assessed with a combination of metrics, including direct cell counts,

**TABLE 3** $ScCO_2$ enrichment isolate physiology

| *Bacillus* sp. strain | 16S rRNA BLAST identification (% identity) | Isolation environment | $O_2$ requirement | Spore formation | Temp range (°C)[a] | pH range[b] | Salinity range (g/liter)[c] | Colony morphology[d] |
|---|---|---|---|---|---|---|---|---|
| MITOT1 | *B. subterraneus* (98.6) | Sandstone core, 1,193.6 m, 39°C; Otway Basin, Australia | Facultative anaerobe | + | 23–37 | 4–8 | 0.5–50 | 0.2 mm, circular, entire, raised, smooth, grayish |
| MIT0214 | *B. cereus* (99.8) | Formation water, 1,528–1,534 m, 55°C; Frio-2, Texas | Facultative anaerobe | + | 23–45 | 4–10 | 0–40 | 5 mm, circular, undulate, raised, smooth, off-white |
| WG1 | *B. amyloliquefaciens* (98.6) | Unconsolidated sandstone core, 1,447 m; King Island, CA | Facultative anaerobe | + | 23–55 | 2–10 | 0–60 | 4 mm, circular, undulate, umbonate, wrinkly, off-white |
| WMR1 | *B. safensis* (99.2) | Unconsolidated sandstone core, 1,447 m; King Island, CA | Facultative anaerobe | + | 23–55 | 4–12 | 0–60 | 2 mm, circular, entire, raised, smooth, yellow-white |
| WMe1 | *B. safensis* (99.1) | Unconsolidated sandstone core, 1,447 m; King Island, CA | Facultative anaerobe | + | 23–45 | 4–12 | 0–60 | 2 mm, circular, entire, raised, smooth, yellow-white |
| WMe2 | *B. megaterium* (99.4) | Unconsolidated sandstone core, 1,447 m; King Island, CA | Facultative anaerobe | + | 23–45 | 4–10 | 0–60 | 2 mm, circular, entire, convex, smooth, yellow-white |

[a] Temperatures tested were 9, 23, 30, 37, 45, 55, and 65°C over 72 h.
[b] pHs tested were 2, 4, 6, 7, 8, 10, and 12 over 123 h.
[c] Salinities tested were 0, 0.5, 1, 10, 20, 30, 40, 50, and 60 g/liter over 72 h.
[d] Colony morphologies were determined after 20 h of growth on LB agar.

changes in cell morphology from spores to vegetative cells, viable cell counts, and optical density.

**Statistical methods.** Logistic regression analyses were performed using JMP Pro v. 10 where growth outcome (growth/no growth) was the dependent variable and incubation time and inoculating spore density were the independent variables. Student's *t* tests were performed in Microsoft Excel.

**Nucleotide sequence accession numbers.** 16S rRNA gene sequences from isolates and clones characterized in this study have been deposited in the NCBI database with accession numbers KP873174 to KP873199.

## RESULTS

**Enrichment cultivation under $scCO_2$.** Enrichment cultivation of subsurface samples in bioreactors containing nutrients, water, and $scCO_2$ yielded isolates from all three GCS sites examined in this study (Fig. 1; Table 2). Enrichment cultures inoculated with filtered formation fluid (Frio-2) or crushed rock cores (Otway or King Island) were evaluated for growth under an $scCO_2$ headspace after 14 to 30 days as evidenced in some cases by an increase in the turbidity of culture medium. All samples were evaluated by epifluorescence microscopy to confirm biomass in turbid cultures and to screen for microbial cells in nonturbid samples (Fig. 1). Microscopy and extraction of total nucleic acids confirmed biomass generation after successive rounds of dilution and growth (Table 2).

The composition of microbial assemblages enriched under an $scCO_2$ atmosphere was determined by analysis of cloned 16S rRNA gene sequences for enrichments from the Frio-2 and Otway samples achieving cell biomass greater than approximately $10^5$ cells/ml. The libraries from the initial enrichment and first passage of the Frio-2 sample revealed a mixed community of microbes dominated by members of the *Bacillus* genera (see Fig. S1 in the supplemental material). DNA isolated from Frio-2 passages 7 and 9 revealed a single dominant *Bacillus* sequence type sharing 99.8% 16S rRNA sequence identity (1,463/1,465 nucleotides) with *B. cereus* ATCC 14579 (see Fig. S1 in the supplemental material).

Biomass was detected in enrichment cultures inoculated with four different core samples from the Otway Basin under a variety of media and environmental conditions (Table 2). However, sparse cell densities ($\leq 10^4$ cells/ml) did not yield PCR-amplifiable DNA. Subsequent rounds of dilution and incubation in $scCO_2$ bioreactors were performed, leading to increased biomass consisting of a mixture of vegetative and spore-like cells for core 3 in MS medium with an oxidized metal supplement (Fig. 1C; Table 2). The 16S rRNA gene was amplified and cloned from DNA extracted from passage 3 of Otway core 3, yielding 26 clones of a single ribotype matching *B. subterraneus* with 98.5% identity (1,502/1,521 nucleotides) (Table 3; see also Fig. S2 in the supplemental material). $scCO_2$ enrichment cultures inoculated with material from the King Island core revealed positive growth in 5 out of 8 incubations (Table 2). 16S rRNA clone libraries of King Island enrichments were not pursued as the core was unconsolidated and thus fully permeated by drilling tracer fluid, which likely contaminated the microbial diversity. The 16S rRNA gene of isolates from the $scCO_2$ enrichment of King Island core material was sequenced following colony purification (Table 3).

**Colony purification and strain characterization.** Since the *Bacillus* spp. identified in clone libraries are known to be predominantly aerobic and heterotrophic/copiotrophic, we attempted to isolate *Bacillus* strains as colonies on LB agar plates from $scCO_2$ enrichment passages of all samples with significant growth ($>10^5$ cells/ml). When growth under $scCO_2$ was observed, depressurized cultures were used as inocula and subsequent colony formation under ambient aerobic conditions (37°C) was observed after 1 to 3 days. Individual colonies with distinct morphologies were colony purified and identified by 16S rRNA gene sequencing and corresponded to *B. cereus* (Frio-2 sample, passages 2 and 7, isolate

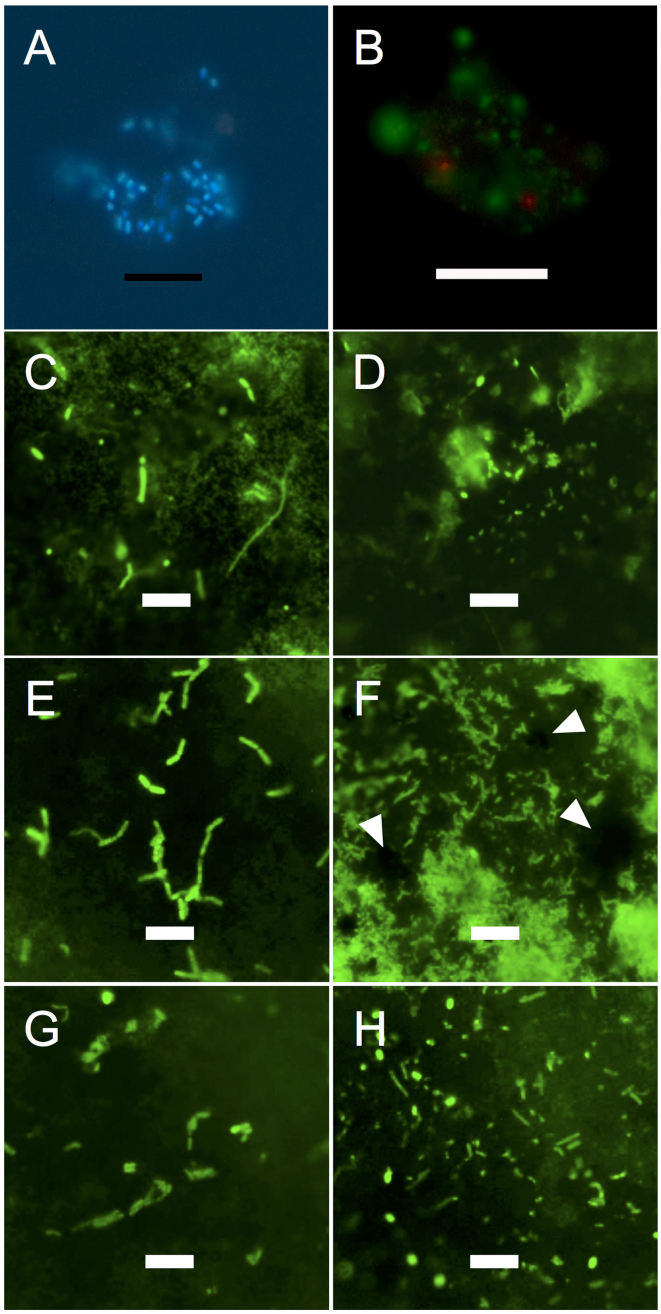


**FIG 1** Epifluorescent microscopy of enrichment cultures grown under $scCO_2$, filter concentrated onto 25-mm 0.2-μm-pore-size membranes, and stained with DAPI (A) or SYTO9 (B to H). Bars, 10 μm. (A) Cells from Frio-2 initial enrichment. (B) Cells from Frio-2, passage 1, counterstained with propidium iodide (red) to identify membrane-compromised cells. (C) Cells from Otway core 3, passage 3, showing larger vegetative cells and smaller cells that may be spores observed within the same sample. (D) King Island initial enrichment, metal reducer medium. (E to H) Images correspond to cultures of MIT0214, MITOT1, WMe2, and WG1, respectively. Cultures in panels E to H were inoculated with spores, with a mixture of mostly vegetative cells and some spores visible after 30 days of growth under $scCO_2$. Black particles (denoted by white arrowheads in panel F) correspond to metal solids from the growth medium for metal reducers.

MIT0214); *Bacillus subterraneus* (Otway core 3, passage 5, isolate MITOT1); and *Bacillus amyloliquefaciens*, *Bacillus megaterium*, and *Bacillus safensis* (2 strains) (King Island core, passage 3, isolates WG1, WMe2, WMe1, and WMR1, respectively) (Table 3; also see Fig. S2 in the supplemental material). Isolates were subjected to physiological characterization and were facultative anaerobes with variable ranges of pH, temperature, and salinity similar to previously characterized *Bacillus* spp. (Table 3). Isolates were subjected to regrowth experiments to confirm bacterial growth under $scCO_2$.

**Growth of *Bacillus* strains MIT0214 and MITOT1 and *Bacillus* type strains under $scCO_2$.** Based on our observations of biomass increases in enrichment cultures, spore formation in isolated strains, and the lethal effects of $scCO_2$ on vegetative cells of isolates and type strains grown under ambient conditions (see Fig. S3 and S4 in the supplemental material), we hypothesized that spores but not vegetative cells would germinate and grow under $scCO_2$. To test our hypothesis, we inoculated growth medium with a range of spore concentrations ($2.4 \times 10^4$ to $9.9 \times 10^6$ spores/ml for isolate MIT0214 and $6.9 \times 10^3$ to $5.6 \times 10^6$ spores/ml for isolate MITOT1), followed by incubation under $scCO_2$ from 1 week to 49 days (Fig. 2), accompanied by antibiotic (see Fig. S5 in the supplemental material) and no-cell controls. As we observed highly variable outcomes (i.e., growth/no growth) in replicate cultures of MIT0214 and MITOT1 incubated under an $scCO_2$ headspace, we used logistic regression (Fig. 2A and B) to determine the relationship between frequency of growth under $scCO_2$, incubation time, and initial spore density. For >20-day incubations of strain MIT0214, 26/78 cultures exhibited increased cell density (average final cell density of $5.6 \times 10^7$ cells/ml), while 0/15 no-cell controls and 0/30 antibiotic controls displayed growth (Fig. 2A; see also Fig. S5 in the supplemental material). For ≥20-day incubations of strain MITOT1, 32/58 cultures exhibited increased cell density (average final cell density of $1.2 \times 10^7$ cells/ml), while no increase in cell density was seen for no-cell controls (0/15) or antibiotic controls (0/4) (Fig. 2B; see also Fig. S5 in the supplemental material). For logistic regression analysis, no-cell controls were input at half the detection level (i.e., at 870 cells/ml), while antibiotic controls were excluded from the model. As expected, growth under $scCO_2$ is significantly affected by both initial inoculum concentration and incubation time (Fig. 2A: $P = 0.045$ and $P = 0.0005$, respectively, for MIT0214, $n = 78$; Fig. 2B: $P = 0.0014$ and $P = 0.023$, respectively, for MITOT1, $n = 73$). We noted that growth of MITOT1 consistently accompanied more reduced conditions in growth medium that contained the resazurin redox indicator dye and oxidized metal supplements (see Fig. S6 in the supplemental material). The incubation times associated with a 50% likelihood of growth in cultures inoculated with at least $1 \times 10^4$ spores/ml are 33 days for MIT0214 and 36 days for MITOT1.

**Growth of *Bacillus* type strains and King Island isolates under $scCO_2$.** After observing growth under $scCO_2$ of isolates MIT0214 and MITOT1, we investigated whether other *Bacillus* strains, including subsurface isolates from the King Island site and additional *Bacillus* type strains, could germinate and grow under $scCO_2$. Spores of three isolates from the King Island core (WG1, WMe1, and WMe2) and three *Bacillus* type strains, *B. mojavensis* JF-2, *B. subtilis* PY79, and *B. cereus* ATCC 14579, were inoculated into growth medium and incubated under $scCO_2$ for 30 days. King Island isolates and *Bacillus* type strains yielded increased cell

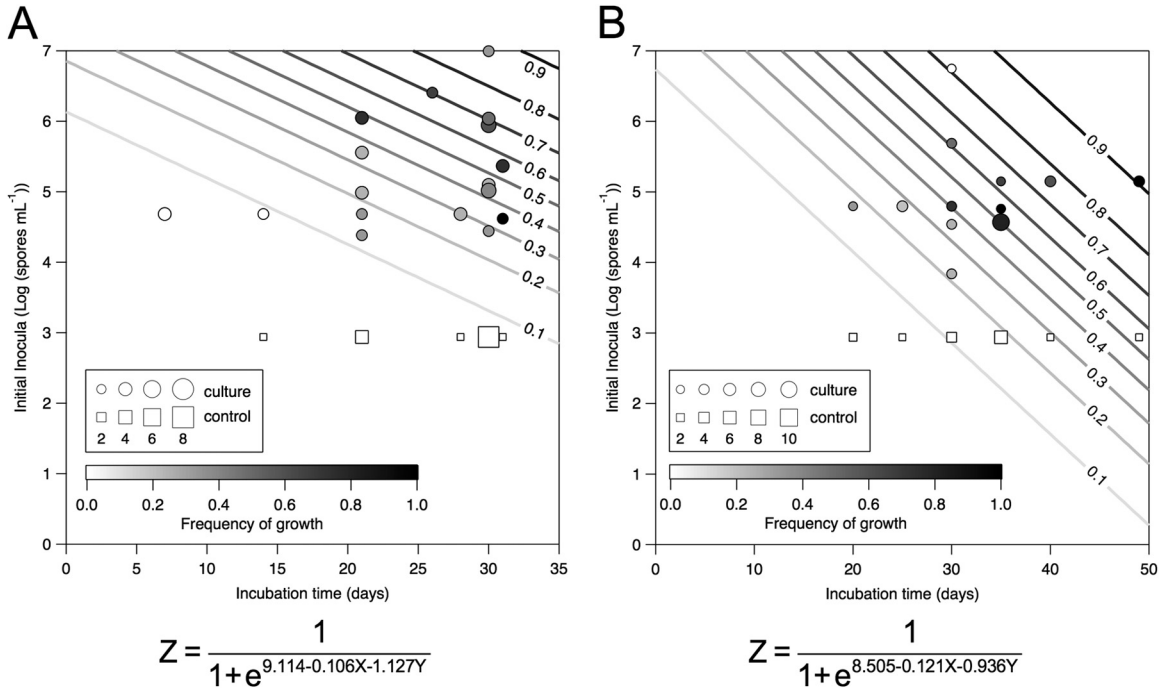


**FIG 2** Logistic regressions of MIT0214 (A) and MITOT1 (B) growth outcomes under $scCO_2$ show a significant increase in the frequency of observed growth with increasing incubation time (MIT0214, $P = 0.0005$; MITOT1, $P = 0.023$) and increasing density of spore inocula (MIT0214, $P = 0.045$; MITOT1, $P = 0.0014$). Model results predicting frequency of growth are represented by contour lines. Experimental data (MIT0214, $n = 78$; MITOT1, $n = 73$) are overlaid on top of the contours with symbol shade indicating frequency of growth and symbol size proportional to number of replicates represented by each point. Circles are cell-containing samples, and squares are no-cell controls that were entered into the model at half the detection limit (870 cells/ml).

densities after incubation (Fig. 3A and B, respectively). However, growth patterns were variable between replicates as previously observed during experiments with strains MIT0214 and MITOT1 (Fig. 2). This unexpected result appears to indicate that the ability to grow in environments containing $scCO_2$ may be widespread among *Bacillus* spp.

**Variable survival of cells and spores after exposure to $scCO_2$.** In contrast to the observed germination and growth of spores in bioreactors containing $scCO_2$, vegetative cells grown under aerobic conditions and ambient pressure were not able to acclimate to $scCO_2$ exposure and grow in our experimental system. Vegetative cells from overnight stationary-phase cultures of isolate *B. cereus*

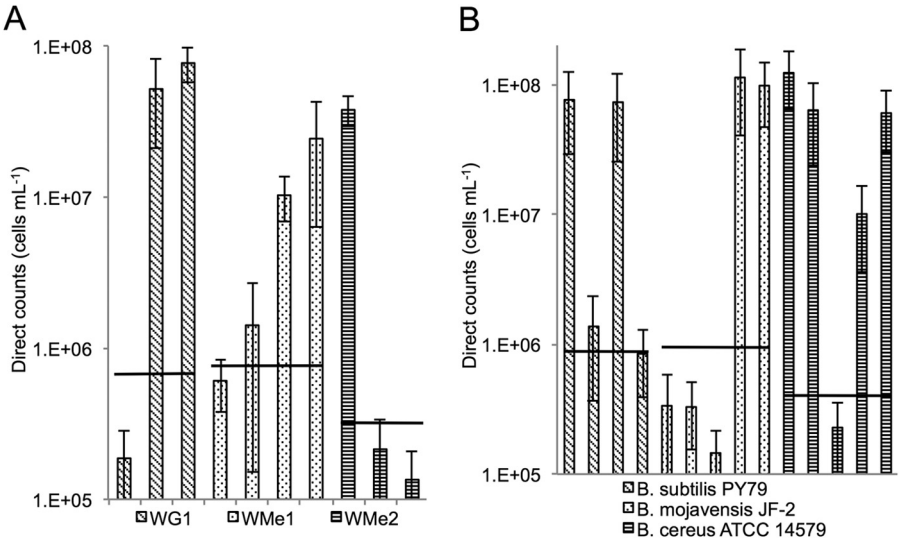


**FIG 3** Microscopic enumeration of cell abundance in cultures grown under $scCO_2$ for King Island isolates (A) and three *Bacillus* type strains (*B. subtilis* PY79, *B. mojavensis* JF-2, and *B. cereus* ATCC 14579) (B). Data shown are total direct cell counts after 30 days of incubation, with horizontal lines representing initial cell counts. Initial spore densities ranged from $3.2 \times 10^5$ to $9.5 \times 10^5$ spores/ml.

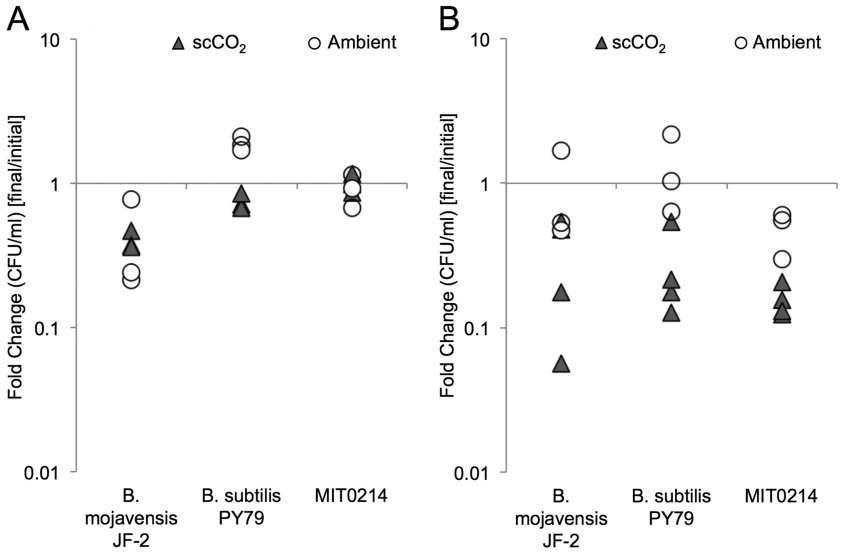


**FIG 4** Change in viable cell count (CFU/ml, final/initial) in spores from three *Bacillus* species exposed to $scCO_2$ for 2 weeks. (A) Spores resuspended in spore preparation buffer (dark triangles) revealed no decrease in viability upon exposure to $scCO_2$ relative to starting spore counts and ambient-atmosphere-incubated controls (open circles). *B. subtilis* spores may have germinated and grown during the incubation periods under ambient conditions. (B) Spores exposed to dry $scCO_2$ (dark triangles) decreased in viability by 66 to 86%.

MIT0214 and type strains *B. cereus* ATCC 14579, *B. subtilis* PY79, and *B. mojavensis* JF-2 were exposed to $scCO_2$ for 6 h at 37°C. Reductions in viable cell counts of 4 to 8 orders of magnitude were observed for all strains exposed to $scCO_2$ relative to controls incubated under ambient conditions (see Fig. S3 and S4 in the supplemental material). Cells surviving exposure to $scCO_2$ were most likely spores based on similar proportions of cells surviving heat-kill treatments (see Fig. S4 in the supplemental material).

Spores from *B. cereus* MIT0214, *B. subtilis* PY79, and *B. mojavensis* JF-2 were robust to $scCO_2$ exposure under both aqueous and desiccated conditions. Spores that were dried and then resuspended in aqueous buffer and exposed to $scCO_2$ showed no significant change in viability after 2 weeks relative to the initial viable count or controls incubated under ambient conditions ($P > 0.05$) (Fig. 4A). While dried spores directly exposed to $scCO_2$ were more susceptible to the killing effect, 14 to 24% of spores remained viable after 2 weeks relative to ambient-incubated controls for *B. mojavensis* and *B. cereus* ($P < 0.05$) and for *B. subtilis* ($P = 0.08$) (Fig. 4B). Strain *B. cereus* MIT0214, isolated early in the project, was used as a model $scCO_2$-tolerant isolate for most analyses. Anecdotally, spores of strain *B. subterraneus* MITOT1 appeared to be less robust than spores of other *Bacillus* spp. tested. In the spore tolerance experiment, the pretreatment protocol to desiccate the spores resulted in total loss in viability for MITOT1, and thus, no data are available for this strain. Overall, this experiment indicated that spores from diverse *Bacillus* strains can withstand both direct and indirect exposure to $scCO_2$, with enhanced survival of spores under aqueous conditions (Fig. 4; also see Fig. S7 in the supplemental material).

**Growth of MIT0214 under variable $CO_2$ and $N_2$ pressure.** We incubated strain MIT0214 under subcritical pressures (<72.9 atm) of $N_2$ and $CO_2$ to observe whether more consistent growth outcomes (growth/no growth) would accompany more permissive growth conditions. At 1-atm pressure, anaerobic growth under $CO_2$ or $N_2$ was reproducible among triplicate incubations with increases in turbidity and viable cell counts revealing lag, log, stationary, and decline phases over a 1-week time frame (see Fig. S8A and B in the supplemental material). Considerably lower germination frequencies were observed under 1-atm $CO_2$ (<19%) than 1-atm $N_2$ (>99%) (see Fig. S8A in the supplemental material). In contrast to reproducible growth observed at ambient pressure, growth at all pressures above 1 atm under either $N_2$ or $CO_2$ revealed positive growth but variability in outcome (i.e., growth/no-growth) between replicates over 1- and 2-week time frames, respectively, similar to the variability observed for cultures incubated under $scCO_2$ (see Fig. S9 in the supplemental material).

## DISCUSSION

We demonstrated that bacterial growth under an $scCO_2$ atmosphere is possible using strains isolated through enrichment cultivation of samples from three sites targeted for geologic sequestration of $CO_2$: filtrate samples from the Frio-2 project in Texas; consolidated rock cores from the Paaratte formation in the Otway Basin, Australia; and an unconsolidated core from the Mokelumne formation at the King Island site in California. In addition, we observed germination and growth under $scCO_2$ of three *Bacillus* type strains isolated from subsurface (*B. mojavensis* JF-2) and surface (*B. subtilis* PY79 and *B. cereus* ATCC 14579) environments. These observations of microbial growth under $scCO_2$ have several implications for geological carbon dioxide sequestration (GCS). (i) Microbial survival and activity are possible, and perhaps likely, at the $CO_2$ plume-water interface, even in areas previously exposed to pure-phase $scCO_2$. (ii) Engineering biofilm barriers or stimulating biomineralization *in situ* at the $CO_2$ plume-water interface may be feasible, despite previously documented lethal properties of $scCO_2$ for nonacclimated microorganisms. (iii) Organisms likely to survive and proliferate after $CO_2$ injection include spore-forming microbes that can withstand the initial $CO_2$ stresses.

Phylogenetic and physiological analyses reveal that strains iso-

lated from the three GCS sites are similar to previously described *Bacillus* isolates that are readily cultured copiotrophs. Anaerobic enrichment medium formulations, although supplemented to target diverse metabolic groups, contained a base of organic carbon (yeast extract and peptone), potentially explaining the dominance of *Bacillus* spp. in our enrichments. While the catabolic pathways for different strains recovered were not determined in this study, previous studies suggest that closely related *Bacillus* spp. may grow anaerobically by fermentation and/or anaerobic respiration. Strain MIT0214 is most closely related to *B. cereus*—a widely distributed bacterium that has been isolated from a diverse range of environments, including sites characterized by heavy metals, deep oil reservoirs, and/or hypersaline conditions (42–45). Strain MITOT1 is closely related to strains *B. subterraneus* and *Bacillus infernus*, both of which were isolated from deep subsurface environments (46, 47). King Island isolates are closely related to strains of *B. megaterium*, *B. safensis*, and *B. amyloliquefaciens*, which have all been isolated from diverse terrestrial environments (48–53). In light of the diversity of *Bacillus* strains able to grow under $scCO_2$, we hypothesize that the ability to grow under high $pCO_2$ is a widespread feature of *Bacillus* spp. and possibly other organisms.

All *Bacillus* spp. isolated in this study were sporeformers capable of facultative anaerobic growth under both $N_2$ and $CO_2$ atmospheres. Notably, we have observed that all strains exhibit the fastest growth under aerobic conditions and atmospheric pressure but are also able to grow anaerobically at elevated pressures beyond the critical point for $CO_2$ (greater than 37°C and 73 atm). Growth dynamics of strain MIT0214 at 1 atm are consistent with anaerobic growth via fermentation where end product inhibition leads to a reduction of cell and spore viability in stationary phase (see Fig. S8A and B in the supplemental material). In strain MITOT1, the observed change in culture oxidation state that accompanied cell growth (as demonstrated by the resazurin indicator) suggests that MITOT1 may be capable of Fe(III) or Mn(IV) reduction (see Fig. S6 in the supplemental material), although whether oxidation state changes occur via fermentation or respiration has not been established in this study. We note that several close phylogenetic relatives of strain MITOT1 are anaerobic metal reducers capable of Fe(III), Mn(IV), Se(VI), and As(V) respiration (46, 47, 54).

Spores of three *Bacillus* strains, including isolate *B. cereus* MIT0214, are tolerant of both direct and indirect exposure to $scCO_2$. Direct exposure of dried spores to $scCO_2$ proved to be a more severe stress, resulting in a 66 to 86% loss in spore viability over a 2-week interval, while no significant loss was observed in spores indirectly exposed via aqueous phase (Fig. 4; also see Fig. S7 in the supplemental material). The decrease in viability of spores directly exposed to $scCO_2$ may be due to its solvating and desiccating properties, as spores in the aqueous phase do not experience the same degree of contact with $scCO_2$ and are protected from dehydration. While it remains to be determined whether the viability decrease is time dependent, our observation of a decrease of $<1$ order of magnitude in survival of desiccated spores exposed to $scCO_2$ suggests that spores in subsurface environments will likely be able to tolerate at least brief periods of direct exposure to pure-phase $scCO_2$. This finding is especially relevant in the GCS context because as buoyant $scCO_2$ migrates toward the cap-rock interface, spores may experience fluctuations in $scCO_2$ contact in areas where formation fluids have been displaced, creating local areas of more extreme and desiccating conditions. Persistence of a subpopulation of spores after direct $scCO_2$ exposure suggests that $scCO_2$ injection during GCS may not effectively sterilize the subsurface, enabling the resumption of microbial activities upon return of an aqueous phase. Our results, and previous demonstrations of spore resistance to $scCO_2$ sterilization (9), suggest that spore-forming microbial taxa may be able to survive and acclimate to *in situ* conditions post-$CO_2$ injection during geologic carbon dioxide sequestration.

Previous work suggests that several features of *Bacillus* physiology are likely responsible for the variable, but predictable, time- and density-dependent growth observed under $scCO_2$. First, germination frequencies under $CO_2$ headspaces are known to be significantly lower than those under aerobic or anaerobic nitrogen headspaces, with only 10 to 30% of *B. cereus* spores germinating under 1 atm $CO_2$ (55). Growth dynamics for isolate *B. cereus* MIT0214 (see Fig. S8A in the supplemental material) were consistent with these findings, displaying a germination frequency at 1 atm $CO_2$ no greater than 19%, in contrast to a 99% germination frequency under 1 atm $N_2$ (see Fig. S8A in the supplemental material). Second, it has been shown that certain stresses, such as pH and oxidative stresses, significantly extend the lag time for germination of *Bacillus* spores (55, 56). We have observed extended lag phases for growth of MIT0214 under 1 atm $CO_2$ compared to 1 atm $N_2$ (see Fig. S8A and B in the supplemental material) and have also observed extended lag times for growth of all isolates at low pH (pH $<6$) (data not shown) during physiological tests (Table 3). The third mechanism that may explain the observed variability in growth has been termed the "microbial scout hypothesis," where activation of cells from dormant bacterial populations (e.g., spores) is stochastic but occurs at a constant low frequency (i.e., $\sim$0.01% per day) independent of environmental cues. This behavior would allow a clonal population to sense and respond to permissive growth conditions without risking activation of the entire dormant population (57). Fourth, it has been shown that modulation of the sporulation conditions has a significant effect on spore physiology and resistance to stress (58, 59), and we suggest that a population of spores may contain a range of this physiological plasticity where some, but not all, spores are able to grow following germination under stressful conditions. Finally, microenvironments within cultures (e.g., associated with biofilms or mineral surfaces) may influence the degree to which cells are exposed to stresses associated with $scCO_2$ (17, 60). Thus, variability in exposure and stress tolerance of spores in combination with extended lag times under conditions of high $pCO_2$ and low pH, and inherently stochastic and suppressed germination frequencies, may account for observed variability of growth under $scCO_2$.

Microbial biomass obtained from ongoing GCS projects supports the notion that spore-forming *Firmicutes* populations, such as *Bacillus* spp., persist and potentially grow in the subsurface after $scCO_2$ injection. Recovery of phospholipid fatty acid (PLFA) from Frio-2 groundwater samples indicated the presence of viable microorganisms after $CO_2$ injection, especially *Firmicutes*, as indicated by the observation of characteristic terminally branched saturated fatty acids. In particular, i15:0, i17:0, and a17:0, which are prominent fatty acids in *B. cereus* (61, 62), were observed in the sample from the Frio-2 project from which *B. cereus* strain MIT0214 was isolated and in samples following $CO_2$ breakthrough (S. Pfiffner, personal communication). Microbial communities recovered in formation waters of the Otway Basin (Aus-

tralia) and Ketzin (Germany) GCS sites (10 to 12 days and 2 days to 10 months post-$CO_2$ injection, respectively) include taxa of the *Firmicutes* (e.g., *Bacilli* and *Clostridia*) among the detectable diversity. Finally, in experimental incubation of formation fluids under $scCO_2$, enrichment of *Firmicutes* (*Clostridiales*) was observed after removal of $scCO_2$, demonstrating the resilience of native *Firmicutes* populations to $scCO_2$ exposure (63).

While some populations of *Bacillus* are well established as members of subsurface biospheres (46, 47), others may be introduced to the subsurface through drilling activities. Although sampling methods for Frio-2 formation water and Otway cores were designed to reduce the possibility of drilling fluid contamination through use of a U-tube sampling device (33) or fluorescein drilling fluid penetration data, respectively, drilling activities remain a potential source for some of the strains recovered in this study. Indeed, the King Island core, which yielded the highest number of $scCO_2$-tolerant isolates, was unconsolidated sandstone and fully penetrated by drilling fluid based on tracer data. Notably, drilling fluid characterized by Mu et al. (22) was dominated by sequences of *Bacilli*.

High concentrations of local dissolved organic carbon at the edges of injected $CO_2$ plumes, as observed during the Frio-2 project (29, 64), may stimulate growth of native or introduced bacteria. Enrichment of dissolved organic carbon near the plume may be due to extraction of the subsurface organic matrix by the nonpolar solvent-like properties of $scCO_2$. We suggest that these enrichments of dissolved organic carbon at the leading edge of the $CO_2$ plume may serve as potential hot spots for microbial activity fueled by anaerobic metabolism. Previous observations that DNA and lipid biomass from *Firmicutes* populations persist in $scCO_2$-exposed environments (21, 22), coupled with the presented evidence that a diverse set of *Bacillus* strains and isolates may grow in the presence of $scCO_2$, water, and nutrients, suggest that microbially mediated processes may continue to occur up to the $scCO_2$ plume-water interface during geologic carbon dioxide sequestration. Understanding how $CO_2$ injection shifts the balance of microbial community structure, metabolic potential, and associated biogeochemical processes is therefore necessary to predict the long-term fate of injected $CO_2$.

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The authors declare no conflict of interest with this work.

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