Analysis of microbial activity under a supercritical CO_2 atmosphere

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1. Abstract

Because the extent and impact of microbial activity in deep saline aquifers during geologic sequestration is unknown, the objectives of this proposal were to: (1) characterize the growth requirements and optima of a biofilm-producing supercritical CO_2-tolerant microbial consortium (labeled MIT0212) isolated from hydrocarbons recovered from the Frio Ridge, TX carbon sequestration site; (2) evaluate the ability of this consortium to grow under simulated reservoir conditions associated with supercritical CO_2 injection; (3) isolate and characterize individual microbial strains from this consortium; and (4) investigate the mechanisms of supercritical CO_2 tolerance in isolated strains and the consortium through genome-enabled studies.

Molecular analysis of genetic diversity in the consortium MIT0212 revealed a predominance of sequences closely related to species of the spore-forming genus *Bacillus*. Strain MIT0214 was isolated from this consortium and characterized by physiological profiling and genomic analysis. We have shown that the strain MIT0214 is an aerobic spore-former and capable of facultative anaerobic growth under both reducing N_2 and CO_2 atmospheres by fermentation and possibly anaerobic respiration. Strain MIT0214 is best adapted to anaerobic growth at pressures of 1 atm but is able to growth at elevated pressures After 1 week growth was observed at pressures as high as 27 atm (N_2) or 9 atm (CO_2) and after 26-30 days growth can be observed under supercritical CO_2. In addition, we have determined that spores of strain *B. cereus* MIT0214 are tolerant of both direct and indirect exposure to supercritical CO_2. Additional physiological characterization under aerobic conditions have revealed MIT0214 is able to grow from temperature of 21 to 45 °C and salinities 0.01 to 40 g/L NaCl with optimal growth occurring at 30˚C and from 1 - 5 g NaCl/L. The genome sequence of *B. cereus* MIT0214 shared 89 to 91% of genes with other genome-sequenced strains with 93.3 to 97.8% nucleotide identity among shared genes. Comparison of the sequence of MIT0214 or a B. *cereus* strain isolated from an oil well in China to *B. cereus* isolates from surface environments revealed a higher proportion of genes involved in Cell wall and capsule biosynthesis and metabolism, metabolism of aromatic compounds, and stress response. Since *Bacillus* species, including *B. cereus* strains, have commonly been recovered from other “extreme” environments including the deep subsurface – the $scCO_2$ tolerance of spores and growth under high pCO_2 conditions is consistent with persistence in a subsurface environment after CO_2 injection.

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2. Executive Summary

Because the extent and impact of microbial activity in deep saline aquifers during geologic sequestration is unknown, the objectives of this proposal are to: (1) characterize the growth requirements and optima of a biofilm-producing supercritical CO_2-tolerant microbial consortium (labeled MIT0212) isolated from hydrocarbons recovered from the Frio Ridge, TX carbon sequestration site; (2) evaluate the ability of this consortium to grow under simulated reservoir conditions associated with supercritical CO_2 injection; (3) isolate and characterize individual microbial strain(s) from this consortium; and (4) investigate the mechanisms of supercritical CO_2 tolerance through genome-enabled studies.

In the work detailed in this report we show that generation of bacterial biomass (i.e. growth) under a supercritical CO_2 atmosphere is possible using enrichment cultures started from samples collected during the Frio 2 project and an isolated strain. This observation of microbial growth under $scCO_2$ has two fundamental implications for geological carbon dioxide sequestration (GCS): (1) Microbial activity is possible, and perhaps likely, at the CO_2 plume water interface, even in areas previously exposed to pure-phase $scCO_2$. (2) Engineering biofilm barriers to grow *in situ* at the CO_2-plume + brine interface is not precluded by the previously documented sterilizing properties of supercritical CO_2.

These two implications for GCS are supported by a series of experiments performed over the duration of this project. First, we observed that the initial enrichment of a sample from the Frio 2 pilot experiment under supercritical CO_2 was dominated by DNA sequences (16S rRNA gene clones) corresponding to strains of the genus Bacilli. Observation of Bacilli in the initial enrichments is not surprising in light of their spore-forming abilities and thus resistance to harsh environmental conditions such as $scCO_2$. An isolated *Bacillus* strain designated MIT0214 emerged from additional passage of the enrichment culture. Although not recovered in community analysis of the initial enrichment, biomarkers of the strain recovered in early passages of the sample suggest it may have been part of the initial enrichment community. We have shown through phylogenetic analysis that strain *Bacillus* MIT0214 is most closely related to *B. cereus* – a widely distributed bacterium that has been isolated from a wide diversity of environments including sites characterized by heavy metals, hydrocarbons and/or hypersaline conditions.

To examine whether strain MIT0214 could serve as a model for microbial activity under a supercritical CO_2 atmosphere we subjected the strain to a battery of physiological characterizations. We determined that MIT0214 is an aerobic spore-former capable of facultative anaerobic growth under both reducing N_2 and CO_2 atmospheres by fermentation and possibly anaerobic respiration. We have observed that strain MIT0214 is best adapted to anaerobic growth at pressures of 1 atm but is able to growth at elevated pressures beyond the critical point for CO_2. Specifically, after 1 week growth was observed at pressures as high as 27 atm (N_2) or 9 atm (CO_2) and after 26-30 days growth can be observed under both a N_2 headspace at 100 atm and under a supercritical CO_2 headspace at a temperature of 37˚C and pressures >71 atm (to 136 atm).

In addition to demonstrating growth under $scCO_2$ we have determined that spores of strain *B. cereus* MIT0214 are tolerant of both direct and indirect exposure to $scCO_2$. As expected, direct exposure of dried spores to $scCO_2$ proved to be the more severe stress resulting in a 66-86% loss in spore viability over a 2 week interval, however persistence of a subpopulation of spores after exposure to dry $scCO_2$ suggests that even direct exposure to supercritical CO_2 during GCS may not effectively sterilize the subsurface, indicating that upon return of an aqueous phase, microbial activities can resume.

Additional physiological characterization under aerobic conditions have revealed MIT0214 is able to grow at temperatures from 21 to 45 °C (but not at 55˚C) and salinities 0.01 to 40 g/L NaCl with optimal growth occurring at 30˚C and from 1 - 5 g NaCl/L. We have noted that MIT0214 spores are able to tolerate temperatures in excess of 80˚C and saturated salt solutions without loss of viability. Since *Bacillus* species, including *B. cereus* strains, have commonly been recovered from other deep subsurface environments – the $scCO_2$ tolerance of spores and growth under high pCO_2 conditions is consistent with persistence in a subsurface environment after CO_2 injection and it is likely that strain MIT0214 would have been able to persist as a spore, but not grow, under the down-hole conditions observed at the Frio 2 site (temperature 55˚C, suspended solids 93 g/L consisting primarily of salts) (Hovorka et al., 2006).

Genomic analysis of strain MIT0214 reveals a shared evolutionary history with other *B. cereus* strains isolated from surface environments including signatures of lateral gene transfer. The genomes of strain MIT0214, and a *B. cereus* strain isolated from an oil well in China (strain Q1) each revealed a significant enrichment in the number of genes assigned to functional categories for producing and maintaining the “Cell Wall and Capsule”, regulating and mediating “Stress Response”, and “Metabolism of Aromatic Compounds” relative to six *B. cereus* isolates from surface environments, which may point to adaptations that facilitate survival in subsurface habitats.

Our findings suggest that strategies to engineer biofilm barriers *in situ* under high pCO_2 conditions should consider addition of bacterial cells as spores rather than as vegetative cells as we have observed that spores survive initial supercritical CO_2 exposure while aerobically-grown vegetative cells do not. Moreover, our observations indicate that survival of some microbial types, such as Bacilli, at the interface between the $scCO_2$ plume and the formation water is likely and that such survival may be mediated heavily by pre-existence of the bacterium as a spore. After survival, we have observed germination of spores and growth of vegetative cells under supercritical CO_2. Understanding how CO_2 injection effects microbial activity *in situ* including the role in mediating biogeochemical cycling is therefore necessary to predict the long-term fate of injected CO_2.

3. Background and Introduction

An enabling technology for geological carbon sequestration is the development of reservoir sealing mechanisms and leak remediation strategies. Biofilm barriers hold promise for both applications, however establishing such barriers *in situ* e.g. after reservoir decommissioning requires that biofilm producing strains are active under high pCO_2 conditions. Biofilm barriers have been developed and deployed as a means to reduce the permeability of aquifers for both flow containment and contaminant remediation e.g. (Komlos, et al, 2004; Seo, et al, 2009). Biofilm barriers are formed when limiting nutrients are supplied to stimulate growth of indigenous or introduced microbial populations in residual pore spaces between sediment grains. The formation of biomass and production of extracellular biopolymers reduces the permeability of the subsurface formation. Work from the Center for Biofilm Engineering at Montana State University has established the potential for using biofilm barriers to reduce the permeability of sandstone to the migration of supercritical CO_2 (Mitchell, et al, 2008; Mitchell, et al, 2009; Cunningham, et al, 2009). However, it remains an open question whether such barriers can be engineered to grow *in situ* post-CO_2 injection such as may be required if a leak is detected after decommissioning of a carbon storage reservoir. $scCO_2$ has been regarded as a sterilizing agent by the Food and Drug Administration (i.e. reducing viable bacterial cells by 6-log units) (Dillow, et al, 1999) although subsequent studies have shown that a small fraction (0.1 to 10%) of *Bacillus mojavensis* cells can survive supercritical CO_2 exposure over a 19 minute interval (Mitchell, et al, 2008).

The goal of our research project was to identify and develop strains capable of microbial activity under supercritical CO_2 in support of the DOE's objective to develop technologies to form "seal mechanisms that can be induced if leakage occurs or proactive mitigation through remediation/plugging of abandoned well-bores" (per the initial request for proposals). To this end we have characterized the diversity of bacteria able to survive under a supercritical CO_2 atmosphere and are investigating the molecular mechanisms of microbial survivability and stress response in $scCO_2$-tolerant bacteria through physiological and genomic profiling.

The specific ***scientific objectives*** of this proposal were to (1) characterize the growth requirements and optima of a biofilm-producing supercritical CO_2-tolerant microbial consortium isolated from hydrocarbons recovered from the Frio Ridge, TX carbon sequestration site; (2) evaluate the ability of this consortium to grow under simulated reservoir conditions associated with supercritical CO_2 injection; (3) isolate and characterize individual microbial strain(s) from this consortium (4) investigate the mechanisms of $scCO_2$ tolerance in isolated strains and the consortium through genome-enabled studies.

In addition, this project included ***educational objectives***, specifically to (1) support PhD thesis research of a candidate in the department of Civil and Environmental Engineering at MIT, (2) support at least three year-long DOE-funded research opportunities for undergraduates in "Geological Carbon Sequestration Microbiology" to provide exposure to the emerging field of Geological Carbon Sequestration as students are evaluating post-baccalaureate career opportunities. The research results presented herein represent the combined efforts of a postdoctoral researcher (project years 1 and 2), a graduate student (project years 1-3), and three MIT undergraduate students.

4. Experimental Methods

Sample collection and storage. Samples from sites targeted for geologic carbon dioxide sequestration were utilized as inocula for microbial enrichment cultures using supercritical CO_2 as the selective agent. Samples from the Frio CO_2 sequestration site near Houston Texas were previously collected as part of the Frio2 project and were shared courtesy of Drs. Tommy Phelps, Oak Ridge National Laboratory and Susan Pfiffner, University of Tennessee. Frio samples consisted of filter-concentrated >1 um size particles from groundwater sampled with the U-tube system (Freifeld et al., 2005; Hovorka et al., 2006) collected prior to the start of CO_2 injection, and at 7.5 hr and 372 days post-injection. Filter samples were maintained at -80C prior to analysis. Additional inocula were collected as grab-samples from various soil and aquatic environments around MIT. Samples for enrichment cultures were prepared in an anaerobic tent with a 95% CO_2 / 5% H_2 atmosphere. Approximately 10uL of hydrocarbon residues associated with surface of glass fiber filters from the Frio 2 samples were used as inocula for enrichment cultures.

Cultivation media. Glucose Yeast Peptone media (GYP) was used for enrichment of Frio samples and consisted of (per L) 6 g glucose, 1 g yeast extract, 1 g tryptic peptone, 1 g sodium acetate, 0.2 g $MgSO_4x7H_2O$, 0.01 g $MnSO_4x4H_2O$, 0.01 g $FeSO_4x7H_2O$, 0.01 g NaCl (Barker, et al 1998). Luria Broth (LB) or Luria Agar (Difco) was used for cultivation of strain MIT0214.

Pressurized incubations. Chambers for high-pressure growth were designed and constructed using 316 stainless steel HPLC column bodies (4, 10 or 19 ml capacity) or 316 stainless steel tubing (4 and 10 mL capacity) as described by Peet, et al (2011, 2012, In Prep). Chambers were fitted with ball valves (Supelco) or quarter turn plug valves (Swagelok or Hylok). The headspace of the stainless steel culture vessels was pressurized with industrial grade N_2 gas (Airgas) or with extraction grade CO_2 gas (Airgas) with a helium headpressure such that the in-tank pressure was 120-136 atm. Pressurization of culture vessels was carried out at a rate of 2-3 atm min^{-1} and headspace volume represented 40-50% of the culture vessel space. Pressurized culture vessels were incubated in a 37˚C warm room with shaking at 100 rpm. Following incubation the final pressure in the culture vessels was noted and samples were degassed at a rate of 3-5 atm^{-1} min. Generally culture vessels with starting headspace pressures of >100 atm lost 5-25 atm of pressure over the course of a multiple-week incubation with greater losses associated with longer incubations. Unless specifically noted, all data reported herein for incubations under supercritical CO_2 maintained headspace pressure such that the final pressure before sample degassing was greater than the supercritical CO_2 pressure threshold of 71 atm. Following degassing cultures were transferred to an anaerobic tent containing a 5% CO_2/5% H_2 atmosphere where samples were held at atmospheric pressure during culture processing and until subsequent passage.

Propagation of enrichments for strain cultivation. Following incubation the contents of enrichment vessels were analyzed for cell growth and microbial community composition upon initial degassing, and again prior to initiation of subsequent passages.

The enrichment cultures from the Frio filters were propagated through serial passages by diluting 10% v/v of the previous culture in fresh growth media under a 95% CO_2/5%H_2 atmosphere, pressurization to 120 atm with CO_2, followed by incubation at 37°C. Frio passages 1-3 were incubated for 15 days, while passage 4 incubated for 60 days; subsequent passages were incubated for 9 to 15 days (Table 1). Samples were taken from each passage for microscopic enumeration and live/dead staining (Invitrogen).

DNA Extraction. DNA extraction from scCO_2-tolerant microbial enrichments derived from the Frio sample was performed using a protocol modified from Lessard et al. for gram-positive bacteria (2004). Briefly 1 mL of cells were concentrated into a pellet by centrifugation (5 min at 6500 x g), removal of supernatant, and frozen overnight at -80° C. Thawed cell pellets were resuspended in 250 μL TE buffer containing fresh lysozyme (10 mg mL^{-1}) and incubated with shaking at 37° C for 6-8 hours. Next, 50 μL of buffer containing 0.5 M EDTA, 50 μL 10% SDS, 50 μL NaCl and 15 μL proteinase K was added to the cell slurry and incubated at 37°C overnight. The following day 50 μL of a sodium perchlorate solution (1 mg/mL) was added to cell slurry, mixed gently, and genomic DNA was extract with one volume of phenol:chloroform:isoamyl alcohol (25:24:1); followed by a 400 μL chloroform:isoamyl alcohol (24:1) extraction. DNA was precipitated in 600 μL isopropanol, washed with 70% ethanol, and resuspended in 100 μL water.

Amplification, cloning, and sequencing of 16S rRNA genes from scCO_2 microbial enrichments. Amplification of 16S rRNA genes from bacterial enrichment cultures was performed using PCR with universal eubacterial primers 515F 5'- GTG CCA GCM GCC GCG GTA A- 3' and 1406R 5'-ACG GGC GGT GWG TRC AA- 3' (Frio passages 1, 2 and 7) and using 27F 5'- AGA GTT TGA TCM TGG CTC AG- 3' and 1492R 5'-TAC GGY TAC CTT GTT ACG ACT T- 3' (Frio passage 9). PCR mixtures (20 μL per reaction) contained 25 to 75 ng of genomic DNA, 1X 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 were an initial 3 minutes at 95°C followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 90 sec; followed by a final extension time of 5 min. PCRs always included a negative and a positive control.

Amplified 16S rRNA gene fragments from Frio samples were gel purified (Qiagen gel extraction kit) and ligated into the pJET1.2 vector (Fermentas) according to manufacture's protocol. Ligation products were transformed into *E. coli* DH5α or *E. coli* Top10 cells and clones were selected for sequencing. For the Frio enrichment sequencing reactions were prepared using Big Dye Terminator 3.1 according to the manufacturer's instructions and sequencing was performed on an ABI 3130 platform. Removal of vector and primer sequences, and manual editing and clustering of operational taxonomic units (OTUs) at 99% nucleotide identity was 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.

Phylogenetic analysis of microbial communities in scCO_2-tolerant microbial enrichments. 16S rRNA gene sequences obtained from passages of the supercritical CO_2 enrichments from the Frio site were aligned using MUSCLE (Edgar, 2004) and maximum likelihood phylogenies were reconstructed using the PhyML package (Guindon and Gascuel, 2003). Stability of the groupings was estimated by bootstrapping on 100 trees using the same package. Trees were visualized using FigTree software, version 1.3.1 (Rambaut, 2008).

Growth dynamics at ambient pressure. Growth curves for MIT0214 at ambient atmospheric pressure were conducted in serum bottles with degassed LB media (Figs. 11-12). The reductant Na_2S (0.25 g/L) was added to purge any residual oxygen after degassing and the redox dye resazurin was added to media to visualize whether oxygen was present. Triplicate cultures were incubated shaking at 37 °C and sampled with sterile needles and syringes through the butyl rubber stoppers. Prior to sampling, needles were flushed 3 times with the headspace gas (either N_2 or CO_2). Cultures were diluted and plated for viability counts. To quantify spore production aliquots were heated to 80 °C for 10 min, prior to plating.

Preparation of spores. Spores were prepared by growing cells from colony purified samples in LB media for 24 hours, followed by a 1:50 dilution into Modified G Medium (Kim and Goepfert. 1974). Modified G Medium contains the following (in g/L): yeast extract 2.0, $CaCl_2.2H_2O$, 0.025, K_2HPO_4 0.5, $MgSO_4.7H_20$ 0.2, $MnSO_4.4H_2O$ 0.05, $ZnSO_47H_2O$ 0.005, $CuSO_4.5H_2O$ 0.005, $FeSO_4.7H_2O$ 0.0005, $(NH_4)_2SO_4$ 2.0, adjusted to pH 7.1 after autoclaving. Cells were incubated shaking for 72 hours to allow formation of spores, and then centrifuged for 15 minutes at 4000 X g. Samples were resuspended and centrifuged 5 times in a 1 mM Phosphate wash buffer with 0.01% Tween20 to prevent aggregation of spores. Samples were heat-killed at 80 °C for 10 minutes to kill remaining vegetative cells. Spores were stored in the wash buffer at 4 °C until further use.

Measuring growth and survival in anaerobic CO_2 and N_2 atmospheres. Experiments testing for the growth or survival of isolated strains under high pressure conditions were conducted in 316 stainless steel pressure chambers (Figs. 6, 7, 14-16), or using a High Pressure Equipment Company (HIP) 1 liter pressure vessel (Fig. 8). Autoclaved culture media added to serum bottles was degassed with a stream of 100% CO_2 or 100% N_2 gas for 30 minutes prior to pressurization. The redox indicator resazurin was added to culture media at 0.001 g/L to verify anaerobic conditions. Culture inocula were dilutions of previous cultures or prepared spores (Figs. 7 to 16). Growth and survival for all strains were measured as a change between initial and final cell densities. Cells were enumerated by a combination of viability counts (determination of colony forming units (CFU) on LB agar), microscopic enumeration of DAPI or SYTO9 stained cells, or optical density measurement (at 600 nm) of culture turbidity.

Comparison of *Bacillus* strain growth under standard conditions and high- and low-pressure CO_2. In order to determine whether Bacilli type strains, that were closely related to those in our scCO_2-tolerant enrichment clone libraries, could survive under a similar range of incubation conditions, cultures of the type strains of *Bacillus subtilis*

PY79, *Bacillus mojavensis* JF-2 (ATCC 39307), *Bacillus cereus* (ATCC 14579) (Tables 5 and 6) were grown overnight (to stationary phase) and diluted approximately 100-fold to 1 x 10^7 cells ml^{-1} in fresh growth media. As a control the $scCO_2$ tolerant biomass from passage 9 (consisting of the isolate MIT0214) was diluted approximately 100-fold to 1 x 10^5 cells in fresh growth media. Type strains and the $scCO_2$ tolerant strain MIT0214 were then incubated under each of three conditions: (1) an ambient atmosphere (P ~ 1 atm), (2) a 95%CO_2/5%H_2 headspace (P ~ 1 atm), or (3) 100% CO_2 headspace (P = 120 atm). Triplicate cultures were incubated for 6 hours or 7 days after which time cell survival was assessed by comparison of initial and post-incubation total cell counts (DAPI), membrane integrity (Invitrogen Live/Dead stain), quantification of spore formation (1), and determination of colony forming units (CFU) on LB agar plates.

Enumeration of cell density. To quantify cell numbers we used several approaches. Viability counts (CFU plating) were carried out using Luria Broth Agar. For direct imaging of cells aliquots of samples were stained with 4',6-diamidino-2-phenylindole (DAPI) or SYTO9 (Invitrogen) for 10 minutes in the dark. 500 µL to 1 mL of sample was then filtered onto 25 mm, 0.2 µm pore size, black, polycarbonate filters (Nucleopore), followed by 2 washes with 1 mL of Phosphate Buffered Saline (PBS). PBS was incubated on the filtered sample for 1 minute to help wash off excess dye. Filters were laid on slides under microscope immersion oil with a cover slip (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 x 10 microscope grid) by the dilution factor and then by 3.46 x 10^4 (as one 10 x 10 grid at 1000X magnification corresponds to 1/3.46 x 10^4 of a 25 mm filter). Samples were visualized on a Zeiss Axioplan fluorescent microscope. Images were captured on a Nikon D100 camera using the NKRemote live imaging software.

Electron microscopy. Cells grown from the archived stock of the ninth-passage were fixed in 2.5% gluteraldehyde, 3% paraformaldehyde with 5% sucrose in 0.1M sodium cacodylate buffer (pH 7.4), pelletted, and post fixed in 1% OsO_4 in veronal-acetate buffer. The cell pellet was stained overnight with 0.5% uranyl acetate in veronal-acetate buffer (pH 6.0), then dehydrated and embedded in Spurrs resin. Sections were cut on a Reichert Ultracut E microtome with a Diatome diamond knife at a thickness setting of 50 nm, stained with uranyl acetate, and lead citrate. The sections were examined using a FEI Tecnai spirit at 80KV and photographed with an AMT CCD camera.

Chemical analysis of culture media (pH). The pH of the ambient and CO_2 saturated media was measured at 22°C using an Orion model 520A pH meter. The pH of media under a supercritical CO_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 media (Table 3) under a CO_2 or N_2 atmosphere and as a function of temperature and pressure. Thermodynamic data was obtained from the Lawrence Livermore National Library (LLNL) database.

Molecular verification of MIT0214. To determine whether strain MIT0214 was associated with the initial enrichment and early passages of the MIT0214 enrichment, a series of PCR assays were developed based on conserved *B. cereus* sequences and unique regions of the MIT0214 genome sequence (genome sequencing described later). This assay was used to screen enrichment genomic DNA preparations from passage 1, 2, 7, and 9 as well as DNA prepared from aerobic cultures grown from archived glycerol stocks of passages 4 through 9 (Table 1). The initial PCR assays developed utilized (1) a *B. cereus* group specific primer with a 16S universal primer and (2) GroEL primers unique to the *B. cereus* group and using three genes unique to MIT0214 and no other *B. cereus* strains. These MIT0214 specific PCR assays used genes for a Mosquitocidal toxin, Salivaricin A and SK12-helicase. The PCR assays were validated with the *B. cereus* ATCC14579 type strain and aerobically grown MIT0214.

Quantification of Extracellular Polysaccharides (EPS). EPS was quantified via the phenol-sulfuric acid method, which quantifies total sugars (Daniels, 1994; Michel, 2009). Cell cultures were grown overnight in LB or Peptone media and then pelleted by centrifugation for 5 minutes at 10,000 x g, followed by aspiration of the supernatant for analysis of free polysaccharides. Cell pellets were resuspended in a 1µM Zwittergent, 1 mM EGTA, pH 2.5 solution and incubated for 45 minutes on the bench, followed by vortexing for 15 minutes. 100 µL of supernatant and cell suspension were each mixed rapidly with 100 µL of 5% phenol (w/v) solution, followed by immediate addition of 500 µL of concentrated sulfuric acid. Samples were incubated in a water bath for 25 minutes at ambient temperature. 100 µL of each sample was added to a polystyrene 96-well plate and the absorbance at 488 nm was read in a UV-Vis spectrophotometer. Cell-bound or free EPS was calculated by using a glucose standard curve of (0, 0.01, 0.05, 0.1, 0.5, 2.0 g/L).

Quantification of Biosurfactant production. Biosurfactant production was quantified via an emulsion layer assay (Kim et al., 2000). Bacterial strains (*B. mojavensis* JF2, *B. cereus* ATCC 14579 and MIT0214) were grown at 37 °C for 48 hours. The optical densities at 600 nm were measured, and cultures were adjusted to 5 cell densities (10^5, 10^6, 10^7, 10^8, and 10^9 cells/mL), based on conversion of OD readings to cell counts (i.e. OD 600nm of 1.0 = 10^9 cells/mL). Cell cultures and a LB media negative control were centrifuged at 4000 rpm for 30 min, followed by aspiration of the supernatant and further centrifugation at 4000 rpm for 15 min. The cell supernatant was filtered through a 0.2 micrometer filter and the emulsion assay was performed. 1 mL of cell free supernatant and 0.5 mL of n-hexdecane were each added to a 6 mL borosilicate glass test tube and vortexed at 2000 rpm for 1 min. The solution was allowed to stand and the height of any resulting emulsion layer was measured after 2 hours and again at 24 hours.

Genome Sequencing. MIT0214 and OT1 genomes were sequenced via the Illumina HiSeq 2000 platform. 2.24×10^6 reads of MIT0214 and 1.38×10^7 reads of OT1 were obtained and trimmed to increase quality (10 bases off the start and 20 bases off the end). Both strains were assembled *De novo* with CLC Genomic Workbench followed by open reading frame calling and functional annotation via the RAST (Rapid Annotation using Subsystem Technology) server (Aziz et al., 2008).

Comparative Genomic Analysis of Bacillus strain MIT0214. Comparison between MIT0214 and closely related *Bacillus* genomes (*B. cereus* ATCC 14579 and *B. cereus* ATCC 10987) and the subsurface extremophilic isolate *B. cereus* Q1 were performed using the RAST sequence based comparison tool to identify shared genes between organisms. The *B. cereus* Q1 strain was selected as it was isolated from a deep subsurface environment, and shared genes with MIT0214 may provide insight into adaptations for subsurface ecosystems. MIT0214's unique genes were visualized with CLC Genomic Workbench using RAST annotations. To search for evidence of HGT, MIT0214 contigs were screened for regions of GC content skew and compared to plasmids from closely related strains (i.e. plasmids pXO1, pXO2, pBc10987, pBc239, pBc53 and pBclin15). Contigs with BLAST hits greater than 100 bases to plasmids were examined in further detail and visualized with CLC Genomic Workbench Software.

5. Results and Discussion

A. Enrichment and characterization of biomass from Frio 2 formation water filters

The Frio experiment in Texas was undertaken to characterize the transport and fate of injected CO_2 during geologic sequestration (Hovorka, 2006). During the Frio 2 project (2006) 1,600 tons of CO_2 were injected into a 6 m thick high-permeability sandstone (Frio saline aquifer, Liberty County Texas) at a depth 1,657 m below ground surface and samples for geochemical and biological analysis were collected at an observation well 30 m down-gradient (Hovorka, 2006). On-site characterization indicated a formation pressure of 146 atm, temperature 53˚C, and 93 g/L of total dissolved solids (primarily Ca^{2+}, Na^{+}, Cl^{-}) (Hovorka, 2006).

Biomass observed in initial enrichments. Supercritical CO_2-tolerant enrichment cultures were started from sterile scrapings of filter residues collected during the Frio 2 experiment. Residues were suspended in GYP growth media and incubated in high-pressure growth chambers containing supercritical CO_2 (120 to 136 atm at 37˚C). Positive growth of a $scCO_2$-enrichment culture from the pre-injection filter sample was indicated by an increase in the turbidity of culture media and confirmed by the presence of cells that stained with nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI) and the Live/Dead membrane integrity probe (Invitrogen) (Table 1, Fig. 1 A, B). Samples from post-injection filters as well as surface-samples of soil and water from the MIT vicinity were negative for growth under $scCO_2$. Microscopy and extraction of total nucleic acids confirmed biomass generation after successive rounds of dilution and growth (Table 1).

Diversity of initial enrichments and passages. The composition of the microbial assemblage enriched under a supercritical CO_2 atmosphere was determined by analysis of cloned 16S rRNA gene sequences. A total of 131 non-chimeric Bacterial 16S rRNA gene sequences (*E. coli* positions 515 – 1406) were obtained by sequencing approximately 48 clones each from the initial enrichment, and the first, seventh and ninth serial passage of the culture (Table 1). Analysis of these clone libraries revealed 15, 26, 1, and 1 sequence types respectively and the chao1 statistical estimator of species richness indicated decreasing bacterial diversity associated with increasing passage (Table 2). The libraries from the first and second passage revealed a mixed community of microbes dominated by members of the Bacillus genera (Fig. 2). The majority of clones from the seventh and ninth passage corresponded to the genus *Bacillus* (97% and 100%, respectively). The microbial assemblage from passages 7 and 9 were dominated by a single *Bacillus* ribotype sharing 99.8% 16S rRNA sequence identity (1463/1465 nucleotides) with *B. cereus* (ATCC 14579) and >97% 16S rRNA sequence identity with ubiquitously distributed environmental and clinical strains i.e. *B. mycoides*, *B. thuringiensis,* and *B. anthracis*. *B. cereus* and closely related strains are widely distributed in soil and sediment environments and the species *B. cereus* has been isolated from a wide diversity of environments including sites characterized by heavy metals, hydrocarbons and/or hypersaline conditions (Xiong, et al. 2009; Singh, et al. 2010; Pandey, et al. 2011; Sriram, et al. 2011).

Detection and Correction of Contamination in CO_2 system. Following identification of MIT0214 as the sole sequence type in passage 9 physiological and genomic characterization of the strain was undertaken to understand the range of conditions under which it could grow (pH, temperature, salinity). However, a systematic flaw in our lab's protocol for gassing/degassing samples and failure to properly follow the standard operating procedure for microscopic examination of no-inoculation controls was uncovered which cast severe doubt on the validity of this earlier data including growth rates under $scCO_2$ and other physiological data reported in quarterly progress reports from project year #2. The source of the contamination was found to be MIT0214 spores in CO_2 tubing for pressurization and depressurization that resulted in systematic contamination of reactors, including negative controls (i.e. un-inoculated reactors). These contamination issues were ameliorated by adding a step for thorough washing of all CO_2 tubing with 10% Bleach, MilliQ water and 100% ethanol before and after every pressurization or depressurization cycle. Implementation of this protocol eliminated contamination in the negative controls to below the limit of detection for microscopic enumeration (i.e. <1 cell per 15 fields or $1x10^3$ cells/ml).

Observation of contamination raised the question of the validity of the diversity observed in the initial enrichments and first passage. However, we have confirmed that these enrichments were incubated in parallel with negative controls that were not contaminated. In addition, two of three samples screened from the Frio 2 site (representing post injection samples) and various soil environments around MIT screened for $scCO_2$ tolerant growth were negative. Thus, we conclude that the early enrichment and initial passage were not compromised by the late-rising contamination issue.

Screens of enrichment MIT0212 and early passages for MIT0214 markers. As the ribotype corresponding to strain MIT0214 was not observed in the clone libraries from the initial enrichment, or the first passage, we developed and employed PCR assays specific for MIT0214 genomic biomarkers to examine whether the strain was part of the initial enrichment community. We screened archived glycerol stocks of enrichment biomass that was preserved from passages 4 to 7 and archived genomic DNA preparations from the initial enrichment and passages 1 to 9 for MIT0214 markers. Aerobically-grown biomass from glycerol stocks of passages 4 through 7 was confirmed to be strain MIT0214. Due to insufficient or possibly degraded genomic DNA PCR assays to detect MIT0214 archived DNA samples were inconclusive (i.e. the markers were not detected even in DNA extractions obtained from passage 7 where MIT0214 was confirmed to be the dominant taxa from earlier 16S rRNA clone libraries and from aerobic re-growth experiments.) However, one faint positive band for the Mosquitocidal toxin gene was detected in Passage #2 which lends some weak support to MIT0214 as a potential member of the initial enrichment and early passages. While at this stage, we cannot confirm that strain MIT0214 was part of the initial enrichment, its selection by the supercritical CO_2 based cultivation system and unique growth properties (i.e. tolerance and growth under $scCO_2$) poise it as a useful cultivatable model for microbial processes under supercritical CO_2.

B. Physiological Characterization of *B. cereus* strain MIT0214.

Characterization of growth optima for Salinity, Temperature and pH under aerobic conditions. We have observed that strain MIT0214 is an aerobic spore former (Fig. 1 C, E, G-I) and a facultative anaerobe growing in a reduced CO_2 atmosphere (98% CO_2/2% H_2) or a reduced nitrogen atmosphere (100% N_2 amended with NaS_2). Physiological characterization under aerobic conditions (1 atm pressure, ambient atmosphere headspace incubated at 37C) has revealed MIT0214 is able to grow at salinities from 0.01 to 40 g/L NaCl in glucose yeast peptone (GYP) media (Fig. 3) and in LB media adjusted with hydrochloric acid to pH values as low as 5.6 (Fig. 4) and appears to have a temperature optimum near 30˚C with growth observed from 21˚C to 45 ˚C but not at 12˚C or 55˚C after 20 hours (Fig. 5).

Tolerance of aerobically-grown MIT0214 to variable pCO_2. Overnight aerobically-grown cultures of MIT0214 and *B. cereus* 14579 were tested for tolerance of different partial pressures of CO_2 i.e. aerobic atmospheric (pCO_2~0.04 atm), 95-98% CO_2/5-2%H_2 at 1 atm and extraction grade CO_2 95-98% CO_2/5-2%He at 100 atm. Cultures were sealed in anaerobic culture vessels (either glass hungate tubes for ambient pressure or stainless steel pressure chambers for elevated pressure) with headspace composition of either 1 atm CO_2, 100 atm CO_2 or 1 atm ambient atmosphere), and incubated for 6 hours at 37 °C, followed by CFU plating to determine viability, (Fig. 6A). No change in viability was observed for control cultures or cultures exposed to 1 atm CO_2 headspace. In contrast, 4-8 log orders of reduction in viability was observed for both strains exposed to 100 atm CO_2 (supercritical headspace) with increased survival for MIT0214 cells relative to ATCC14579 cells exposed to $scCO_2$ (Fig. 6A). To test whether the increased relative survival of MIT0214 cells during $scCO_2$ treatment was due to a higher proportion of spores in MIT0214 relative to ATCC14579 an aliquot of the initial inoculum from each culture was exposed to a heat treatment (80 °C for 10 minutes) in order to kill all vegetative cells, but leave spores viable. The number of cells surviving the heat-kill treatment agrees with the number of cells surviving the scCO2 treatment, and supports a model of spore-survival but vegetative cell killing during short-durations of $scCO_2$ exposure (6 hr) (Fig. 6B). We concluded that vegetative cells of MIT0214 grown under aerobic ambient conditions are killed by $scCO_2$, however the spores from these strains suspended in liquid media remained viable after $scCO_2$ exposure.

In order to test longer-term viability of spores under $scCO_2$, a stock of spores was prepared using the Modified G Medium protocol (Kim & Goepfert, 1974) and maintained at 4 °C in buffer. Spores were exposed to 1 atm CO_2 and 100 atm CO_2 at 37 °C, with triplicate samples plated at 1 day, 7 days and 14 days, (Fig. 7). The CFU counts indicated that spores of both strains do not show loss in viability at up to two-week time scales. These spore stocks were used to carry out additional experiments that demonstrate germination and growth of spores under $scCO_2$ (see section *Characterization of growth of MIT0214 under $scCO_2$).*

Survival/inactivation of MIT0214 spores upon direct and indirect exposure to CO_2. In previous experiments, all spores and cultures have been suspended in aqueous growth media, and thus indirectly exposed to $scCO_2$, which exists as a buoyant phase above the

aqueous phase, and mimics conditions at the interface between $scCO_2$ and formation water during geologic CO_2 sequestration. However, spores in geologic formations may experience direct exposure to $scCO_2$ which would represent a more extreme and desiccating environment than at the $scCO_2$ aqueous interface. Survival of MIT0214 spores during exposure to dry CO_2 seemed likely due to this strain's ability to contaminate the tubing used for $scCO_2$ delivery to our culture reactors. To test whether *Bacilli* spores could tolerate direct contact with dry $scCO_2$ we exposed prepared spores of MIT0214, *B. subtilis* and the oil-field isolate *B. mojavensis* JF2 to $scCO_2$ indirectly in an aqueous phase buffer, as well as directly by drying spores overnight at 70˚C and then exposing them $scCO_2$ (Fig. 8 A and B, respectively). After 2 weeks spores incubated with the aqueous phase again showed no significant change in viability relative to initial conditions or ambient controls ($p>0.05$ for all three strains). In contrast, when spores were directly exposed to $scCO_2$, there was a significant decrease in spore viability relative to ambient-incubated controls ($p<0.05$) for *B. mojavensis* and MIT0214 where 14-24% of initial spores remained viable after a 2-weeks under $scCO_2$. A parallel decrease did not reach statistical significance for *B. subtilis* ($p=0.08$). Whether this decrease is time-dependent remains to be determined however, observation of <1 order of magnitude decrease in spore survival over a 2 week interval suggests that spores in subsurface environments will likely be able to tolerate at least brief periods of exposure to pure-phase $scCO_2$.

Characterization of anaerobic growth of MIT0214 under ambient pressure. To determine whether strain MIT0214 was capable of anaerobic growth under CO_2 or N_2, $1x10^5$ spores were used to inoculate LB media with and without nitrate as a potential alternate electron acceptor. Growth in shaking glass hungate tubes at 37 °C occurred under both anaerobic headspaces, with enhanced biomass when supplemented with nitrate (Fig. 9A). Anaerobic growth by MIT0214 under both N_2 and CO_2 headspace, in various media formulations (LB or MS with or without nitrate) was confirmed with a follow-up experiment (Fig. 10).

Growth dynamics for MIT0214 under ambient, 1 atm N_2 and 1 atm CO_2 conditions were characterized over 16-17 days (Figs. 11, 12A and B). The characteristic growth curve for an ambient headspace is rapid growth over the first 7 hours with no apparent lag phase, concurrent with spore numbers dropping to below detection due to spore germination. After an exponential growth phase sporulation begins (day 2) followed by relatively little loss in total viable cells over 2 weeks. In contrast, anaerobic cultures under N_2 and CO_2 headspace show a slower initial growth rate, lower maximum cell density, and a decline of viability with time where final viable cell counts (cfu/ml) are at or below that of the initial inoculum. This "decline phase" is likely due to accumulation of toxic end-products of fermentation as is commonly observed during fermentative growth. Optical density (600nm) confirmed that biomass density remained near peak levels throughout the experiment (Fig.12B). CO_2 cultures but not N_2 cultures, revealed a 24-hour lag before growth and this is consistent with previously observed dynamics of growth under CO_2 (Enfors and Molin, 1980). While this apparent lag was not observed in the second growth curve experiment, these early dynamics were confounded by a brief intrusion of oxidative potential as evidenced by emergence of a slight pink color in the resazurin dye of sealed cultures from time point 0 to 7 hours in N_2

and CO_2 samples. However the redox indicator returned to clear for the rest of the experiment indicating true anoxic conditions. Follow up work to clarify the nature and cause of this early phase lag is planned.

Germination and sporulation rates appeared to be strongly effected by headspace composition. During the initial growth period (0 to 99 hours for aerobic cultures) spores from the initial inoculum dropped to below-detection as the spores germinated in the aerobic culture – based on a detection limit of 10 cfu/ml and an initial spore density of $4x10^4$ cfu/ml this indicates that at least 99.98% of spores germinated under aerobic conditions – which is typical for aerobic Bacilli (Roth and Lively, 1956). In the N_2 incubations a 100-fold higher proportion of spores did not germinate (i.e. 99% germinated while 1% remained dormant), while 49% of spores in the CO_2 incubated culture germinated – which may reflect an over-estimation of the germination rate under CO_2 due to the brief intrusion of oxidative potential described earlier. This decreased rate of germination is characteristic of *Bacillus cereus*, which has previously been documented to have a germination rate of 10-30% under CO_2 (Enfors and Molin, 1978). In both anaerobic experiments the sporulation stage evidenced by an increase in spore density in aerobic cultures around day 4 was not observed over the 16 to 17 day experiment.

Pressure optima of MIT0214. To determine whether MIT0214 could grow optimally under elevated pressures of N_2 or CO_2 spores were diluted into growth media to $1x10^5$ cells /ml and subjected to varied pressures of N_2 or CO_2 headspace (from 1 to 100 atm initial pressure corresponding to 1 to 68 atm final pressure) in stainless steel pressure chambers for 1 week. As previous experiments conducted at 1 atm had been performed in glass hungate tubes we confirmed that similar growth was observed in 1 week in stainless steel vessels (Fig. 13). Pressure chambers were depressurized and change in cell biomass was quantified through viability counts (Fig. 14 A, B and Fig. 15 A) and OD (Fig. 14 C, D). After 1 week growth at 1 atm was consistent among triplicate incubations with increases in turbidity while viable cell counts tended to be similar to starting conditions, likely due to inhibitory effects of fermentation endproducts as suggested by the growth dynamics (Figs. 11, 12). In contrast to the 1 atm control samples, growth at all pressures above 1 atm exhibited high variability within triplicates (i.e. from 0 to 2 of the triplicates showing increased turbidity). The maximum final pressures of N_2 headspace associated with growth during the 1-week incubations were 27 atm and 26 atm from two separate experiments (Fig. 14) and the highest pressure of CO_2 examined (9-10 atm) also supported growth after 1 week (Fig. 15) extending the known range of *B. cereus* pressure tolerance to growth under CO_2 from 3 atm to 9 atm (Enfors and Molin, 1980).

Characterization of growth of MIT0214 under $scCO_2$. We had previously observed that aerobically-grown cultures of MIT0214 were rapidly inactivated by $scCO_2$ (Fig. 6 A and B). To determine whether MIT0214 spores incubated under $scCO_2$ could germinate and grow, mimicking conditions associated with the initial enrichment, we added spores to GYP growth media at a starting concentration of $2.5x10^6$ cells /ml and subjected them to a $scCO_2$ headspace in stainless steel pressure chambers for 26 days (Fig. 16A). Pressure chambers were depressurized and change in cell biomass was quantified through viable and direct counts. After 26 days 2 of 3 replicates revealed an increase in biomass relative

to starting spore density with observed cell densities of $2.2x10^7$ and $4.8x10^7$ cells/ml. Parallel viable cell counts did not show a significant increase consistent with dropping culture viability during late stages of fermentation as observed previously in growth curves under 1 atm CO_2 (Figs. 11 and 12). The ability of MIT0214 to generate biomass under $scCO_2$ was confirmed using replicate incubations with inocula consisting of either a passage of one of the samples that grew in the first experiment diluted to an initial density of $4.2x10^4$ cells/ml (Fig. 16B) or from spores diluted to an initial density of $2.3x10^5$ cells/ml (Fig. 16C). After 30 days 3 of 3 replicates from the passage inoculum and 3 of 4 replicates from the spore inoculum demonstrated growth with greater than 100-fold increase in biomass where average cell densities for positive growth were $4.6x10^7$ cells/ml ± $3.3x10^7$ (for passage) and $9.9x10^7$ cells/ml ± $7.3x10^7$ (for spores). Culures that were positive for growth were associated with a majority of rod-shaped vegetative cells while no rod shaped cells were observed in the single culture from the spore inoculum that did not grow. Microscopic enumeration of no-inoculation controls incubated in parallel with both experiments had no detectable biomass (i.e. $<1 \times 10^3$ cells/ml). Additional work is currently in progress to characterize the growth dynamics of MIT0214 under $scCO_2$.

EPS and Biosurfactant production. As cells in biofilms have been shown to have increased resistance to $scCO_2$ (Mitchell et al., 2008) we are seeking to determine whether $scCO_2$ tolerant microorganisms produce EPS or surfactants to minimize stress from $scCO_2$. EPS is a significant component of biofilms, and biosurfactant production in *Bacillus cereus* has been positively associated with biofilm formation (Hsueh, et al. 2006). We have validated an assay for quantification of EPS in MIT0214 via the phenol-sulfuric acid method (Michel et al., 2009; Masuko et al., 2005), which quantifies total sugars that are cell-bound or dissolved in the growth media. We have used this approach to characterize EPS levels in aerobically grown cultures incubated in LB media and a modified media based on GYP containing only peptone and salts to reduce background due to sugar monomers in the media-only control (Figs. 17 A-C). Under ambient aerobic conditions MIT0214 is a net consumer of polysaccharides, and net EPS production isn't evident. We have also used an emulsion assay to characterize biosurfactant activity in MIT0214 culture supernatants, finding significantly less biosurfactant activity associated with aerobic cultures of MIT0214 than associated with the known biosurfactant producer *B. mojavensis* JF-2 (formerly *B. licheniformis*) (Lin, et al. 1993) (Fig. 18). In future experiments we will examine whether CO_2 and $scCO_2$ incubated cultures of MIT0214 produce more free EPS and biosurfactant activity than high-pressure N_2 and ambient-incubated controls.

C. MIT0214 Genome Sequencing and Analysis

Sequencing, Assembly, and Annotation. Strain MIT0214 was subjected to whole genome sequencing at the MIT BioMicroCenter using Illumina GA2 sequencer. A total of 1.94×10^6 high quality 72 base reads were generated. Comparing the total base reads to the average size of known Bacilli genomes (~5.5 megabases) indicated that coverage was greater than 25x for the complete genome. Reads were assembled into regions of continuous sequence (contigs) using the *de novo* assembly tools in CLC Genomic

Workbench, and targeted Sanger sequencing to reduce gaps in the draft assembly. Using this approach we have determined that the genome of strain MIT0214 is approximately 5,620,449 base pairs with 35% GC-content. We have annotated the draft assembly of the MIT0214 genome by the Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al., 2008) which revealed 5640 coding sequences where 3888 of the coding sequences were assigned putative functions and 1752 were annotated as hypothetical proteins.

Comparison to closely related strains. MIT0214 shows a high proportion of similar genome features with other genome- sequenced *B. cereus* group type strains (*B. cereus* ATCC 14579, *B. cereus* ATCC 10987, *B. cereus* Q1 and *B. anthracis* Ames). These strains share 89 to 91% of genes with the MIT0214 genome with 93.3 to 97.8% nucleotide identity among shared genes. MIT0214 and close relatives also had similar GC content, genome size, and total gene number (Table 5). Since genes unique to MIT0214 may shed light on its ecology relative to other *B. cereus* strains we examined the gene content of genomic regions unique to MIT0214 (Fig. 3). MIT0214 contains 310 unique genes when compared to the three most closely related genome-sequenced strains (Fig. 19) with a core or "shared" genome of 4763 genes. Examples of genome regions bearing novel content include a region containing CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) sequences that may mediate phage resistance. In addition, several unique Choline binding proteins were observed and have been implicated in cell adhesion and hydrophobicity of the cell surface in other systems (Swiatlo, 2002) (Fig. 20). Finally, unique hypothetical proteins were observed in proximity to a pXO1 protein, possibly indicating novel gene content transferred through plasmid vectors to MIT0214. The pXO1 plasmid is widely distributed among *B. cereus* strains, however MIT0214 lacks certain elements of pXO1 such as the toxin genes found in *B. anthracis*. Genome regions with a GC% differing from the genome average GC (34.9%) may have originated from lateral transfer from a different species (Fig. 20). The genome region containing the choline binding protein GC content is 30.1%, while the region bearing both plasmid-associated and hypothetical genes have a GC content of 29.3% which raises the possibility of potential acquisition of these genes via horizontal gene transfer.

The genome sequenced strain *B. cereus* Q1 is an oil-well isolate from China (Xiong, et al, 2009) and as such may share genomic adaptations to the deep subsurface with *B. cereus* MIT0214 if their isolation origins reflect a natural history in the deep biosphere. We compared the SEED subsystem distribution found in MIT0214 (Fig. 21) and Q1 with six surface isolates (*B. cereus* ATCC 14579, ATCC 10987, B4264, G9241, and *B. anthracis* Ames and Sterne), and found enrichment in three subsystems in MIT0214 and Q1 relative to the known surface-isolates (Table 5). These subsystems are 1. cell wall and capsule, 2. stress response, and 3. metabolism of aromatic compounds. Subsystems where these two strains were depleted relative to surface isolates are: 1. cofactors, vitamins, prosthetic groups, pigments, 2. potassium metabolism, 3. miscellaneous, and 4. RNA metabolism. Comparison of the gene content between MIT0214 and Q1 reveals 46 shared genes that are not found in closely related *B. cereus* strains ATCC 14579 and ATCC 10987 (Table 6). While the majority of these genes encode hypothetical proteins, one interesting protein is a choline binding protein, a

protein involved in adhesion and membrane hydrophobicity. In Fig. 22, panel B, several unique choline binding proteins were identified to be part of a region that may have been horizontally transferred.

While a small portion of MIT0214's genome reveals unique content that may have been horizontally transferred to MIT0214 as in Fig. 21, we also have identified a potential pool of shared genes based on similarity to plasmids in other closely related *Bacillus* strains (Fig. 22). 1.8% of the MIT0214 genome matches plasmid-borne genes. These genes cluster on several different contigs, and frequently match plasmids (and strains) such as pXO1 (from *B. antrhacis* Ames), pBc10987 (from *B. cereus* ATCC 10987), and pBc239 (from *B. cereus* Q1). The genes in Fig. 22 annotate as cell wall / membrane proteins and type II secretion (A), pXO1 proteins and DNA transfer and regulation (B), pXO1 proteins (C), and phage and DNA transcription (D). These annotations of these genes, their similarity to plasmid sequences, and their differing GC content (in some cases), indicate that strain *B. cereus* MIT0214 has had access to a shared pool of genetic elements with other closely related strains over its natural history.

6. Conclusions

We have shown that bacterial biomass generation (i.e. growth) under a supercritical CO_2 atmosphere is possible using enrichment cultures started from samples collected during the Frio 2 project and an isolated strain. This observation of microbial growth under $scCO_2$ has two fundamental implications for geological carbon dioxide sequestration (GCS): (1) Microbial activity is possible, and perhaps likely, at the CO_2 plume water interface, even in areas previously exposed to pure-phase $scCO_2$. (2) Engineering biofilm barriers to grow *in situ* at the CO_2-plume + brine interface is not precluded by the previously documented sterilizing properties of supercritical CO_2.

These two implications for GCS are supported by a series of experiments performed over the duration of this project. First, we observed that the initial enrichment of a sample from the Frio 2 pilot experiment under supercritical CO_2 was dominated by DNA sequences (16S rRNA gene clones) corresponding to strains of the genus *Bacillus*. Observation of Bacilli in the initial enrichments is not surprising in light of their spore-forming abilities and thus resistance to harsh environmental conditions such as $scCO_2$. An isolated *Bacillus* strain designated MIT0214 emerged from additional passage of the enrichment culture. Although not recovered in community analysis of the initial enrichment, biomarkers of the strain recovered in early passages of the sample suggest it may have been part of the initial enrichment community. We have shown that strain *Bacillus* MIT0214 is most closely related to *B. cereus* – a widely distributed bacterium that has been isolated from a wide diversity of environments including sites characterized by heavy metals, hydrocarbons and/or hypersaline conditions (Xiong, et al. 2009; Singh, et al. 2010; Pandey, et al. 2011; Sriram, et al. 2011).

To examine whether strain MIT0214 could serve as a model for microbial activity under a supercritical CO_2 atmosphere we subjected the strain to a battery of physiological characterizations. We determined that MIT0214 is an aerobic spore-former capable of facultative anaerobic growth under both reducing N_2 and CO_2 atmospheres by fermentation and possibly anaerobic respiration by denitrification. We have observed that strain MIT0214 is best adapted to anaerobic growth at pressures of 1 atm but is also able to growth at elevated pressures beyond the critical point for CO_2. Specifically, after 1 week growth was observed at pressures as high as 27 atm (N_2) or 9 atm (CO_2) and after 26-30 days growth can be observed under both a N_2 headspace at 100 atm and under a supercritical CO_2 headspace at a temperature of 37˚C and pressures >71 atm (to 136 atm).

In addition to demonstrating growth under $scCO_2$ we have determined that spores of strain *B. cereus* MIT0214 are tolerant of both direct and indirect exposure to $scCO_2$. As expected, direct exposure of dried spores to $scCO_2$ proved to be the more severe stress resulting in a 66-86% loss in spore viability over a 2 week interval, however persistence of a subpopulation of spores after exposure to dry $scCO_2$ suggests that even direct exposure to supercritical CO_2 during GCS may not effectively sterilize the subsurface, indicating that upon return of an aqueous phase, microbial activities can resume.

Additional physiological characterization under aerobic conditions have revealed MIT0214 is able to grow at temperatures from 21 to 45 °C (but not at 55˚C) and salinities

0.01 to 40 g/L NaCl with optimal growth occurring at 30˚C and from 1 - 5 g NaCl/L. We have noted that MIT0214 spores are able to tolerate temperatures in excess of 80˚C and saturated salt solutions without loss of viability. Since *Bacillus* species, including *B. cereus* strains, have commonly been recovered from other deep subsurface environments – the $scCO_2$ tolerance of spores and growth under high pCO_2 conditions is consistent with persistence in a subsurface environment after CO_2 injection and it is likely that strain MIT0214 would have been able to persist as a spore, but not grow, under the down-hole conditions observed at the Frio 2 site (temperature 55˚C, suspended solids 93 g/L consisting primarily of salts) (Hovorka et al., 2006).

Genomic analysis of strain MIT0214 reveals a shared evolutionary history with other *B. cereus* strains isolated from surface environments including signatures of lateral gene transfer. The genomes of strain MIT0214, and a *B. cereus* strain isolated from an oil well in China (strain Q1) each revealed a significant enrichment in the number of genes assigned to functional categories for producing and maintaining the "Cell Wall and Capsule", regulating and mediating "Stress Response", and "Metabolism of Aromatic Compounds" relative to six *B. cereus* isolates from surface environments, which may point to adaptations that facilitate survival in subsurface habitats.

Our findings suggest that strategies to engineer biofilm barriers *in situ* under high pCO_2 conditions should consider addition of bacterial cells as spores rather than as vegetative cells as we have observed that MIT0214 spores survive initial supercritical CO_2 exposure while aerobically-grown vegetative cells do not. Moreover, our observations indicate that survival of some microbial types, such as Bacilli, at the interface between the $scCO_2$ plume and the formation water is likely and that such survival may be mediated heavily by pre-existence of the bacterium as a spore. After survival, we have observed germination of spores and growth of vegetative cells under supercritical CO_2.

After CO_2 injection high local dissolved organic carbon concentrations have been observed at the leading edges of CO_2 plumes (Hovorka, 2006; Kharaka, 2006) likely due to extraction of the organic matrix of the subsurface by the solvent-like properties of supercritical CO_2. These enrichments of dissolved organic carbon at the leading edge of the plume are potential hot spots for microbial activity fueled by anaerobic metabolism. Microbial processes in subsurface environments can catalyze both mineral dissolution and nucleation (Barker, 1998; Cunningham, 2009; Mitchell, 2010) and CO_2 reduction by acetogenesis or methanogenesis pending availability of appropriate biological catalysts, pH, Eh and electron donors (Onstott, 2005). On post injection timescales the release of metal cations from the weathering of silicate minerals is predicted to be the rate-limiting step in mineral trapping of CO_2 through the precipitation of metal-carbonates (Gaus, 2005; Gaus, 2010) and bacterial activity can increase this rate through production of chelating agents and etching of mineral surfaces (Barker, et al, 1998; Ferris, et al, 1996; Mitchell, et al, 2009) and thus control rates of mineral trapping of CO_2 in carbonates. Our evidence of a complex consortium of $scCO_2$-tolerant microbes and characterization of an isolate adapted to conditions associated with $scCO_2$ indicate that the microbially-mediated processes described above can occur up to the CO_2 plume-water interface. Understanding how CO_2 injection shifts the balance of these microbial processes is therefore necessary to predict the long-term fate of injected CO_2

7. Tables

Table 1. Passages performed in the isolation of strain MIT0214. The '+' symbols indicate the samples that were subjected to genomic DNA isolation and characterization of 16S rRNA gene diversity.

Isolation of *Bacillus* MIT0214

Passage	Duration	Percent of previous culture used as inoculum	DNA yield (ng/mL)	Cloned 16SrRNA genes	Molecular Detection of MIT0214* (+/-)
Initial enrichment	14 days	NA	703	+	Inc.
1	16 days	15 %	1435	+	Inc.
2	15 days	10 %			DNA+
3	15 days	10 %			Inc.
4	60 days	10 %			Gly+
5	15 days	10 %			Gly+; Inc.
6	12 days	10 %			Gly+
7	9 days	10 %	2301	+	16S+; Gly+; Inc.
8	9 days	10 %			Not tested
9	9 days	10 %	1768	+	16S+; Gly+

* Marker Detection Key
gly = 0214 marker observed (+) in biomass grown from glycerol stock
DNA = 0214 marker observed in archived DNA extract
Inc. = inconclusive results from DNA screen (passage 0, 1, 3, 5, 7).
16S = 0214 observed as 16S rRNA sequence type in clone library

Table 2. Ribotypes identified from 16S rRNA analysis of the initial enrichment and passages 1, 2, 7.

Community Change via passage				
Passage	Total Sequences (non-chimeric)	Chimeras Removed	Chao1 Mean	Chao 1 95% Confidence
Initial enrichment	34	10	29.5	12.9 to 99.3
1	45	3	78.5	43.5 to 708.5
7	31	3	2	27.5 to 31.8
9	21	0	1	27.5 to 31.8

Table 3. Observed and predicted pH as a function of GYP culture media composition [a], headspace gas, temperature and pressure.

	Temperature (C)	Pressure (atm)	Predicted pH [b]	Observed pH [c]
CO_2	37	1	5.3	5.1
CO_2	37	120	3.8	3.9 to 4.5
pN_2	37	1	7.0	7.0
pN_2	37	120	7.0	ND

ND: Not determined

[a] Growth media components (g/kg): Acetate 0.72, Na^+ 0.2839, Cl^- 0.607, Fe^{2+} 0.002, Mn^{2+} 0.0025, Mg^{2+} 0.0196, and SO_4^{2-} 0.08575. 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 Phreeq-C calling the llnl database.

[c] pH measured by Orion model 520A pH meter (P = 1 atm) or visualized at (P=120 atm) via indicator strip (EMD Chemicals).

Table 4. Comparison of MIT0214 and closely related/ geologically relevant *B. cereus* group strains.

	B. cereus MIT0214	*B. cereus* Q1	*B. anthracis* Ames	*B. cereus* ATCC 14579	*B. cereus* ATCC 10987
GC content (%)	34.9%	35.5%	35.4%	35.3%	35.5%
No. of plasmids (size)	TBD	2 (53kb & 239kb)	2 (95kb & 182kb)	1 (15kb)	1 (208kb)
Genome size (Mb)	5.62 Mb	5.51 Mb	5.23 Mb	5.43 Mb	5.43 Mb
No. of Coding Sequences	5640	5646	5667	5561	5924
Genes shared with MIT0214	-	5032 (89.2%)	5001 (88.7%)	5086 (90.2%)	5034 (89.3%)
Average Nucleotide Identity with MIT0214	-	93.6%	93.2%	97.9%	93.3%

Table 5. Subsystems that are differentially represented in the MIT0214 and Q1 genomes relatives to surface strains. Comparison of the percent of total SEED subsystems of both MIT0214 and *B. cereus* Q1 that show more than 2 standard deviations from the mean of 6 surface strains (*B. cereus* ATCC 14579, ATCC 10987, B4264, G9241, *B. anthracis* Ames and Sterne). *B. cereus* isolates associated with the deep subsurface (MIT0214 and Q1) show enrichment of three subsystems that may represent important functions for adaptation to the subsurface: 1. cell wall and capsule, 2. stress response and 3. metabolism of aromatic compounds.

6 Surface isolates % (Std. Dev.)	MIT0214 %	Q1 %	SEED Level 1 Subsystems
7.59 (0.32)	5.89	6.27	Cofactors, Vitamins, Prosthetic Groups, Pigments
4.52 (0.43)	**5.86**	**5.68**	Cell Wall and Capsule
0.972 (0.09)	0.47	0.48	Potassium metabolism
9.67 (0.45)	7.38	8.35	Miscellaneous
5.99 (0.11)	4.51	3.66	RNA Metabolism
3.34 (0.14)	**3.93**	**3.86**	Stress Response
0.397 (0.047)	**0.56**	**0.51**	Metabolism of Aromatic Compounds

Table 6. 46 genes are shared between MIT0214 and Q1 and not found in the two surface-isolated *B. cereus*.

Contig	Length (AA)	Function
1	42	FIG01225132: hypothetical protein
5	304	FIG01226470: hypothetical protein
13	47	conserved hypothetical protein
16	40	FIG01225200: hypothetical protein
23	38	FIG01229872: hypothetical protein
26	46	FIG01225893: hypothetical protein
52	102	possible DNA-binding protein
52	176	hypothetical protein
60	241	membrane protein, putative
64	177	FIG01228319: hypothetical protein
77	161	FIG01226296: hypothetical protein
86	51	FIG01236245: hypothetical protein
87	41	FIG01229129: hypothetical protein
95	40	FIG01229392: hypothetical protein
111	45	FIG01228577: hypothetical protein
112	192	hypothetical protein
113	74	FIG01226613: hypothetical protein
114	38	FIG01231881: hypothetical protein
114	85	hypothetical homeodomain-like
115	326	FIG01225259: hypothetical protein
121	42	hypothetical protein
121	120	FIG01225113: hypothetical protein
123	139	FIG01226855: hypothetical protein
138	136	conserved hypothetical protein
138	138	hypothetical protein
141	120	hypothetical protein
144	141	FIG01245493: hypothetical protein
159	137	FIG01233818: hypothetical protein
164	39	FIG01227524: hypothetical protein
170	46	FIG01234055: hypothetical protein

171	39	FIG01226717: hypothetical protein
182	186	FIG01226586: hypothetical protein
187	310	hypothetical protein
187	1032	putative salivaricin A modification enzyme; amino acid dehydration
191	39	FIG01226938: hypothetical protein
192	142	CDS_ID OB2928
202	58	FIG01227923: hypothetical protein
210	167	Translation initiation factor 3
52	98	hypothetical protein
75	570	Mosquitocidal toxin
102	247	FIG01228363: hypothetical protein
121	118	prophage LambdaBa04, DNA-binding protein
141	64	hypothetical integrase, catalytic domain, homeodomain-like
172	386	Alpha-D-GlcNAc alpha-1,2-L-rhamnosyltransferase (EC 2.4.1.-)
187	364	Choline binding protein A *
202	92	pXO1 ORF14-like protein

8. Figures

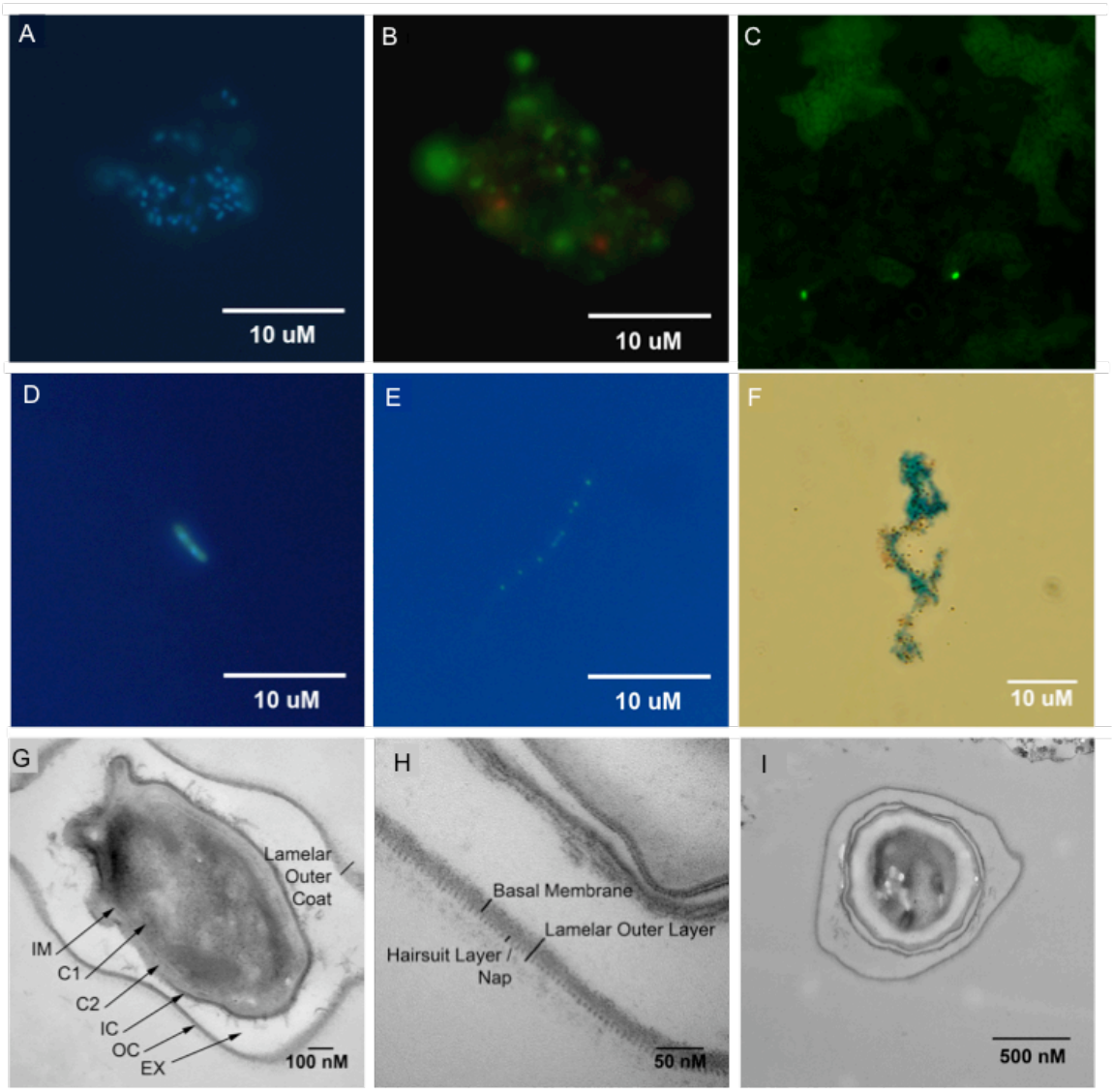


Figure 1. Fluorescent stained biomass from the initial enrichment of the Frio2 sample and the first passage (A) DAPI stain (initial enrichment) and (B) Invitrogen Live/Dead stain (first passage) reveal clusters of cells with a diameter of approximately 0.5-1 µm connected by an extracellular material that may be extracellular polysaccharide (EPS). Scale bar 10 µm. (C) Culture of strain MIT0214 from passage #9 grown under 1atm CO_2 exhibits similar aggregation after 15 days. Bright cells are most likely spores. (D) DAPI stained MIT0214 isolate showing dividing cells; (E) DAPI stain of MIT0214 spores; (F) Aggregate of MIT0214 spores stained with malachite green. Cells are embedded in matrix, possibly EPS. (G-I) Thin section TEM showing spore structural elements of MIT0214. *B. cereus* MIT0214 cells grown under a supercritical CO_2 headspace were rod-shaped and formed spores (G) Structural components of the spore are noted: Inner membrane (IM), inner dense layer (C1), less dense cortical layer (C2), inner (IC) and outer (OC) coats, exosporium (EX) and ribosomal aggregates (R). (H) Cross section of MIT0214 spore outer coat reveals the ultrafine structure of the lamellar coat seen. The basal membrane lamination consists of 3 dark bands separated by two light bands including the outer fine structure (i.e. 'hairsuit' or nap layer) that surrounds the lamellar outer coat layer. An angular cross section of the nap layer shows the structured pattern of the lamellar protrusions on the surface.

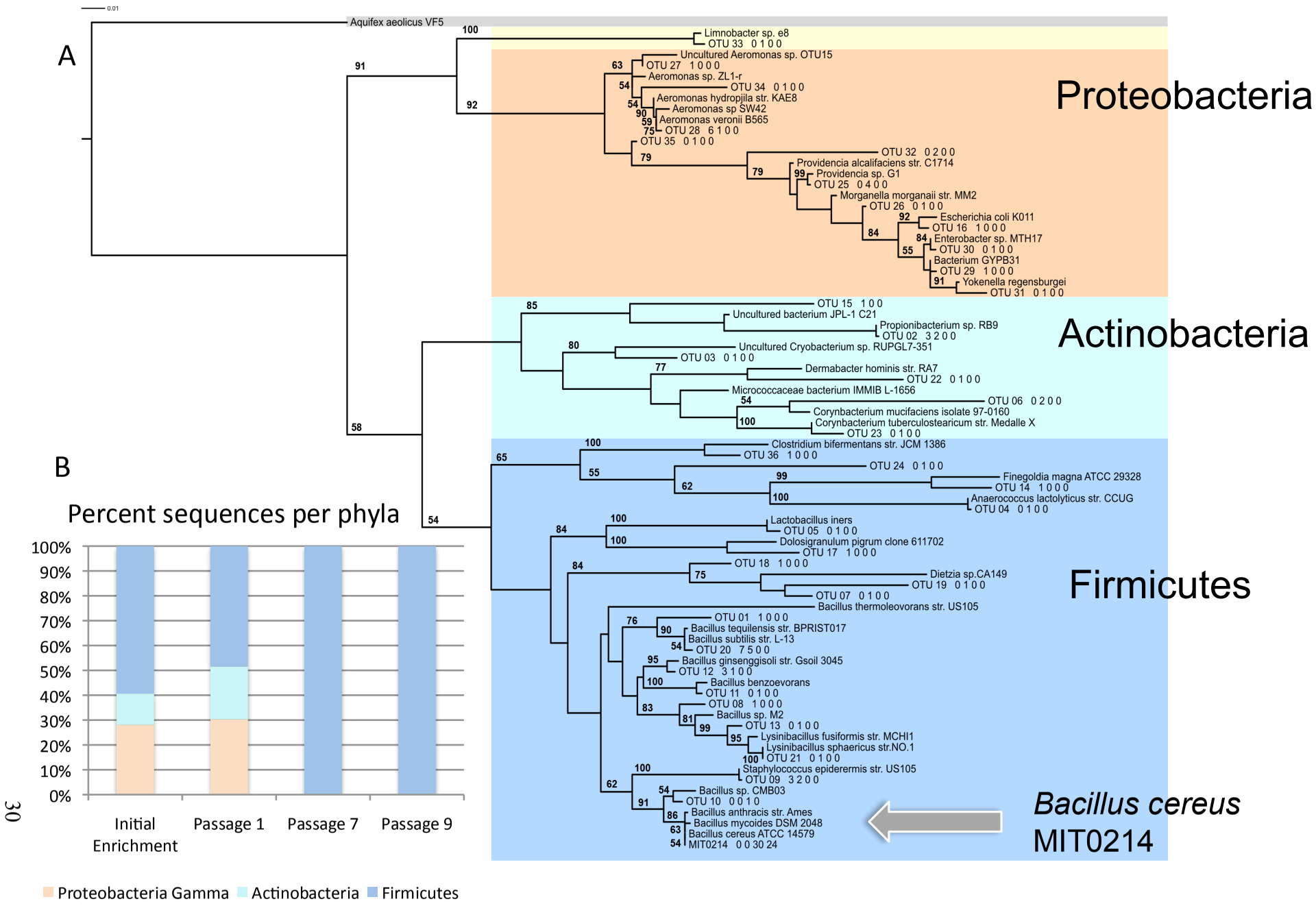

A
0.01
Aquifex aeolicus VF5
Limnobacter sp. e8
OTU 33 0 1 0 0
Uncultured Aeromonas sp. OTU15
OTU 27 1 0 0 0
Aeromonas sp. ZL1-r
OTU 34 0 1 0 0
Aeromonas hydropjila str. KAE8
Aeromonas sp SW42
Aeromonas veronii B565
OTU 28 6 1 0 0
OTU 35 0 1 0 0
OTU 32 0 2 0 0
Providencia alcalifaciens str. C1714
Providencia sp. G1
OTU 25 0 4 0 0
Morganella morganaii str. MM2
OTU 26 0 1 0 0
Escherichia coli K011
OTU 16 1 0 0 0
Enterobacter sp. MTH17
OTU 30 0 1 0 0
Bacterium GYPB31
OTU 29 1 0 0 0
Yokenella regensburgei
OTU 31 0 1 0 0
Proteobacteria
OTU 15 1 0 0
Uncultured bacterium JPL-1 C21
Propionibacterium sp. RB9
OTU 02 3 2 0 0
Uncultured Cryobacterium sp. RUPGL7-351
OTU 03 0 1 0 0
Dermabacter hominis str. RA7
OTU 22 0 1 0 0
Micrococcaceae bacterium IMMIB L-1656
OTU 06 0 2 0 0
Corynbacterium mucifaciens isolate 97-0160
Corynbacterium tuberculostearicum str. Medalle X
OTU 23 0 1 0 0
Actinobacteria
B
Percent sequences per phyla
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Initial Enrichment
Passage 1
Passage 7
Passage 9
Proteobacteria Gamma
Actinobacteria
Firmicutes
Clostridium bifermentans str. JCM 1386
OTU 36 1 0 0 0
OTU 24 0 1 0 0
Finegoldia magna ATCC 29328
OTU 14 1 0 0 0
Anaerococcus lactolyticus str. CCUG
OTU 04 0 1 0 0
Lactobacillus iners
OTU 05 0 1 0 0
Dolosigranulum pigrum clone 611702
OTU 17 1 0 0 0
OTU 18 1 0 0 0
Dietzia sp.CA149
OTU 19 0 1 0 0
OTU 07 0 1 0 0
Bacillus thermoleovorans str. US105
OTU 01 1 0 0 0
Bacillus tequilensis str. BPRIST017
Bacillus subtilis str. L-13
OTU 20 7 5 0 0
Bacillus ginsenggisoli str. Gsoil 3045
OTU 12 3 1 0 0
Bacillus benzoevorans
OTU 11 0 1 0 0
OTU 08 1 0 0 0
Bacillus sp. M2
OTU 13 0 1 0 0
Lysinibacillus fusiformis str. MCHI1
Lysinibacillus sphaericus str.NO.1
OTU 21 0 1 0 0
Staphylococcus epiderermis str. US105
OTU 09 3 2 0 0
Bacillus sp. CMB03
OTU 10 0 0 1 0
Bacillus anthracis str. Ames
Bacillus mycoides DSM 2048
Bacillus cereus ATCC 14579
MIT0214 0 0 30 24
Firmicutes
Bacillus cereus
MIT0214

Figure 2. Maximum likelihood phylogenetic tree of 16S rRNA sequences (positions 515 to 1406 *E. coli*) from clone libraries prepared from biomass from passages initial enrichment, 1, 7 and 9. Numbered columns after OTU names indicate frequency of sequence type by libraries respectively. Type strains are indicated by species name and genbank sequence accession number wherever possible. Bootstrap values of over 50 (expressed as percentages of 100 replications) are shown at branch points. Colors represent bacterial phyla. The scale bar on upper left represents the unit length of the number of nucleotide substitutions per site. The bar chart represents the distribution of sequence types among the three phyla associated with enrichments and passages.

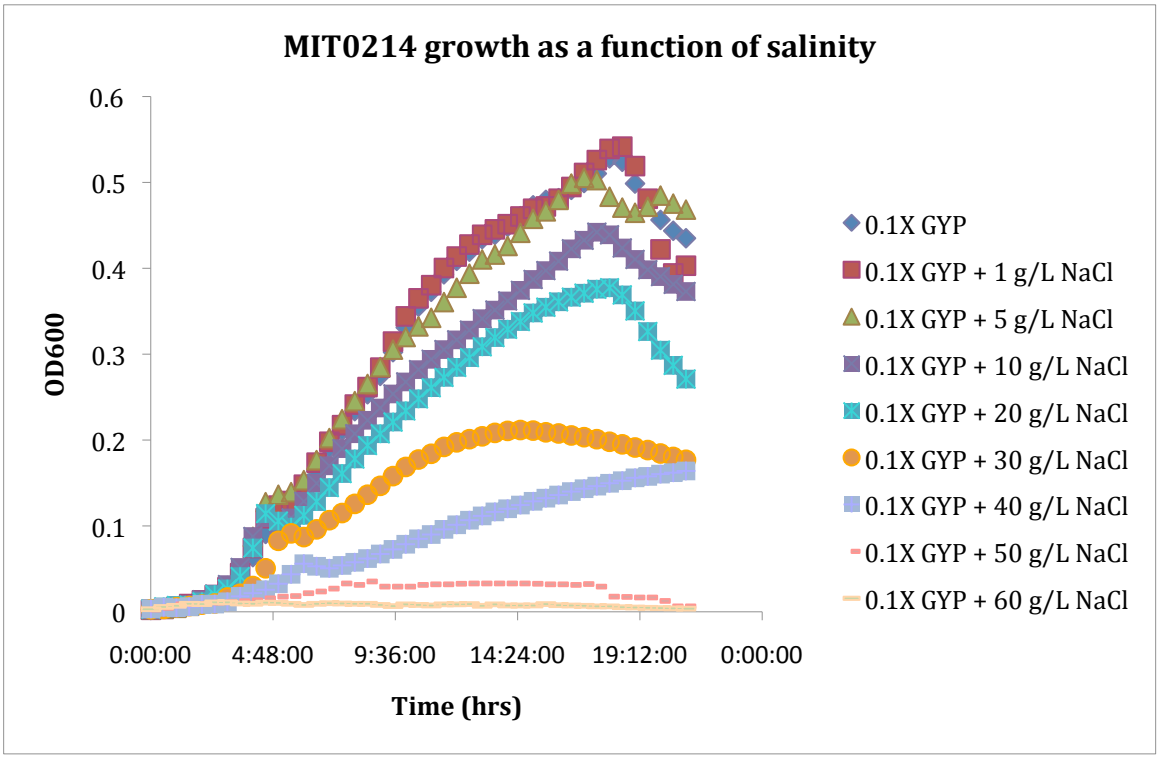


Figure 3. MIT0214 aerobic growth under variable salinities in 0.1 X GYP media at 37˚C.

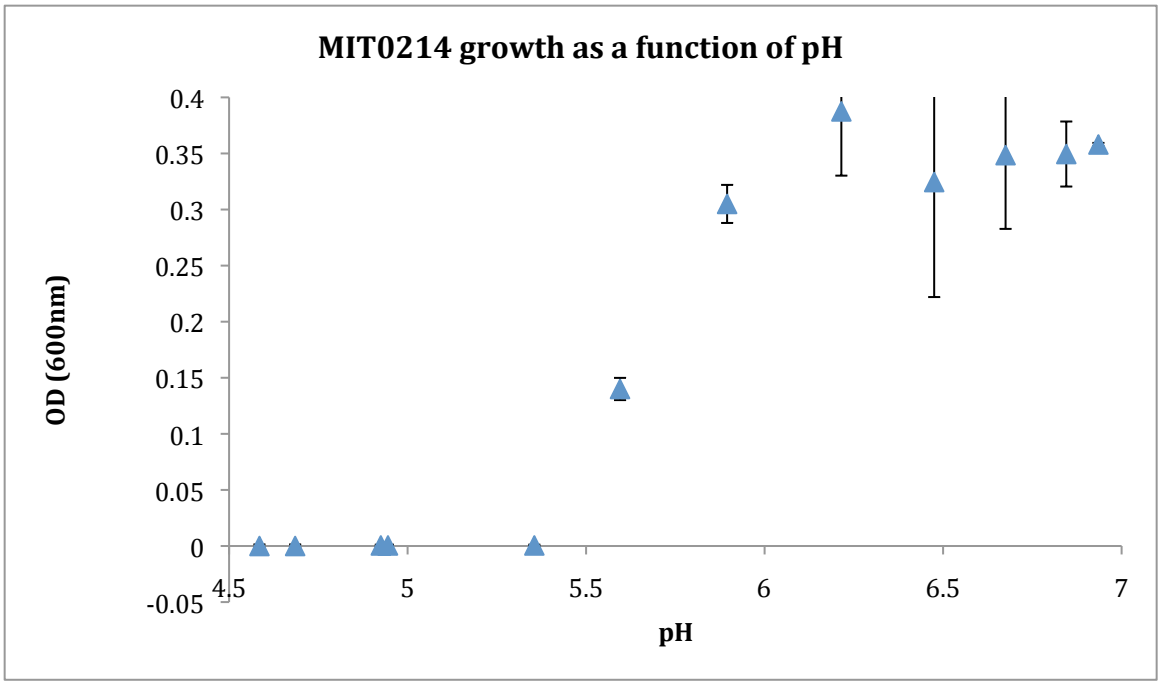


Figure 4. MIT0214 aerobic growth under variable pH in LB media at 37˚C.

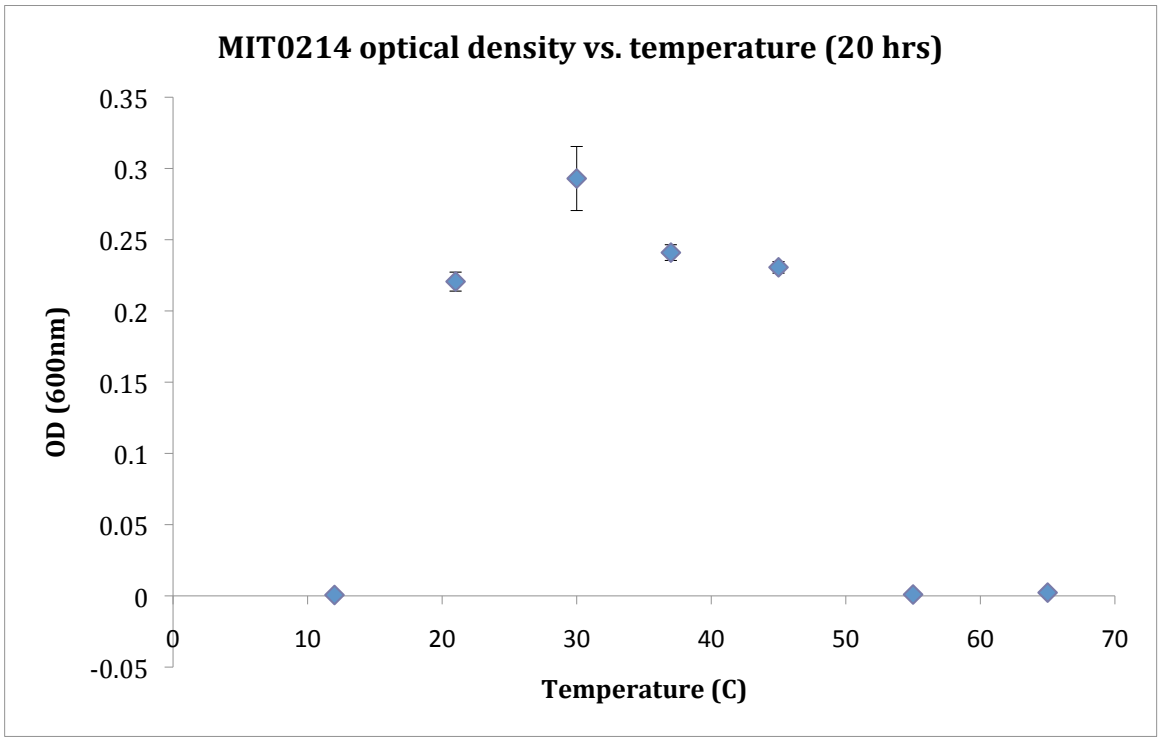


Figure 5. MIT0214 aerobic growth as a function of temperature in LB media.

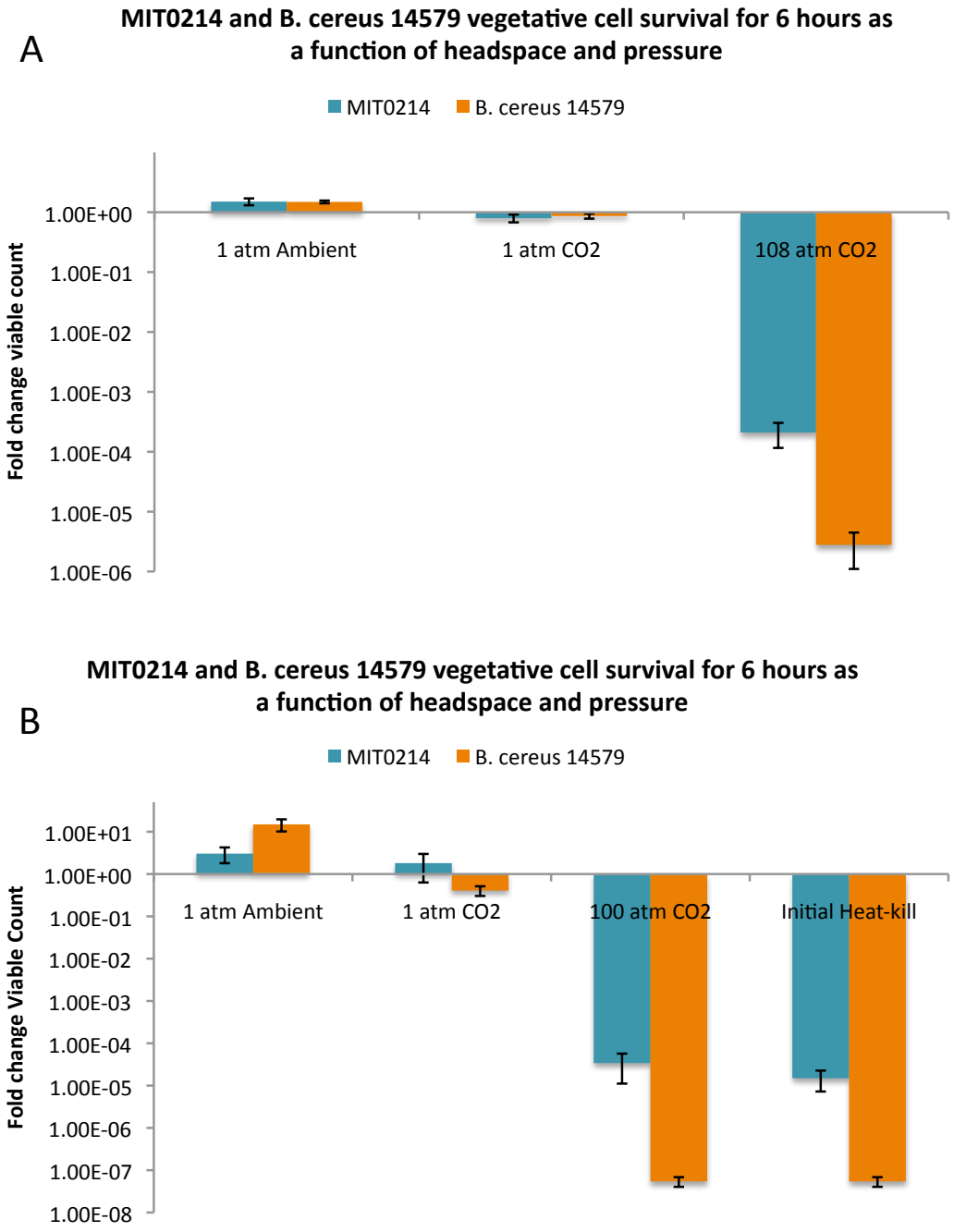


Figure 6. Short-term inactivation and survival of early stationary phase cultures of *B. cereus* MIT0214 (blue) and *B. cereus* ATCC14579 (orange). (A) Cultures were exposed to 1 atm ambient headspace, 1 atm CO_2 or 100 atm CO_2 for 6 hours. (B) The experiment in (A) was repeated with an additional treatment of "heat killing" i.e. incubation of cells at 80˚C for 10 minutes to select for spores. The fraction of cells surviving the heat kill matches the fraction that survive 100 atm CO_2 exposure indicating that the cells surviving $scCO_2$ exposure are spores and pointing to a higher fraction of spores in MIT0214 cultures relative to *B. cereus* 14579 after the same growth interval.

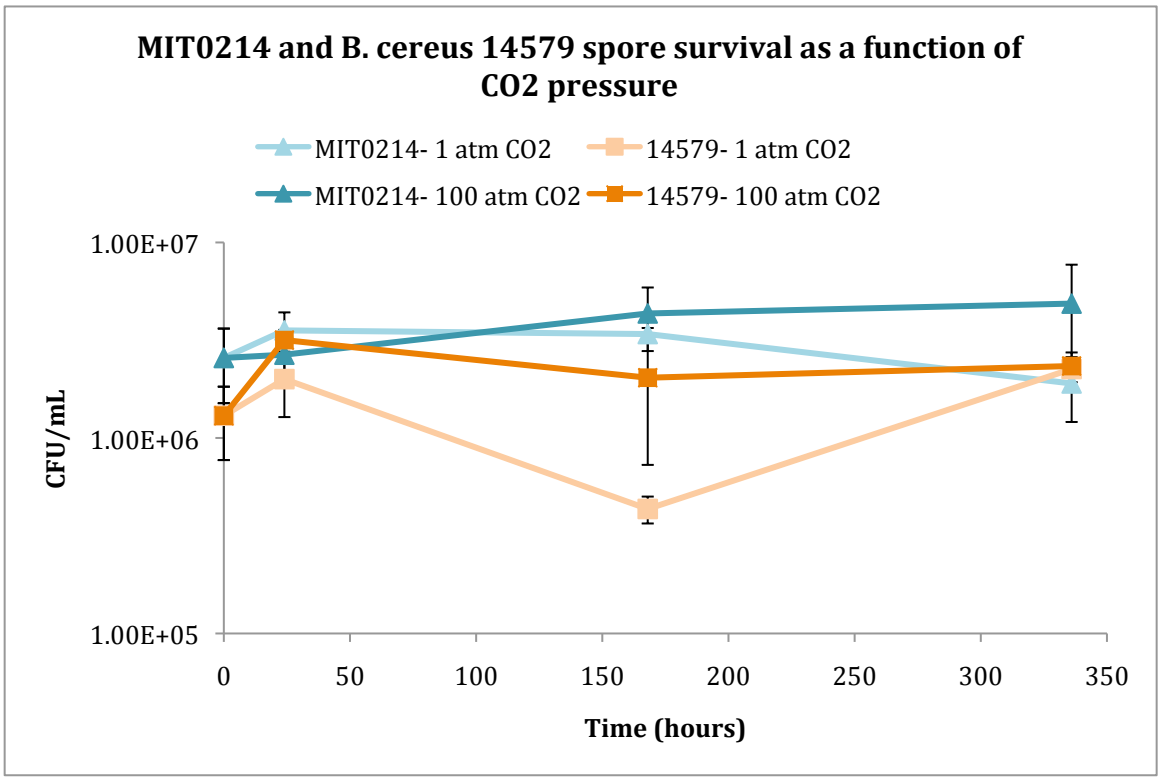


Figure 7. Intermediate-term survival of spores from MIT0214 (blue shades) and its closest relative, *B. cereus* ATCC 14579 (orange shades) under 1 atm CO_2 and 100 atm CO_2 headspace. Triplicate cultures per timepoint were inoculated with prepared spores (based on Kim & Goepfert, 1974). Reactors were pressurized, incubated for 1, 7 or 14 days followed by plating on LB to obtain viable cell counts (CFU/mL). No significant loss in viability was detected after 2 weeks incubation ($p>0.05$).

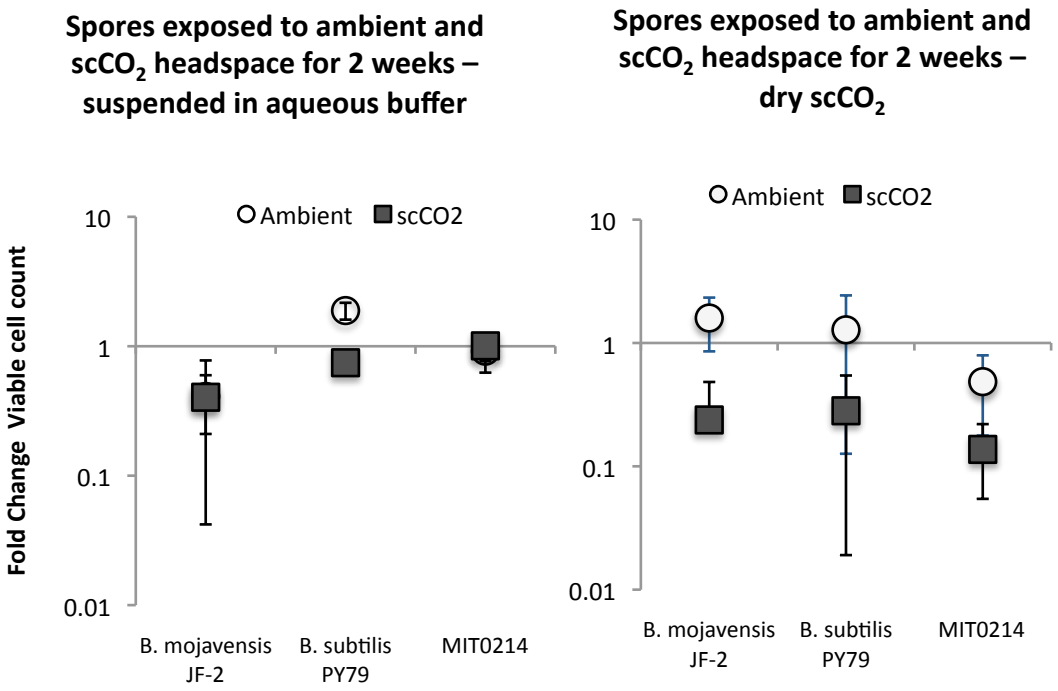


Figure 8. Change in viable cell count (cfu/ml) in spores from three *Bacillus* species exposed to $scCO_2$ for 2 weeks. Spores were dried in an oven for 72 hours at 70°C prior to exposure. (A) Spores resuspended in spore-preparation buffer (dark squares) revealed no decrease in viability upon exposure to $scCO_2$ relative to starting spore counts and ambient atmosphere incubated controls (open circles). (B) Spores exposed to dry $scCO_2$ (dark squares) decreased in viability by 66-86%.

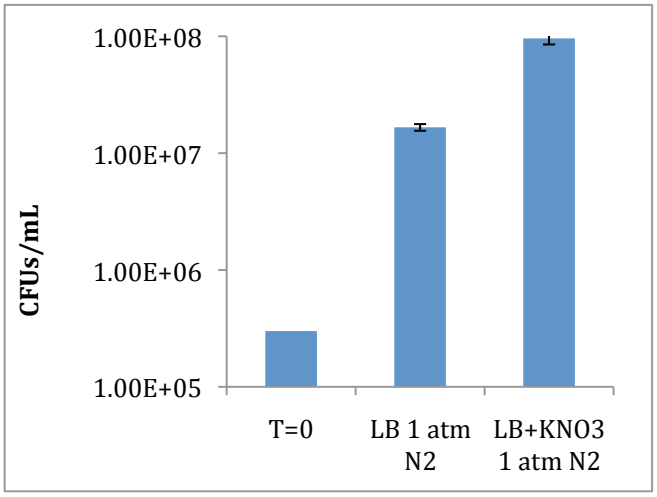


Figure 9. MIT0214 growth under anaerobic conditions at ambient pressure with N_2 headspace with and without addition of nitrate. Cultures were grown in glass Hungate tubes with 100 rpm shaking at 37 °C for 24 hours.

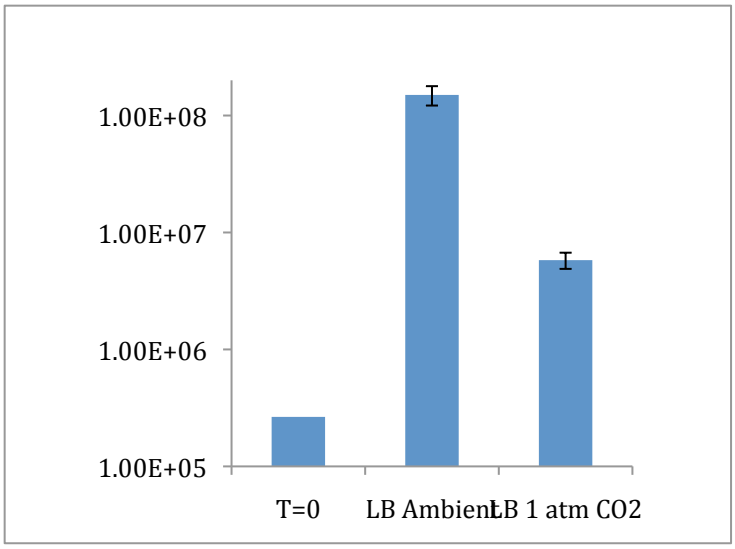


Figure 10. MIT0214 growth under anaerobic conditions at ambient pressure with CO_2 headspace. Cultures were grown in glass Hungate tubes with 100 rpm shaking at 37 °C for 48 hours.

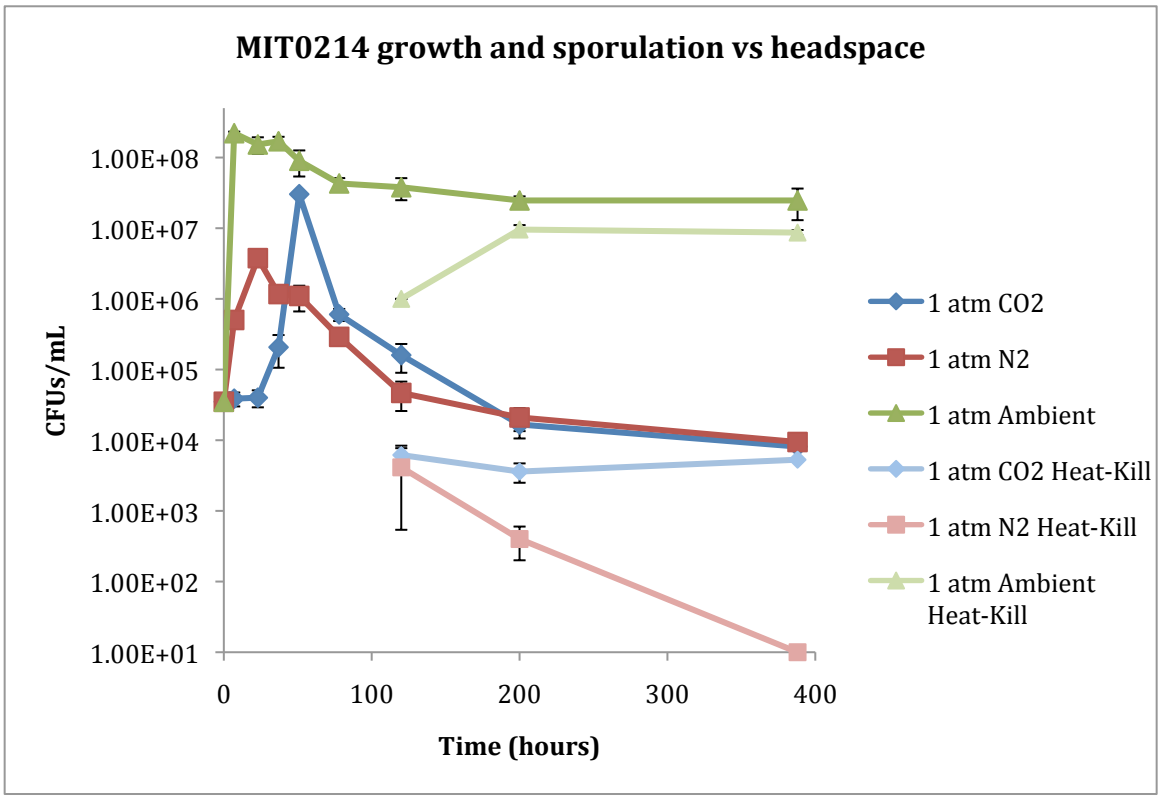


Figure 11. Growth curve of MIT0214 under 1 atm ambient, 1 atm N_2 and 1 atm CO_2 headspace over 388 hours. Triplicate cultures were incubated with shaking at 100 rpm at 37 °C and sub-sampled for CFU counts. Heat-killing of aliquots was used to determine spore numbers from 120 hours to the end of the experiment.

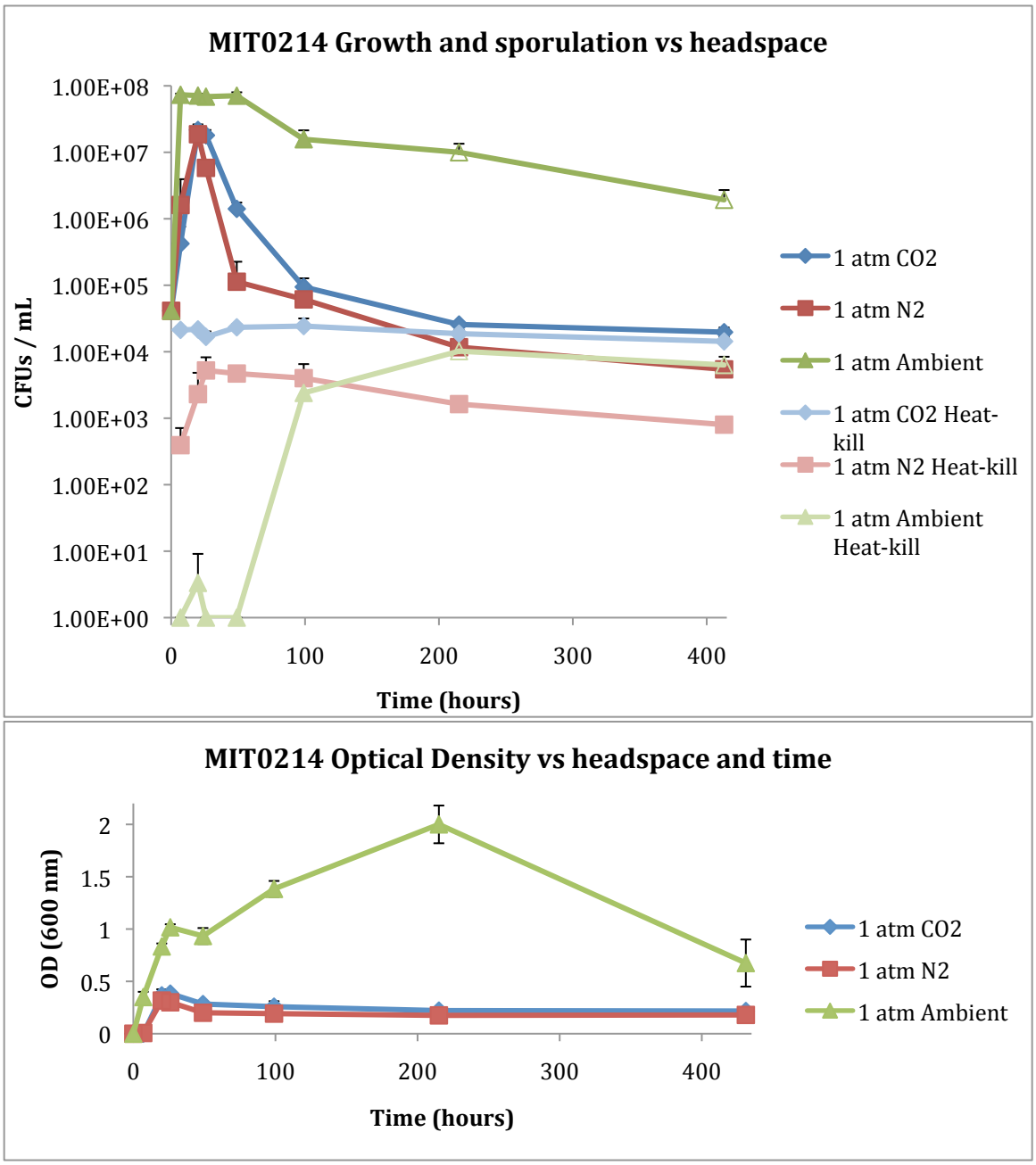


Figure 12. Growth of MIT0214 under ambient, 1 atm N_2 and 1 atm CO_2 headspace over 388 hours. Triplicate cultures were incubated with shaking (100 rpm and 37 °C) and sub-sampled for CFU counts. Heat-killing of aliquots was used to determine spore numbers. After inoculation of anaerobic samples the redox indicator briefly turned light pink indicating microaerophilic conditions. The dye returned to clear (anoxic) by the next time point (7 hrs). By the 215 hour time-point, all three ambient/aerobic samples showed contamination with a second CFU morphology, and thus quantification of MIT0214 viable counts and spores for this condition is likely confounded by competitor effects (open symbols). Additional work to validate growth dynamics of the strain under aerobic

and anaerobic conditions are in progress. (B) Optical Density (600 nm) values for the experiment described in section A. Note: the 431 hour time point for the 1 atm Ambient samples does not accurately represent culture optical density as the culture had become clumpy and heterogeneous.

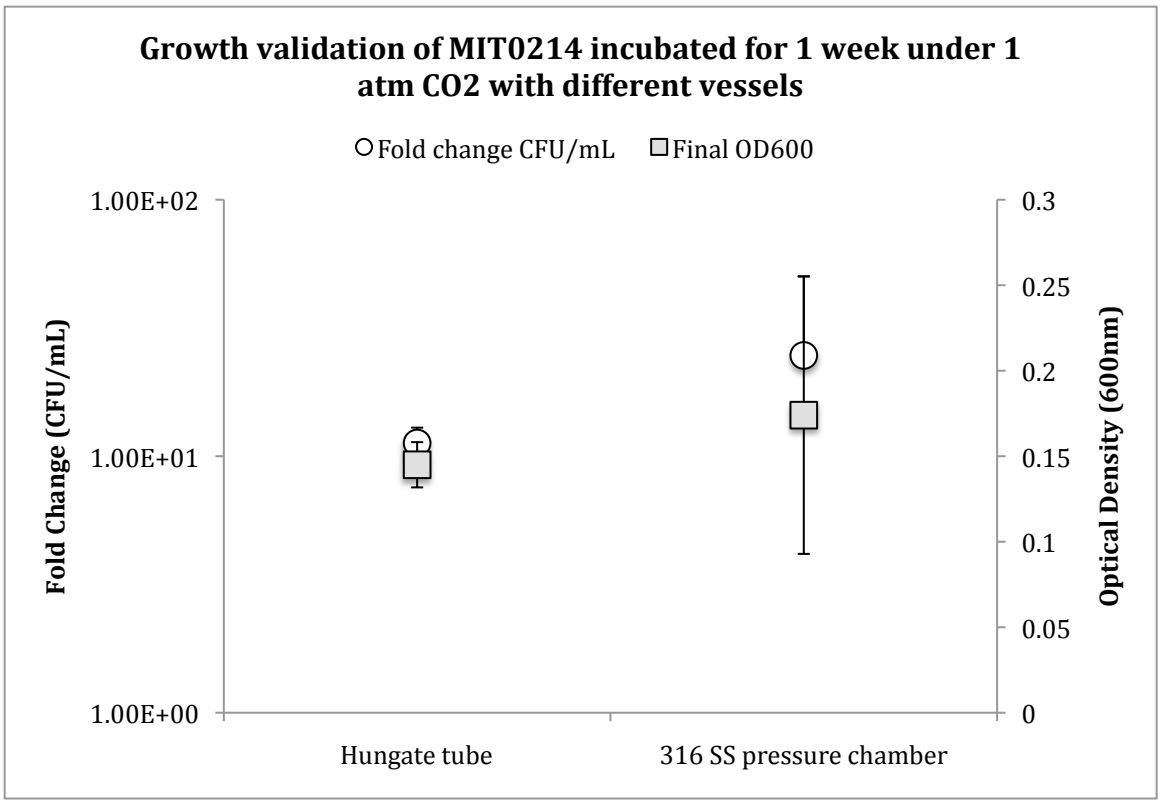


Figure 13: Validation of Hungate tubes and 316 Stainless steel pressure vessels for anaerobic growth of MIT0214. Fold change of CFU counts are plotted on the left axis (open circles) while final culture optical density is plotted on the right axis (gray squares). Error bars represent absolute uncertainty of 1 standard deviation for fold change values and 1 standard deviation for OD. Hungate tubes (N=3) show less variability in both viability counts and OD than steel pressure vessels (N=5) although averages are not significantly different.

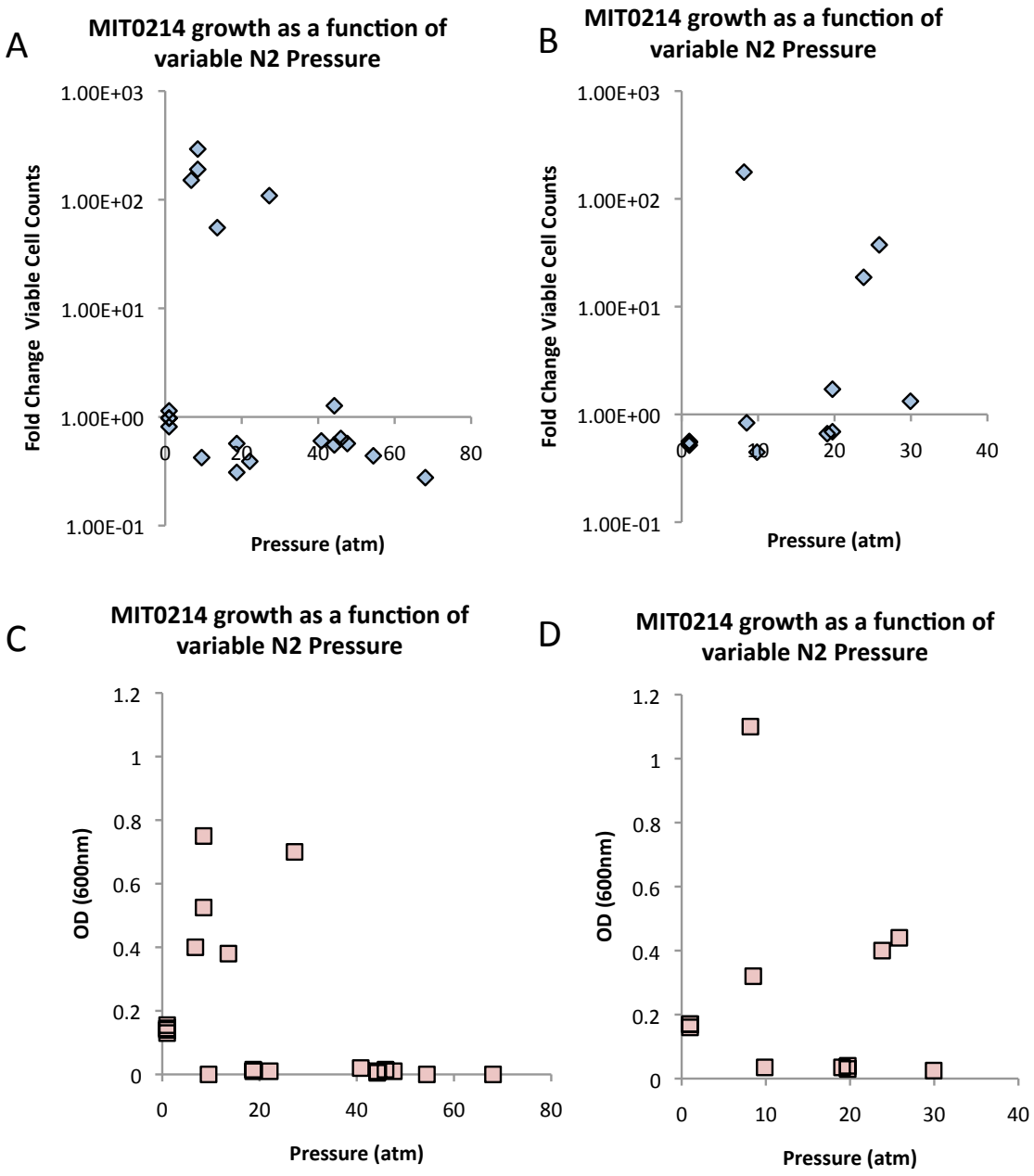
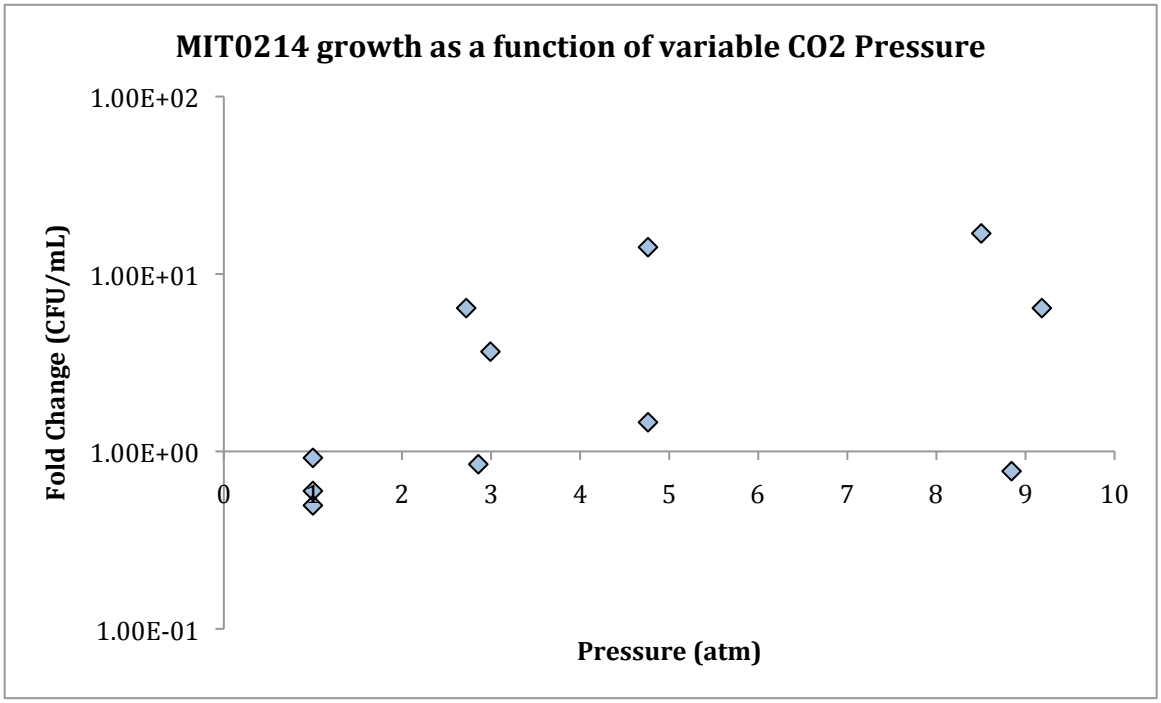


Figure 14. A & C Biomass and optical density of MIT0214 under variable pressure N_2 after 1 week incubation as change in viable cell counts relative to initial conditions (1 x 10^5 cfu/ml) and optical density (600nm) plotted versus the final observed pressure in the incubations. Initial pressures were from 1 to 100 atm. Growth results show some inconsistency with three samples at approximately 20 atm failing to grow, while one sample at 27 atm showing growth. We observed that the 1 atm N_2 samples show elevated OD600 values, without an increase in CFU counts consistent with loss of viable cell numbers in the late stages of fermentation. However, the OD600 values remain elevated and are reliable measures of growth. (04/17/12). B & D The observations were repeated for starting pressures from 1 atm to 30 atm. MIT0214 under variable pressure N_2 after 1 week incubation. Results also show inconsistent growth under pressure as three samples

between 19 and 20 atm did not appear to grow based on both CFU counts and OD600 values, while two samples between 23 and 26 atm showed confirmed growth with both CFU and OD600 counts.

A



B

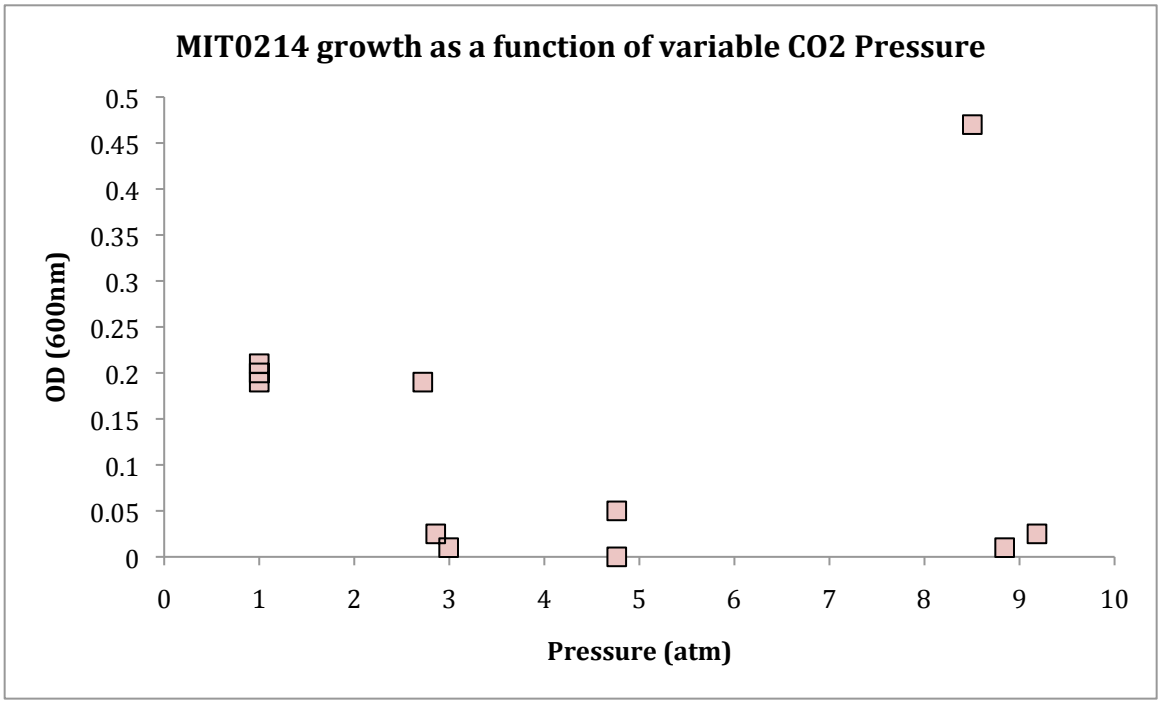


Figure 15. Growth of MIT0214 under variable pressure CO_2 after 1 week incubation.

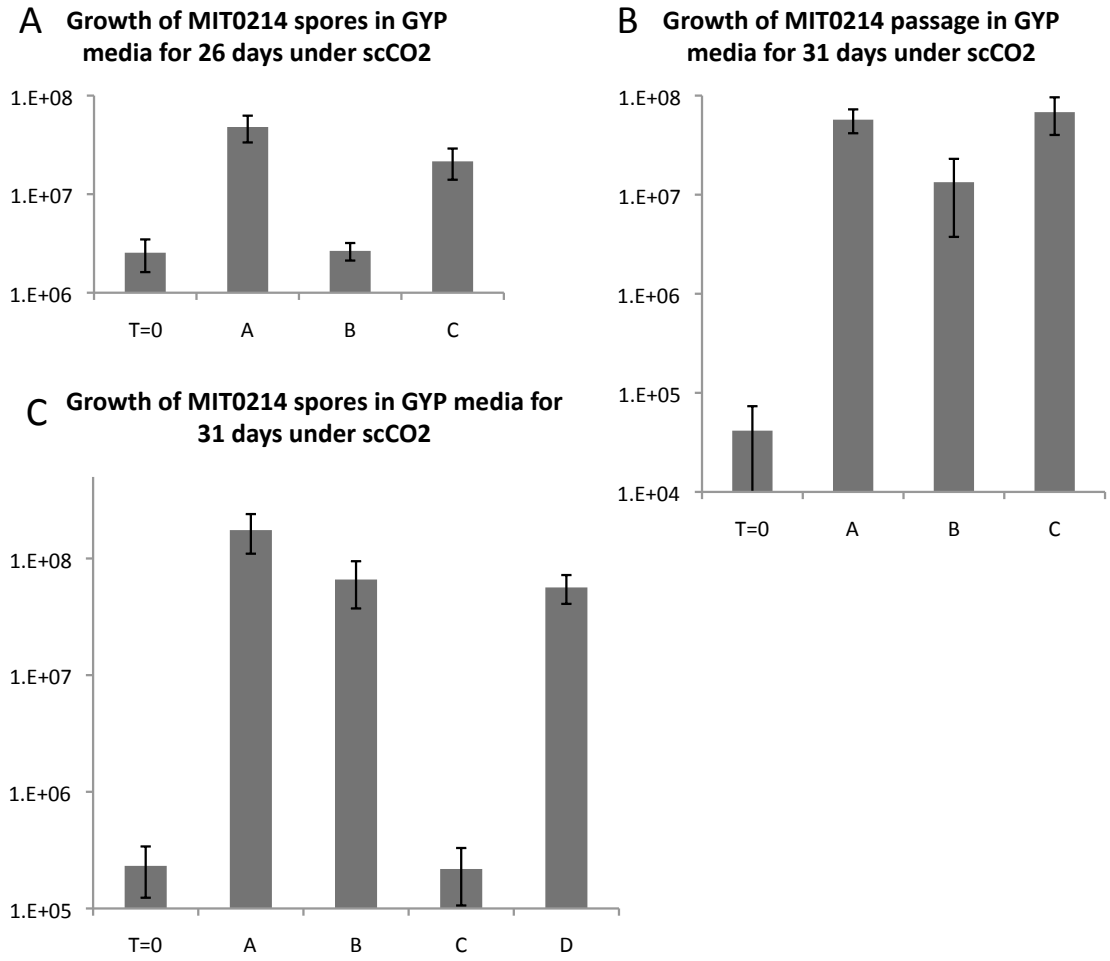


Figure 16. Growth of MIT0214 under supercritical CO_2 in three experiments. (A) Two of three replicates reveal 10 to 20-fold increases in biomass density in cultures inoculated with 2.5 x 10^6 spores after 26 days. (B) Biomass from Replicate A was used to inoculate a second passage at a starting cell concentration of $4.2x10^4$ cells/ml which was incubated for 31 days. All replicates manifested growth. (C) Spore stock was used to inoculate fresh cultures and three of four replicates revealed growth. In all cases, no inoculation controls had no observable biomass after the incubation periods (i.e. <1 x 10^3 cells/ml).

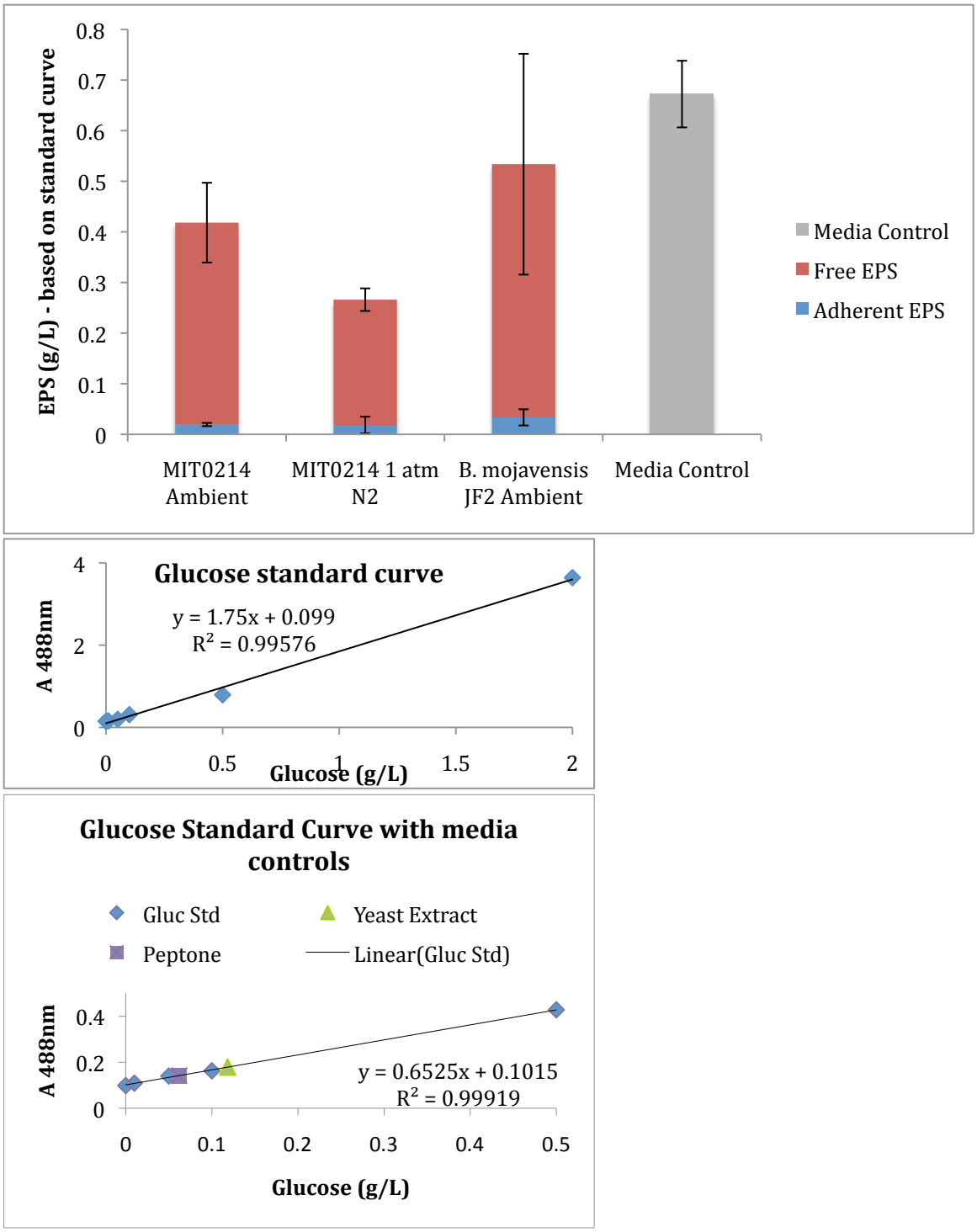


Figure 17. (A) Adherent and free EPS of MIT0214 grown under an ambient headspace and N2 headspace, compared to *B. mojavensis* JF2 grown under an ambient headspace. Neither strain shows significant EPS formation compared to the media control. Error bars show 1 standard deviation of triplicate data points. (B) Standard curve calibrated to glucose. (C) Repeated standard curve with peptone or yeast-extract media illustrating lower background for peptone-based media.

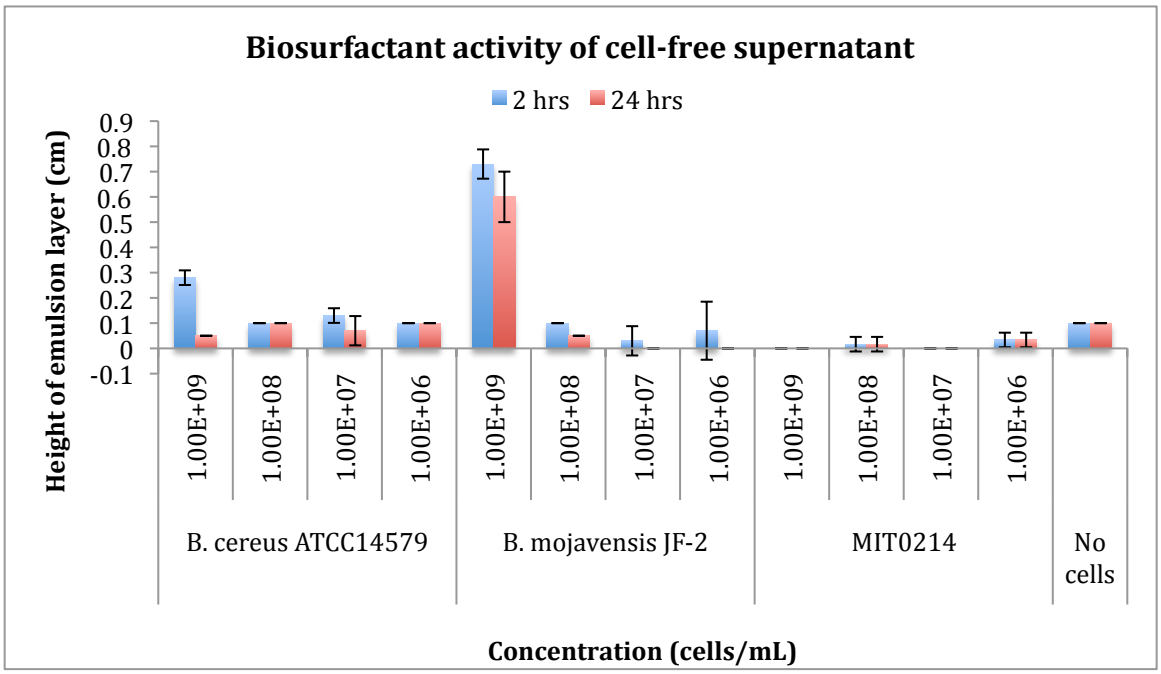


Figure 18. Biosurfactant activity of aerobically-grown MIT0214, *B. cereus* 14579 and *B. mojavensis* JF2 in LB media measured using an emulsion layer assay. Only *B. mojavensis* at 10^9 cells/mL shows significantly higher biosurfactant activity than the media control.

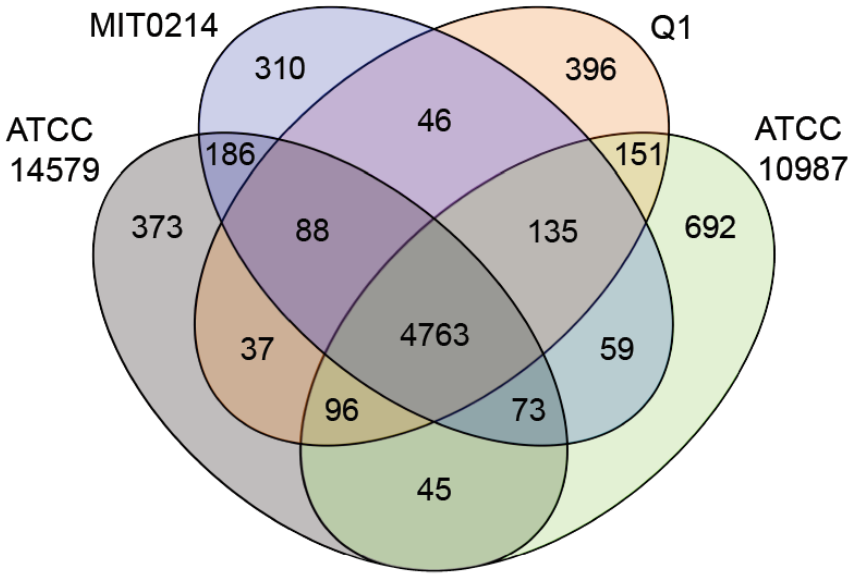


Figure 19. Venn Diagram illustrating the distribution of shared and unique genes among MIT0214 and three closely related *B. cereus* group strains: *B. cereus* ATCC 14579, *B. cereus* ATCC 10987, and *B. cereus* Q1.

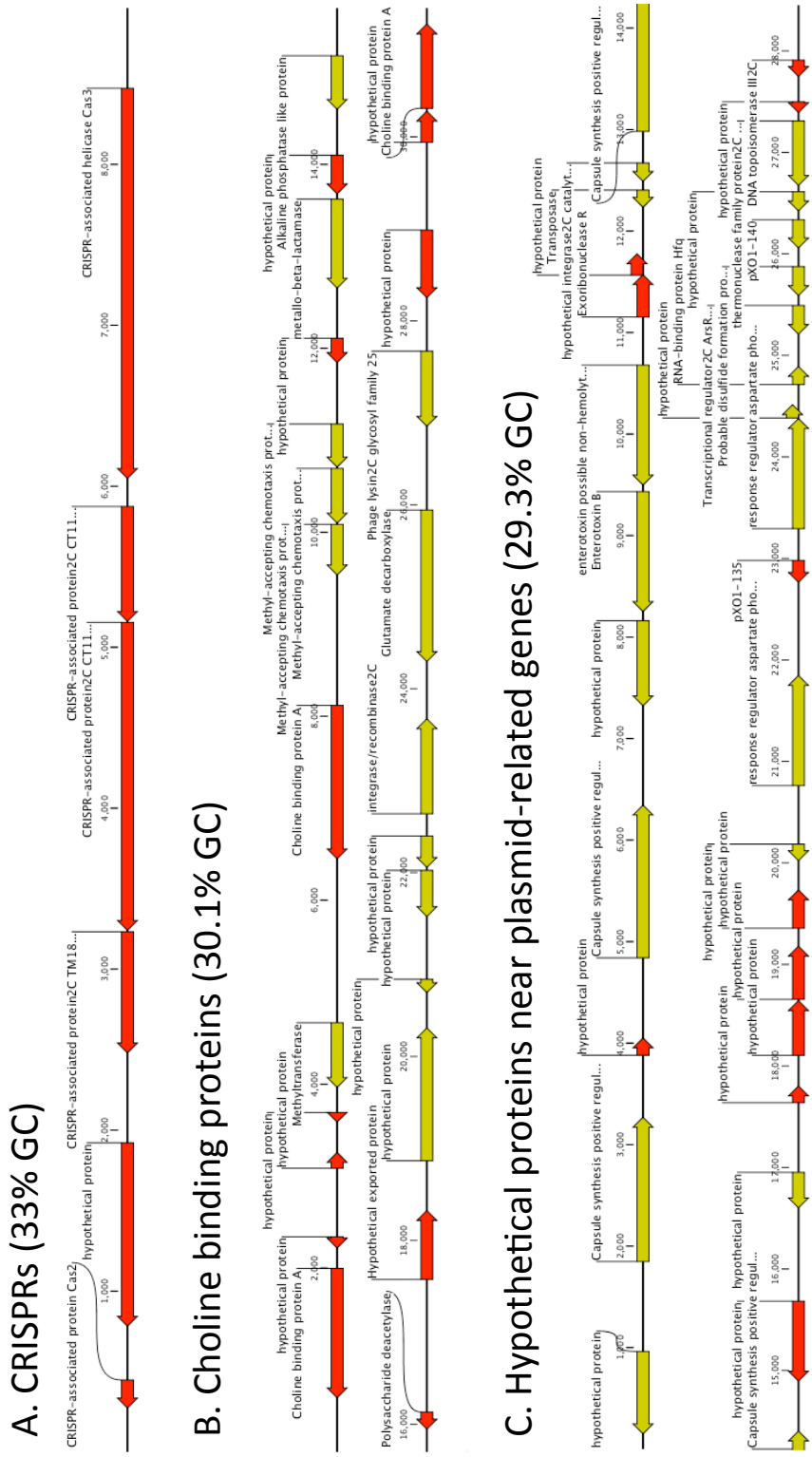


Figure 20. Contigs bearing novel gene content in MIT0214. Genes unique to MIT0214 are indicated in red while yellow indicates the gene is shared with another closely related *Bacillus* strain. These genome regions with a GC% differing from the genome average GC (34.9%) may have originated from lateral transfer from a different species. A) Contig 28 annotates as CRISPR genes, B) Contig 49 reveals several unique Choline binding proteins. C) Contig 284 contains unique hypothetical proteins and a pXO1 protein.

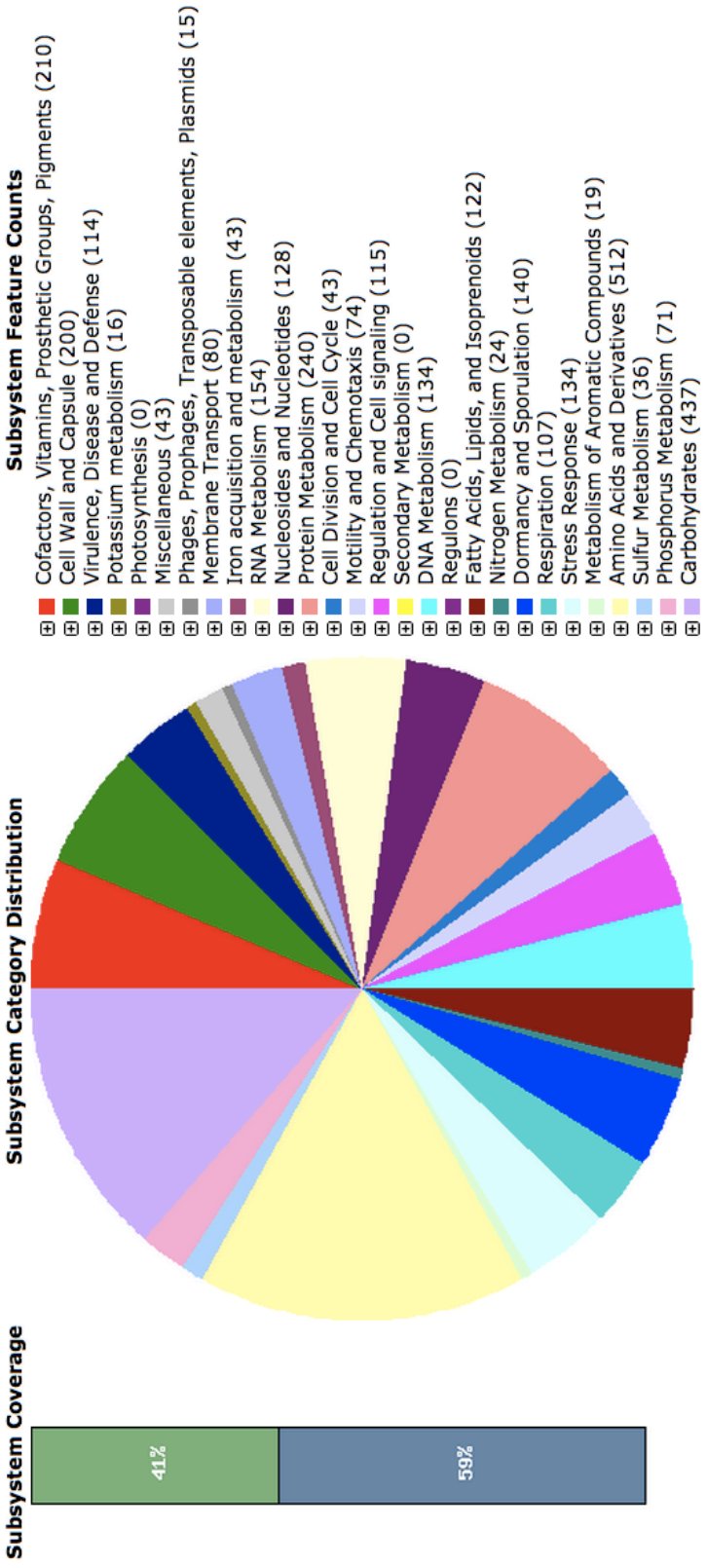


Figure 21. SEED Level 1 subsystem distribution of MIT0214 via RAST.

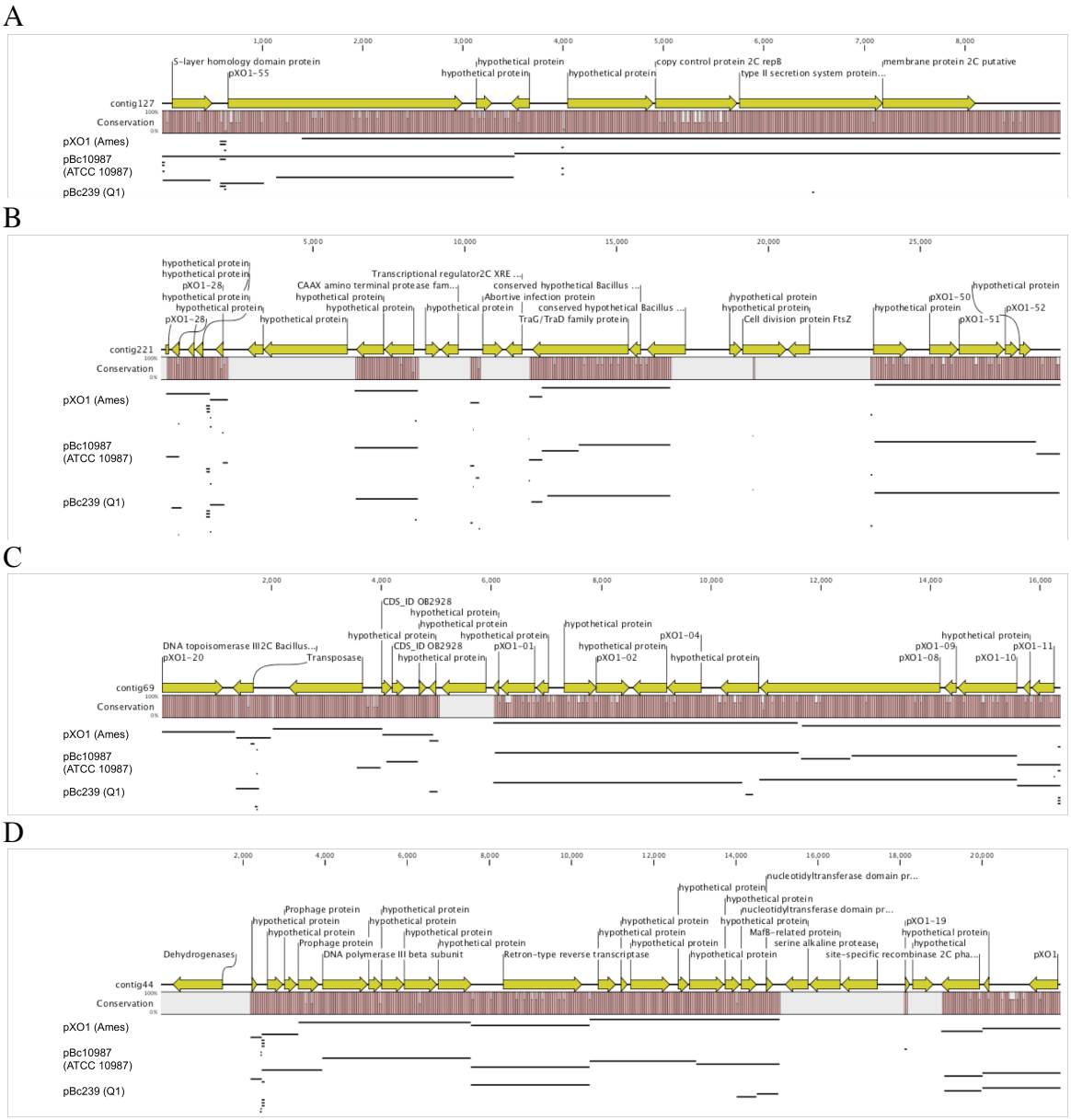


Figure 22. MIT0214 contigs with BLAST hits from plasmids of closely related *Bacillus* strains. 1.8% of the MIT0214 genome matches plasmid-borne genes. Plasmids are shown in the column to the left of the contigs with BLAST hit coverage displayed as black lines below the contig and sequence conservation. A) Contig 127, genes relevant to wall/ membrane proteins and type II secretion (35.4% GC). B) Contig 221, genes annotated as pXO1 proteins, DNA transfer and regulation. Lower than average GC content may indicate recent HGT (31.4% GC). C) Contig 69, genes annotated as pXO1 proteins (33.9% GC). D) Contig 44, genes annotated as phage and DNA transcription (33.8% GC). These plasmid-like regions highlight that MIT0214 has interacted with related strains and maintains regions that may aid in further horizontal gene transfer.

9. References

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10. List of Acronyms and Abbreviations

16S rRNA	16S ribosomal RNA
atm	unit of pressure corresponding to 1 standard Atmosphere
BLAST	Basic local alignment search tool
CCS	Carbon Capture and Storage
cfu/ml	Colony Forming Unit per milliliter (proxy for biomass)
CO_2	Carbon Dioxide
DAPI	4',6-diamidino-2-phenylindole (fluorescent DNA stain)
EPS	Extracellular Polysaccharide
GC%	Proportion of sequence comprised of guanine (G) and cytosine (C).
GCS	Geological Carbon Sequestration
GYP media	Glucose Yeast Peptone Media
LB media	Luria Broth or Luria Agar
OD600	Optical Density or absorbance at 600 nm wavelength
Redox	Reduction/Oxidation Potential
rpm	Rounds Per Minute (proxy for centripetal force)
N_2	Nitrogen (gas)
OTU	Operational Taxonomic Unit (proxy for microbial species)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism analysis
$scCO_2$	Supercritical Carbon Dioxide