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Dr. Neil Summer (PI)

President

Ecolyse, Inc. (Phage Biocontrol)
11142 Hopes Creek Road, College Station, TX 77845
Phone: (979) 694-6500
Email: Neil@ecolyse.com

Duns: 82-653-6968

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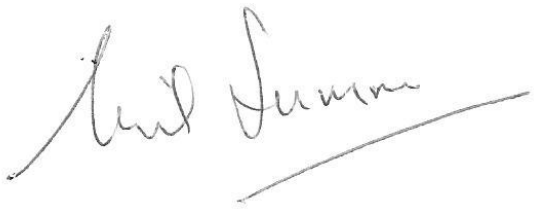

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ABSTRACT

Efficiency and safety within the oil and gas (O&G) industry is often compromised due to microbiological activity. Bacterial growth has been responsible for many instances of biofouling, reservoir souring, as well as the corrosion of concrete and metal surfaces. In particular, sulfate reducing bacteria (SRB) cause significant problems in the petroleum industry through microbially influenced corrosion (MIC) of metal pipelines and infrastructure, and by degradation of petroleum in reservoirs (reservoir souring) and storage facilities. We are actively investigating the potential use of phage for controlling SRB. Phage are natural, bacteriolytic agents that are highly specific for bacterial hosts and harmless to all other life forms, including humans. Because of their extreme host specificity, the use of phage requires knowledge of the specific bacterial targets in any given sample. The types of bacteria which are present in petroleum samples and handling facilities are currently unknown, except at a very superficial level. Funds from RPSEA were utilized to evaluate both the diversity of sulfate reducing bacteria within pipes and reservoirs, to isolate and culture SRB from these same samples, and to evaluate the capacity of the phage to control SRB under laboratory conditions.

Project scope included the evaluation of the bacterial populations present in two locations of direct interest to RPSEA: an oil brine mud sample from a crude oil salt storage cavern (COSC) and oil and water samples taken from an on shore pipeline (OSP). Both sites receive oil from the deep water Gulf of Mexico. The diversity of bacteria in the samples was determined both utilizing a traditional method- DGGE, and by using a cutting edge, next generation sequencing approach based on pyrosequencing analysis of the 16s amplicon.

Bacteroidetes, *Haloanaerobium*, *Halocella*, *Clostridia* and salt-tolerant sulfate reducing members of the Deltaproteobacteria including *Desulfobotulus* and *Desulfonauticus* were found to dominate the COSC oil brine. In contrast, the OSP pipeline was found to be dominated by highly aggressive SRB strains as well as iron reducing bacteria. Individual SRB were also cultured from each of these locations. Phages were isolated that showed virulent activity against the SRB cultures. Application of phage to either mixed or pure cultures inhibited growth of the SRB and thus Phage biocontrol treatment holds promise for the oil and gas industry by reducing the need for toxic chemical biocides.

TABLE OF CONTENTS

FIGURES and/or TABLES

EXECUTIVE SUMMARY

Pipeline corrosion and reservoir souring are major issues that result in elevated costs, increased health risks, and a host of operating problems for the petroleum industry (146, 147). Sulfate-reducing bacteria (SRB) pose a particular threat to the industry due to their ability to reduce sulfates to sulfides, releasing sulfuric acid and hydrogen sulfide (H_2S) as byproducts (146, 147). H_2S gas is not only extremely toxic and flammable, but it causes souring of the petroleum product, resulting in reduced quality and increased handling costs. Current technologies used to control SRB include mechanical scraping of biofilms formed in pipelines with 'pigs' and widespread application of chemical biocides. Chemical biocides are expensive, toxic to humans and the environment, and are not always effective. Improved biocides should be less toxic and more effective.

New biocides are being developed herein based on bacteriolytic agents called phage. Phage are the abundant and diverse natural viral predators of bacteria and are composed of protein and DNA (15), (18, 51, 126). There is considerable interest in developing phage products for human and agricultural purposes (54, 73, 83). Commercial phage products are available in the United States for controlling plant pathogenic bacteria on tomatoes and peppers as well as *E. coli* O157:H7 levels on slaughterhouse cattle. Importantly, these products are considered non-toxic, application does not require protective gear, and there is no wait time to handle or consume vegetables or cattle after application.

The biggest challenge facing the development of phage-based biocides is matching active phage to the target bacteria. This is because each phage recognizes and kills only a few types of bacteria and the bacterial populations present in oilfield systems are poorly understood (15). The limited number of types of bacteria that a given phage will infect is termed the phage host range. Even though it is known that the desired targets are the H_2S producing SRB, the SRB includes many different types of otherwise unrelated bacteria (8, 149). Only a few phage capable of infecting SRB have been described in the scientific literature and these were not oil industry associated SRB (34, 119, 120, 150). This means that research into target bacteria present in the oil field, as well as *de novo* phage isolation will be required prior to the production of phage for biocidal use. RPSEA funding was utilized to address these limitations. Bacterial populations from several locations were elucidated. SRB present in these samples were isolated and matched to lytic phage. Finally, the phage were demonstrated to limit SRB growth in liquid cultures.

These results are very promising. However, development of effective phage products will ultimately depend on identifying or developing phage with expanded host ranges.

BACKGROUND AND RATIONALE

The corrosion-promoting capacity of microorganisms has been recognized for over 50 years, a process often referred to as MIC, for microbial influenced corrosion (89). The most commonly used approaches to controlling MIC include the physical removal of microbial biofilms by pigging and the application of chemical biocides. However, despite active monitoring and rigorous chemical biocide applications, incidences of MIC associated pipeline failures continue to be reported (84). Often, these same biocides are shown to control MIC-associated organisms quite well in a laboratory situation following established industry practices, but their efficacy drops over time and following dilution within the product downstream. Biocide efficacy testing is usually performed by treating rapidly growing cells in defined growth media. The majority of studies utilize bacteria capable of producing either acids or H_2S, and are classified based on these phenotypes as acid producing bacteria (APB), or sulfate reducing bacteria (SRB), respectively. Treatment efficacy is usually scored by monitoring changes in population density of planktonic cells, determined by conducting a dilution series and calculating bacterial density using the most probably number (MPN) approach. There are several limitations to this approach, necessitating the need to develop more accurate monitoring and testing procedures in order to better identify truly effective treatment regimes.

Biocide efficacy assays typically assume that all SRB or APB will respond similarly to chemical treatments and thus that the specific identity of any SRB or APB is not important. This is almost certainly a gross simplification. In medical microbiology, there is a more general understanding that different bacteria can be resistant or sensitive to different antibiotics, and even to more general disinfectants. Culture swabs are routinely obtained from patients in order to evaluate bacterial antibiotic sensitivity. The problem for the O&G industry is compounded by confusion over what the terms “APB” and “SRB” actually encompasses. The terms “SRB” and “APB” are phenotypic, rather than genotypic, classifications, and numerous types of unrelated groups of bacteria are capable of acid production or sulfate reduction. The SRB, for example, are not a homogenous group of bacteria (149). Bacterial clades where sulfate reduction occurs include members of the Firmicutes, the delta subgroup of the Proteobacteria, Deferribacter, and Nitrospira as well as several archaeal clades (149). The genetic hallmarks defining SRB are the genes encoding the DsrA and DsrB dissimilatory sulfate reductase subunits (88, 148). It needs to be emphasized, however, H_2S-generating sulfur and thiosulfate reducing bacteria lack DsrA/B genes. The relative abundance and importance of sulfate, sulfur and thiosulfate reducing bacteria in generating H_2S in the oilfield is unknown. Various molecular tools such as qPCR, microarrays, and next generation “high throughput” DNA sequencing technologies have been introduced to more accurately dissect bacterial populations of relevance to the oilfield. (75) These advanced techniques have revealed that there is a larger microbial consortium that may be involved in MIC. This information is extremely viable to understand and develop a greater understanding of the resulting MIC corrosion mechanism, by understanding what bacteria are present and more directly what impact they have on the observed corrosion.

Another issue is that while biocide efficacy tests focus of planktonic cells, the organisms actually responsible for any given MIC incidence are often growing in a biofilm. Planktonic and biofilm populations may have markedly different responses to chemical treatments. Much of this may be due to the structure of the biofilm. For example, the exopolysaccharide matrix produced by biofilm organisms may exclude and/or influence the penetration of antimicrobial agents. (28, 143) Furthermore, bacteria in a biofilm may not be rapidly growing and thus can escape short term chemical exposures. Poor penetration into, and reduced mass transport of the biocide chemicals within the biofilm results in significantly less concentration of active biocide at the base of the biofilm where the problem actually exists.

Ultimately, advances in the technologies used to understand and monitor microbial corrosion mechanism need to be translated into superior field mitigation methods. MIC related issues are severe enough to justify testing highly experimental or novel approaches (92, 146). An approach that we are investigating is the use of bacteriolytic phage, the natural, highly diverse and highly abundant viral predators of bacteria (44, 73, 140, 157). The most pressing issues affecting the use of phage is the extreme host specificity that phage exhibit (15). Any one type of phage typically infects only one or a few strains of a specific bacterial species. Extreme host specificity makes phage harmless to non-target organisms. However, the downside of extreme host specificity is that developing phage treatments requires detailed knowledge of the types of bacteria that are causing the problem. The application of genomic technologies, specifically high throughput shotgun sequencing of environmental DNA samples, for bacterial identification will allow for the rational application of phages for the control of problem bacteria (31). Phages have been identified against a few SRB, specifically *Desulfovibrio vulgaris* and *Desulfovibrio aespoeensis*. (34, 49, 119, 120, 130, 134, 150) Because of the extraordinarily high genetic diversity of phages, there are no universally conserved sequences present in all phage that can be used as a molecular marker to assay for the presence of phage (17, 18, 52).

We are developing an approach to research, develop, and screen novel treatment programs designed to combat MIC within the petroleum industry. The potential for using phage to control SRB populations within the oilfield is being investigated. Towards this end, population studies of oilfield samples were conducted and the dominant SRB identified and scored. We previously reported isolating a phage capable of forming plaques on a lawn of a mixed co-culture of a *Desulfovibrio* and *Haloanaerobian* isolated from one of these environmental samples (130). The SRB from these samples were isolated in pure culture and it was demonstrated to be the actual host of phage ϕCOSC.D. Application of ϕCOSC.D to culture media prior to inoculation with the mixed Desulfovibrio and *Haloanaerobian* host was demonstrated to control growth of the SRB for extended periods of time. Future experimental plans include utilization of a genetically defined, yet highly mixed populations of bacteria in a dynamic flowcell system providing a more accurate representation of a field situation in which biofilm development occurs.

REPORT DETAILS

Experimental Methods:

Culture medium. All SRB pure and mixed cultures were propagated per NACE TMO194-94 Standard recommended Modified Postgates B Broth (MPB) consisting of, per L, 0.5 g KH_2PO_4, 1.0 g NH_4Cl, 1.0 g Na_2SO_4, 1.0 g $CaCl_2$-$6H_2O$, 2.0 g $MgSO_4$-$7H_2O$, 5.0 mL 60% syrup $NaC_3H_5O_3$, 1.0 g Yeast Extract, 2.5 g $NaC_2H_3O_2$, 0.1 g $C_2H_4O_2S$, 0.1 g $C_6H_7NaO_6$, 0.5 g $FeSO_4$-$7H_2O$, 25 g NaCl and supplemented with either 5 or 20 g/L Instant Ocean for 2.5% salinity and 6% salinity, respectively, and adjusted to pH of 7.2 prior sterilization (Appendix B). GHB and APB populations were cultured utilizing the NACE TMO194-94 Standard recommended Phenol Red Dextrose (PRD) consisting of, per L, 1.0 g Beef Extract, 10.0 g Peptone, 0.018 g Phenol Red, 5.0 g Dextrose, and 37.8 g Instant Ocean. The pH was adjusted to 8.13 with HCl and autoclaved at 121°C for 15 minutes.

Bacterial Enrichment Cultures. Approximately 4 L of anoxic black sediment with associated headspace of brine water was collected from efflux brine pond formed by a salt cavern oil storage system. Oil and H_2S was obvious in the sample. For bacterial enrichments, 0.5 ml of

the sample was inoculated into to 4.5 ml MPB, supplemented to 1%, 2.5%, 6%, and 10% salinity. Colony isolation was performed by plating liquid cultures on MPB supplemented with 16 g/L Bacto agar for bacterial colony isolation (103, 106). All bacterial incubations were conducted at 22^{o}C.

Isolation of pure SRB strains. All SRB host and phage manipulations were conducted in a Forma anaerobic chamber in a 90% N_2, 5% CO_2, 5% H_2 gas mixture. The SRB present in mixed culture COSC.D1 was isolated in pure form by preparing 10 fold serial dilutions of the mixed culture and immediately plating on MPB agar plates (130). Well-separated black colonies appeared after three weeks incubation at 22^{o} C in the anaerobic chamber. These colonies were subject to repeated dilution and plating until only black SRB colonies were observed.

Bacterial DNA Isolation. DNA was extracted from the raw sample bacterial pellets, which contain a mixture of small solids and bacterial cells, and enrichment culture with a commercially available soil DNA isolation kit (SoilMaster™ DNA Extraction Kit, Epicenter Biotechnologies) following the manufacturers recommendations. For colony PCR, DNA was isolated from single colonies using the Lyse and Go PCR Reagent (Pierce Chemicals), following the manufacturers recommendations.

16s rDNA PCR and Temperature Gradient Gel Electrophoresis, TGGE. Population structures of the samples was determined by analysis of the 16s ribosomal sequences by temperature gradient gel electrophoresis (TGGE) analysis followed by sequencing individual bands. PCR reactions were performed on the isolated DNA with primers (16s.F-GC-clamp, 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') and (16s.R, 5'-CCCCGTCAATTCCTTTGAGTTT3') and PCR reagents according to the manufacturer's recommendation (Taq DNA polymerase with ThermoPol buffer, New England Biolabs catalog. number M027L) and cycle conditions of 1 min- 30 sec- 1 min for 30 cycles). The bands were resolved by TGGE using the BioRad Dcode Universal Mutation Detection System (BioRad). PCR products were resolved on an 8% urea, 8% acrylamide, 1.25X TAE gel with a temperature range of 48^{o} to 63^{o} C at a ramp rate of 1.3^{o}C/hour, for 21 hours. The gel was stained with ethidium bromide and imaged. Band were excised and the DNA was extracted by maceration of the acrylamide slice, addition of 10 mM Tris pH 7.5, freezing overnight and pelleting of the acrylamide by centrifugation. The supernatant was used as a template in PCR reactions using (16s.F-forward 5'-CCTACGGGAGGCAGCAG-3') and 16s.R (16s.R, 5'-CCCCGTCAATTCCTTTGAGTTT3') primers and the same conditions that GC-clamp PCR was performed above. The 16s genes from pure cultures were amplified by colony PCR using the 16s.F and 16s.R PCR primers. All PCR products were purified using the QIAquick PCR purification kit (Qiagen catalog number 28106) and were sequenced with the 16s.F primer using Applied Biosystems (AB1) BigDye Terminator Cycle Sequencing Kit version 3.1 (catalog number 4337455) according to the manufacturer's recommendation. The sequences were resolved at the Institute for Plant Genomics and Biotechnology at Texas A&M University. Sequence chromatograms were trimmed and edited using Sequencher (GeneCodes). Comparisons to sequences in the public database were made using blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis of completely sequence SRB. DsrA protein sequences and 16s rRNA gene sequences were extracted from each whole genome entry. The program ClustalX 1.83 (http://www.clustal.org) was used to generate neighbor-joining trees with bootstrap values of 1000.

Identification of prophage elements in SRB genome entries. Putative prophage elements were identified in the genome entries of SRB species by screening the protein database of each species for contiguous segments at least 30 kb encoding proteins with functional annotations limited to a combination of phage associated functions and hypothetical proteins of unknown function (16, 131). Phage associated functions included phage morphogenesis, regulatory, and lysis proteins, along with the DNA metabolism proteins most commonly encoded in phage genomes. Representative entries in each block were compared to proteins in the public protein database using BLAST. The left and right most boundaries of each element were arbitrarily defined as the left and right most genes whose product exhibited significant similarity to phage proteins present in the Caudovirales protein database.

Phage Isolation. Phage were isolated using a standard phage enrichment method (132-134). Briefly, an extract was prepared from the original BM sediment by filtration through decreasingly small pore size filters, ultimately through a 0.22 mm pore size sterile filter unit. This BM extract was mixed 1:1 with MPB and inoculated with 100 ml of a week old culture of OBM2. Following seven-day incubation at 22^{o}C without agitation, chloroform was added to 0.1% of the sample volume and bacterial cell debris removed by a combination of centrifugation and filtration through a 0.22 mm pore size filter. Agar overlays were prepared my mixing 100 ml of a four-day culture of host with MPB top agar (MPB, made without Fe, augmented with 0.7 g/L agar) and pouring over MPB agar plates (made without Fe). 10 ml drops of the phage enrichment were spotted onto the lawn. Clearings were observed following seven day incubation at 22^{o}C. The concentration of phage in the sample was determined by addition of 100 ml of serially diluted phage stock into the MPB top agar/ bacterial host suspension prior to overlaying on MPB agar plates. The ability of phage ϕCOSC.D to form plaques on the lawn of the pure SRB host was tested as described for the mixed host culture, except that a four-day old stock of pure host was used as the plating host (130).

Efficacy Study in Liquid Media. Phage efficacy studies were conducted in 100 ml of 6% MPB broth in 250 ml orange-capped bottles. Each bottle was inoculated with 200 ml of host bacteria, corresponding to 10^4 SRB and either no phage (control) 0.01 ml phage, 0.1 ml phage, or 1 ml phage. Bottles were incubated at 22^{o}C for nine days with gentle shaking (92 rpm). SRB growth was monitored visually for the production of black iron sulfide and by serial dilution counting in microtitier plates. Phage prophylactic experiments were conducted by preparing 10-fold serial dilutions of the mixed host in 6% MPB media in 10 ml serum vial. Phage treated samples also received 0.1 ml of a phage lysate containing 10^7 pfu/ml ϕCOSC.D. Vials were incubated at room temperature and SRB growth scored.

Pyrosequence analysis of environmental samples, enrichment cultures and pure strains. DNA was isolated from either one ml of enrichment culture, or from 1 gram of sediment from environmental samples using the UltraClean Microbial DNA Isolation Kit (BioExpress G-3200-50). DNA was subject to bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) using primers G.28F 5'TTTGATCNTGGCTCAG and G.519r 5' GTNTTACNGCGGCKGCTG (31). Resulting sequences were trimmed and quality scored. All sequences passing quality score were compared using BLASTn to a ribosomal database to make classification. Identity values were used to make assignments to the appropriate taxonomic levels based on the following cutoffs: Sequences with identity scores, to known or well characterized 16S sequences, greater than 97% identity (<3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family and between 85% and 90% at the order level, 80 and 85% at the class and 77% to 80% at phyla level.
DNA was isolated from one ml of pure culture using the UltraClean Microbial DNA Isolation Kit (BioExpress G-3200-50). The 16s coding region was amplified from each DNA sample using

primers 16s.F (CCTACGGGAGGCAGCAG) and 16s.R (CCCCGTCAATTCCTTTGAGTTT) using the AcuPrime PCR master mix (Invitrogen) following the manufactures protocol. PCR products were sequenced using BigDye terminators (ABI) at Eton Bioscience Inc (California).

Identification of prophage elements in SRB genome entries.
Putative prophage elements were identified in the genome entries of SRB species by screening the protein database of each species for contiguous segments at least 30 kb encoding proteins with functional annotations limited to a combination of phage associated functions and hypothetical proteins of unknown function (16, 131). Phage associated functions included phage morphogenesis, regulatory, and lysis proteins, along with the DNA metabolism proteins most commonly encoded in phage genomes. Representative entries in each block were compared to proteins in the public protein database using BLAST. The left and right most boundaries of each element were arbitrarily defined as the left and right most genes whose product exhibited significant similarity to phage proteins present in the Caudovirales protein database.

Phylogenetic analysis of completely sequence SRB.
DsrA protein sequences and 16s rRNA gene sequences were extracted from each whole genome entry. The program ClustalX 1.83 (http://www.clustal.org) was used to generate neighbor joining trees with bootstrap values of 1000.

Results and Discussions:

Impact to Producers

Phage have similar inhibitory effects on active SRB cultures as do currently used chemical biocides. Furthermore phage biocontrol treatments are naturally "green" and have a longer lasting inhibitory effect; thus, implying that phage based biocontrol can provide a better treatment option for the petroleum industry to counter microbially influenced corrosion and possibly reservoir souring.

Technology Transfer Efforts

Ecolyse has given more than 4 presentations at RPSEA conferences and meetings. Continued commercialization efforts are underway with RPSEA members. See Appendix G for a partial list of presentations.

A. Sample Collection: COSC and OSP

COSC Sample Details

Approximately 4 L of a high-salinity black mud (BM) sample from a brine pond congruous with a gulf coast crude oil salt storage cavern COSC. The sample was composed of black mud (BM) and sand, with a heavy petroleum component. The sulfate reducing bacteria (SRB) activity was obvious, as evidenced by H_2S emissions from the sample.

OSP Sample Details

The second set of samples were collected from an on shore pipeline (OSP) that receives crude oil from pipelines originating from off shore sources in the Gulf of Mexico. The pipeline is approx. 36 miles of 12", 0.562 wall pipe. This pipeline has a history of

rapid MIC incidences, in particular at girth welds downstream of a certain production platform. Corrosion was exasperated following an eight month line shut- due to Hurricane Katrina. Fluids transported by this line consists of approximately 75% produced water with a total dissolved solid (TDS) between 120,000 and 140,000 ppm, and 25% crude oil approximately. Three production streams feed into OSP. One of the production lines carries crude and other production fluids from production systems in different formations that come into a platform and commingles with local production. Further, production feeds into this line as well. A service company treats the pipeline continuously with corrosion inhibitor and a second separately treats the production field. What chemicals are used on these two platforms and whether any chemical incompatibilities may exist is unknown. In the final analysis, the total fluids being transported through OSP are commingled from numerous production zones within different formations from the Gulf of Mexico.

A total of eight samples were collected from the pipeline. Seven of these were taken in a timed sequence in front of a pigging run. The eighth sample is the wax and biofilm solids from the pigging run. For each time point, 500 ml of water and associated oils were collected. At each time point, the alkalinity, temperature, pH, dissolved H_2S, CO_2, and O_2 levels of the sample were measured (Table 1, OSP Field Chemistry Analysis). Additionally, a full water analysis was also performed for each sample (Appendix A). Sulfate levels ranged from 43.1 to 54.5 ppm. This is greater than the minimum concentration required for maintaining regular microbial activity. Sulfide was detected in samples at 2.4 ppm. Sulfide may be biogenic or abiotic in origin, evolved as the by-product of SRB-mediate sulfate reduction or via the geothermal degradations of sulfur containing rock. Volatile fatty acid levels were less than 2 ppm. Commonly used corrosion inhibitors were present at undetectable levels.

Sample	Sampling Point	Alkalinity	TOSP (*F)	pH	Dissolved H_2S	Dissolved CO_2	Dissolved O_2
1	920 Barrels	480 ppm	82.4	6.0	0.2 ppm	160 ppm	< 20ppb
2	4400 Barrels	546 ppm	86.0	6.5	0.1ppm	170 ppm	< 20ppb
3	9300 Barrels	456 ppm	82.4	6.0	0.3ppm	160 ppm	< 20ppb
4	12,000 Barrels	598 ppm	78.8	6.0	0.2 ppm	120 ppm	< 20ppb
5	21,400 Barrels	436 ppm	82.4	6.0	0.0 ppm	160 ppm	< 20ppb
6	26,100 Barrels	480 ppm	84.2	6.0	0.0 ppm	160 ppm	< 20ppb
7	34,000 Barrels	500 ppm	84.2	6.0	0.0 ppm	130 ppm	DNP
8	26,100 Barrels.c	nd	nd	nd	nd	nd	nd

Table 1. Field Chemistry Analysis of the OSP pig samples analyzed immediately following sample collection, utilizing the field test kits by Hach for alkalinity and Chemetrics for H_2S, CO_2, and O_2. . Sample 26,100 Barrels. c is from a coupon sample. nd, not determined.

Both SRB and APB were detected by MPN analysis in the planktonic phase of every sample taken, ranging from 10^4 to 10^5 SRB/ml and 10^1 to 10^2 APB/ ml sample (Table 2). The ATP assays indicated microbial levels of between 10^5 to 10^7 cells per ml

sample. Taken together, this data suggests that there exists a high level of contamination and/or the presence of a biofilm in the system.

OSP Sample	Combined	Sample	GHB	APB	SRB	ATP Analysis
OSP.1	OSP.A	920 Barrels	2.5 X 10^2 /mL	2.5 X 10^1 /mL	4.5 X 10^4 /mL	7.8 X 10^5 /mL
OSP.2	OSP.B	4,400 Barrels	2.5 X 10^1 /mL	2.5 X 10^1 /mL	1.5 X 10^5 /mL	1.3 X 10^6 /mL
OSP.3	OSP.A	9,300 Barrels	9.5 X 10^1 /mL	2.5 X 10^1 /mL	2.5 X 10^5 /mL	9.1 X 10^5 /mL
OSP.4	OSP.B	12,000 Barrels	9.5 X 10^1 /mL	9.5 X 10^1 /mL	9.5 X 10^4 /mL	4.9 X 10^6 /mL
OSP.5	OSP.B	21,400 Barrels	2.5 X 10^2 /mL	4.5 X 10^1 /mL	4.5 X 10^5 /mL	1.3 X 10^7 /mL
OSP.6	OSP.C	26,100 Barrels	2.5 X 10^3 /mL	2.5 X 10^1 /mL	2.5 X 10^4 /mL	6.8 X 10^5 /mL
OSP.7	OSP.B	34,000 Barrels	4.5 X 10^2 /mL	1.5 X 10^2 /mL	4.5 X 10^4 /mL	2.2 X 10^6 /mL
OSP.8	OSP.D	26,100 Barrels.c	0.9 X 10^0 /in^2	0.4 X 10^0 /in^2	9.5 X 10^2 /in^2	nd

Table 2. Enumeration of bacteria in OSP samples 1 through 8 by culture-based MPN and an ATP quantification assay. GHB, general heterotrophic bacteria; APB, acid-producing bacteria; SRB, sulfate-reducing bacteria. The data presented in this table is a quantification of the viable planktonic bacterial population (suspended bacteria) and the viable sessile bacteria population (attached bacteria). GHB and APB levels were assayed by monitoring growth in PRD at 14 days incubation at 30°C. SRB levels were assayed by monitoring growth in MPB after 28 days of incubation at 30°C. ATP assays were conducted utilizing the Luminultra^{tm} Quench-Gone Aqueous kit^{tm} (QGA). nd, not determined (due to small sample volume and high oil content).

B. SRB Isolation

Isolation of mixed and pure SRB cultures from the COSC and OSP samples

The initial efforts to isolate phage were based on classical methodologies that require pure cultures of each bacterial host to utilize as bait in phage hunts and to prepare overlays for plaque visualization. The traditional method used to isolate bacteria in pure culture is the streak plate method, in which bacteria are subject to multiple rounds of colony isolation. The assumption is that each colony originates from a single bacterial cell and develops into a clonally homogeneous population. Initial efforts to purify SRB strains from the COSC and OSP samples involved preparing spread plates directly onto solid agar MPB plates. Simultaneously, aliquots were inoculation of aliquots directly into liquid MPB media. For bacteriological and DNA based diversity analysis, the 8 WD-73 samples were clustered into four groups, OSP.A (samples 1 and 3), OSP.B (samples 2,4,5,7), OSP.C (sample 6), and OSP.D (sample 8) (Table 2). Black growth, indicative of SRB activity, was observed only in the liquid media and not detected on solid media plates. When aliquots of the SRB positive liquid enrichments cultures were used to prepare spread plates, black SRB colonies, as well as white non-SRB colonies, did form. The initial black colony formation required between three and six weeks of incubation. Colonies of both the non-SRB and SRB were subject to numerous rounds of colony purification. The non-SRB strains from COSC white colonies on MPB were readily purified to homogeneity. In contrast, even after between 10 to 20 rounds of colony streaking, SRB from both the COSC and OSP samples categorically propagated only as co-culture with the non-SRB. The partially purified SRB cultures from the COSC sample were referred to as COSC (for “brine mud oil, black”) COSC.A1, COSC.A2, COSC.B1, COSC.B2, COSC.C2, COSC.D and COSC.2. The purified non-SRB cultures were named (BmoW for “brine mud oil, white”) BmoW.31, BmoW.21, BmoW.23, BmoW.11, BmoW.13 and BmoW.4.

COSC OSP Bacterial Strains	Media Salinity	Sequence ID	ϕCOSC2	ϕCOSCD	SRB phage**

COSC.2	6%, 10%	*D. halophilus* (99%)	*+	-	-
COSC.D1	6%, 10%	*D. halophilus* (99%)	-	*+	-
OSP.A1	2.5%,	*D. senesii* (99%)	-	-	-
OSP.C1	2.5%,	*D. caledoniensis* (99%)	-	-	-
OSP.D1	2.5%,	*D. senesii* (99%)	-	-	-
OSP.A	2.5%,	nd	-	-	-
OSP.B	2.5%,	nd	-	-	-
OSP.C	2.5%,	nd	-	-	-
OSP.D	2.5%,	nd	-	-	-
COSC.A1	6%, 10%	*Haloanaerobium*, Deltaproteobacteria SRB	-	-	-
COSC.A2	6%, 10%	*Haloanaerobium*, Deltaproteobacteria SRB	-	-	-
COSC.B1	6%, 10%	*Haloanaerobium*, Deltaproteobacteria SRB	-	-	-
COSC.B2	6%, 10%	*Haloanaerobium*, Deltaproteobacteria SRB	-	-	-
COSC.C2	6%, 10%	*Haloanaerobium*, Deltaproteobacteria SRB	-	-	-
COSC.D1 (COSCD1 source)	6%, 10%	*Haloanaerobium*, Deltaproteobacteria SRB	-	*+	-
COSC.2 (COSC2 source)	6%, 10%	*Halanaerobium praevalens, Desulfovibrio halophilus, Desulfovibrio frigidus, Bacteroides thetaiotaomicron*	*+	-	-
BmoW.31	6%, 10%	nd*	-	-	-
BmoW.21	6%, 10%	*Clostridium* spp.	-	-	-
BmoW.23	6%, 10%	*Clostridium* spp.	-	-	-
BmoW.11	6%, 10%	*Clostridium* spp.	-	-	-
BmoW.13	6%, 10%	*Clostridium* spp.	-	-	-
BmoW.4	6%, 10%	*Haloanaerobium*	-	-	-

Table 3. Summary of OSP and COSC bacterial strain and phage isolation. Bacterial strain, culture media salinity, taxonomic identification, and SRB phage sensitivity. ** Ecolyse SRB phage collection tested: (fDala.1, fDala.2, fDala.3, fDala.4, fDala.CJ1, fEBS14.1, fEBS14.2, fEBS14.3, fEBS14.4, fEBS14.5, f EBS14.6, fEPF27, fEPF27.2, fEPF27.3, fEPF27.4, fWRF 20.1, fWRF20.2, fWRF20.3, fPDS.7).

The observation that SRB could be propagated from the OSP and COSC samples, but only as co-cultures strongly suggested that culture conditions were not adequate for SRB growth as pure cultures and that the non-SRB is compensating for the limiting step in SRB growth. This could possible due to nutrient limitations or due to physiological stress. Physiological stresses that might impede SRB pure culture isolation include residual oxygen or a sub-optimal reducing environment. Multiple types of media and supplements were assayed in an effort to determine if nutrient limitations were responsible for the purification recalcitrance (Appendix B). Media modifications evaluated included vitamin supplementation (thiamine, B12, PAPA), additional reducing agents and oxygen scavengers (resazurin, cysteine HCl) and even crude oil (data not show) in an effort to improve pure culture recovery.

Once conditions for culturing SRB were better established, then pure strains of SRB from the COSC, OSP, as well as numerous additional oilfield samples were isolated. Sequence analysis of the 16s rRNA gene in each of these indicated that COSC2 and COSC2 are most similar to *Desulfovibrio halophilus.* The 16s region sequenced was found to be 100% identical to nucleotides 364 to 877 of *Desulfovibrio* sp. (in GenBank entry GI:1297336). Both of these species are 99% identical to the salt tolerant *Desulfovibrio* species, *Desulfovibrio halophilus* (159) OSP.A and OSP.D are most similar to *D. senesii*, OSP.C is most similar to *D.*

caledoniensis. Because of project time constraints, these partially purified cultures were utilized in downstream experimentation.

C. Bacterial Diversity from COSC and OSP

Bacterial Diversity Analysis Using Traditional Methods: DGGE

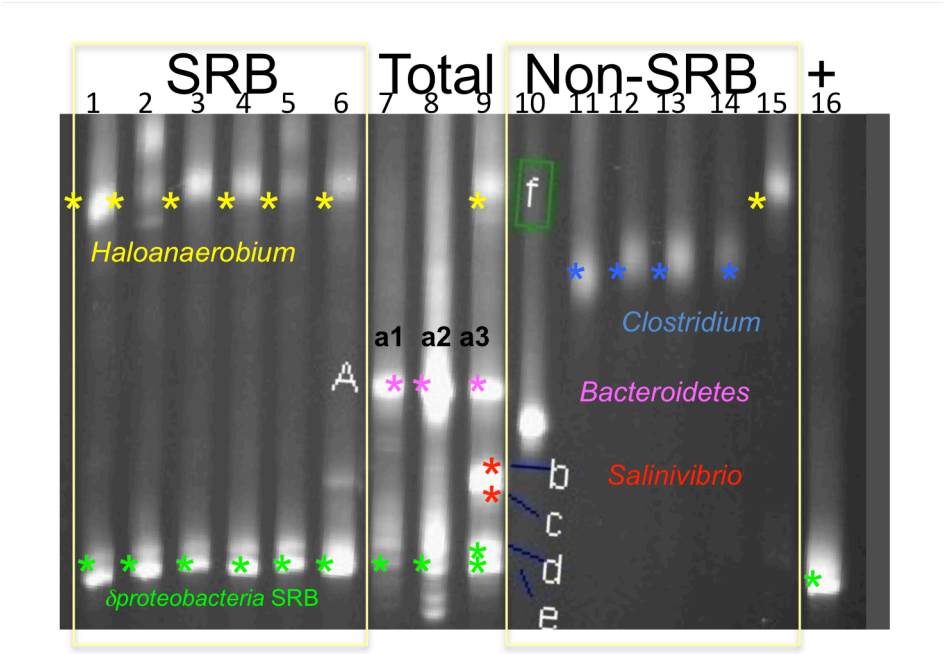

Figure 1. TGGE Population analysis of uncultured and cultured bacteria present in the COSC sediments. Image is of an ethidium bromide stained, 8% urea, 8% acrylamide gel on which the 16s rRNA gene sequences PCR amplified from bacterial DNA samples were resolved by temperature gradient gel electrophoresis. Lanes 1 through 6: SRB strains (COSC.A1, A2, B1, B2, C2, and D1); Lanes 10 through 15: non-SRB (BmoW.31, 21, 23, 11, 13, and 4) strains. Lanes 7, 8, 9: total bacteria from COSC sample at time of collection (T1), following a 2 month storage of the sample at 4°C (T2) or following enrichment for 7 days in 10% salinity MPB media (T3). Lane 16: TGGE product from pure Desulfovibrio vulgaris DNA template. Bands labeled A, (a1, a2, a3) b, c, d, e and f were excised from this gel for sequence analysis. Taxonomic assignments based on these sequences are color-coded.

The initial approach used to simultaneously evaluate bacterial diversity in the COSC samples and to compare cultivated strains to the wild type populations were conducted by denaturing gradient gel electrophoresis (DGGE) based methodology called TGGE (temperature gradient gel electrophoresis (Figure 1). This approach is based on determining the 16s rRNA complexity in a sample by electrophoresis of the 16s rRNA PCR product on a denaturing gel. Resolution into more than one band implies a mixed rRNA sequences are in the sample, suggestive of more than one type of bacteria. DNA was isolated from six the colony-purified SRB cultures (COSC.A1, A2, B1, B2, C2, D), and six colony-purified non-SRB cultures (BmoW.31, 21, 23, 11, 13, 4). As a control, 16s PCR products using total DNA from the COSC sample as well as from a pure culture of *Desulfovibrio vulgaris* str. Hildenborough as template was analyzed simultaneously. For analysis of the total bacterial population in the COSC sample, total bacterial DNA was isolated from the anoxic brine sediment following three storage conditions: T1 (within one week of collection of the sample and storage at 4^{o}C, considered to be the most similar to the initial environmental sample), T2 (following a two month incubation of the unprocessed environmental sample at 4^{o} C) and T3 (following incubation of 1 ml of the sample for 7 days in MPB 10% salinity media at 22^{o}C).

As expected, the total bacterial fractions T1, T2, and T3 are complex, with multiple bands. All three samples possessed a brighter band (A) with a medium migration rate (bands a1, a2, and a3) as well as several bands that exhibited the fastest migration rates. Samples T1 and T2, corresponding to bacterial DNA isolated from the unprocessed sample at one week and two months storage at 4^{o}C, were more similar to each other then to sample T3, which had undergone incubation in MPB 10% salinity media for one week. Sample T3 possessed novel high intensity bands clustered at two positions on the gel, bands b, c, and f. Each of the SRB cultures resolved at least two distinct bands while only a single band was present for each of the non-SRB cultures. For comparison, the product derived from amplification of the 16s gene from *D. vulgaris* migrated as a single, fast moving species that co-localized on the gel with the fastest migrating bands from the SRB cultures and the total bacterial population samples. This suggests that the SRB cultures contain a population that includes at least one member similar to *D. vulgaris* and one member similar to the non-SRB pure culture BmoW.4. Bands from three of non-SRB, colony purified cultures BmoW.31, BmoW.21, and BmoW.23 were found to be 100% identical with each other and were determined to be Clostridial species whose closest characterized relative was identified as forming a lactate degradation pathway along with SRB including *Desulfobulbus* spp. in sulfidogenic bioreactors (158). The mixed SRB cultures were determined to be a deltaproteobacterial SRB and a *Haloanaerobium*. This was determined by pyrosequencing the COSC.2 culture was found to be composed of *Desulfovibrio* species, primarily *D. halophilus* but also very low levels of *D. frigidus* as well as a *Haloanaerobium* most similar to *H. praevalens.*

Bacterial genera present in the different samples was determined by excising seven of the bands resolved by TGGE (Figure 1 bands a1, a2, a3, b, d, e, and f), re-amplification of the 16s PCR product, and sequencing. This resulted in sequences TGGE.A1, TGGE.A2, TGGE.A3, TGGE.B, TGGE.C TGGE.D, TGGE.E, TGGE.F, respectively. Sequences derived from the non-cultured bacterial genera in the total bacterial populations were analyzed, bands a1, a2, and a3 represent the major band present in both the newly isolated and cultured in the mixed bacterial population samples. The closest relatives of these bacteria present in the public database were also identified by culture-independent 16s analysis performed on other hypersaline environments, including the anoxic hypersaline mats from the Mediterranean and hypersaline sediments from the Great Salt Lakes, Utah (69, 91). These bacteria appear to be members of the Bacteroidetes. The sequence derived from band c, which became evident only following total enrichment culture indicated they were derived from *Salinovibrio* sp. *Salinovibrio* are gammaproteobacteria somewhat related to pathogenic *Vibrio* spp. *Salinovibrio* spp are

facultative anaerobes frequently isolated from saline soils, lakes, and even salt preserved foods. These bacteria are not considered at target organism for control. The slowest and fastest migrating bands from enrichment culture T3 co-migrated with bands from the purified bacterial cultures. The sequence from the slowest migrating band, f was found to be only two nucleotides different from pure culture strain BmoW.4. These were found to be 99% identical to *Haloanaerobium* species and uncultured clones identified in several metagenomic analysis of high salinity, anoxic environments. *Haloanaerobium* species have been isolated from other high salinity, petroleum associated locations and have also been demonstrated to engage in interspecies hydrogen transfer (19, 94). Some *Haloanaerobium* have been shown to be sulfur and thiosulfate reducing bacteria but are not sulfate reducing bacteria (110). Visual analysis of chromatograms from the mixed SRB cultures clearly indicated that a *Haloanaerobium* sequence was present in all of the isolated SRB cultures for which sequence data was obtained (COSC. A1, A2, B1, C2). Finally, two of the sequences obtained from the fastest migrating bands in sample T3, TGGE.D and TGGE.E were found to be 88% identical to each other with 460 out of 522 nucleotides matching. A comparison with the public database revealed that they were both likely derived from sulfate reducing bacterial members of the deltaproteobacteria. The sequence of TGGE.E was found to be >97% identical to genebank entries from numerous uncultured deltaproteobacteria isolated from a variety of high saline environments including the anoxic sediments of a Mediterranean saltern hypersaline microbial mat from Puerto Rico,(60) and a hypersaline lake in Tibet (60, 63, 91). The most closely related bacterium from a cultured sample was found to be Desulfovibrionales enrichment culture clone SLAb1-3 (12). This sulfate reducing bacteria was isolated using enrichment in lactate-arsenate medium from Searles Lake in California, a salt saturated, alkaline, high arsenate and high borate content body of water. The sequence of TGGE.D was found to be 99% identical (511/513 bp) to *Desulfohalobium retbaense* DSM 5692 16s rRNA sequence. *D. retbaense* DSM 5692 is a deltaproteobacteria isolated from saline sediment from Retba Lake in Senegal, Africa (98). Other *Desulfohalobium* species include the sulfate reducing bacteria *Desulfohalobium utahense*, isolated from the high salinity Great Salt Lake (62).

Bacterial Diversity Analysis Using Metagenomics Approach

SRB Diversity in COSC and OSP Samples.

The low resolution of bacterial populations afforded by the utilization of DGGE was determined to be insufficient for elucidating bacterial diversity of the RPSEA samples. Therefore, a new approach was utilized based on a metagenomics approach first established for medical samples. This approach, called 454 or pyrosequencing, is based on shotgun sequence analysis of 16s rRNA amplicons (Figure 2).

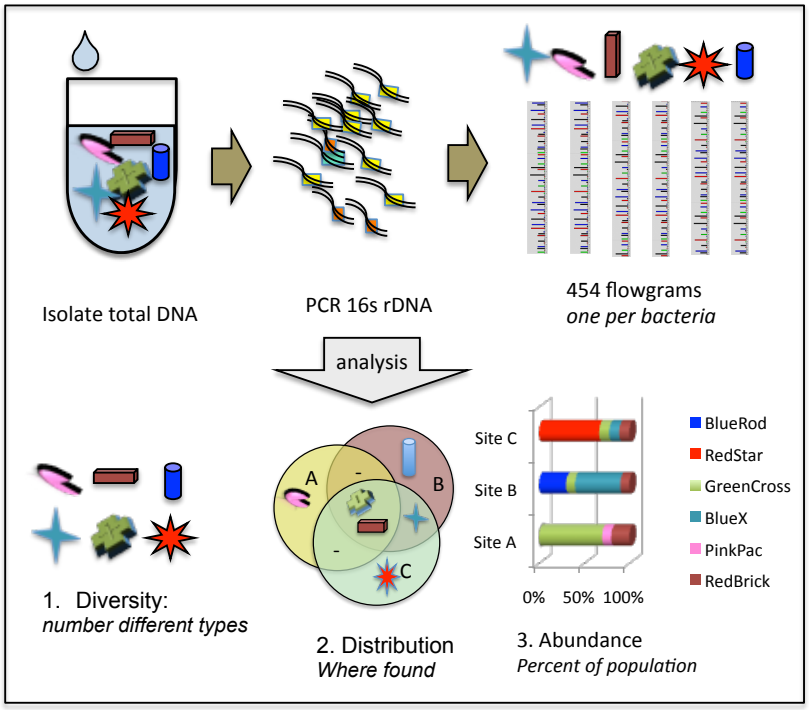

Figure 2. **Overview of the Pyrosequencing workflow and data analysis pipeline.**
A. Generating the data: Total DNA is isolated from an environmental sample containing numerous different types of bacteria, where each type might be present at widely different percentage of the population. A 454 library is then generated from the DNA utilizing a PCR based method. Sequencing reactions are conducted at a scale of between 2,000 to 10,000 sequences; each sequence being generated from a single bacteria in the starting sample.
B. Analyzing the data: The raw sequences are matched to potential source bacterial identifications by comparing to a ribosomal database derived from known bacterial strains, as well as from other environmental shotgun sequences. The frequency of the occurrence of any given species is directly correlated to the concentration of those bacteria in the starting sample. Identified bacteria are matched to a database of metabolic traits of relevance to the oil industry. Then, the diversity (how many types), distribution (where found) relative abundance (percentage of overall population) can be evaluated.

Pyrosequencing results in thousands of individual DNA sequences from each sample, each individual DNA sequences originated from a single bacterial cell in the starting sample (Figure 2). Shotgun pyrosequencing of 16s amplicons is far superior to DGGE, or cultivation methods such as MPN analysis, because the method is less biased and provides details on a much higher number of individual bacteria. These traits confer statistical robustness to the data. Even unculturable bacteria comprising as little as 0.01% of the population (1 cell per every 100 cells) can be robustly identified 99% of the time through sequencing as few as 1,000 bacterial cells.

Low-level pyrosequencing runs were performed on samples COSC (12/4), COSC (2/25), OSP.A, OSP.B, OSP.C, and OSP.D. Between 2,447 and 5,763 individual bacteria were analyzed from each sample, for a total of 22,499 bacteria screened in all (Table 3, Appendix C). From these 22,499 bacterial sequences, 246 sequence clusters were generated, each one corresponding to an operational taxonomic unit, or species. The number of species identified in each sample varied from 70 to 108. Most bacterial species identified were present as “low abundance” organisms, with 223 of the 246 species being present at less than 1% of the total population in any one sample. Only 23 species were present at greater than 1% of the population of any one sample (Table 4). The most abundant bacteria overall were members of anaerobic fermentative and sulfidogenic genera such as *Halanaerobium, Cytophaga, Halocella, Pelobactera, Desulfohalobium, Desulfonauticus,* and

Desulfovibrio. Several species, including *Haloanaerobium saccharolyticum*, *Desulfovibrio gabonensis*, as well as uncharacterized *Anaerophaga, Peptostreptococcaceae*, and *Lactobacillus* species, were present in all six samples.

Sample:	COSC 12/4 Eco-4	COSC 2/25 Eco-5	OSP.A Eco-8	OSP.B Eco-9	OSP.C Eco-10	OSP.D Eco-11	Total
# Bacteria Analyzed:	2573	3810	2776	5130	5763	2447	22,499
# Species Identified:	62	108	70	105	77	105	246
Metabolism: abundance (% of population) ; diversity (number of species)							
Sulfidogens	9.66% ; 13	10.08% ; 23	54.1% ; 14	51.75% ; 26	75.67% ; 24	54.5% ; 23	42.6% ; 47
SRB	*9.62% ; 12*	*10.02% ; 21*	*8.13% ; 10*	*4.54% ; 21*	*3.33% ; 19*	*6.49% ; 13*	*7% ; 36*
Ferm + APB	7.24% ; 15	69.01% ; 24	18.75% ; 16	22.87% ; 19	16.35% ; 14	14.12% ; 18	24.7% ; 37
Biodeg	62.85% ; 6	15.57% ; 9	5.26% ; 5	1.97% ; 11	0.87% ; 8	10.25% ; 16	16.1% ; 22
Fe(III)RB	0% ; 0	0.03% ; 1	15.74% ; 3	21.1% ; 7	5.09% ; 4	14.46% ; 6	9.4% ; 9
other	20.31% ; 28	5.48% ; 51	6.21% ; 32	2.39% ; 42	2.05% ; 27	6.58% ; 42	7.02% ; 131

Table 4. Metagenomic analysis of COSC and OSP Bacterial Populations. The total number of bacteria analyzed from each sample is given, along with the corresponding number of unique species identified. These species were classified by metabolic traits, shown both as a percent of the total population and the number of unique species that exhibit the indicated metabolic trait. Metabolic abbreviations: APB, acid producing bacteria; BioDeg, biodegradation of atypical substrates (for example, hydrocarbons), Fe(III)RB, iron reducing bacteria; ferm, fermentative; NRSOB, nitrate reducing, sulfur oxidizing bacteria; sulfidogen- hydrogen sulfide generating; SRB, sulfate reducing bacteria. Taxonomic breakdown at a Class level is also given, with percent of total population and number of unique species.

Metabolic Assignments

Metabolic assignments were given to 145 of the 246 species identified in the six samples (Table 4). This included all bacteria present in at least 1% of the total population of at least one sample. In all, over 98% of the bacteria present in the six samples received some level of metabolic classification (Table 4). The metabolic annotations are included in the table containing all identified species in the project (Appendix C).

Sulfidogenisis and Iron Reducing Bacteria

The metabolic pathways of most interest to the oilfield community are those that generate significant levels of hydrogen sulfide. **Sulfate reducing bacteria** (**SRB**) are particularly aggressive at sulfide production and are the group of bacteria most commonly implicated oil filed biogenic sulfide production (8). However, sulfur- and thiosulfate- reducing bacteria (SuRB and TRB, respectively) can also generate significant levels of H_2S and contribute to corrosion and souring (3) (80).Compared to SRB, the TRB are harder to classify based on taxonomically as they are members of bacterial genera that can include non-tSRB members. Examples of sulfidogenic TRB commonly found in oilfield samples include *Halanaerobium congolense*, as well as some *Thermoanaerobacter*, and *Spirochaeta.*

All six RPSEA samples contained significant levels of sulfidogenic bacteria, both in terms of relative abundance in the total population and in terms of species diversity. Overall, over 42% of all bacteria identified, encompassing 47 unique species, were capable of hydrogen sulfide generation. True, sulfate-reducing bacteria were the most abundant sulfidogens in the two COSC samples, composing around 10% of each sample. SRB present in the COSC samples included *Desulfobotulus, Desulfonauticus, Desulfovibrio*, and *Desulfocaldus, Desulfosalina*.

In contrast to the COSC sample, even though "true SRB" were present in the OSP samples, the most abundant sulfidogenic species detected in all four OSP combined samples was most closely related to a thiosulfate, and sulfur reducing species, *Halanaerobium congolense*. *H. congolense* was first identified in a produced water sample from a Congo offshore oil field (111).

Iron reducing bacteria

Many bacteria have the capacity to directly solubilize iron. For example, *Shewanella* species produce chelators that solubilize Fe(III) oxides (Lovley et al, 2004). Additionally, *Shewanella* is capable of growing in corrosive biofilms. *Shewanella* has also been shown to remove the protective H_2 film layer that normally protects iron surfaces from corrosion under anoxic conditions. The OSP samples were particularly enriched in two iron reducing bacterial genera: *Pelobacter* and *Geobacter*. Along with *Desulfuromonas* species, which are both sulfidogenic and iron reducing, nine different iron reducing species were found to comprise between 5% and 21% of all bacteria in the sample. In contrast, iron-reducing bacteria constituted only negligible percentage of the COSC sample populations.

Fermentative and Acid Producing Bacteria

Acid producing bacteria (APB) are of specific interest to the oilfield community as acid production directly and aggressively promotes corrosion. Several metabolic pathways result in the production of acids, including fermentative pathways that generate organic acids such as lactic acid and acetic acid, as well as those that generate sulfuric acid as a byproduct of the oxidation of inorganic sulfur compound oxidation. It should be noted that not all fermentative pathways result in acidification, and the identification of a bacteria as acid producing does not necessarily indicate acidification of bulk fluids. In particular, the link between fermentative pathways and environmental acidification requires more research to clarify. Because of this, fermentative and acid producing bacterial levels are reported together, even though not all bacteria included in this classification contribute to bulk acidification.

A total of 37 species were annotated as acid producing bacteria and/or fermentative. The percentage of the population in the four OSP samples ranged from 14% to almost 25%, with between 14 to 19 species annotated as such in each sample. The majority of these were Halanaerobium species. With in the COSC samples, the population that emerged from the sample after two months storage at 4^{o}C was significantly enriched in *Halocella*, which increased from 4% of the population in COSC 12/4 to 51% of the population by 2/25. This population profile switch was balanced by a reduction in the relative levels of hydrocarbon degrading *Cytophaga* in the sample, which declined from 62% to 12% during the two months of storage.

Hydrocarbon Degrading Bacteria

Hydrocarbons, including alkanes, alkenes, aromatic hydrocarbons, and waxes, are found naturally in great variety in crude oil and other petroleum compounds. Due to their structural diversity, most bacteria lack the capacity to utilize petroleum hydrocarbons as food sources. Each type of hydrocarbon-degrading microorganism is likely to be capable of metabolizing a few specific types of hydrocarbons. The sample with the highest proportion of biodegrading species was the initial COSC sample, at 62% of the population- primarily due to the already mentioned abundance of Cytophaga in this sample. Abundant genera in the samples include *Marinobacter* and *Paenibacillus*.

Species	COSC 12/4	COSC 2/25	OSP. A	OSP. B	OSP. C	OSP. D	Metabolism Lookup	Ref
Halanaerobium congolense	0	0	44.92	46.84	72.18	46.71	Sulfidogen, TRB, Ferm	(111)
Cytophaga sp	62.3	12.5	1.04	0.51	0.15	0.94	BIoDeg HC, oilfield	(113)
Halocella sp	4.59	51.6	1.12	0.14	0.67	0.65	Ferm, oilfield	(108)
Pelobacter sp	0	0	11.38	19.69	4.83	12.38	Fe(III)RB	(76)
Halanaerobiaceae sp	0	0.37	8.32	8.21	2.73	4.54	Ferm	(99)
Halanaerobium saccharolyticum	1.75	9.69	2.52	3.8	3.51	2.57	Ferm, promotes sulfidogens	(68)
Halanaerobium sp	0	2.7	3.75	6.34	4.55	3.56	Ferm, promotes sulfidogens	(61)
Halanaerobium acetethylicum	0	2.23	1.08	3.76	4.26	1.8	Ferm, promotes sulfidogens	(99)
Desulfohalobium retbaense	0	0	5.66	2.36	0.58	3.02	Sulfidogen, SRB	(125)
Pseudomonas sp	7.93	1.5	0.79	0.45	0.17	0.57	BioFilm	(109)
Desulfonauticus autotrophicus	1.17	6.25	0.04	0.02	0	0	Sulfidogen, SRB	(85)
Geobacter sp	0	0	4.32	1.15	0.17	1.72	Fe(III)RB	(76)
Paenibacillus sabinae	0.12	1.92	2.09	0.66	0.29	2.17	BioDeg PAH	(64)
Desulfobotulus sp	6.45	0.55	0	0	0	0	Sulfidogen, SRB	(121)
Deinococcus sp	5.01	0.37	0	0	0	0	unkwn radiation resistant	(26)
Marinobacter sp	0	0	0	0.21	0.09	4.82	BioDeg HC	(154)
Desulfovibrio sp	0	0.05	1.12	1.01	1.14	1.19	Sulfidogen, SRB	(8)
Leptotrichia sp	3.85	0.31	0.04	0.04	0	0	pathogen	(33)
Arcobacter sp	0.04	0	0.29	0.29	0.72	1.63	NRSOB	(57)
Desulfovibrio gabonensis	0	0.03	0.54	0.41	0.17	1.19	Sulfidogen, SRB	(139)
Sphingomonas sp	0	0	1.73	0.27	0.1	0.16	BioDeg phenanthrene	(55)
Peptostreptococcaceae	0	0.21	0.25	0.1	0.2	1.14	BioFilm MIC	(13)
Desulfuromonas palmitatis	0	0	0.9	0.25	0.1	0.57	Sulfidogen, SuRB Fe(III)RB	(76)
Mariprofundus ferrooxydans	0	0	1.08	0.33	0.12	0.29	Fe(II)OX MIC iron corroding	(86)
Desulfocaldus sp	0.74	0.87	0	0	0.02	0.04	Sulfidogen, SRB	(88)

Desulfosalina sp	0.47	0.52	0	0	0.09	0.08	Sulfidogen, SRB	(1)
Caminicella sp	0	0	0.86	0.04	0.2	0.04	Ferm thermophile spore-former	(4)
Paenibacillus sp	0.08	0.26	0.29	0.16	0.05	0.25	BioDeg PAH	(50)
Desulfovibrio capillatus	0	0	0	0.1	0.7	0.2	Sulfidogen, SRB	(90)

Table 5. Bacterial species present at more than 1% (green) of the total population of samples COSC (one week post collection), COSC (one month post collection), and OSP.A, OSP.B, OSP.C, and OSP.D are listed, along with the percent abundance in that sample and metabolic assignments. Metabolic abbreviations: Ferm, fermentative; SuRB, sulfur reducing bacteria; TRB, thiosulfate reducing bacteria; BioDeg, biodegradation of atypical substrates such as HC (hydrocarbons), PAH (polyaromatic hydrocarbons), and complex polysaccharides; Fe(III)RB, iron reducing bacteria; NRSOB, nitrate reducing, sulfur oxidizing bacteria. Yellow are MIC associated species.

Conclusions From Bacterial Diversity Analysis

The rationale and practical use of bacteriophage to control problem bacterial populations requires a working knowledge of the target bacteria (Table 5). For human pathologically significant bacteria, a large body of literature on types and abundance of bacteria that cause specific problems is available. The strikingly difference in the bacterial population following a one-week enrichment in culture media was not unexpected. It should be noted, however, that while all identification methods are inherently biased, culture-based methods are considered to be particularly prone to bias (31). However, the population of bacteria identified in this sample, even after culturing, was similar to bacteria isolated from other anoxic, high saline locations ranging from Tibet to Africa and North America. This suggests that it should be possible to generate reasonable predictions of the SRB population profile of high saline, oil rich environments, which will be important for genera specific control.

D. Analysis of Temperate SRB Phage Diversity

Phylogenetics of Sequenced SRB

The diversity of SRB identified in the COSC and OSP samples raised concerns about whether or not phage capable of infecting all these SRB types could be identified. The metabolic capacity for energy extraction from sulfate reduction is present in isolated lineages within several taxonomically diverse clades, including some members of the Gram-negative class Deltaproteobacteria, some members of the Gram positive Firmicute family Peptococcaceae, as well as several deep branching bacterial classes such as the Thermodesulfobacteria and Thermatogales. Therefore, the relevance of this collection of SRB phage to all SRB organisms needed to be established by analyzing the diversity of SRB. The collection of SRB genomes was extracted from the public database. Only “true SRB” were analyzed, as defined as bacteria encoding recognizable dissimilatory sulfite reductase genes A and B (DsrAB) homologues were included. This, therefore, does not include other sulfur-respiring organisms, for example sulfur and thiosulfate reducing bacteria such as Haloanaerobium (110) . A total of 32 whole genome entries were identified as originating from sulfate reducing bacteria (Appendix D). The DsrA and 16s rRNA sequences from the sulfate-reducing archaea, *Archaeoglobus,* was used as the outgroup. With 24 entries, the majority of SRB whole genome entries were members of the Deltaproteobacteria, and the order Desulfovibrionales was most frequently samples (17 members included). Only five members of the have been sequenced. It has been recently observed that some members of the Peptococcaceae possess DsrAB genes that appear to be the result of a recent lateral gene transfer from a Proteobacteria (70, 88, 149, 160). In order to determine if sequenced Peptococcaceae include representatives of members with vertically and

horizontally acquired DsrAB genes, a neighbor joining tree was made from the DsrA proteins sequences and 16s rRNA gene sequences from the fully sequenced sulfate reducing bacteria. The tree results are consistent with published DsrA and 16s trees (70, 88, 149, 160), and show that three of the sequenced Peptococcaceae species have the vertically transmitted DsrA gene and two have the horizontally acquired DsrA genes Only one genome entry, the Nitrospira, *Thermodesulfovibrio yellowstonii* DSM 11347, originated from a non-Proteobacteria, non-Firmicute clade.

SRB Prophage Identification

In order to extend the information on SRB phage, prophage elements in the genomes of 32 sulfate reducing bacteria were identified. A total of 46 prophage elements were identified in 22 of the 32 genomes (Appendix D, Appendix E, Appendix F). Many of these contained genes encoding structural proteins with significant amino acid sequence similarity to the most common types of bacteriophage (lambda, Mu, and P2). The majority of these, 25, were predicted to encode phage with contractile tails (myophage), similar to P2 and Mu. Ten of the prophage elements are likely to encode a flexible tailed phage, similar in morphology to lambda. Finally, two of the prophage were predicted to encode the structural genes of a short tailed, podophage morphology. Morphological predictions could not be made for the remaining elements. This indicates that tailed phage are common among the SRB. These phage sequences will be used to develop molecular tools to identify at least the most abundant phage types.

The search criteria limited prophage elements only to those encoding proteins related to phage already present in the public database. As it is not uncommon for newly discovered phages to encode primarily proteins not related to any in the public database, it is not expected that this criteria will result in a comprehensive list and there are undoubtedly as-yet unidentified prophage elements in at least some of the SRB genomes surveyed. Identified SRB prophage elements were mostly related to P2-like and Mu-like myophage, followed by lambdoid siphophage and last by podophage. This distribution of phage morphotypes may be biased is due to the search criteria used. Due to the complexity of contractile tail morphogenesis, P2- and Mu-like myophage encode more structural proteins then do siphophage or podophage, making them more likely to be recognized. Podophage encode the least number of structural proteins and may have the smallest genomes of the three phage morphologies, as a result podophage prophage elements were most likely to have been overlooked. The most significant impediment to using an annotation-dependent method is that the RefSeq annotations may be incomplete or inaccurate. Despite there being hundreds of phage sequences in the public database, and a list of protein types that are frequently encoded by a phage, protein functional annotations based on similarity searches against proteins of known function still result in only limited and partial annotations. In part, this is due to some phage genes having undergone such extreme divergence that authentic homologous proteins share no amino acid similarity. The extreme diversity of homologous proteins is most obvious for phage major capsid proteins, which share the same fold despite there being hundreds of unrelated sequences . Additionally, phage may possess any one of many analogous but unrelated proteins that carry out the same function, the most obvious examples of this being the presence of either a Clp-type or herpesvirus-type protease in the prohead protease protein (23).

E. Isolation of Novel Phage Active Against COSC SRB

Phage Enrichments

Due to project timeline constraints, the SRB/non-SRB co-cultures established from the COSC sample were utilized for phage isolation. Two bacteriophage, ϕCOSCD and ϕCOSC2, were isolated from the same BM sample that the host strains were isolated from. These phage form large, clear plaques on lawns of COSCD and COSC2, respectively (Figure 3). Plaques took an average of 5 days to appear on bacterial overlays. This time frame is consistent with the length of time it takes for bacterial growth in the overlay to be apparent. A dilution series of the phage was made to determine the concentration of phage in the enrichment. The titer of phage in the original enrichment was found to be 1.5 X 10^5 plaque forming units (pfu)/ml enrichment. In an effort to generate a high-titer lysate, 9 agar plates were prepared with overlays containing 10^4 pfu phage with the host bacteria. Following a 10 day incubation at room temperature, a 40 ml lysate was prepared from these plates. Spot titering indicated that the lysate contained 10^8 pfu/ml ϕCOSC2. Upon isolation of COSCD and COSC2 SRB as pure cultures, it was demonstrated that the cognate phages formed plaques on lawns of the purified SRB (data not shown).

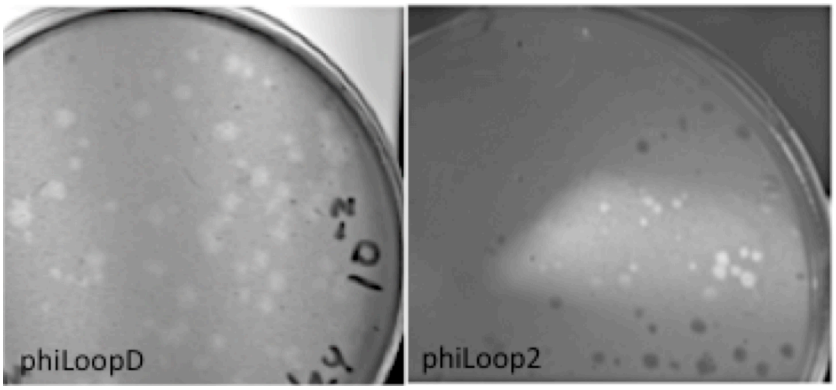

Figure 3. **Phage active against mixed host lawns of COSCD and COSC2. phiCOSCD:** Soft agar overlay containing the initial COSCD mixed culture (*Desulfovibrio halophilus* and *Halanaerobium preaevalens*) and phage phiCOSC.D. **phiCOSC2:** Soft agar overlay containing the initial COSC2 mixed culture (*Desulfovibrio halophilus* and *Halanaerobium preaevalens*) and phage phiCOSC.D.Plaques in this lawn are indicative of phage activity.

Phage Host Range Analysis

Host range analysis was performed by spotting 1.5 X 10^4 pfu of ϕCOSCD and ϕCOSC2 onto overlays prepared from five of the COSC SRB cultures. No clearings were observed on any of the overlays except on the specific enrichment host, e.g. ϕCOSC2 forms plaques only on overlays of host COSC2 and ϕCOSCD forms plaques only on overlays of host COSCD. A collection of SRB phages assembled against collections of oilfield SRB was screened for activity against the complete COSC and OSP host collections. These phages included ϕDala.1, ϕDala.2, ϕDala.3, ϕDala.4, ϕDala.CJ1, ϕEBS14.1, ϕEBS14.2, ϕEBS14.3, ϕEBS14.4, ϕEBS14.5, ϕ EBS14.6, ϕEPF27, ϕEPF27.2, ϕEPF27.3, ϕEPF27.4, ϕWRF 20.1, ϕWRF20.2, ϕWRF20.3, ϕPDS.7, ϕCOSC2, ϕCOSCD. None of these additional SRB phage demonstrated plaque-

forming activity against the COSC samples. Similarly, phage ϕCOSCD and ϕCOSC2 failed to form plaques on overlays of 16 additional sulfate reducing bacteria, including additional de novo SRB isolates as well as ATCC strains of *Desulfovibrio longus*, *D. gabonensis, D. desulfuricans, D. vulgaris,* and *D. alaskensis* (data not shown).

Isolation and propagation of phage against a mixed culture.

The relative ease in which phages were isolated using mixed cultures for enrichments was unexpected. Phage capable of infecting both types of bacteria are not expected to exist, given the narrow host range of phage. both a Firmicute, such as *Haloanaerobium* and a Deltaproteobacteria like *Desulfovibrio,* are not expected to exist so it is unlikely that the phage infects both bacteria. If only one member of the bacterial population serves as a host, then plaques should not be visible on agar overlays due to over growth of the non-host bacteria. Alternatively, the presence of clear plaques is indicative of the strong co-culture dependence of the different bacteria, and the phage- mediated killing of one type results in growth inhibition the other. This has implications beyond the scope of this project. The manipulation of phage and bacteria in pure culture is the hallmark of traditional approaches to microbiology. While the classical "one bacteria/ one disease" model is extremely useful, many diseases, in particular chronic wounds are the result of microbial communities rather then single pathogens (153). Biofilm and biofilm communities are known to play an important role in industrial microbiology and these systems have more in common with a chronic wound model then what is thought of as a typical, acute bacterial infection. Bacteria in mixed culture do not necessarily respond to bactericidal treatments the same was as the same bacteria in a pure culture and thus mixed culture experiments might be expected to be a better indicator of treatment response as the in situ populations.

The relative ease in which functional, active phage were isolated against a newly established SRB culture, and the abundance of prophage elements in the genomic sequences of SRB, indicate that phage active against members of even a complex SRB community are abundant. These results supports our original contention that it is possible to cultivate phages against members of a sulfate reducing, petroleum enriched environments and provides a framework with which to develop their exploitation as environmentally benign anti-microbials. (94, 118)

Phage ϕCOSCD Morphology

Particles of phage ϕCOSCD were visualized by electron microscopy (Figure 4). The phage were revealed to have icosahedral heads and short tails, indicating that they are of the podoviridae morphology. The head size was calculated to have an average diameter of 70 nm. This morphology is typical of tailed phage.

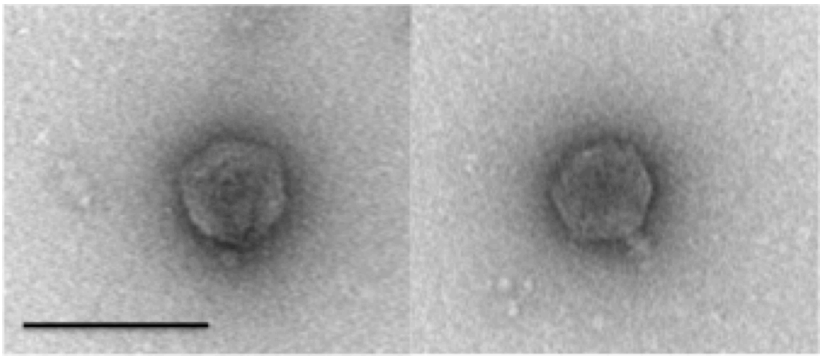

Figure 4. Electron micrograph of phiCOSCD particles, revealing a short tailed (podoviridae) morphology. Scale bare is 100 nm.

Phage Control of SRB Efficacy Testing

Phage control in mixed host cultures

The ability of phage ϕCOSC2 to control host COSC2 (mixed) SRB levels was determined in liquid cultures. To conduct this experiment, 8 duplicate serial 10-fold dilutions of host COSC2 were prepared in 10 ml culture bottles (Figure 5). The starting inoculum of the serial dilutions was determined to be 10^{10} cfu/ml. Immediately following host inoculation, 100 ml of phage ϕCOSC2 stock (at 10^7 pfu/ml) was added to four of the eight dilution series. Growth of SRB was scored after 30 days. Black precipitates indicate positive SRB growth. After 30 days of growth, the untreated inoculums had grown in cultures diluted to 10^{10} fold. In contrast, 3 of the four phage treated dilutions exhibited growth only to the 10^3 dilutions. This indicated that phage to host rations, or multiplicity of infection, of 10 or greater was sufficient to eliminate all SRB in the starting sample.

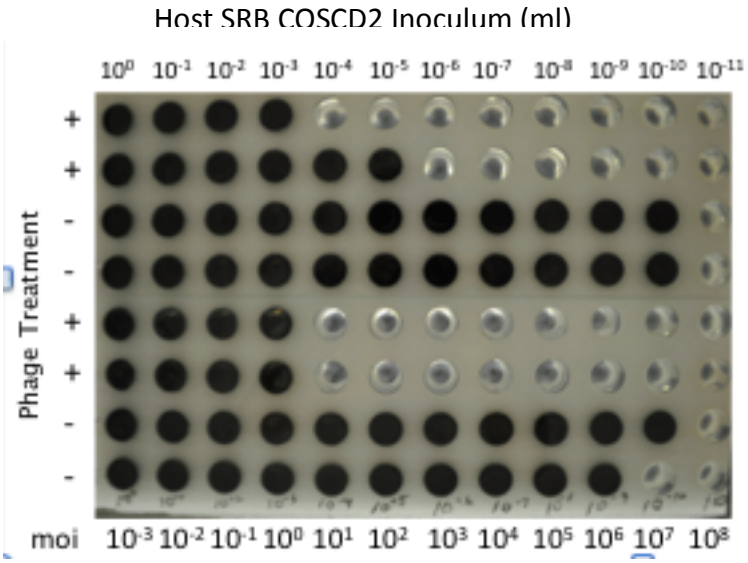

Figure 5. ϕCOSC2 Efficacy Trial. Effect of different phage/ host ratios. Host COSC2 was serial diluted to 10^{-11}. Phage treated samples (+) were inoculated with 10^7 ϕCOSC2 while the no phage control (-) did not receive phage. Image of cultures at 30 days after inoculation.

Control of pure *Desulfovibrio* strain COSC.D by phage ϕCOSC.D

Phage ϕCOSC.D was shown previously to control H_2S produced by mixed culture COSC.D (130). In order to determine if phage ϕCOSC.D is capable of controlling H_2S production by pure *Desulfovibrio* strain COSC.D, serial dilutions of pure culture were set up in 6% MPB in serum bottles. The serial dilutions correspond to inoculation levels of 10^9 bacteria down to an end point dilution. One set of bottles were challenged with 10^6

phage plaque forming units (pfu) while a duplicate control set was not challenged with phage (Figure 5). The cultures were incubated and the rate of SRB growth scored by the appearance of black iron sulfide precipitate. At the end of 31 days incubation, the series of control bottles that did not receive phage treatment showed evidence of growth all the way down to the 10^9 dilution, indicating that the original culture contained around 10^9 bacterial cells per ml. In contrast, the series of bottle challenged with 10^6 pfu each exhibited growth only in the initial bottle that had received one ml of 10^9 bacteria per ml. All of the remaining bottles failed to show any evidence of SRB growth as evidenced by H_2S production, even after more than three months incubation following treatment (Figure 6).

10^9 10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1 10^0 $<10^0$ $<10^0$

Figure 6. Control of Desulfovibrio strain COSC.D by phage ϕCOSC.D. Note that volume differences are due to the removal and testing of samples for phage levels at various time points (data not shown).

Several additional phage efficacy trials gave similar results, with low levels of phage controlling SRB growth for extended periods. However, the capacity of low moi to control host levels was not achieved with every trial. When the experiment was repeated, moi of 1 or greater was required to control host levels for 30 days (Figure 7). In this experiment, four replicates of phage treated and control (no phage treatment) samples were set up in parallel. After 30 days incubation, SRB growth was evident in phage treated samples where the initial phage moi was 10 or less. The reason for the variations in phage efficacy is unclear. However, phage treatment was sufficient to eradicate the SRB host and the effect was observed, even after several months incubation (Figure 7 and data not shown). A difference between the two experiments was the concentration of host in the initial inoculum; experiment 2 levels were 10 fold higher then in experiment 1. This might be correlated to an even higher concentration of H_2S in the initial inoculum, which might have inactivated at least some of the phage, resulting in an even lower effective moi. Clearly, more research is needed to understand the root cause of the variability in the effectiveness of phage control of SRB.

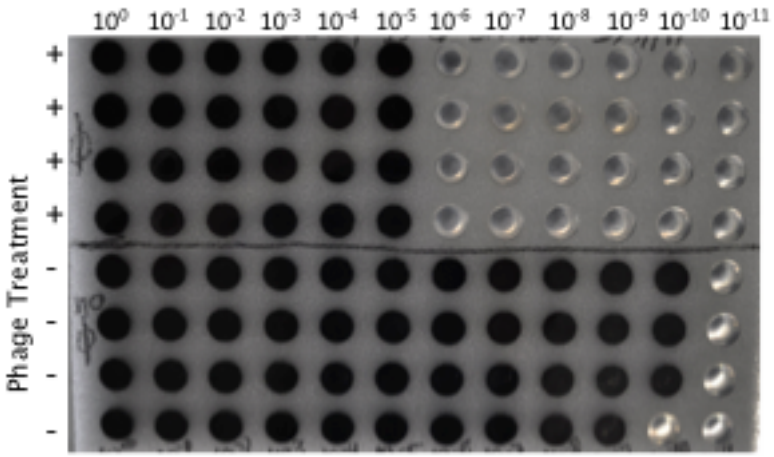

Figure 7. ϕCOSCD2 Efficacy Trial. Effect of different phage/ host ratios. Host COSCD2 was serial diluted to 10^{-11}. Phage treated samples (+) were inoculated with 10^{6} ϕCOSC2 while the no phage control (-) did not receive phage. Image of cultures at 30 days after inoculation. Image is of the culture vials, black indicates SRB growth.

Biocide treatments may not completely kill bacterial populations in part because of the capacity of bacteria to recover from damage or due to the presence of a sub-population of phenotypically resistant cells. A phage preparation was shown to effectively control SRB populations for several months following treatment. The use of phage is complicated by the extremely diverse populations of SRB present in oilfield samples. New approaches must be developed to take advantage of the bacteriolytic nature of phages while over coming the challenges of limited host range

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Conclusions

The conclusion should not simply reiterate what was already stated in the "Results and Discussion" section, but should summarize what has been performed in the project, and include any implications of how the successes are relevant to technology development in the future.

- RPSEA funds were obtained for use in evaluating the potential of utilizing bacteriolytic phage as SRB control agents
- Understanding target bacterial populations is critical for developing phage based control products
- Two sample locations were chosen for analysis: COSC and the OSP
- Bacterial population analysis was conducted on the COSC and OSP samples
- 47 species of sulfidogenic bacteria, constituting 36% of all bacteria in the samples, were identified
- 36 species were classified as "true sulfate reducing bacteria"
- Iron reducing bacteria were also present in significant numbers in the OSP samples
- SRB strains were cultured from the COSC and OSP samples
- 46 new prophage elements were identified in the genomes of sequenced SRB
- New phages capable of killing the COSC SRB were isolated
- The effective dose of phage required to control SRB growth varied dramatically between experiments.
- The reasons for the variable dose requirements are not understood and are the subject of future research efforts.
- Phage treatments resulted in the extended control of SRB levels, as compared to untreated samples, and thus holds promise as a biopesticide for use in the petroleum industry to reduce the need for chemical biocides.

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LIST OF ACRONYMS AND ABBREVIATIONS

Key words: sulfate reducing bacteria, phage, high saline environment, oil and gas industry, chemical biocides

crude oil salt storage cavern (COSC)

on shore pipeline (OSP)

microbial influenced corrosion (MIC)

temperature gradient gel electrophoresis (TGGE)

most probable number (MPN)

acid producing bacteria (APB)

sulfate reducing bacteria (SRB)

Modified Postgate's B Broth (MPB)

APPENDICES

Appendix A. Full water analysis for OSP samples.

Analysis	OSP Sample 3 9300 Barrels	OSP Sample 5 21,400 Barrels	OSP Sample 7 34,000 Barrels
Aluminium	0.283 ppm	4.44 ppm	3.23 ppm
Barium	38.9 ppm	19.6 ppm	18.3 ppm
Bromide	<0.060 ppm	<0.060 ppm	<0.060 ppm
Cadmium	<0.006 ppm	<0.006 ppm	<0.006 ppm
Carbonate	0.00 mg/L	0.00 mg/L	0.00 mg/L
Chloride	70,500 ppm	71,800 ppm	76,300 ppm
Chromium	<0.005 ppm	<0.005 ppm	<0.005 ppm
Copper	<0.010 ppm	<0.010 ppm	0.637 ppm
Fluoride	52.1 ppm	55.2 ppm	55.7 ppm
Iron	3.87 ppm	5.51 ppm	4.16 ppm
Lead	0.053 ppm	0.039 ppm	0.027 ppm
Magnesium	800 ppm	735 ppm	449 ppm
Manganese	0.410 ppm	0.447 ppm	0.531 ppm
Nickel	<0.005 ppm	<0.005 ppm	<0.005 ppm
Nitrate-N	12.6 ppm	13.8 ppm	<0.060 ppm

Nitrite-N	<0.060 ppm	<0.060 ppm	<0.060 ppm
Phosphate	<0.090 ppm	<0.090 ppm	<0.090 ppm
Potassium	383 ppm	330 ppm	385 ppm
Sodium	22,100 ppm	18,000 ppm	22,100 ppm
Sulfate	45.2 ppm	54.5 ppm	43.1 ppm
Zinc	0.221 ppm	0.221 ppm	0.577 ppm
Alkalinity	336 mg/L CaCO3	360 mg/L CaCO3	352 mg/L CaCO3
Conductance	142.0 mS	136.6 mS	147.8 mS
Specific Gravity	1.066	0.937	1.014
Sulfide	2.4 ppm	2.4 ppm	2.4 ppm
TDS	145,000 ppm	142,000 ppm	149,000 ppm
Corrosion Inhibitor	**PPM**	**PPM**	**PPM**
Imidazoline	< 50 ppm	< 50 ppm	< 10 ppm
Quaternary Amines	< 50 ppm	< 50 ppm	< 10 ppm
VFA	**PPM**	**PPM**	**PPM**
Acetate	< 2 ppm	< 2 ppm	< 2 ppm
Formate	< 2 ppm	< 2 ppm	< 2 ppm
Butyrate	< 2 ppm	< 2 ppm	< 2 ppm
Proprionate	< 2 ppm	< 2 ppm	< 2 ppm

Samples collected at approximately 9300, 21,400, and 34,000 barrels of water received at GIT. There was no immediately detectable carbon source. Upstream data is required to confirm this. Corrosion inhibitor was less than 50 ppm in the samples collected at GIT. No significant corrosion risks were identified in the analysis above except for the concentration of chloride in the system. Lab Ref. No. 10010-REP-001

Appendix B. SRB Media Component Comparisons

Component, per L	[1]D.aespoeensis	[2]MPB	[3]D.marrakechis	[4]D. longus
KH2PO4	0.15	0.5 g	0.5 g	none
K2HPO4	none	none	0.5 g	none
NH4Cl	1	1.0 g	0.5 g	1.0 g
Na2S04		1.0 g	none	2.0 g
CaCl2*6H20	1.0 g (*2H2O)	0.1 g	0.05g (*2H2O)	1.0 g (*2H2O)
MgS04 (anhydrous)	0.3 (*7H20)	1.0 g	none	1.0 g *7H2O
MgCl2*6H2)	0.5	none	0.3 g	none
KCl	0.67	none	0.1 g	none
KHCO3	none	none	none	0.3 g
cystiene hydrochloride *after autoclave*	10 ml	none	0.25 g	0.5 g
MOPS buffer		none	none	3.0 g
trace mineral solution	10 mL	none	1 ml	none
resazurin, 0.1% *after autoclave*	2 ml	none	1 ml	none
NaHCO3 *after autoclave*	none	none	to 30 mM	none
Na2S*9H2O *after autoclave*	10 ml	none	to 1.7 mM	none
l21 Vitamine *after autoclave*	10 mL	none	1 ml	none
vitamin B12 stock after autoclave	1 ml	none	none	none
thiamine stock after autoclave	1 ml	none	none	none
Na Lactate (60%)	5 ml	4.7 mL	none	6.0 ml
Yeast Extract	0.5 g	1.0 g	0.1 g	1.0 g
Sodium acetate	none	2.5 g	none	1.0 g
Na thioglycolate/ Mercaptoacetic Acid	none	0.1 g	none	none
Sodium ascorbate	none	0.1g	none	none
FeS04*7H20	none	0.5g	none	1 ml of 0.4%
NaCl	11.9	25 g NaCl	0.4 g	none
Instant Ocean				
For 2.5% MPB	none	*5 g*	none	none
For 6% MPB	none	*20 g*	none	none

Appendix C. Bacterial Diversity of COSC and OSP samples.

Species	COSC 12/4	COSC 2/25	OSP. A	OSP. B	OSP. C	OSP. D	Metabolism Lookup	Ref
Acetobacter syzygii	0	0.03	0	0	0	0		
Acetobacterium tundrae	0	0.03	0	0.02	0	0	APB	(6)
Acidobacterium sp	0	0.03	0	0	0.07	0	APB	(53)
Acinetobacter johnsonii	0	0	0	0	0	0.04		
Actinomycetales sp	0	0.05	0	0	0	0		
Alkalibacter sp	0	0	0	0	0	0.12	Biodeg sugars alkaliphile	(40)
Alkalibacterium sp	0	0.03	0	0	0	0	alkaliphile	
Alkaliflexus imshenetskii	0	0	0.29	0.08	0	0.41	alkaliphile	
Alkaliflexus sp	0	0	0	0.02	0	0	alkaliphile	
Allochromatium palmeri	0	0	0	0	0	0.04		
Anaerophaga sp	0.04	0.03	0.22	0.06	0.15	0.2	Ferm thermophile	(30)
Anaerophaga thermohalophila	0	0	0.07	0.04	0.03	0.12	Ferm thermophile	(30)
Aquicella sp	0	0.03	0	0	0	0		
Arcobacter sp	0.04	0	0.29	0.29	0.72	1.63	NRSOB	(57)
Arenimonas sp	0	0	0.07	0.1	0.03	0		
Arhodomonas sp	0	0.03	0	0	0	0		
Avibacterium sp	0	0	0.07	0.02	0	0		
Bacillus azotoformans	0	0	0	0	0	0.04		
Bacillus licheniformis	0.62	0.05	0	0	0	0		
Bacillus sp	0	0.03	0	0	0	0	Diverse	(136)
Bacteroides graminisolvens	0.04	0	0	0	0	0	Ferm	(95)
Bacteroides sp	0.04	0.05	0	0.02	0	0.04	Ferm	(104)
Beggiatoa sp	0	0	0.61	0.04	0.1	0.12	SOB, white mats hydrocarbon seeps	(116)
Blastomonas sp	0	0	0	0.02	0	0		
Bosea sp	0	0	0.04	0.02	0	0		
Caldilinea sp	0	0	0	0	0	0.04	filamentous	(72)
Calditerrivibrio nitroreducens	0	0	0	0	0	0.04		
Caminicella sp	0	0	0.86	0.04	0.2	0.04	Ferm thermophile spore-former	(4)
Campylobacteraceae sp	0	0	0	0.02	0	0		
Candidatus Alysiosphaera europeae	0	0	0	0	0	0.04		
Candidatus Magnetobacterium sp	0	0	0	0	0	0.04		
Candidatus Protochlamydia sp	0	0.03	0	0	0	0		
Cellulophaga sp	0	0	0.07	0.02	0	0		
Chromatiaceae sp	0	0	0	0	0	0.04		
Clostridia sp	0	0	0	0	0	0.04		

Clostridiales sp	0	0.16	0.18	0.02	0.05	0.25		
Clostridiisalibacter paucivorans	0	0	0	0.02	0	0.04		
Clostridium difficile	0	0	0.04	0	0	0.04		
Clostridium paradoxum	0	0	0	0	0	0.04		
Clostridium sp	0	0.21	0.11	0.06	0	0.16	Ferm Diverse	(101)
Coccinistipes vermicola	0	0.08	0	0	0	0		
Corynebacterium sp	0	0	0	0	0.02	0	heterotroph	(82)
Croceitalea eckloniae	0.04	0	0	0	0	0		
Cytophaga fermentans	0	0.03	0.04	0.04	0.02	0.04	subsurface community	(7)
Cytophaga sp	62.3	12.55	1.04	0.51	0.15	0.94	BIoDeg HC oilfield	(113)
Dehalobacter sp	0	0.05	0	0	0	0	BioDeg dichlorobenzene deg	(93)
Deinococcus navajonensis	0.35	0	0	0	0	0	radiation resistant	(26)
Deinococcus sp	5.01	0.37	0	0	0	0	radiation resistant	(26)
Desulfobacteraceae sp	0.12	0.08	0	0	0	0.04	SRBa SRB	(8)
Desulfobacterium sp	0.04	0	0	0	0	0	SRBa SRB BioDeg Benzene	(117)
Desulfobotulus sp	6.45	0.55	0	0	0	0	SRBa SRB	(121)
Desulfobulbus sp	0.12	0.1	0	0.02	0	0	SRBa SRB	(100)
Desulfocaldus sp	0.74	0.87	0	0	0.02	0.04	SRBa SRB	(88)
Desulfocapsa sp	0	0	0.4	0.04	0	0.08	SRBa SRB	(88)
Desulfococcus oleovorans	0	0	0	0	0.02	0	SRBa SRB BioDeg HC alkane	Gi:
Desulfohalobiaceae sp	0	0.34	0	0.02	0.02	0	SRBa SRB	(39)
Desulfohalobium retbaense	0	0	5.66	2.36	0.58	3.02	SRBa SRB	(125)
Desulfohalobium utahense	0.12	0.21	0.04	0	0.14	0.04	SRBa SRB	(62)
Desulfomicrobium sp	0	0	0	0.12	0.03	0.04	SRBa SRB	(151)
Desulfonatronospira thiodismutans	0	0.16	0	0	0	0	SRBa SRB	(122)
Desulfonatronovibrio hydrogenovorans	0	0	0.11	0.12	0.24	0.49	SRBa SRB	(122)
Desulfonatronovibrio sp	0	0	0.07	0.06	0.02	0	SRBa SRB	(122)
Desulfonauticus autotrophicus	1.17	6.25	0.04	0.02	0	0	SRBa SRB	(85)
Desulfonauticus submarinus	0	0.03	0	0	0	0	SRBa SRB	(5)
Desulfonospora sp	0	0	0	0.02	0.03	0		
Desulfopila aestuarii	0	0	0	0.02	0	0	SRBa SRB	(137)
Desulfosalina propionicus	0.04	0.1	0	0.02	0	0	SRBa SRB	(69)
Desulfosalina sp	0.47	0.52	0	0	0.09	0.08	SRBa SRB	(1)
Desulfothermus naphthae	0.23	0.03	0.04	0.04	0.03	0	SRBa SRB	(88)
Desulfothermus okinawensis	0	0.45	0	0	0	0	SRBa SRB	(96)
Desulfotomaculum geothermicum	0	0.05	0	0	0	0	SRBa SRB	(29)
Desulfotomaculum sp	0	0.03	0	0	0	0	SRBa SRB	(42)
Desulfovermiculus halophilus	0.08	0.08	0	0	0.03	0	SRBa SRB	(10)

Desulfovibrio alkalitolerans	0	0	0	0.04	0.02	0	SRBa SRB Alkaliniphile municipal heating water	(2)
Desulfovibrio bastinii	0	0	0	0	0.02	0	SRBa SRB	(79)
Desulfovibrio capillatus	0	0	0	0.1	0.7	0.2	SRBa SRB	(90)
Desulfovibrio dechloracetivorans	0	0.03	0	0.02	0.02	0.04	SRBa SRB	(135)
Desulfovibrio gabonensis	0	0.03	0.54	0.41	0.17	1.19	SRBa SRB	(139)
Desulfovibrio gracilis	0	0	0	0.02	0.02	0	SRBa SRB	(79)
Desulfovibrio indonesiensis	0	0	0	0.02	0.02	0.04	SRBa SRB	(115)
Desulfovibrio senezii	0	0	0.11	0.02	0	0	SRBa SRB	(141)
Desulfovibrio sp	0	0.05	1.12	1.01	1.14	1.19	SRBa SRB	(8)
Desulfovibrio tunisiensis	0	0	0	0.04	0	0	SRBa SRB oilfield refinery	(11)
Desulfovibrionales sp	0.04	0.03	0	0.02	0	0	SRBa SRB	(88)
Desulfuromonadales sp	0	0	0	0.02	0.02	0.04	SRBa SuRB Fe(III)RB	(76)
Desulfuromonas acetexigens	0	0	0	0.02	0	0	SRBa SuRB Fe(III)RB	(102)
Desulfuromonas palmitatis	0	0	0.9	0.25	0.1	0.57	SRBa SuRB Fe(III)RB	(76)
Desulfuromonas sp	0	0	0.04	0.08	0	0.16	SRBa SuRB Fe(III)RB	(76)
Desulfuromonas svalbardensis	0	0	0	0	0	0.29	SRBa SuRB Fe(III)RB	(145)
Desulfuromonas thiophila	0	0	0	0	0.02	0.04	SRBa SuRB Fe(III)RB	(36)
Desulfuromusa sp	0	0	0	0	0	0.04	SRBa SuRB Fe(III)RB	(77)
Dethiobacter sp	0.04	0	0	0	0	0.04	SRBa TRB SuRB	(124)
Devosia sp	0	0	0	0.02	0	0	heterotroph	(113)
Dorea sp	0	0	0.04	0	0	0		
Ectothiorhodospiraceae sp	0.12	0.16	0	0	0	0		
Elizabethkingia miricola	0	0	0.07	0	0	0		
Endozoicomonas elysicola	0	0	0	0.02	0	0		
Enterococcus sp	0	0.03	0	0	0	0	APB	(37)
Erythrobacter sp	0.04	0	0	0	0	0		
Eubacterium sp	0	0	0	0	0	0.04	Ferm acetate butyrate ethanol	(114)
Eudoraea adriatica	0	0	0.07	0	0	0		
Eudoraea sp	0	0	0	0	0	0.04		
Exiguobacterium sp	0	0	0	0	0.12	0		
Fervidobacterium sp	0	0	0	0.02	0	0		
Flavobacterium sp	0.27	0.03	0	0	0	0	BioDeg HC community member	(14)
Flexibacter sp	0	0.05	0	0	0	0	BioDeg HC oil degrading consortium bacteria	(113)
Flexistipes sinusarabici	0.04	0.21	0.18	0.1	0.09	0.16	Ferm, similar to Fe(III)RB	(74)
Fusibacter sp	0	0.03	0	0	0	0.04	SRBa SuRB	(3)
Fusobacterium sp	0	0	0	0	0.02	0	unkwn anaerobe pathogens	38GI:
Gemmatimonas sp	0	0	0	0	0	0.04	unkwn	(156)
Geobacter metallireducens	0	0	0	0.02	0	0	Fe(III)RB	(76)
Geobacter sp	0	0	4.32	1.15	0.17	1.72	Fe(III)RB	(76)

Geotoga petraea	0	0	0.04	0.06	0.02	0	Ferm	(56)
Geotoga subterranea	0	0.03	0	0	0	0		
Gracilibacillus halotolerans	0.54	0.37	0	0	0	0		
Halanaerobacter chitinivorans	0.04	0	0	0	0	0		
Halanaerobacter lacunarum	0.04	0.1	0	0	0	0	Ferm	(99)
Halanaerobiaceae sp	0	0.37	8.32	8.21	2.73	4.54	Ferm	(99)
Halanaerobiales sp	0.08	0.79	0	0	0	0	Ferm	(99)
Halanaerobium acetethylicum	0	2.23	1.08	3.76	4.26	1.8	Ferm promotes SRB a methanogens	(99)
Halanaerobium congolense	0	0	44.92	46.84	72.18	46.71	SRBa TRB Ferm promotes sulfidogens	(111)
Halanaerobium fermentans	0	0.29	0.04	0.1	0.03	0	Ferm promotes sulfidogens	(71)
Halanaerobium lacurosei	0	0.03	0.04	0.02	0	0	Ferm promotes sulfidogens	(20)
Halanaerobium praevalens	0	0.08	0.07	0.02	0	0.04	Ferm promotes sulfidogens	(61)
Halanaerobium saccharolyticum	1.75	9.69	2.52	3.8	3.51	2.57	Ferm promotes sulfidogens	(68)
Halanaerobium salsuginis	0	0	0	0.04	0.02	0	Ferm promotes sulfidogens	(99)
Halanaerobium sp	0	2.7	3.75	6.34	4.55	3.56	Ferm promotes sulfidogens	(61)
Halocella cellulolsilytica	0	0.16	0	0	0	0.04	Ferm	(108)
Halocella sp	4.59	51.68	1.12	0.14	0.67	0.65	Ferm oilfield	(108)
Halomonas alimentaria	0	0	0	0	0.07	0	BioDeg HC halophile haloalkaliphilic	(155)
Halomonas sp	0.04	0.42	0	0.02	0	0.25	BioDeg HC halophile haloalkaliphilic	(27)
Halomonas ventosae	0	0	0	0	0	0.12	BioDeg HC halophile haloalkaliphilic	(27)
Halothermothrix orenii	0.04	0.21	0	0	0	0.04	BioDeg starch thermophile	(21)
Halothiobacillus halophilus	0	0.03	0	0	0	0	BioDeg starch thermophile	
Halothiobacillus hydrothermalis	0	0.13	0	0	0	0	APB SOX	(97)
Halothiobacillus sp	0	0.03	0	0	0	0.04	APB SOX	(97)
Idiomarina sp	0.04	0.03	0	0	0	0		
Ignavibacterium album	0.12	0.05	0	0	0	0		
Imtechium assamiensis	0	0	0.07	0	0	0	Biofilm Biodeg PCB	(78)
Kaistia soli	0	0	0.18	0.02	0.02	0		
Kordiimonas sp	0	0	0	0.02	0	0		
Kurthia gibsonii	0	0	0	0.02	0	0	unkwn	(65)
Lactobacillus alvei	0	0.03	0	0	0	0	APB	
Lactobacillus crispatus	0.08	0	0	0	0	0	APB	(129)
Lactobacillus hamsteri	0.04	0	0	0	0	0	APB	(129)
Lactobacillus plantarum	0	0.03	0	0	0	0.04	APB	(112)
Lactobacillus sp	0	0	0	0.02	0	0	APB	(129)
Lactococcus lactis	0	0.03	0	0	0.02	0	APB	(129)
Lactococcus sp	0	0.05	0.04	0.02	0	0	APB	(129)
Leptotrichia sp	3.85	0.31	0.04	0.04	0	0	pathogen	(33)
Leuconostoc lactis	0.04	0	0	0	0	0		

Levilinea sp	0	0	0	0.02	0	0	oilfield produced waters Alaska	(104)
Lewinella nigricans	0	0	0	0	0	0.04		
Loktanella tamlensis	0	0.05	0	0	0	0		
Maribius sp	0	0.03	0	0	0	0		
Marinilabilia salmonicolor	0	0	0	0.02	0	0	BioDeg Agar	(138)
Marinobacter bacchus	0	0	0	0	0	0.16	BioDeg HC	(48)
Marinobacter excellens	0	0	0	0.02	0	0	BioDeg HC	(45)
Marinobacter hydrocarbonoclasticus	0	0	0	0	0	0.04	BioDeg HC	(41)
Marinobacter sp	0	0	0	0.21	0.09	4.82	BioDeg HC	(154)
Marinobacterium georgiense	0	0	0	0	0	0.04	BioDeg HC	(41)
Marinobacterium litorale	0	0	0	0.06	0.1	0.12	BioDeg HC	(41)
Marinobacterium sp	0	0	0	0.02	0	0.98	BioDeg HC	(41)
Marinobacterium stanieri	0	0	0	0	0	0.04	BioDeg HC	
Mariprofundus ferrooxydans	0	0	1.08	0.33	0.12	0.29	Fe(II)OX MIC iron corroding	(86)
Maritimibacter alkaliphilus	0	0.05	0	0	0	0		
Methylobacter sp	0	0	0	0	0	0.04	methylT	(24)
Methylocaldum sp	0.04	0	0	0	0	0.04	methylT	(24)
Methylocella sp	0	0	0	0.02	0.02	0	methylT	(24)
Methylophaga sp	0	0.03	0.25	0.04	0	0	methylT	(24)
Microbulbifer sp	0.31	0.1	0	0	0.02	0		
Microcella sp	0.12	0.03	0	0	0	0		
Natroniella sp	0	0.03	0	0	0	0		
Nevskia sp	0	0	0	0.04	0.03	0.08		
Nitrincola sp	0	0	0	0	0.02	0		
OD1 uncultured	0.04	0	0	0	0	0	uncultured anaerobe	(32)
Paenibacillus abekawaensis	0	0	0.04	0	0	0		
Paenibacillus anaericanus	0	0	0	0.04	0.02	0.12		
Paenibacillus campinasensis	0	0	0.04	0	0	0		
Paenibacillus graminis	0	0.08	0	0	0	0		
Paenibacillus sabinae	0.12	1.92	2.09	0.66	0.29	2.17	BioDeg PAH	(64)
Paenibacillus sp	0.08	0.26	0.29	0.16	0.05	0.25	BioDeg PAH	(50)
Paludibacter sp	0.19	0	0	0	0	0	Ferm	(142)
Parvularcula sp	0	0	0.04	0	0	0		
Pelobacter acetylenicus	0	0	0	0.1	0.07	0	Fe(III)RB	(76)
Pelobacter carbinolicus	0	0	0	0	0	0.12	Fe(III)RB	(76)
Pelobacter propionicus	0	0	0	0.02	0	0.08	Fe(III)RB	(76)
Pelobacter seleniigenes	0	0	0.04	0.08	0.02	0.04	Fe(III)RB	(76)
Pelobacter sp	0	0	11.38	19.69	4.83	12.38	Fe(III)RB	(76)
Pelobacter venetianus	0	0	0	0.04	0	0.12	Fe(III)RB	(76)
Pelotomaculum sp	0	0	0.04	0	0	0	Ferm	(58)
Peptostreptococcaceae sp	0	0.21	0.25	0.1	0.2	1.14	BioFilm MIC	(13)

Phormidium sp	0.04	0	0	0	0	0	photoT oil tolorent	(25)
Pigmentiphaga daeguensis	0	0	0	0	0	0.04		
Prolixibacter bellariivorans	0	0	0	0	0.02	0.12		
Prosthecochloris vibrioformis	0	0.03	0	0	0	0	photoT	
Prosthecomicrobium sp	0	0	0	0.02	0	0		
Proteiniphilum sp	0.19	0	0	0	0	0	Ferm	(22)
Proteus vulgaris	0	0	0	0.02	0	0		
Pseudomonas sp	7.93	1.5	0.79	0.45	0.17	0.57	BioFilm MIC	(109)
Pseudoruegeria sp	0	0.08	0	0	0	0		
Psychrobacter sp	0	0	0	0	0	0.04	BIoDeg HC oil oilfield	(107)
Psychroflexus sp	0.08	0	0	0	0	0		
Rhizobium sp	0	0	0.04	0	0	0	NiF	(43)
Rhodobacter sp	0	0.03	0	0	0	0.04	photoT	(81)
Rhodobacteraceae sp	0	0.08	0	0	0	0		
Rhodobacterales sp	0.04	0	0	0	0	0		
Rickettsia sp	0.08	0	0	0	0	0		
Roseobacter sp	0	0.05	0	0	0	0		
Rubrimonas sp	0	0	0	0	0	0.04		
Ruminococcus sp	0	0	0.04	0	0	0		
Salinibacter sp	0.16	0.29	0	0	0	0		
Sediminimonas qiaohouensis	0	0.03	0	0	0	0		
Sneathiella glossodoripedis	0	0	0.29	0.08	0.05	0.04		
Sphingobacteria sp	0	0	0	0.02	0	0		
Sphingobacterium sp	0.27	0.03	0.32	0.08	0.07	0.2	generalist aerobic	(67)
Sphingomonadaceae sp	0	0	0.18	0.06	0.03	0.04		
Sphingomonas sp	0	0	1.73	0.27	0.1	0.16	BioDeg phenanthrene (water insoluble)	(55)
Spirochaeta bajacaliforniensis	0	0.03	0.11	0	0.02	0.08	SRBa TRB SuRB Ferm	(38)
Spirochaeta sp	0.04	0	0.29	0	0.02	0.04	Ferm Divers some SRBa TRB H2S resistant	(80)
Staphylococcus epidermidis	0	0.03	0	0	0	0		
Streptococcus mutans	0.04	0.13	0.18	0	0	0.04		
Sulfurospirillum arcachonense	0	0	0	0.02	0	0	NRSOB	(36)
Sulfurospirillum sp	0	0	0	0.02	0.03	0	NRSOB	(57)
Sulfurovum lithotrophicum	0.04	0	0	0	0	0	SOB	(59)
Synechococcus sp	0	0.16	0	0	0	0	photoT	(47)
Syntrophaceticus schinkii	0	0.03	0	0	0	0	syntrophic acetate-oxidizing; co-methanogen-syntroph	(152)
Syntrophomonas sp	0	0	0	0.02	0	0	Syntroph BioDeg FA	(87)
Syntrophus gentianae	0	0	0.11	0.02	0.02	0.04	BioDeg syntroph alkane	(117)
Syntrophus sp	0	0.08	0	0	0	0.08	BioDeg syntroph alkane	(46)

Telmatospirillum siberiense	0	0.03	0.14	0.04	0	0.04		
Thermoanaerobacter cellulolyticus	0	0.03	0	0	0	0		
Thermoanaerobacter sp	0.04	0	0	0.02	0	0.04	Ferm ethanol thermophile	(35)
Thermodesulforhabdus norvegica	0	0.03	0	0	0	0	SRBa SRB thermophile	(9)
Thermothrix thiopara	0	0.03	0	0	0	0		
Thermotogales sp	0	0.03	0.32	0.02	0.05	0.16		(56)
Thermovirga sp	0	0	0	0.04	0.03	0.25	Biofilm Pipeline	(128)
Thermus sp	0	0.03	0	0	0	0	Fe(III)RB thermophile Taq polymerase source	(66)
Thiohalorhabdus denitrificans	0.23	0.21	0	0	0	0	NRSOB neutrophile halophile	(123)
Thiomicrospira sp	0	0.03	0	0	0	0.04	SOB neutrophile	(123)
Tindallia sp	0.04	0.05	0	0	0	0	Ferm alkaliphile	(105)
Trichococcus sp	0	0.03	0	0	0	0	APB Ferm	(127)
Vagococcus lutrae	0	0	0	0	0.02	0		
Verrucomicrobiales sp	0	0	0	0	0	0.04		
Verrucomicrobium sp	0.04	0.03	0	0	0	0	Ferm	(144)
Zooshikella ganghwensis	0	0.03	0	0	0	0		

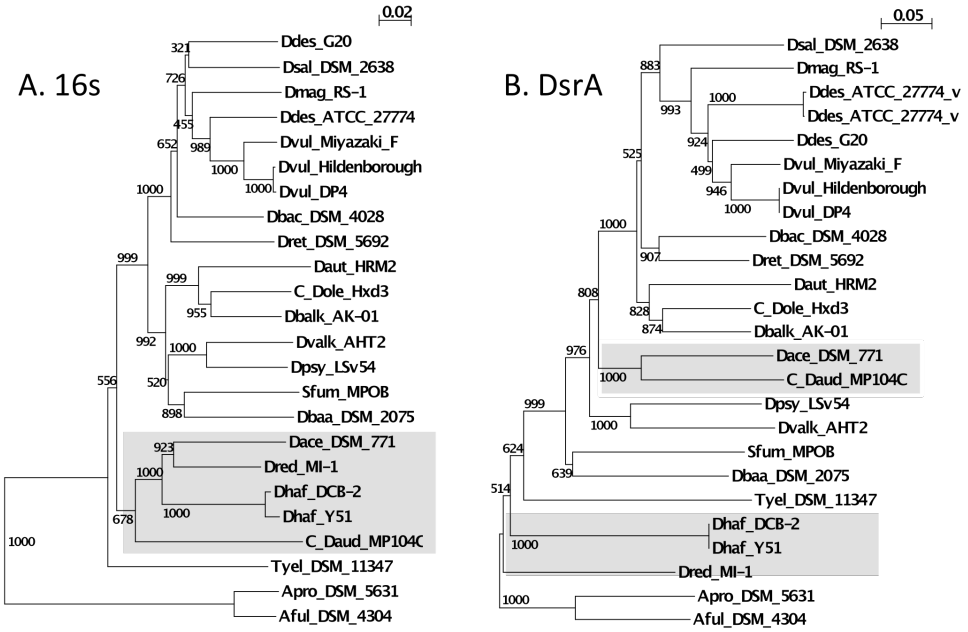
Anaerobic alkane-degrading bacterium Desulfococcus oleovorans Hxd3 (DSM 6200) was isolated from an oil/water mixture from an oil production plant. [GI:158508843]

Appendix D. Genomic Details of 32 Completely Sequenced SRB.

Table 1. Complete genome entires of 32 Sulfate-Reducing Bacteria						
Genus species strain	**Refseq**	**Genome length, Mbp**	**Proteins**	**Annotated integrases, phage proteins**	**Prophage regions > 30 kb**	**DsrA**
Peptococcaceae						
Desulfotomaculum reducens MI-1	NC_009253	3.6	3276	25, 34	2	YP_001114514.1
Desulfotomaculum acetoxidans DSM 771	NC_013216	4.5	4470	23, 16	1	YP_003189665
Desulfitobacterium hafniense Y51	NC_007907	5.7	5137	0, 0	none*	YP_516542
Desulfitobacterium hafniense DCB-2	NC_011830	5.2	4883	6, 26	1	YP_002456756
Candidatus Desulforudis audaxviator MP104C	NC_010424	2.3	2157	11, 10	none	YP_001718320
Nitrospira						
Thermodesulfovibrio yellowstonii DSM 11347	NC_011296	2.2	2033	1, 1	none	YP_002249783
Deltaproteobacteria						
Desulfarculales						
Desulfarculus baarsii DSM 2075	NC_014365	3.6	3277	15, 37	3	YP_003806924
Desulfobacterales						
Desulfatibacillum alkenivorans AK-01	NC_011768	6.5	5252	11, 16	2	YP_002433449
Desulfobacterium autotrophicum HRM2	NC_012108	5.6	4867	20, 14	none	YP_002605460
Candidatus Desulfococcus oleovorans Hxd3	NC_009943	3.9	3265	8, 1	none	YP_001530549
Desulfotalea psychrophila LSv54	NC_006138	3.5	3116	6, 6	2	YP_064533
Desulfovibrionales						
Desulfohalobium retbaense DSM 5692	NC_013223	2.9	2479	6, 4	none	YP_003197124
Desulfonatronospira thiodismutans ASO3-1	NZ_ACJN00000000	4.1	3663	43, 5	none	ZP_07017788
Desulfomicrobium baculatum DSM 4028	NC_013173	3.9	3436	14, 6	none	YP_003156822
Desulfovibrio vulgaris subsp. vulgaris DP4	NC_008751	3.4	2941	9, 48	4	YP_967975
Desulfovibrio vulgaris str. 'Miyazaki F'	NC_011769	4	3180	2, 34	3	YP_002435969
Desulfovibrio desulfuricans subsp. desulfuricans str. ATC	NC_011883	2.9	2356	14, 14	1	YP_002480868
Desulfovibrio magneticus RS-1	NC_012796	5.2	4625	2, 3	none	YP_002951737
Desulfovibrio salexigens DSM 2638	NC_012881	4.2	3807	9, 11	1	YP_002990392
Desulfurivibrio alkaliphilus AHT2	NC_014216	2.8	3185	14, 5	none	YP_003689626
Desulfovibrio piger ATCC 29098	NZ_ABXU00000000	2.8	3114	0,0	none*	BAB55561.1
Desulfovibrio vulgaris RCH1	NZ_ACQX00000000	3.7	3311	14, 44	2	ZP_04792716
Desulfovibrio aespoeensis Aspo-2	NZ_ADDI00000000	3.6	3294	13, 26	3	ZP_06233091
Desulfovibrio sp. FW1012B	NZ_ADFE00000000	4.1	3737	12, 27	2	ZP_06370881
Desulfovibrio fructosovorans JJ	NZ_AECZ00000000	4.7	4159	20, 21	3	ZP_07335109
Desulfovibrio desulfuricans subsp. desulfuricans str. G20	NC_007519	3.7	3780	3, 32	3	YP_387022
Desulfovibrio vulgaris str. Hildenborough	NC_002937	3.6	3380	11, 26	7	YP_009626
Desulfovibrio sp. 3_1_syn3	NZ_ADDR00000000	3.6	3244	21, 37	1	ZP_07356420
Syntrophobacterales						
Syntrophobacter fumaroxidans MPOB	NC_008554	4.9	4064	11, 29	2	YP_848143

Completely sequenced sulfate reducing bacteria present in the NCBI public database.
Information in each column:
1. Entries, grouped by taxonomic proximity.
2. Refseq identifiers are given.
3. The genome size, in million base pairs (Mbp)
4. Total number of annotated protein coding genes,
5. The number of proteins annotated as integrases, and phage related. None indicates that the protein entries were incomplete*
6. The number of prophage elements identified
7. Corresponding DsrA protein accession numbers.

Appendix E. Comparison of the phylogenetic analysis of A



Comparison of the phylogenetic analysis of A. 16s rRNA gene sequences and B. DsrA protein sequences from 24 sulfate reducing prokaryotes for which whole genome sequences are available. A neighbor-joining tree was generated using ClustalX with a bootstrap value of 1000. Shaded boxes correspond to members of the Peptococcaceae. Genus, species abbreviations are as follows: Desulfotomaculum_reducens MI-1 (Dred_MI-1), Desulfotomaculum acetoxidans DSM 771(Dace_DSM_771), Desulfitobacterium hafniense Y51 (Dhaf_Y51), Desulfitobacterium hafniense DCB-2 (Dhaf_DCB-2), Candidatus Desulforudis audaxviator MP104C (C_D_aud_MP104C), Thermodesulfovibrio yellowstonii DSM 11347 (Tyel_DSM_11347), Desulfarculus baarsii DSM 2075 (Dbaa_DSM_2075), Desulfatibacillum alkenivorans AK-01 (Dbalk_AK-01), Desulfobacterium autotrophicum HRM2 (Daut_HRM2), Candidatus Desulfococcus oleovorans Hxd3 (C_Dole_Hxd3), Desulfotalea psychrophila LSv54 (Dpsy_LSv54), Desulfohalobium retbaense DSM 5692 (Dret_DSM_5692), Desulfonatronospira thiodismutans ASO3-1 (Dthi_ASO3-1), Desulfomicrobium baculatum DSM 4028 (Dbac_DSM_4028), Desulfovibrio vulgaris subsp. vulgaris DP4 (Dvul_DP4), Desulfovibrio vulgaris str. 'Miyazaki F' (Dvul_Miyazaki_F), Desulfovibrio desulfuricans subsp. desulfuricans str. ATCC 27774 (Ddes_ATCC_27774), Desulfovibrio magneticus RS-1 (Dmag_RS-1), Desulfovibrio salexigens DSM 2638 (Dsal_DSM_2638), Desulfurivibrio alkaliphilus AHT2 (Dvalk_AHT2), Desulfovibrio piger ATCC 29098 (Dpig_ATCC_29098), Desulfovibrio vulgaris RCH1 (Dvul_RCH1), Desulfovibrio aespoeensis Aspo-2 (Daes_Aspo-2), Desulfovibrio sp. FW1012B (Dsp_FW1012B), Desulfovibrio fructosovorans JJ (Dfru_JJ), Desulfovibrio desulfuricans subsp. desulfuricans str. G20 (Ddes_G20), Desulfovibrio vulgaris str. Hildenborough (Dvul_Hildenborough), Desulfovibrio sp. 3_1_syn3 (Dsp_3_1_syn3), Syntrophobacter fumaroxidans MPOB (Sfum_MPOB), Archaeoglobus fulgidus DSM 4304 (Aful_DSM_4304), Archaeoglobus profundus DSM 5631 (Apro_DSM_5631)

Appendix F. Prophage Elements Identified in 32 SRB Genomes

Lysogen	Nucleotide range	Locus tag range	Morph
D. reducens MI-1	1) 1258679 to 1305344	integrase (Dred_1182) to cell wall hydrolase (Dred_1228).	Myo
D. reducens MI-1	2) 2814935 to 2849119	lysozyme (Dred_2589) to integrase (Dred_2625)	Sipho
D. acetoxidans DSM 771	1) 4384776 to 4424989	XRE tx reg (Dtox_4206) to reverse transcriptase (Dtox_4258)	Myo
	note: maybe more but disrupted by integrases, XRE transcription factors, insertion sequences		.
D. hafniense DCB-2	1) 1514420 to 1561628	XRE/ Cro (Dhaf_1407) to glycosyltransferase moron (Dhaf_1469)	Sipho
D. baarsii DSM 2075	1) 1745473 to 1783984)	peptidgoglycan binding prt (Deba_1569) to phage repressor (Deba_1611).	Myo
D. baarsii DSM 2075	2) 2000510 to 2053639	PAAr repeat protein (Deba_1789) to phage protein (Deba_1843)	Myo
D. baarsii DSM 2075	3) 2838573 to 2902396	head prt (Deba_2544) to phage prt (Deba_2610) phage remnents, addiction modules	ukn
D. alkenivorans AK-01	1) 4488357 to 4524522	hyp prt (Dalk_3540) to repressor (Dalk_3582)	ukn
D. alkenivorans AK-01	2) 5668762 to 5725292	SP6gp43 (Dalk_4541) to DNA methylase (Dalk_4614)	ukn
D. oleovorans Hxd4	1) 3493451 to 3547434	integrase (Dole2906) to integrase (Dole_2954), no identified virion genes.	ukn
D. psychrophila LSv54	1) 1746547 to 1787685	T5orf172 dmn prt (DP1566) to integrase (DP1604)	Sipho
D. psychrophila LSv54	2) 2262798 to 2321973	Large phage protein (DP1991) to integrase (DP2040)	ukn
D. vulgaris sb. vulgaris DP4	1) 1049047 to 1083146	integrase (Dvul_0852) to integrase (Dvul_0877), maybe truncated	Podo
D. vulgaris sb. vulgaris DP4	2) 1272991 to 1315732	integrase (Dvul_1037) to conserved phage tail assemply prt (Dvul_1082)	Sipho
D. vulgaris sb. vulgaris DP4	3) 1745393 to 1781803	txn reg (Dvul_1443) to integrase (Dvul_1487) P2-like	Myo
D. vulgaris sb. vulgaris DP4	4) 3198595 to 3232809	methylase (Dvul_2741) to integrase (Dvul_2782) P2-like	Myo
D. vulgaris str. 'Miyazaki F'	1) 865833 to 901608	phage rep (DvMG_0710) to glycosyl transferase (DvMF_0756) Mu-like.	Myo
D. vulgaris str. 'Miyazaki F'	2) 1558929 to 1597453	Phage repressor (DvMF_1261) to DNA methylase (DvMF_1308) Mu-like	Myo
D. vulgaris str. 'Miyazaki F'	3) 2016313 to 2055510	Phge prt (DvMF1634) to integrase (DvMF1687)	Sipho
D. desulfuricans ATCC 27774	1) 277508 to 306161	integrase (Ddes_0222) to Gp5 dmn prt (Ddes_0263) P2 like	Myo
D. salexigens DSM 2638	1) 2324343 to 2361506	Late control D prt (Desal_2111) to integrase (Desal_2156) P2 like	Myo
D. vulgaris RCH1	1) 544429 to 578809	tail fiber (DevalDRAFT_0507) to phage repressor (DevalDRAFT_0552)	Myo
D. vulgaris RCH1	2) 1192658 to 1225822	integrase (DevalDRAFT_1100) to tail fiber (DevalDRAFT_1141)	Sipho
D. vulgaris RCH1	*3) 3451485 to 3468963	baseplate J (DevalDRAFT_3071) to TerL (DevalDRAFT_3097) Tail genes only	Myo
D. vulgaris RCH1	*4) 3501484 to 3527858	Mor (DevalDRAFT_3125) to MugpT (DevalDRAFT_3149) Lysis/ head genes only.	ukn
D. aespoeensis Aspo-2	1) 427760 to 489469	phage prt (DaesDRAFT_0381) to glycosyl hydrolase (DaesDRAFT_0448)	Myo
D. aespoeensis Aspo-2	2) 632255 to 666932	integrase (DaesDRAFT_0561) to Gp5 domain protein (DaesDRAFT__0618)	Myo
D. aespoeensis Aspo-2	3) 2787013 to 2820438	tail protein (DaesDRAFT_2565) to Mor (DaesDRAFT_2616) Mu-like	Myo
Desulfovibrio sp. FW1012B	1) 1543396 to 1573274	TMP (DFW101DRAFT_1368) to phage repressor (DFW101DRAFT_1398) Mu-like	Myo
Desulfovibrio sp. FW1012B	2) 2721197 to 2781228	phageprt (DFW101DRAFT_2373) to integrase (DFW101DRAFT_2464)	ukn
D. fructosovorans JJ	1) 33508 to 75894	integrase (DesfrDRAFT_0031) to P4 primase (DesfrDRAFT_0080)	Sipho
D. fructosovorans JJ	2) 138295 to 187786	primase (DesfrDRAFT_0127) to integrase (DesfrDRAT_0190)	Sipho
D. fructosovorans JJ	3) 1358186 to 1388840	repressor (DesfrDRAFT_1175) to hypothetical protein (DesfrDRAFT_1219) Mu-like	Myo
D. desulfuricans str. G20	1) 939853 to 980902	integrase (Dde_0912) to tail fiber (Dde_0954)	ukn
D. desulfuricans str. G20	2) 1918124 to 1987712	tail fiber (Dde_1868) to phage related protein (Dde_1936)	Myo
D. desulfuricans str. G20	3) 3321832 to 3359581	hyp nov (Dde_3338) to tail fiber (Dde_3393) Mu-like, IS insertions.	Myo
D. vulgaris str. Hildenborough	1) 233752 to 270425	P4 primase (DVU0236) to integrase (DVU0189)	Myo
D. vulgaris str. Hildenborough	2) 1561263 to 1595902	tfa (DVU1483) to integrase (DVU1527)	Sipho
D. vulgaris str. Hildenborough	3) 1771104 to 1822728	tfa (DVU1695) to integrase (DVU1757)	Podo
D. vulgaris str. Hildenborough	4) 2252215 to 2293693	tail fiber (DVU2153) to integrase (DVU2197).	Sipho
D. vulgaris str. Hildenborough	5) 2936472 to 2977150	integrase (DU2828) to primase (DVU2881)	Myo
D. vulgaris str. Hildenborough	6) 1205411 to 1240251	tfa (DVU1099) to Cro/Ci (DVU1144) Mu-like	Myo
D. vulgaris str. Hildenborough	7) 2801478 to 2833113	TnpA (DVU2688) to tfa (DVU2731) Mu-like.	Myo
Desulfovibrio sp. 3_1_syn3	1) 1748161 to 1780719	integrase (HMPREF0326_01510) to P4 primase (_015445)	ukn
S. fumaroxidans MPOB	1) 2282818 to 2320999	phage repressor (Sfum_1866) to transposase (Sfum_1901)	Myo
S. fumaroxidans MPOB	2) 4644739 to 4685731	integrase (Sfum_3796) to tail fiber (Sfum_3838)	Myo
SRB genomes that lack identifiable P2, lambda, or Mu related prophage elements: Desulfitobacterium hafniense Y51, Candidatus Desulforudis audaxviator MP104C, Thermodesulfovibrio yellowstonii DSM 11347, Desulfohalobium retbaense DSM 5692; Desulfonatronospira thiodismutans ASO3-1; Desulfomicrobium baculatum DSM 4028; Desulfovibrio magneticus RS-1; Desulfurivibrio alkaliphilus AHT2; Desulfovibrio piger ATCC 29098; Desulfuromonas acetoxidans DSM 684			

Prophage identified in the completely sequence genomes of sulfate reducing bacteria. Nucleotide range corresponds to base pairs of putative prophage, based on the corresponding genome entry listed in Table 1. Locus tag range denotes the predicted gene products from the leftmost and rightmost genes of the prophage element that exhibit similarity to a known phage associated gene product. Morphology abbreviations are contractile-tailed myophage (Myo), flexible-tailed siphophage (sipho), and short-tailed podophage (podo). Elements for which morphological prediction could not be made are listed as unknown (ukn).

Appendix G. Partial List of Presentations

Replacing Chemical Biocides with Targeted Bacteriophage. June 23, 2010. RPSEA Technical Conference. Houston, TX.

Replacing Chemical Biocides with Bacteriophage. June 26, 2011. RPSEA Conference. Houston, TX.

Replacing Chemical Biocides with Bacteriophage. June 31, 2011. RPSEA Environmental Health & Safety. Houston, TX.

Ecolyse SRB Phage Report. November 21, 2011. RPSEA Ultra Deep Water Conference. The Woodlands, TX.