Sulfate-reducing bacteria (SRB) constitute a group of diverse, primarily chemoorganotrophic microorganisms.  Sulfur, present in abundance in the anoxic primordial Earth, can assume a wide range of oxidation states from its most oxidized form, sulfate (SO42-), to its most reduced form, hydrogen sulfide (H2S).  Due to its redox versatility and availability, early microbes utilized sulfur chemistry to drive metabolic processes.  This is exemplified by the SRB, which produce a proton motive force to drive production of ATP through the reduction of sulfate to hydrogen sulfide.  This is then released as a waste product of the dissimilatory sulfate reduction pathway.  SRB are anaerobic because oxygen is not the final electron acceptor. (1) However, a small number of species may be tolerant of some levels of oxygen, and hence are aerotolerant. Because of their chemical requirements, SRB are commonly found in freshwater sediments, sewage, and other environments with a high concentration of sulfate and a low concentration of oxygen, including the human oral cavity. (2)

        Research on members of the genus *Desulfovibrio* has elucidated many of the key metabolic processes in SRB.  An electron transport chain consisting of cytochromes, flavoproteins, and iron-sulfur proteins is used to generate a proton gradient across the bacterial cell membrane.  Most SRB are chemoorganotrophs that utilize electrons from diverse sources that vary greatly among SRB, but commonly include lactate or pyruvate.  Chemolithotrophic SRB utilize hydrogen gas as electron donors. (3) The terminal electron acceptor is sulfate from environmental sources.  Sulfate is first reduced to sulfite (SO32-), a process that requires ATP hydrolysis.  Sulfite is next reduced to sulfide (S2-), which is released as hydrogen sulfide, utilizing a second molecule of ATP.  This post-electron-transport-chain reductive process incorporates eight electron transfers, use of two molecules of ATP, and is catalyzed by four enzymes. (4) ATP is ultimately synthesized through the dissipation of the proton motive force as protons move down their concentration gradient through an ATP synthase integral protein.

SRB have evolved along with the sulfur-oxidizing bacteria and they contribute greatly to the balance of sulfur, sulfate, and sulfide in the sulfur cycle. (5) In deep layers of sediment, sulfur compounds are present at low concentrations. Therefore, interdependence between SRB and sulfur-oxidizing bacteria evolved to ensure each group has access to enough of its required resource, produced by the other. (6) The study of SRB is thus an examination of the coevolution of two groups. Furthermore, an understanding of the resulting metabolic interdependence is essential for the successful cultivation or reduction of these organisms.

The study of SRB also has numerous industrial applications. SRB are present in hydrothermal vents, and therefore can have impacts on the oil and gas industries. (7)Because one byproduct of sulfate reduction is hydrogen sulfide, which can react with sources of iron in machinery for extracting crude oil to form a precipitate, SRB can cause industrial inefficiencies. (8) They also thrive in industrial wastewater. The contamination of the environment or equipment with a precipitate, color, or hydrogen sulfide smell makes the study and control of SRB a priority for these industries. Estimates of the industrial damage construed by SRB to iron equipment corrosion and reduction in heat transfer efficiency has risen from $600 million annually in 1985 to several billion dollars annually at the present time, signifying that SRB biocorrosion is a major economic hindrance to industry that has not yet received an adequate solution. (9, 10)

Furthermore, although SRB are slow growing, they are able to withstand unfavorable conditions. Some species even form endospores and can therefore exist in a dormant state during anti-SRB treatment.(4)The study of SRB and how to remove them from an environment is a challenging priority for oil industries and water treatment plants that warrants further attention.

This project centers on isolation of SRB from environmental samples such as freshwater sediment.  Although SRB thrive in a multitude of environments, this source was selected as a common representative location of SRB in the state of Vermont, where SRB contribute a key role to the sulfur cycle as described above.  Moist sediments contain abundant sulfates and potentially low concentrations of oxygen, suitable conditions for the growth of SRB.  After extraction of sediments and surrounding fresh pond water, SRB were enriched in a Winogradsky column.  Winogradsky columns represent pond cross-sections and self-sufficient ecosystems in which organisms in the sealed column form microenvironments based on availability of nutrients and diverse metabolisms.  After weeks of growth, the column develops both an oxygen gradient (aerobic zones descend into anaerobic) as well as a sulfur gradient (reduced sulfide exist in high concentrations low in the column).  Furthermore, sources of energy differ from light-harvesting phototrophs at the top, to chemotrophs in the depths of the dark soil.  With a large diversity of microorganisms separating into pseudo-discrete components, the use of a Winogradsky column allows for the enrichment of SRB in the deep anoxic zone.  Laboratory culture conditions sought to reproduce the natural conditions for SRB through anaerobic culture and biochemical selections, allowing for SRB to be cultured and isolated from these sources.  In closing, this work is significant because an understanding and optimization of SRB cultivation will allow ease of experimentation of inhibitory compounds, which may be applied in both industrial and municipal settings to halt the growth of SRB.

**Materials and Methods**

SRB Enrichment

        Pond sediment and water were collected from a site in Ripton, VT (from Tim Allen).  SRB were enriched and isolated from Winogradsky columns prepared earlier in the semester at Middlebury College.   As described by Allen and Spatafora, (11) columns were generated by homogenizing 400 mL of pond sediment with supplements that included 10 g calcium carbonate (CaCO3) as a ready carbon source, 5 g shredded paper as a long-term carbon source, 20 g calcium sulfate (CaSO4) as a source of sulfur, 20 g dipotassium phosphate (K2PO4) as a potassium and phosphorus source, and 2 g of yeast extract to provide miscellaneous vitamins, minerals, and macromolecules.  The supplemented mud was packed into a 250 mL graduated cylinder and 60 mL of pond water was added to the column.  Winogradsky columns were sealed and grown for ten weeks at room temperature with abundant sunlight prior to the cultivation of SRB.

SRB should be enriched primarily in the anoxic sediment in the lower layers of the column.  Core samples of this sediment were removed from the base of the column.  Samples were transferred to an inoculation needle and deep media was directly inoculated. Triple Sugar Iron (TSI) Agar (**VENDOR**) and Sulfide Indole Motility (SIM) media (**VENDOR**), which contain ferrous sulfate as both a sulfate source and an indicator, were used to culture putative SRB samples.  The reaction of iron with hydrogen sulfide, a byproduct of SRB metabolism, produces a black ferric sulfide precipitate in the presence of SRB. After incubation for 24-36 hours at 37°, black growths in the anaerobic zone of the deep were aseptically extracted and streaked onto SIM agar plates.  Plates were incubated anaerobically at 37°. Anaerobic culture conditions were generated with GasPak (BD) in sealed bags.

Confirmation of SRB Isolation

        Putative cultures of SRB were subjected to various biochemical tests to confirm whether the cultivated microbes were sulfate-reducers.  Analyses included oxidase and catalase tests, H2S gas production, motility determination, as well as gram staining.

**Results**

The first evidence of SRB occurred during sample collection. Removal of the Winogradsky column seal revealed a distinct rotten eggs smell due to the presence of hydrogen sulfide gas. After culturing samples from the Winogradsky columns using TSI and SIM deeps, the expected black growth formed anaerobically along with gas bubbles, signifying the production of hydrogen sulfide and fermentation products. The production of hydrogen sulfide was further evident by the rotten eggs smell when the lids of the deeps were removed. SIM and TSI deeps displayed evidence of motility because black precipitates were formed away from the stab line. Anaerobic growths were selected using an inoculation loop and SIM plates were streaked for isolation. After incubation under anaerobic conditions for 24 to 36 hours, black colonies in the media were again selected using a loop and tested to verify their identity as SRB.

Because hydrogen sulfide production does not necessarily indicate sulfate reduction, further tests were executed to determine whether the hydrogen sulfide producers isolated from the SRB-enriched sediment were SRB.  While SRB constitute a diverse group of bacteria, all SRB share several common traits.  Tests evaluated these conserved traits, such as a gram negative cellular envelope. Further tests, summarized in the table below, were used to support the identity of the isolated organisms as SRB. The experimentally isolated SRB were compared to positive and negative controls (Table 1). SRB were successfully isolated, as shown in the last column of Table 1.

(12)

Although SRB growth on SIM and TSI media was successful, SRB were unable to be cultured under other conditions.  Supplementing enriched media, such as lysogeny broth (LB) or tryptic soy agar (TSA), with iron sulfate as an indicator and sulfate source and a preferred carbon source like lactate or ethanol did not produce any growth of SRB.  Additionally, thioglycolate broth, a medium containing reducing agents to remove oxygen and generate anoxic conditions below the surface, did not support the growth of SRB when supplemented similarly.

**Discussion**

SRB were successfully isolated from a sample of pond sediment, as the isolated bacteria share all of the characteristics of typical SRB. The first method used in the isolation of SRB was enrichment in a Winogradsky column that generated proper nutrient conditions to grow SRB in the bottom zone of sediment. In subsequent cultures, only anaerobically growing hydrogen sulfide producers were selected for isolation and further evaluation. Although hydrogen sulfide production is a defining characteristic of SRB, further tests were carried out to confirm the presence of other typical SRB characteristics. Isolated SRB were strictly anaerobic and were determined to not contain catalase, an enzyme responsible for the conversion of toxic hydrogen peroxide to water and oxygen to protect the cell from oxidative damage. Strict anaerobes do not typically encode catalase as the presence of toxic environmental oxidizing compounds is limited.

This method of isolating SRB from enrichment culture differs from methods commonly found in the literature. Many researchers have grown SRB from environmental sources on enriched media supplemented with  iron sulfate and a preferred carbon source, typically lactate or acetate. (13, 14) (15) We were unsuccessful in cultivating SRB on solid LB or TSA with these supplements as well as in supplemented thioglycolate broth.  It is possible that SRB were outcompeted in liquid culture by facultative anaerobic organisms from the soil samples that could utilize the oxic zone atop the broth as well as the lower anoxic regions.  Shukla and Reed also claim that SRB growth in broth medium is not a recommended method of isolation because the change in optical density does not occur for a number of days. We also noted that solutions of iron frequently generated gray precipitates of ferric iron compounds when filter sterilized into media at 50˚ C, possibly precipitating with an essential anion and therefore removing it from the available nutrients.  Furthermore, solid iron compounds may demonstrate oligodynamic properties, inhibiting the growth of certain microbes and accounting for the slow and reduced growth of all organisms on media containing traces of precipitated iron. (16)

The method of sampling the Winogradsky columns proved to be effective at isolating similar hydrogen sulfide producing colonies as isolated by Iversonusing complex media. (17) This method was effective because enriching for SRB prior to isolation avoided the main challenge for previous SRB isolation in the literature, ensuring that SRB were not outcompeted by other organisms. Therefore, 3% NaSO3.7H2O was not required to inhibit the growth of competing organisms, as proposed by Butlin and Adams. (6)

The identification and isolation of SRB using ferric sulfide as a marker of growth suggests that detection of SRB contamination is a straightforward process. Therefore, further research should prioritize cost-efficient decontamination methods over diagnostic methods. This updated focus of research could assist in the efficiency of SRB remediation efforts considering the vast cost of these methods. Furthermore, the increased understanding of the requirements of SRB produced by this work in terms of the range of their preferred growth conditions may facilitate the establishment of laboratory culture conditions for diverse SRB. The ability to culture many types of SRB under laboratory conditions is requisite for the development of broad-spectrum SRB removal methods because it will both allow for efficient experimentation as well as provide information about essential compounds for SRB inhibition.  Future research will seek to develop broadly acting anti-SRB compounds that are both cost effective and efficient, reducing the economic burden of SRB on municipal and industrial machinery and increasing the productivity of many modern processes from wastewater treatment to oil extraction.

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