

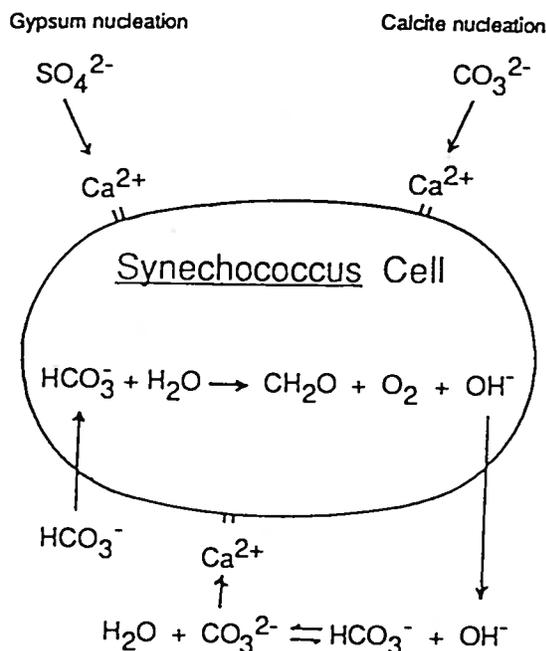
**Calcite Formation by Sulfate Reducing Bacteria Enriched from a
Microbial Mat in Great Sippewissett Salt Marsh:
Is It Possible?**

**Min-Ken Liao, Hope College, Holland, MI. U.S.A.
liao@hope.edu**

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INTRODUCTION

Bacterial biomineralization is a diverse and widespread phenomenon that results from cellularly mediated physiological processes. For example, the role of cyanobacteria in large-scale precipitation of calcium carbonate in natural environments has been well-studied (Thompson and Ferris, 1990; Thompson et al. 1997). It has been proven that *Synechococcus* is responsible for direct alkalization of its microenvironment resulting from phototrophic way of life (Shown below) (Thompson and Ferris, 1990).

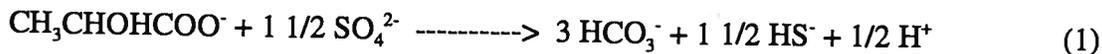


Pseudomonas fluorescens ATCC13525 was also reported to produce exocellular calcite to combat the presence of elevated amounts of calcium in its surroundings (Anderson et al. 1992).

Previous studies on the microorganism mediated calcite formation suggest that the increase of the ANC (acid neutralizing capacity) in the microenvironment and the presence of abundant calcium ion in the environment are the key factors for calcite formation. Therefore, it is reasonable to hypothesize that in a given condition where the calcium ion concentration is high, any microorganism that can increase the pH of its surrounding microenvironment can form calcite. To eliminate the effects from phototrophic and aerobic respiration, I chose Sulfate Reducing Bacteria grown anaerobically in the dark to test the hypothesis.

This project consists of two parts: (1) Enrichment of the Sulfate Reducing Bacteria (SRB), and (2) Examination of the calcite formation by SRBs. We designed two enrichment schemes: (1) Lactate was provided as the carbon and electron source and sulfate as the electron acceptor, (2) Ethanol was provided as the carbon and electron source and sulfate as the electron acceptor. These are based on the following rationales.

(1) If the SRBs oxidize lactate completely, the predicted dissimilation pathway would be:



The ΔANC (acid neutralizing capacity) will be 4 (per lactate) in the neutral to alkaline range. That is, for each complete reaction, four protons from the environment will be removed by base of the products generated from the complete lactate oxidization of SRBs. In result, the pH of the microenvironment might increase and the condition becomes favorable for calcite formation.

(2) If the SRBs can use lactate and the complete oxidization of lactate is a two-step process, the predicted dissimilation pathway would be:



$$\Delta\text{ANC}=1 / \text{lactate}$$



$$\Delta\text{ANC}=3 / \text{lactate}$$

The total ΔANC would be 4, which is equal to the one-step complete oxidation. The pH of the surrounding microenvironment will increase and the condition is favorable for the calcite formation.

(3) If the SRBs oxidize lactate incompletely, the predicted dissimilation pathway would be:



$$\Delta\text{ANC}=1 / \text{lactate}$$

(4) If the SRBs can use ethanol and the complete oxidization of ethanol is a one-step process, the predicted dissimilation pathway would be:



$$\Delta\text{ANC}=3 / \text{ethanol}$$

The ΔANC value suggests that it is possible that the pH of the microenvironment will increase and the condition is favorable for the calcite formation.

To simulate calcite formation in the real environment, a mixed SRBs enrichment culture was used, instead of a pure culture. The SRBs were enriched from microbial mats collected at Great Sippewissett Salt Marsh for two reasons: (1) The habitat of the salt marsh is well studied (Nicholson et al. 1987), (2) SRBs were abundantly present in the habitat (Microbial Diversity Courses, Previous student research reports).

MATERIALS AND METHODS

Sampling and sulfate reducing bacteria enrichment. The microbial mats were collected from the Great Sippewissett Salt Marsh. Approximately 1 gram of the upper four layers (green, pink, gray, and black) were inoculated into enrichment medium. Saltwater medium (reduced) with appropriate supplements was used. To enrich SRBs that can utilize lactate, 10 mM lactate and 20 mM sodium sulfate were supplemented. To enrich SRBs that can utilize ethanol, 20 mM ethanol and 20 mM sodium sulfate were supplemented. To investigate the possibility of calcite formation, 10mM of calcium ion given as CaSO_4 was added to each medium. The enrichments were carried out under anoxic conditions at 22°C in the dark to limit the growth of cyanobacteria., which seem to be able to survive fermentatively under anoxic conditions in the dark. (Jorge L.M. Rodrigues. Project report 1998).

High Pressure Liquid Chromatography (HPLC) analysis. The consumption of lactate and the generation of acetate was followed by HPLC analysis. One milli-liter was taken from the enrichments each day and the cell-free extracts were stored at -70°C until HPLC analysis was performed. The HPLC was equipped with a cation exchange column (Shodex, IonPak KC-811 resin gel.) 22 micro-liters of sample and external standards (5 mM of lactate and 5 mM of acetate) were separated with 0.1% H_3PO_4 as eluent in isocratic elution. The flow rate was 1 ml/min, the UV-detection wavelength was 210 nm, and the column oven temperature was kept at 60°C.

***In situ* 16S rRNA Hybridization.** To identify SRBs in the enrichments, *in situ* hybridization was performed. The procedure for sample preparation was provided as a Course handout prepared by Scott Dawson. Samples were hybridized with SRB2 (5' CGYGCGCCRCTYACT 3') probe to specifically identify SRBs and were also counterstained with DAPI which interacts with all living cells.

Checkerboard Hybridization Analysis. To further identify the microbial community in the enrichments, checkerboard DNA hybridization analysis was performed. The PCR reactions were carried out on the genomic DNA extracted from the lactate/sulfate and ethanol/sulfate enrichments to amplify the 16S rRNA genes. The primers used in this experiments were Dig-UF (5' LAGAGTTTGATYMTGGC 3') and Universal reverse (5' GYTACCTTGTTACGACTT 3'). The resulting PCR products were subjected to checkerboard hybridization analysis. The probes used in the analysis are listed in Figure 1.

DNA Sequence Analysis on the Enrichments. Genomic DNA of the two enrichment cultures, lactate/sulfate and ethanol/sulfate, were extracted for further cloning and sequencing. One milliliter of each culture was used for genomic DNA extraction. The extraction procedure was provided by Dr. Bruce Paster as a Microbial Diversity course handout. The extracted DNA was further purified using Promega Genomic DNA Purification Kit to remove any potential inhibitors that will interfere with the PCR amplification. The procedure of this purification was provided by the supplier. Promega TaqBead Hot Start Polymerase was used for 16S rRNA gene amplification. The standard PCR reaction

mixture was prepared with 2.5 mM MgCl₂ as the final concentration, and the amplification reaction was initiated at 55° as annealing temperature. The primers used for this amplification were universal 16S forward (5' AGAGTTTGATYMTGGC 3') and reverse (5' GYTACCTTGTTACGACTT 3').

The PCR products amplified from two enrichment cultures were purified, concentrated, and blunt-ended using enzyme *Pfu* (72°C, 30 minutes). Blunt end ligation and cloning reaction were performed using Invitrogen Zero-Blunt Kit. The procedure was provided by the supplier. PCR amplifications were performed on the Kanamycin resistant transformants to examine the presence of the inserting gene. Primers used in these amplifications were TOPO-forward (5' CCACTAGTAACGGCCGCC 3') and TOPO-universal (5' CGGCCGCCAGTGTGATG 3'). RFLP (Restriction Fragment length polymorphism) analysis was performed to characterize the inserts. The enzymatic reactions (*HinP* I and *Msp* I) were prepared following the standard procedure provided in the Course handout. Plasmid DNA containing different RFLP patterns were sent to be sequenced.

Scanning Electron Microscopy (SEM). Scanning electron microscopy (SEM) was used to determine the presence, or the absence, of calcite. Two samples were prepared: lactate/sulfate and ethanol/sulfate enrichment. Both were in their third enrichment. One milli-liter of each one-week-old culture was concentrated. The samples were then prepared following the protocol provided by MBL Central Microscopy Facility with a few slight modifications: One 30 minutes fixation in 2% glutaraldehyde, two 15 minutes washes in 0.1 M Na-cacodylate, and a series of ethanol dehydration steps, 50%, 70%, 85%, and 95% ethanol for 10 minutes each. The samples were placed in 100% ethanol overnight at 4°C then taken to the Microscopy Facility for the rest of the preparation. Two samples were dried in a Tusimis Samdri 780-A critical point dryer then coated with carbon and gold/palladium (60:40%) using Tousimis Samsputter 2a. JOEL JSM-840 scanning microscope(20 KV) was used to examine the samples. The samples were also prepared by a different way. One milli-liter of each enrichment was concentrated into a final volume of 10 µl. The cell suspensions were smeared onto two glass slides which were placed in a 55°C oven overnight to dry. This method will not damage calcite, if any, from by bacteria. However, the shapes of bacteria will be altered.

RESULTS

***In situ* Hybridization Analysis.** The results of *in situ* hybridization indicate the abundant presence of SRBs in both enrichments (Fig 2 and Fig 3). The top panel is the result of SRB staining. A positive staining renders cell red in color. The middle panel is the result of DAPI counterstaining. A positive staining renders cell blue in color. The bottom panel is the overlay of the previous two staining. The double staining render cells pink/purple in color. The results of the lactate/sulfate enrichment is shown in Figure 2 and the results of the ethanol/sulfate enrichment is shown in Figure 3. It is clearly shown that in both enrichments, there are more than 50% SRBs present in the microbial community.

Substrate consumption and acetate production. The results of HPLC analyses indicate that lactate in the lactate/sulfate enrichment was converted almost by 100% to acetate in 48 hours (Table 1). The starting lactate concentration was 10 mM. At the end of the second day, approximately 10 mM acetate was detected. The acetate concentration remained stable for the rest of the experimenting period (Fig. 4a). The 1:1 ratio of the lactate consumption and acetate production suggests that the predicted dissimilation equation (4) is probably correct.

On the other hand, not all the ethanol in the ethanol/sulfate enrichment was converted to acetate (Table 2). Due to the lack of the proper column and detector for ethanol, it is difficult to determine its true dissimilation equation. The starting ethanol concentration was 20 mM. At the end of the second day, approximately 5 mM acetate was detected. The acetate concentration remained stable for the rest of the experimenting period (Fig. 4b).

In both enrichments, the acetate concentrations stayed the same for the last six days of the experiment indicating that the conversion was complete in 48 hours and that there were no microorganisms in the enrichments that could consume acetate.

Checkerboard Analysis. The results of the checkerboard analysis is shown in Figure 1. They further confirmed the presence of the SRBs in both enrichments. Also present are some bacteria of the Beta group, low G+C Gram positives, some *Planctomyces*, and some spirochetes. The patterns from two enrichments are very similar indicating that the composition of these two microbial communities may be very similar.

DNA Sequence Analysis. Approximately 100 Kanamycin resistant transformants were obtained from the cloning of the PCR products amplified from the lactate/sulfate enrichment sample. 20 of them were screened for the presence of the insert. Among these 20 clones, 3 of them contain the insert of the right size. RFLP analysis indicated that there were two distinctly different band patterns. Plasmids DNA containing these two RFLP patterns were sequenced (WH124 and WH125). WH124 showed 100% similarity to *Desulfovibrio acrylicus* and WH125 99% to *Desulfovibrio acrylicus* (Table 3) (Figure 5).

Approximately 50 Kanamycin resistant transformants were obtained from the cloning of the PCR products amplified from the ethanol/sulfate enrichment. 16 of them were screened for the presence of the insert. Among these 16 clones, 12 of them contain the insert of the right size. RFLP (Restriction Fragment Length Polymorphism) was performed to characterize these inserts. There were three different major RFLP band patterns among these 12. Plasmid DNA containing three of the most common RFLP patterns were sequenced (WH122, WH123 and WH161). The 16S rRNA gene sequence of WH122 showed 99.3% similarity to *Pelobacter venetianus*, WH123 96.3% to *Desulfovibrio salexigens* and WH161 93% similarity to *Desulfovibrio salexigens*. WH 123 and WH 161 showed 97.4% similarity (Table 3) (Figure 5).

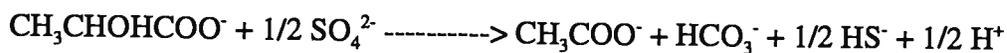
The results from the RFLP analysis and DNA sequencing suggested that after three enrichment steps, the microbial communities in these two enrichments were reasonably homogeneous.

Examination of the Calcite Formation using SEM. The samples prepared using the standard fixation/hydration method did not work as predicted. Due to the lack of experience in sample treatment, the cells were washed away during the fixation procedure. As the end of the course was near, a quick method was used. The cells were precipitated and smeared onto a glass slide. Since the purpose of the SEM analysis was to detect the presence of calcite, the shapes and states of the cells were not the main concern. SEM-EDS analysis was performed on both the lactate/sulfate and ethanol/sulfate enrichments. The presence of calcite was detected on the specific spot on an individual cell and its surrounding field. The readings were compared. In both cases of lactate/sulfate and ethanol/sulfate enrichments, there was no significant calcium detected (Fig. 5). However, the cell shapes were surprisingly clear. The vibrio-shaped *Desulfovibrio* cells were present in the lactose/sulfate enrichments (Fig. 6). The calcium readings focused on the cell and its surrounding showed no significant difference indicating that there was calcite present in the microenvironment. The rod-shaped cells present in the ethanol/sulfate enrichments are shown in Figure 6.

DISCUSSION

The results obtained from *in situ* 16S rRNA hybridization and the Checkerboard hybridization analysis clearly indicate the presence of the sulfate reducing bacteria in the enrichment. Results from *in situ* 16S rRNA hybridization not only provided a direct evidence for the presence of SRBs but also provided the information about cellular morphology. In the lactate/sulfate enrichment, most of the cells interacted with SRB probe were vibrio or rod in shape. However, in the ethanol/sulfate enrichment, most of the cells which interacted with SRB probe were short-rod in shape. Vibrio-shaped cells were almost undetectable in the ethanol/sulfate enrichment. This observation revealed that the members of these two microbial communities were different. The Checkerboard hybridization analysis clearly showed the presence of a series of other microorganisms. However, the information obtained from the checkerboard hybridization analysis was not sufficient enough to support any quantitative study on the microbial communities.

The HPLC analysis on the lactate/sulfate enrichment indicated that the SRBs in the microbial community oxidize lactate incompletely. The dissimilation pathway is:



The stoichiometry of lactate conversion to acetate is 1:1. 10 mM lactate was converted to approximately 10 mM acetate. There were no acetate oxidizing bacteria present in the enrichment to consume the acetate

produced by lactate dissimilation. This dissimilation pathway only provides ΔANC 1 which only causes a slight change of pH, if any, in the microenvironment.

The HPLC analysis on the ethanol/sulfate enrichment indicated that the predicted equation failed to explain the dissimilation pathway carried out by SRBs in the microbial community. Two assumptions can be made from this result: (1) SRBs do oxidize ethanol yet the reaction is incomplete. Only 25% of ethanol was converted to acetate. (2) SRBs do oxidize ethanol and the reaction is complete. The stoichiometry of ethanol and acetate is 4:1. The predicted dissimilation equation needs to be altered in order to explain the observed results. The first assumption can not be tested because the laboratory is not equipped to detect the remaining ethanol. The second assumption can be proven correct if the dissimilation pathway is:



$$\Delta\text{ANC} = 9 / 4 \text{ ethanol} = 2.25 / \text{ethanol}$$

In this case, the ΔANC is slightly smaller than the value in the predicted in equation (5). In result, the pH of the microenvironment will still increase more dramatically than lactate/sulfate enrichment which proceeds as predicted in equation (2).

DNA sequence results specifically indicate that both media successfully enriched sulfate reducing bacteria. The 16S rRNA gene of *Desulfovibrio acrylicus* was amplified from the lactate/sulfate enrichment. *Desulfovibrio acrylicus* was first isolated by van der Maarel, et al. in 1996 due to its unique ability to reduce acrylate. The cells were reported to be short-rods to vibrios in shape and were motile. Cells have of a Gram-negative cell wall structure (van der Maarel, et al. 1996). The *Desulfovibrio* species belongs to *Eubacteria*, *Proteobacteria*, delta subdivision. The species was reported to grow best in basal media with lactate and sulfate, which was the exact way the enrichment was done in this project. DNA sequencing results also agreed with the results obtained from *in situ* hybridization analysis. The distinctive vibrio-shaped *Desulfovibrio acrylicus* was clearly seen in lactate/sulfate enrichments. The SEM microscopic analysis also revealed the presence of *Desulfovibrio acrylicus* (Fig 6).

The 16S rRNA genes of *Desulfovibrio salexigens* and *Pelobacter venetianus* were amplified from the ethanol/sulfate enrichment. *Desulfovibrio salexigens* was classified as a Gram-negative sulfate-reducing bacteria. The species was reported to be able to utilize organic substrates, like lactate, pyruvate, ethanol, and in many cases also malate and fumarate. Electron donors are incompletely oxidized to acetate. This information supports the first assumption that SRBs do oxidize ethanol yet the reaction is incomplete (The Prokaryotes). The cellular morphology of *Desulfovibrio salexigens* also agrees with what was revealed from the *in situ* hybridization analysis and SEM observation. Species in the *Pelobacter* Genus as strictly anaerobic, Gram-negative, nonsporeforming, rod-shaped bacteria that use only a very limited number of substrates. However, *Pelobacter venetianus* was one of the three species in this genus that does not show any resemblance to other fermenting Gram-negative strict anaerobes; instead,

they appeared to be highly related to several strains of sulfur-reducing anaerobes (The Prokaryotes). The fact that *Pelobacter venetianus* is able to utilize ethanol as a metabolic substrate and produce acetate explains the growth in the ethanol/sulfate enrichment. The cellular morphology of the ethanol/sulfate enrichment observed from *in situ* hybridization analysis and the SEM agree with the published cellular morphology of *Desulfovibrio salexigens* and *Pelobacter venetianus*.

The EDS analysis indicated that there was no calcite formed in the microenvironment. This preliminary result suggested that SRBs are able to form calcite is a null hypothesis. This information agrees with the prediction made by Necholson, et al. 1987. They proposed that the formation of stromatolites from the microbial mats at Great Sippewissett Marsh is unlikely to take place (Nicholson, et al. 1987). "The high permeability of sandy sediments, rapid changes in water level due to tidal inundation, its location at the air-sediment interface and near a tidal channel, and the presence of bioturbators (nematodes, worms, etc.) despite the presence of hydrogen sulfide, do not bode well for preservation of individual cells for any long period of time, although the persistence of pigmented buried layers at depth is intriguing." (Nicholson, et al. 1987) Though it has been proposed that the formation of stromatolites can be an abiotic process (Grotzinger, J., and D. H. Rothman 1996), the evidences of stromatolites formed by microbial mats are still strong. This report only suggests that under this particular experimental condition, calcite formation can not be observed. However, with another enrichments and other sets of experimental designs, calcite formation might be observed in the laboratory.

FUTURE STUDIES

1. Positive control: Cyanobacteria should be isolated from the microbial mats and used in this study as a positive control.
2. Water chemistry: The changes of calcium ion concentration, HCO_3^- concentration, and pH value over the time of experiment should be assayed.
3. Buffering capacity: To examine the effect of pH changes, the buffering capacity should be decreased.
4. Ethanol detection: To examine the consumption of ethanol, different detecting device is needed.
5. SEM: The samples should be prepared in the standard way.
6. TEM: Thin-section transmission electron microscopy should be done to physically visualize the forming of calcite around an individual cell.
7. Different microorganisms and samples from different sites can also be tested.

REFERENCES

- Anderson, S., V. D. Appanna, J. Huang and T. Viswnatha 1992. A novel role for calcite in calcium homeostasis. *FEBS* 308 (1): 94-96
- Awramik, S. 1984. Ancient stromatolites and microbial mats. *Microbial Mats: Stromatolites*. pp.1-22
- Grotzinger, J., and D. H. Rothman 1996. An abiotic model for stromatolite morphogenesis. *Nature* 383: 423-4252
- Nicholson, J., J. Stolz and B. Pierson 1987. Structure of a microbial mat at Great Sippewissett Marsh, Cape Cod, Massachusetts. *FEMS Microbiol. Ecol.* 45:343-364
- Schink, B. 1992. Chapter 186: The Genus *Pelobacter*. In: *The Prokaryotes*. Eds.: Balows, A, H. Truper, M. Dworkin, W. Harder and K-H. Schleifer, New York: Springer-Verlag.
- Summer, D. 1997. Late Archean calcite-microbe interactions: Two morphologically distinct microbial communities that affected calcite nucleation differently. *Palaios* 12:302-318
- Thompson, J. B. and F. G. Ferris 1990. Cyanobacterial precipitation of gypsum, calcite, and magnesite from natural alkaline lake water. *Geology* 18: 995-998
- Thompson, J.B., S. Schultze-Lam and T. Beveridge 1997. Whiting events: Biogenic origin due to the photosynthetic activity of cyanobacterial picoplankton. *Limnology and Oceanography* 42(1): 133-141
- van der Maarel, M., S. van Bergeijk, A. van Werkhoven, A. Laverman, W. Meijer, W. Stam, and T. Hansen 1996. Cleavage of dimethylsulfoniopropionate and reduction of acrylate by *Desulfovibrio acrylicus* sp. nov. *Arch. Microbiol.* 166: 109-115
- Widdel, F. and F. Bak. 1992. Chapter 183: Gram -negative mesophilic sulfate-reducing bacteria. In: *The Prokaryotes*. Eds.: Balows, A, H. Truper, M. Dworkin, W. Harder and K-H. Schleifer, New York: Springer-Verlag.

Figure 1. Checkerboard result. The result of ethanol/sulfate enrichment is shown on lane 4 and lactate/sulfate enrichment is shown on lane 5. The probes used in this analysis are shown on the right-hand panel.

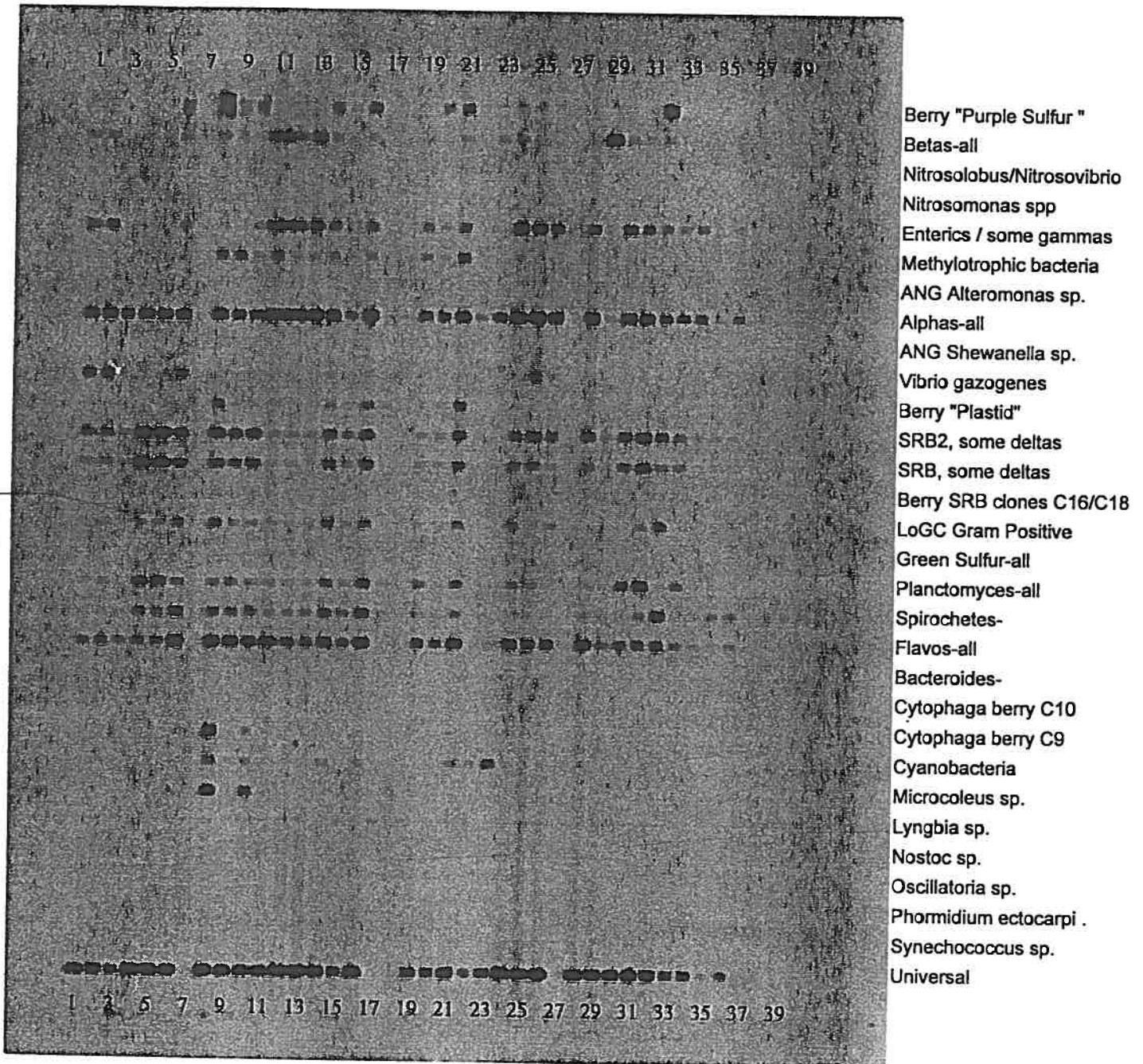


Figure 2. *In situ* hybridization analysis on lactate/sulfate enrichment using SRB probe.



Figure 3. *In situ* hybridization analysis on ethanol/sulfate enrichment using SRB probe.



Table 1. The consumption of the substrate lactate and the production of acetate from the lactate/sulfate enrichment.

LACTATE ENRICHMENT		
DAY	[lactate] mM	[acetate] mM
0	10	0
1	9.43	1.08
2	0	10.70
3	0	8.89
4	0	10.61
5	0	9.25
6	0	9.16
7	0	8.60
8	0	10.51

Table 2. The production of acetate from the ethanol/sulfate enrichment.

ETHANOL ENRICHMENT	
DAY	[acetate] mM
0	0
1	0.57
2	5.73
3	5.83
4	4.80
5	4.34
6	5.29
7	4.34
8	5.10

Figure 4a. Lactate consumption and acetate production in lactate/sulfate enrichment.

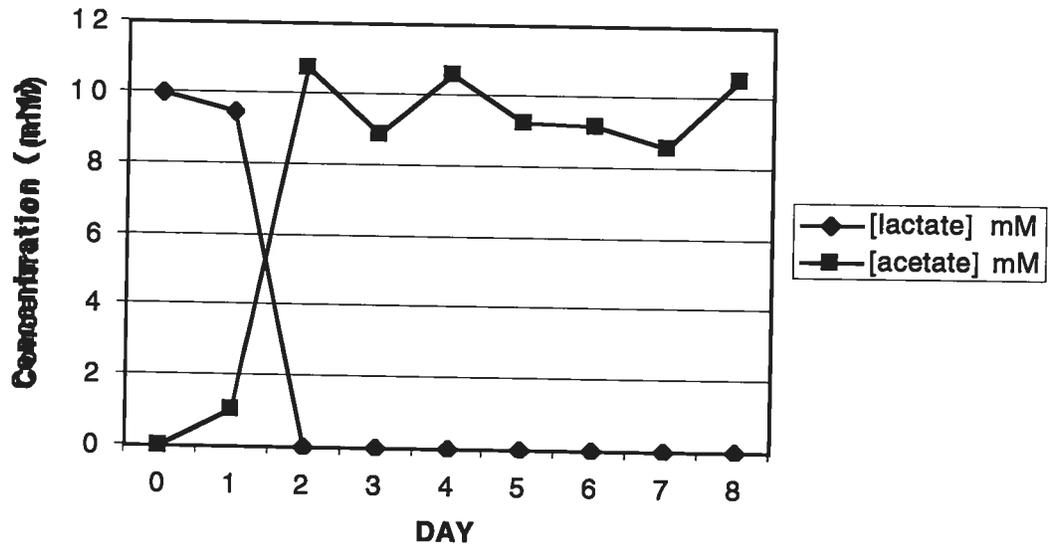


Figure 4b. Acetate production in ethanol/sulfate enrichment.

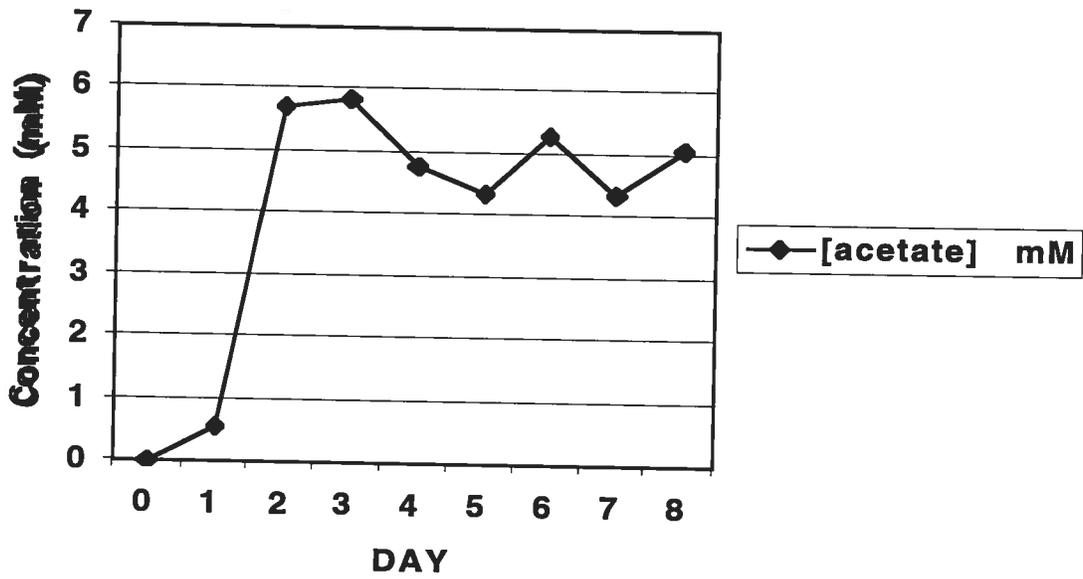


Table 3. DNA sequences of 16S rRNA genes of WH122, 123, 124, 126, and 161.

	WH 124	WH 125	WH 122	WH 123	WH 161
	lactate/sulfate	lactate/sulfate	ethanol/sulfate	ethanol/sulfate	ethanol/sulfate
<i>Desulfovibrio acrylicus</i>	100%	99%			
<i>Desulfovibrio salexigens.</i>				96.3%	
<i>Pelobacter venetianus</i>			99.3%		93%

Figure 5. Phylogenetic analysis of WH122, 123, 124, 125 and 161.

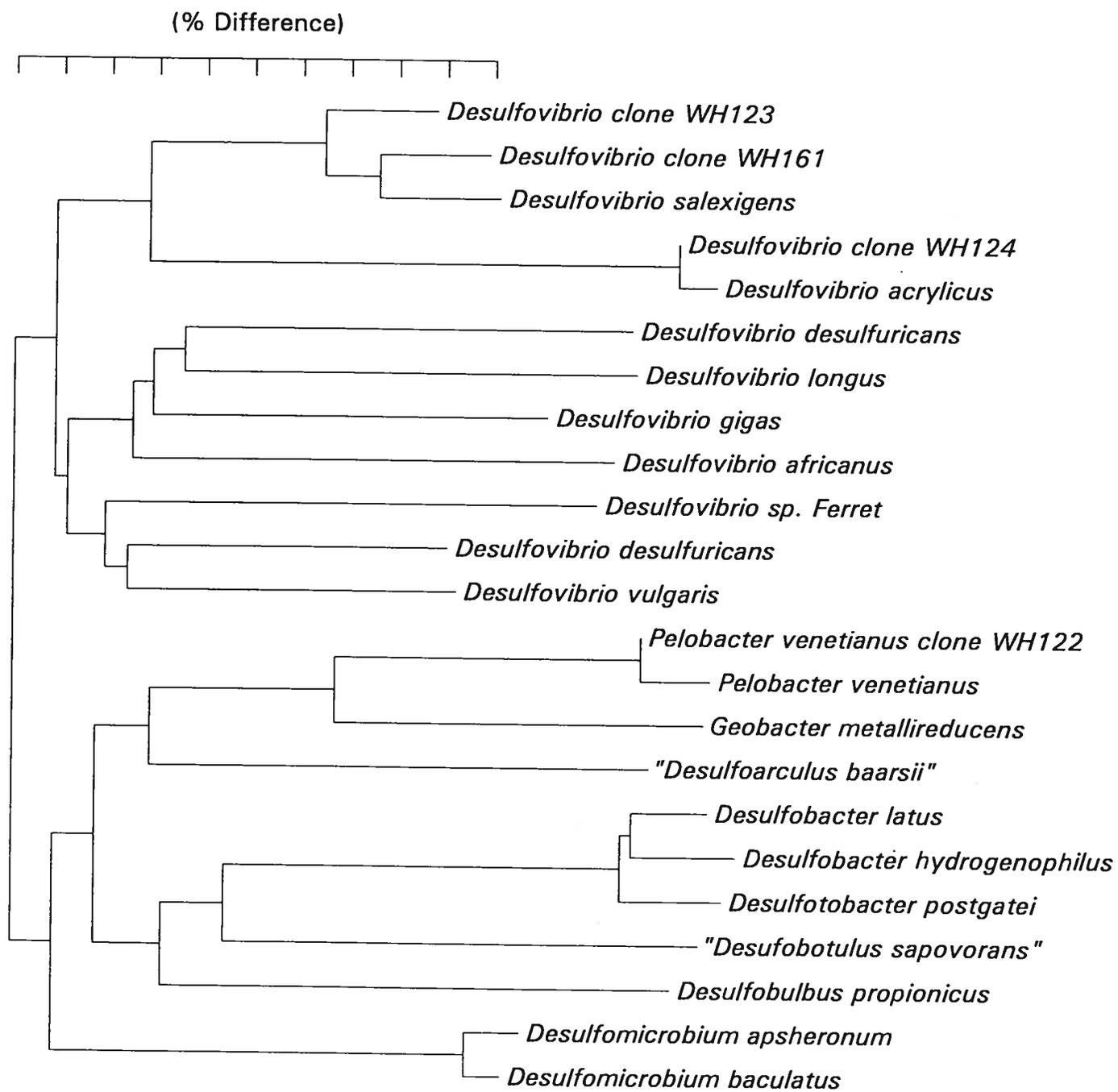


Figure 7. SEM result on ethanol/sulfate enrichment (top panel) and lactate/sulfate enrichment (bottom panel).

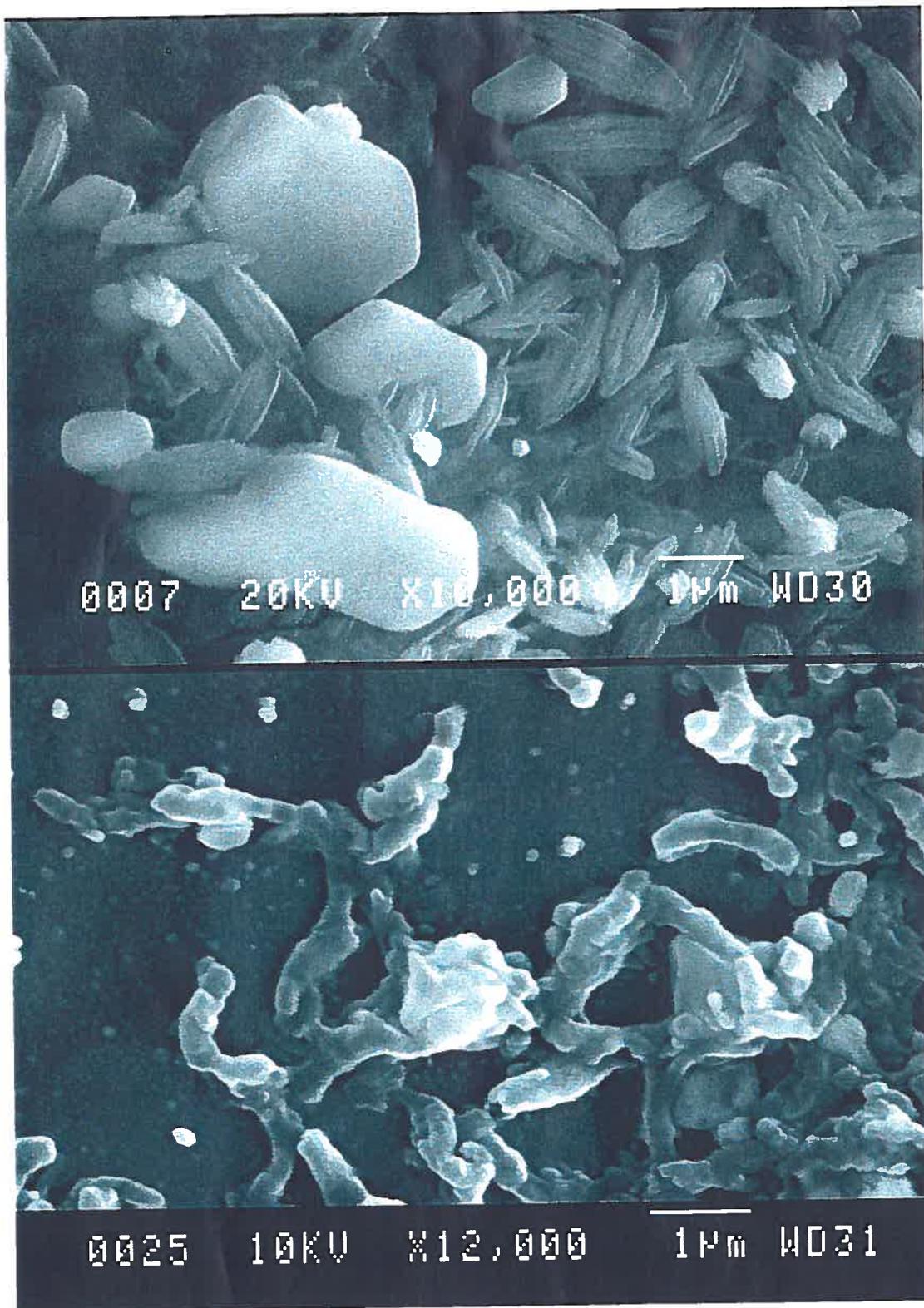


Figure 6. SEM-EDS analysis on the cell surface of SRB.

