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Linking Organic Matter Hydrolysis Rates with Diversity of Sulfate Reducing Bacteria in Marine Sediments

Subproject

Searching for Mesophilic Sulfate Reducing Archaea in Marine Environments

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Abstract

The goal of the project was to investigate relationship between activities of extracellular enzymes and phylogeny of sulfate reducing bacteria in common marine sediments. Three hydrolytic enzyme activities (α-, β-glucosidases, aminopeptidase) were measured in the sediment core recovered from Buzzards Bay, MA (depth 14 m). The values were in the lower range reported so far for different marine environments. All three enzymes showed tendency of decreased activity with depth. Some positive correlation between activities and DNA content in sediments was observed. PCR amplification of dissimilatory sulfite reductase gene (DSR) was used as a tool to investigate phylogenetic diversity of sulfate reducing bacteria. DSR gene was successfully amplified for all investigated isolates of sulfate reducing bacteria. The primer annealing in environmental samples was not specific enough to obtain sharp bands on the gel. Separation of obtained PCR products by gel electrophoresis containing PEG-bisbenzimide conjugates (H.-A.-Yellow and H.-A.-Red) need to be optimized. In future studies the approach combining DSR and 16sRNA genes amplification could allow to investigate relationship between activity and phylogeny of the bacteria capable of dissimilatory sulfate reduction process in nature. Among 16 enrichments of Archaea capable of sulfate reduction under mesophilic conditions, five showed slow growth with hydrogen (3), methanol (1) and lactate (1). Methane and sulfide accumulations were not observed. Amount of cells was too low to obtain bright signal with FISH using general Archaeal primer Arch915R.

INTRODUCTION

Sulfate reducing bacteria (SRB) in marine environments responsible for up to 50% of total organic matter mineralization at the sea floor (Jørgensen, 1982). These bacteria are very versatile group of microorganisms in respect to utilized substrates (from hydrogen and low molecular weight fatty acids to aromatic compounds) (Widdel, 1987). Sulfate is not the only electron acceptor used by SRB; they can reduce iron and manganese hydroxides, and even can grow as fermentors. Measurements of sulfate reduction rates combined with quantification of SRB by rRNA slot-blot hybridization technique in coastal marine sediments (Sahm et al., 1999) showed high abundance of SRB near the surface. At the same depth sulfate reduction rates measured with ^{35}S were the lowest. These and other data suppose differential adaptations of SRB to their natural habitats, and the presence of stratified SRB community in marine sediments.

In natural habitats SRB seems to use preferentially low molecular weight fatty acids, and acetate is the most probable candidate (Widdel, 1987). Thus, it is reasonable to suspect that activities of SRB in environment can be regulated by the availability of these substrates. All these compounds are not the primary products of organic matter degradation, and their production depends on the first stages of organic carbon mineralization, namely hydrolysis. Bacteria are known for their ability to digest various organic compounds with specific extracellular hydrolases (Chrost, 1991). Only monomers and oligomers (below to 600 Da size) can be incorporated by bacterial cells and enter physiological pathways. Therefore, extracellular enzymatic hydrolysis is the key and primary process in the degradation of organic material since it settles at the sea floor.

Since SRB represent probably one of the most important groups of microorganisms responsible for organic matter mineralization, their activities and diversity should have close relationship with quality and availability of

different functional groups of organic matter. The goal of this study was to relate diversity of sulfate reducing bacteria with measurements of exoenzymatic hydrolytic activities in marine sediments.

Activities of three hydrolases (α-, β-glucosidase and aminopeptidase) were used in this study as a reference activities. First group of enzymes hydrolyses two groups of saccharides: α-glucosidic bonds occur in storage polymers like starch and glycogen and relatively easy degradable by different organisms; β-glucosidic bonds are typical for polymers as cellulose, slowly degradable preferentially by bacteria. Aminopeptidase has a broad specificity and cleaves nearly all L-peptides. Peptide bonds occur in different compounds, mostly in proteins. Proteins are the first degradable functional group of organic matter, that is why usually measured aminopeptidase activities in marine sediments are 1-2 orders of magnitude higher glucosidase activities (e.g., Boetius et., 1995). Method used in the study was developed by Hoppe (1983) and Boetius & Lochte (1994) and based on the principle that extracellular enzymes (free or bound to cell and particle surfaces) cleave fluorogenic substrates liberating the fluorochrom, and the fluorescence can be measured over time. Activities are measured at the saturation level of substrate, and represent V_{max}.

Instead of using general SRB probe to investigate the diversity of SRB community, we used PCR primer set targeting highly conserved region in dissimilatory sulfite(=bisulfite) reductase gene. It was shown, that even phylogenetically distant groups of microorganisms capable of the process of dissimilatory sulfate reduction can be targeted by this primer pair (Wagner et al., 1998).

The goal for the second part of the project was to isolate mesophilic sulfate reducing Archaea form different marine environments (Buzzards Bay, MA., Sippewissett Marsh anoxic mud, Southern Guayamas Basin). Phylogenetic diversity in the domain Archaea is subdivided into two main lineages

Euryarchaeota and Creanarchaeota. There is some evidence on the presence of the third kingdom within this domain - Korarchaeota. Representatives of Archaea occupy various habitats worldwide: oxic vs. anoxic, hypertermophilic vs. psychrophilic, they can thrive in neutral as well as in strongly acidophilic conditions. Crenarchaeta was shown to be predominant group in Antarctic waters (DeLong et al., 1994) and was found in a variety of other nonextreme environments like freshwater sediments, and subsurface ocean waters. So far, the only sulfate reducer *Archaeoglobus fulgidus* have been isolated and characterized. Worldwide distribution of Archaea in the ocean could be a good evidence that some groups of Archaea are capable of dissimilatory sulfate reduction process in temperate environments.

MATERIALS AND METHODS

Sampling procedure

Several sediment samples were used in this study. Sediment core from Buzzards Bay, MA was recovered using push sediment core, and after 12 hours of storage at 4°C was sectioned with an interval 2-5 cm. Sampling station was located in the vicinity of Woods Hole, MA at the depth about 14 meters, bottom temperature was 21°C. Sampled sediments were oxidized and had gray color, which testify to the overall conversion of hydrogen sulfide into pyrite phase. For the enrichment of mesophilic sulfate reducing Archaea samples from Southern Guayamas Basin (Rebecca Roost, sulfide mount covered by Beggiatoa mat, 27° 08.'89N 111°24.'63W, Dives 3207&3209) were used: a) upper 5 cm sediment from mesophilic site (T=30°C) taken by yellow push core, b) chimney rock material, c) Beggiatoa mat. Additionally, a sample of black iron sulfide enriched marsh sediment was taken from within the Great Sippewissett Salt Marsh in Sippewisset, MA.

Culture conditions

Mesophilic sulfate reducing Archaea were cultivated under strictly anaerobic

conditions using anaerobic techniques. Modified Widdel media (Widdel and Hansen, 1992) for the isolation of sulfate reducing bacteria was used in the study, and appropriate protocol for preparing the media was applied (Appendix I). The only electron acceptor - sulfate - was used in all enrichments. Approximately 1:1 diluted inoculum (about 1ml) was added to 50 ml of basal media. Electron donors included 300 pKa (H_2/CO_2), CH_4, Na-Ac (15 mM final concentration), lactic acid (30 mM final concentration), methanol (30 mM final concentration). Antibiotic treatment was used to inhibit activity of prokaryotes (Appendix I). To exclude development of methanogenic Archaea bromoethanesulfonic acid (BESA) was added to the final concentration of 50 mM in the enrichments containing hydrogen and methanol as electron donors. Enrichment flasks were incubated at 30°C with shaking and without shaking, and in the dark. Bacterial growth was observed by visual inspection of turbidity in enrichment cultures.

Extracellular enzymatic assays

Laboratory protocols described by Hoppe (1983), and Boetius and Lochte (1994) were used to measure extracellular enzymatic activities in water samples, and sediments. Shortly, the principle of the method is that extracellular enzymes (free or bound to cell and particle surfaces) cleave the nonfluorescent substrates, labeled with methylcoumarinylamide (MCA) or methylumbelliferone (MUF), liberating fluorochrome, which can be measured by fluorometry. The activity of enzymes can be calculated with a calibration curve, since the hydrolysis of bonds and the liberation of MCA (or MUF) are equimolar. The fluorescence of MUF and MCA was detected at 365 nm excitation, and 445 nm emission. The most efficient excitation/emission ratio was checked using standards at high concentrations. Measurements were done by means of Photon Technology International Quanta MasterTM Spectrometer using Felix Software.

Model substrates used in this study were α, β-glucosides(MUF), and leucine(MCA). Final concentrations of these substrates in sediment samples were 100 μM α, β-glucosides(MUF), and 500 μM leucine(MCA); in bottom water samples they were 100 μM α, β -glucosides(MUF), and 200 μ M for

leucine(MCA). Incubations have been done in sediment slurries, and subsamples were taken each 30-100 minutes. Samples for further fluorescence measurements were stored frozen at -20°C until were analyzed. Substrate concentrations were chosen at the saturation level (A.Boetius, personal communication). High specific activities of α-glucosidase in the upper 0-8 cm of Buzzards Bay station did not allow to get reproducible results. Measurements should be done at the lower time intervals.

Genomic DNA isolation from environmental samples and PCR protocol

DNA from the environment was isolated using MoBio UltraClean Soil DNA Isolation Kit. DNA concentrations in environmental samples were estimated as a measure of optical density at the wave length 260 nm using Eppendorf BioPhotometer 6131. DNA concentrations for PCR amplification were about 50 ng per reaction. For PCR amplification the primers encoding most of the α and β subunits of dissimilatory sulfite reductase (DSR) gene present in bacteria and Archaea (*Archaeoglobus fulgidus*) capable of dissimilatory sulfate reduction were used as designed by Wagner et al. (1998): forward primer DSR1F 5′-AC[C/G] CAC TGG AAA GCA CG-3′ (GC content 58.8%) and reverse primer DSR4R 5′-GTG TAG CAG TTA CCG CA -3′ (GC content 52.9%). Due to the mistake of manufacturing company wrong sequence with one mismatch for the forward primer DSR1F (right would be 5′-AC[C/G] CAC TGG AAG CAC G-3′) was used in this study, which probably influenced specificity of primer annealing. Primers were dissolved to a concentration of 20 pmol/l.

For successful PCR amplification, 2 μl of each primer solution, about 50-100 ng of DNA, 5 μl of 10X PCR buffer, 4 μl 25 mM MgCl2, 2 μl of 2.5 mM each dNTP, 2.5U Taq polymerase were combined in final volume of 50 μl. Amplification was done using GeneAmp ™PCR System 9700 - after initial denaturation at 95°C for 5 minutes, amplification was carried out for 30 cycles each consisting of 30 sec at 94°C, 1 min at 60°C, and 2 min at 72°C. The reaction was completed by final extension at 72°C for 5 min. PCR products were stored at -20°C.

PCR products were separated using 1.25% LE agarose gel in 1X TBE buffer at constant voltage 200V using conventional electrophoresis apparatus. Processed gels were stained in ethidium bromide solution overnight.

Separation of PCR-amplified DNA fragments of DSR gene using agarose gel electrophoresis containing bisbenzimide-PEG

Due to the high nucleotide content of DSR encoding gene (about 1.9 kb) the use of conventional DGGE method to separate amplified DNA products was impossible. It has been shown that electrophoresis in agarose gel containing bisbenzimide-PEG (polyethylene glycol) leads to the separation of PCR-amplified DNA fragments due to the effect of the PEG bound to the DNA through the bisbenzimide (Wawer et al., 1995). Sequence similarities ranging between 70 and 90% could be resolved by this method. To our knowledge, there was no report of separating long DNA fragments (up to 1.9 kb in our case) using this method. Two different bisbenzimide-PEG conjugates (H.A.-Yellow, and H.A.-Red - Hanse Analytic, GMBH, Bremen, Germany) were used in this study. 1U of H.A.-Yellow (or H.A.-Red) per ml of 1.5% LE agarose was dissolved in water bath at 60-65°C and stored for 1 hour in the dark. Reaction was accomplished at 120-150V for about 2 hours in conventional electrophoresis apparatus. Gels were stained in ethidium bromide solution overnight.

Fluorescence in situ hybridization (FISH)

FISH was combined with DAPI and acridine orange staining. Enrichment aliquots (app. 1.5 ml) were fixed in 37% formaldehyde at final concentration 3.7% overnight. Next morning 1 ml of fixed cell suspension was filtered through 0.2 μm GTTP membrane filter, incubated for 1 min in mixture 50%EtOH/2%NaCl, and supernatant was discarded under low vacuum. Cy-3 labeled general archaeal probe S-D-Arch-0915-aA-20 (5'-GTG CTC CCC CGC CAA TTC CT-3') was used for hybridization. Hybridization and washing conditions were mostly the same as described by Zarda et al. (1992). Hybridization and wash buffers compositions were as following: 0.9M NaCl, 20 mM Tris-HCl (pH=8), 0.1%SDS. Hybridization

for 2 hours was done at 46°C, washing buffer composition was the same as hybridization buffer, washing has been done twice for 20 min at 48°C.

RESULTS AND DISCUSSION

Extracellular enzymes

Results of the measurements of extracellular enzymatic activities are presented in Table 1.

Table 1

Extracellular enzymatic activities and DNA content in Buzzards Bay surface sediments

Sample	Exoenzymatic activity nmol cm^{-3} hr^{-1}			DNA content*** $\mu g\ g_{w.w.}^{-1}$
	α-glucosidase	β-glucosidase	Aminopeptidase	
Bottom water	b.d.l.*	0.050	0.135	-
Core Water	b.d.l.	0.050	0.027	-
Fluff layer	0.059	High**	0.868	3.267
0-1 cm	0.079	High	7.95	5.799
1-2 cm	0.038	High	7.979	5.189
2-3 cm	0.138	High	7.005	79.022
3-5 cm	-	High	6.966	5.336
5-8 cm	0.125	High	3.995	6.164
8-12 cm	0.111	0.679	3.220	5.065
12-15 cm	0.047	0.184	2.135	16.102
15-18 cm	0.022	0.812	1.238	2.387

*-below detection limit

**- High – activity measured was too high to be determined correctly in the first 30-40 minutes of incubation experiment. Sampling should be more often.

***- DNA concentration was measured as absorbance at 260 nm

Among three exoenzymes, aminopeptidase activity was the highest and α-glucosidase was the lowest. This result seems to be explained by the fact that availability of labile α-polysaccharides in sediments is low, because they were mostly degraded in the water column. In general, activities of exoenzymes show the relative importance of different functional groups in total composition of organic matter in Buzzards Bay sediments. Exoenzymatic activities correlate well with concentrations of their inducible substrates more than with bacterial biomass (e.g., Boetius, 1995). Surprisingly, we did not get significant increasing in activities for the fluff layer, which is supposed to be the most metabolically active layer in marine sediments. Otherwise, the maximum values for all three hydrolytic activities were obtained for the layer 0-5 cm. There is some correlation between these values and the increasing of DNA content. Below 12 cm activities as well as DNA content is 3-5 times lower the upper layers. Apparently, sampled sediments can be separated into three main zones in respect to their metabolic potential: the upper 0-1 cm with low activity, layer 1-10 cm with high activity (enhanced activity probably explained by strong association of enzymes with solid sediment matrix), and the lower part of the core with low values. Activities measured for the above laying water are significantly lower the activities observed in the sediments (several times lower). Based on these preliminary data we can suppose that even within 10 upper cm of marine sediments organic matter undergoes significant transformation, which can have influence on the activity and metabolic diversity of sulfate reducers, if this group plays a key role in organic matter mineralization in the Buzzards Bay sediments. Comparison of exoenzyme activities in the Buzzards Bay sediments with other marine environments is presented in Table 2.

DSR gene amplification and further analysis of PCR products by PEG-Bisbenzimide gel electrophoresis

Former results showed that DNA sequences obtained for more then 20 different isolates of sulfate reducers are very similar to one another (49% to 89% identity), and their inferred evolutionary relationships are nearly identical to those inferred

on the basis on 16S rRNA. DSR gene operon encoding two subunits has size of about 1.9-kb DNA. Before applying PCR amplification for the environmental samples of interest, we needed to develop appropriate protocol for PCR amplification. This was accomplished with the isolates of lactate utilizing sulfate reducers. Single colonies from agar shake tubes were picked up and amplified with appropriate set of primers (see Materials and Methods) (Fig.1).

Table 2

Comparison of exoenzyme hydrolytic activities in different marine environments (0-1 cmbsf)

(after Coolen and Overmann, 2000 with additions)

Sample	Water depth, m	Exoenzyme activity V_{max}, nmol cm^{-3} hr^{-1}	
		β-glucosidase	aminopeptidase
NE Atlantic, Biotrans area	4,500	0.089	15
NW Mediterranean	1,230	ND	576
Ionean Sea	993-4,617	0.33-2.13	109-284
Urania Basin, Mediterranean	3,600	0.120	0.323
Barents Sea, Arctic	1,013	0.2-0.4	67-144
Laptev Sea, Arctic	796-3,427	0.02-0.36	21-116
Amudsen Basin, Arctic	4,480	0.017	73.4
Lomonosov Ridge, Arctic	3,413	0	19.6
Ross Sea, Antarctic	567, 439	80.5-265.8	1,312.2-2,728.2
Buzzards Bay, MA.	**14**	**>>0.679**	**7.979**
Lowes Cove, Maine	0	23.2	12
Dangast, Wadden Sea	0	16.6	86.0

The same PCR protocol applied for the environmental DNA from Buzzards Bay sediments gave positive results for all sediment layers besides the lower 15-18 cm (Figure 2). At the same time, signal was very smeared, and in all amplified DNA we observed non-specific primer annealing in the range of 250-400 bp, which could be explained by non appropriate primers set used in the study (see

Materials and Methods) (Figure 3). Changing of primer and template DNA concentrations did not give better resolution. Our PCR protocol can be used in future with correct set of primers and seems to be appropriate for the amplification of DSR gene in natural samples.

Since obtained PCR products were about 1.9 kb size, use of conventional DGGE method was impossible, because at present, only fragment up to 600 bp can be distinguished by this method. Instead, we tried to apply different method, which in theory can be used for separation of PCR-amplified DNA fragments which are identical in length, but differ in base composition. The limitations of the method, that only changes affecting bisbenzimide binding sites (which consist of at least four consecutive A/T bases –H.A-Yellow conjugate) alter mobility of the fragments on a gel. This can be overcome by the use of both conjugates. Results obtained are shown in Fig.4, identical pattern was shown with H.A-Red conjugate. Due to limited time, optimization conditions were not tested. This approach seems to be promising for future, and can be optimized using low endosmosis agarose, different buffers and their concentrations (TBE vs. EDTA), and different electrophoresis apparatus (e.g., see comments by Wawer et al., 1995).

Isolation of mesophilic sulfate reducing Archaea from different marine environments

Enrichment plan is given in Table 3. All enrichments were done with the addition of antibiotics to inhibit activities of other prokaryotes. Bromoethanesulfonic acid (BESA) at final concentration 50 mM was added into enrichment cultures growing with H_2 and CH_3OH to inhibit methanogenes. After 4th day of incubation visual inspection of turbidity showed positive signal in four enrichment bottles, two of them are with H_2/CO_2 mixture. These were inocula from Buzzards Bay (depth 15-20 cm), yellow push core (Southern Guayamas Basin). Turbidity was also observed in enrichments with lactate (Buzzards Bay sediment sample, 12-15 cm), and with methanol (yellow push core from Guayamas Basin). After 12th day one else enrichment with hydrogen/carbon dioxide mixture showed positive growth – sample from Buzzards Bay (12-15 cm). Thus, after 15 days totally five

enrichments showed positive signal (Fig. 5). After 12 days of growth neither hydrogen sulfide (H_2S smell – sensitivity 30 μM) nor methane (GC FI) were detected in all positive enrichments. Inspection of enrichments under phase and epifluorescence microscopy did dot show any living bacteria. At the same time, in all enrichments with hydrogen we observed slow H_2 consumption. DAPI and acridine orange staining gave very low cell numbers, and cells were mostly associated with inorganic matrix. FISH with a general archaeal primer did not show any signal in all five enrichments tested. No transfers have been done. Interestingly, all enrichments from strictly anoxic Sippewisset Marsh sample did not show any growth.

Table 3

Enrichment scheme used in the study (sulfate is the only electron acceptor)

+/- turbidity check

E-donor / Sample	CH_3OH 30 mM	CH_3COOH 15 mM	Lactate 30 mM	H_2/CO_2	CH_4
Buzzards Bay sediment 12-15 cm			+	+	
Buzzards Bay sediment 15-18 cm				+	
Anoxic Sippewisset Mars	-	-	-	-	-
Beggiatoa mat (S.Guayamas)	-			-	
Chimney rock (S.Guayamas)				-	-
Yellow push core	+	-		+	

Thus, appearance of turbidity in our enrichments was probably associated with some side reactions going on in media influenced by antibiotics. At the same time, hydrogen consumption and low numbers of metabolically active cells seems to be the evidence of slow bacterial growth. Experiments using this isolation

scheme with hydrogen as electron donor should be continued for longer time. Other electron acceptors (sulfur, polysulfides, thiosulfate) should be tested. Experiments with and without hydrogen sulfide scavengers (e.g., ferrous iron) could help to investigate the role of sulfide as a poisoning agent.

CONCLUSIONS

1. **α-, β-glucosidase and aminopeptidase activities measured in Buzzards Bay surface sediments are in the lower range reported so far for marine environments**
2. **DSR gene was successfully amplified during PCR for pure SRB cultures, and showed positive signal for surface sediments. Study of DSR gene expression in natural environments can be very useful in our understanding of functional activities of bacteria (not only SRB) mediated dissimilatory sulfate reduction**

Comment: Applied in this study approach can be used to isolate mesophilic SR Archaea, but such efforts need patience and time, and are not recommended for 2 weeks project

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APPENDIX I

Media used for isolation of sulfate reducing Archaea

Basal SW-base solution:
For 1 liter:
Distilled water 1 liter
NaCl 20 g
$MgCl_2$ $6H_2O$ 3 g
$CaCl_2$ $2H_2O$ 0.15 g
KH_2PO_4 0.2 g
KCl 0.5 g
Na_2SO_4 3 g
100x NH_4Cl solution (25 g/l) 10 ml

Mixture is autoclaved for 30 min at 120°C, and cooled under N_2/CO_2, then add 1 ml of EDTA-chelated trace metals (according to Widdel and Hansen, 1992), 30 ml of CO_2 saturated $NaHCO_3$ solution (100 ml dist. Water and 8.5 g $NaHCO_3$), adjust pH to 7.2

As reductant use 1.5 ml of 1M Na_2S $9H_2O$ for 1 liter medium.

Add vitamin mixture (according to Widdel and Hansen, 1992) 0.1 ml per 1 liter.

Growth stimulating factors (0.1 ml for 100 ml basal media):
Isobutyric acid 0.5 ml
Valeric acid 0.5 ml
Carpoic acid 0.2 ml
Succinic acid 0.6 g
Adjust pH to 9 by NaOH solution and autoclave (added directly into enrichment flasks)

Inhibition antibiotics mixture for 100 ml (filter sterilized, added directly into reaction flasks):
Ampicillin 1g
Tetracyclin 1 g
Phosphomycin 1 g
Rifampicin 1 g
Final concentration in reaction flasks should be 100 μg/ml

Stock solution of substrates (autoclaved):
Sodium acetate 1.5 M
Sodium lactate 3 M
Methanol 3 M
Add 1 ml for 100 ml media

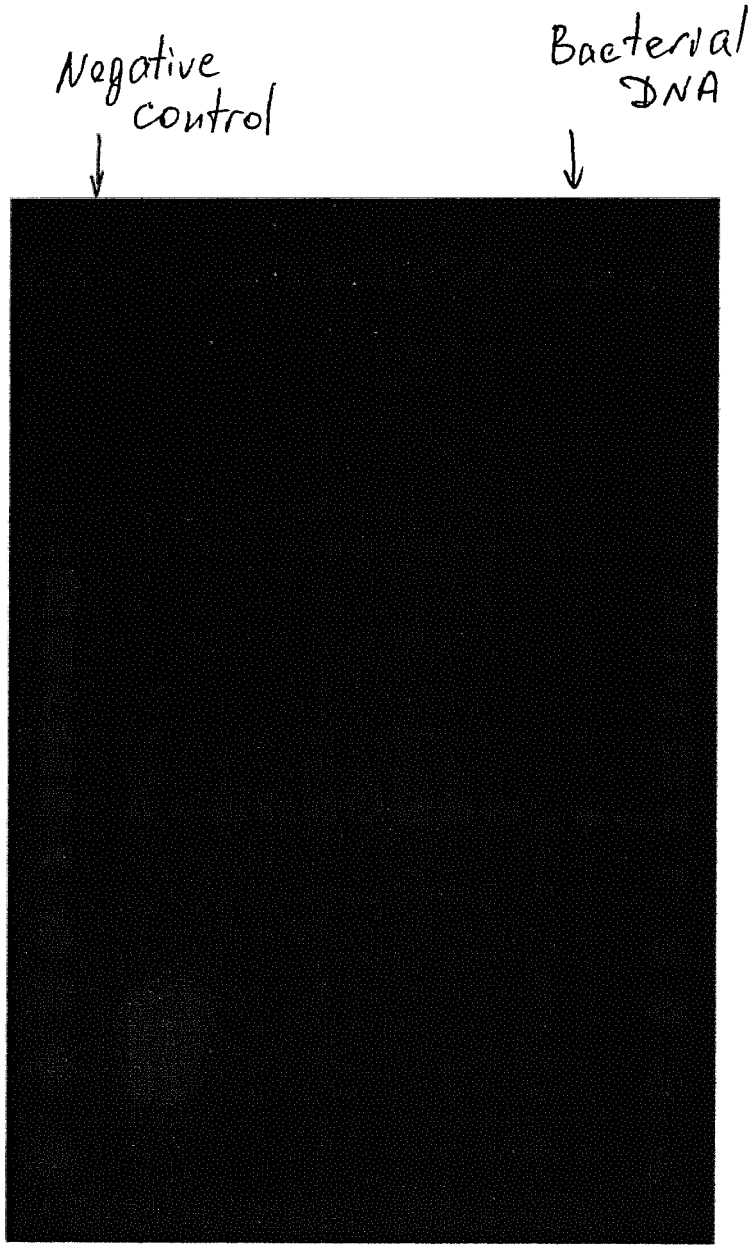

Figure 1

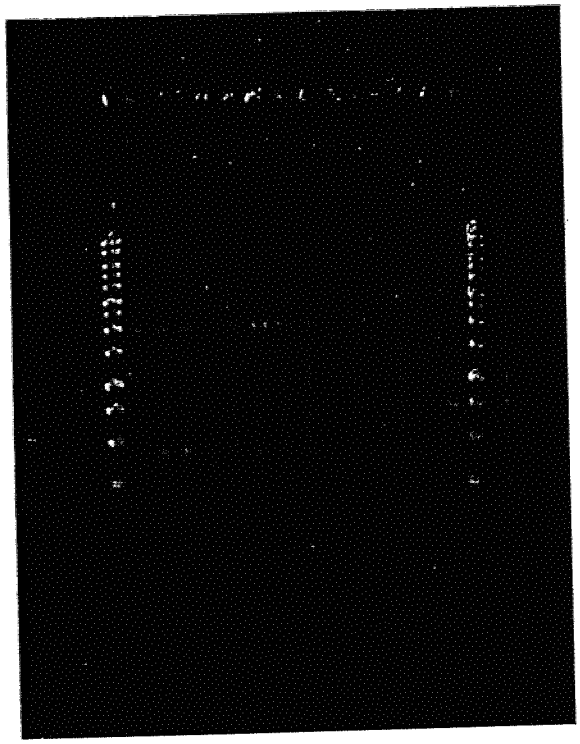

Figure 2

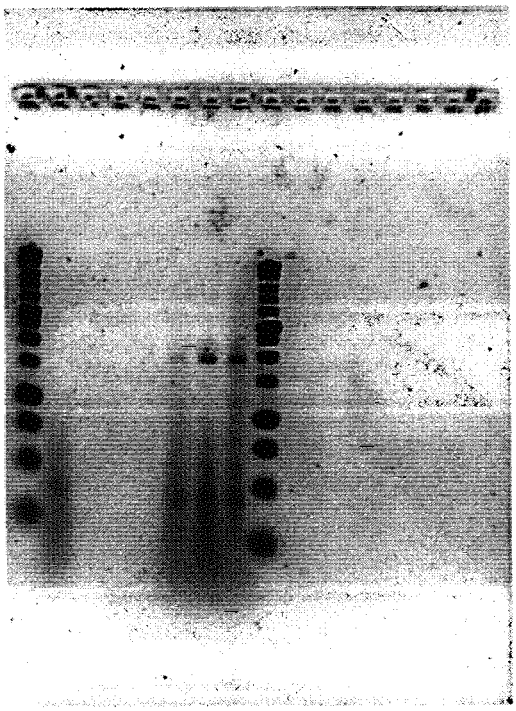

Figure 3

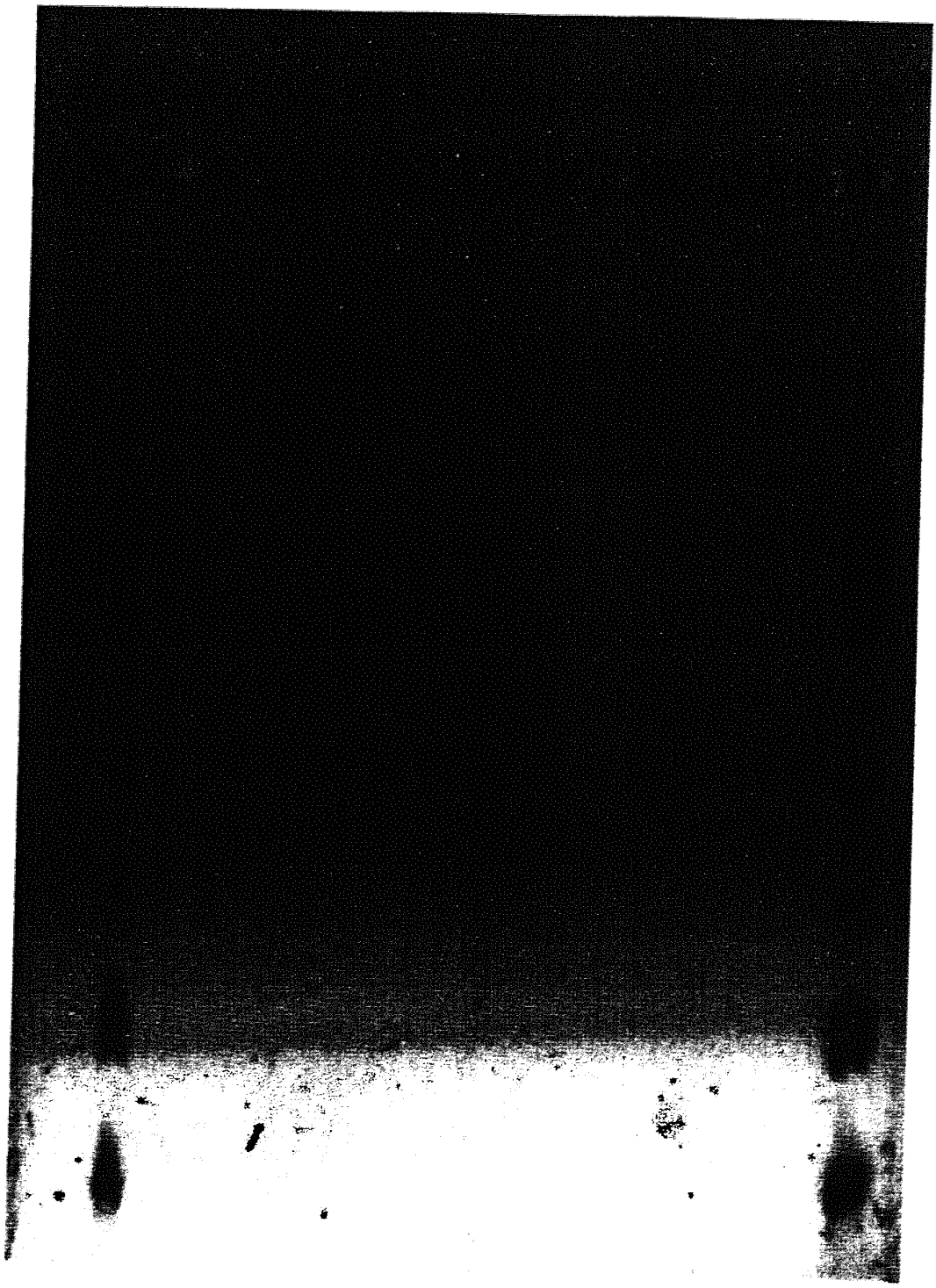

Figure 4


I
BB
YP
YP
BB
BB
Control
30 mM Lactate
30 mM CH_3OH+ BESA
H_2+ BESA
H_2+ BESA
H_2+ BESA

Figure 5