Agar degrading bacteria - a small study

Lars Riis Damgaard
Dept. of Microbial Ecology
University of Aarhus
Denmark

Abstract. Speric macroscopically visible vacuoles were observed to develop 0.25% agar. The vacuoles were devoid of agar inside and enlarged until all agar had disappeared. The growth seemed independent of oxygen and the presence of sulfide or 3-mercaptopropionate. Microscopic examination revealed to morphotypes: long thin rods and shorter fat rods. Growth mode in different agar concentrations was examined by trapping streakings of inocula under glass slides and study colony development microscopically.

Introduction.

Agar is produced from algae and is an extremely common material in microbiological laboratories. Agar is mostly expected to be an inert stable solid substrate for growt of microorganisms, but the degradation of agar is a well-known phenomenon (Reichenbach and Dworkin,1981). That agar-like substances are present in nature from where they are extracted, makes such an utilization a probable, but mostly an unwelcome phenomenon.

Materials and Methods.

Tubes: Originally for a different experiment, the following gradient tubes were made: Aliquots of 1 ml of 12.5% molten agar was tapped into 20 ml test tubes, which were placed in a 60°C water bath. Four ml of purple sulfur bacteria medium containing 5mM was added after being preheated for 10 min. in a 40°C water bath. The test tubes were then flushed with 80/20 N_2/CO_2, closed airtight and cooled to roomtemperature in a water bath so that a solid plug of 1.5% agar containing 4mM sulfide or MPA was formed. The 1.25% agar was placed in a 60° water bath while still hot after autoclaving. A similar medium but without sulfide or MPA was warmed up to 40°C in a water bath and mixed anaerobically with the 1.25% agar to yield a top agar with a concentration of 0.25%. Eight ml of this agar was then poured anaerobically on top of the plug in each of the test tubes to form a slush agar overlay. The test tubes were then left for three days to allow opposing gradients of oxygen and sulfide or MPA to establish through the top agar before inoculations. Nitrate gradient tubes were made by adding an anoxic 3 ml water phase containing 2 mM $NaNO_3$ after inoculation and subsequently flushing the headspace with 80/20 N_2/CO_2 before closing the tubes airtight. That means that the top agar in the nitrate gradient tubes initially contained som oxygen as they were left for 3 days under atmospheric air.

The inoculum was sediment collected June 24 from Oyster Pond. Inoculations were made by filling approximately 0.2 ml of inoculum into a Pasteur pipette which had previously been pulled in a flame. The pipette was then inserted into the agar down to the top of the agar plug and retracted while slowly dispensing the inoculum in the entire length of the top agar.

Microscopic inspection of growth was performed by pulling a Pasteur pipette in a flame, sucking out material with it from discrete locations in the tube and examining it under the microscope.

Trapped colonies. In order to be able to inspect the growth of the colonies a setup for trapping colonies under cover-slips was made. Agar was washed in distilled water 4 times and made up in concentrations of 0.25, 0.75, and 1.5% in purple sulfur bacteria medium pH 7.2 without sulfide. This medium was used as it had already proved to sustain growth of the agar degraders in the tubes. A sterile microscopy slide was placed in a Petri dish in which was poured fifteen ml of 0.25, 0.75, or 1.5% agar. The agar covered the slide with an approximately 1 mm thick layer. Inocula were taken sterilely from the colonies developing in the tubes and the number of cells was

counted in a counting chamber. On the basis of this count the inoculum was diluted down to 3000/0.1 ml and 30/0.1 ml. Plates were streaked with the diluted inoculum and a flame-sterilized 24×50 mm cover-slip was placed on the agar over the slide. The Petri dishes were incubated at 30°C in the dark. Growth was observed under a light microscope.

Results.

Growth in the tubes began as small ovoid spheres that grew larger until they merged with other spheres or touched the glass wall of the tube (Fig. 1). The interior of the spheres was a clear liquid. Growth contiuned until all slush agar had disappeared. The cells observed from the tubes were characteristic long slender rods. This corresponds well with the fact that *Cytophaga sp.* have been shown to be able to degrade agar (Reichenbach and Dworkin,1981). The orientation of the cells seen under the microscope suggested that the cells grew very orderly side by side (data not shown).

Growth was observed in 0.75% agar where colonies enlarged their radius approx. 0.5 mm/day. The 0.25% agar turned out to be too thin to be stable. The 1.5% agar had some colonies, but the colony development was much slower than on the 0.75% agar. On the 0.75% agar two cell types were visible: 1) fat rods, that developed in tightly packed colonies which formed characteristic wave patterns (Fig. 2A) and 2) long slender rods that individually infiltrated the agar matrix. (Fig. 2B, C, and D). No clear zone without agar or cells was observed in any of the two types.

Discussion.

The characteristic pattern of spheres devoid of cells and agar in the tubes could not be repeated under the cover slips. This fact and the fact that the side-by-side growth pattern could not be observed under the cover slips suggest that either different organisms were observed in tubes versus under cover slips or that the state of the agar is very crucial in the development of growth. The fact that no growth of colonies was observed in the 0.25% agar and much more slow growth was observed in the 1.5% agar indicate that agar concentration is important.

The metabolism is most likely fermentation as the growth in tubes seemed independent of oxygen versus nitrate. Whether the nitrate tubes were completely anoxic is not clear though, but as they were tightly sealed and the headspace had been flushed with N_2/CO_2 a small oxygen consumption should be able to remove all oxygen in a short time.

The setup of the cover slip covered agar should be improved to yield an even thinner agar layer, making microscopy better.

Literature:

Reichenbach, H. and M. Dworkin.1981. The Order Cytophagales(with Addenda on the Genera *Herpetosiphon*, *Saprospira*, and *Flexithrix*). In: Starr, M.P. et al. (eds): The Procaryotes. 1. Ed. Springer Verlag Berlin. 1981.

Legend:

Fig. 1. Growth of agar degraders in gradient tubes. The growth shows as spheres that merge or disappear upon touching the glass wall of the tube.

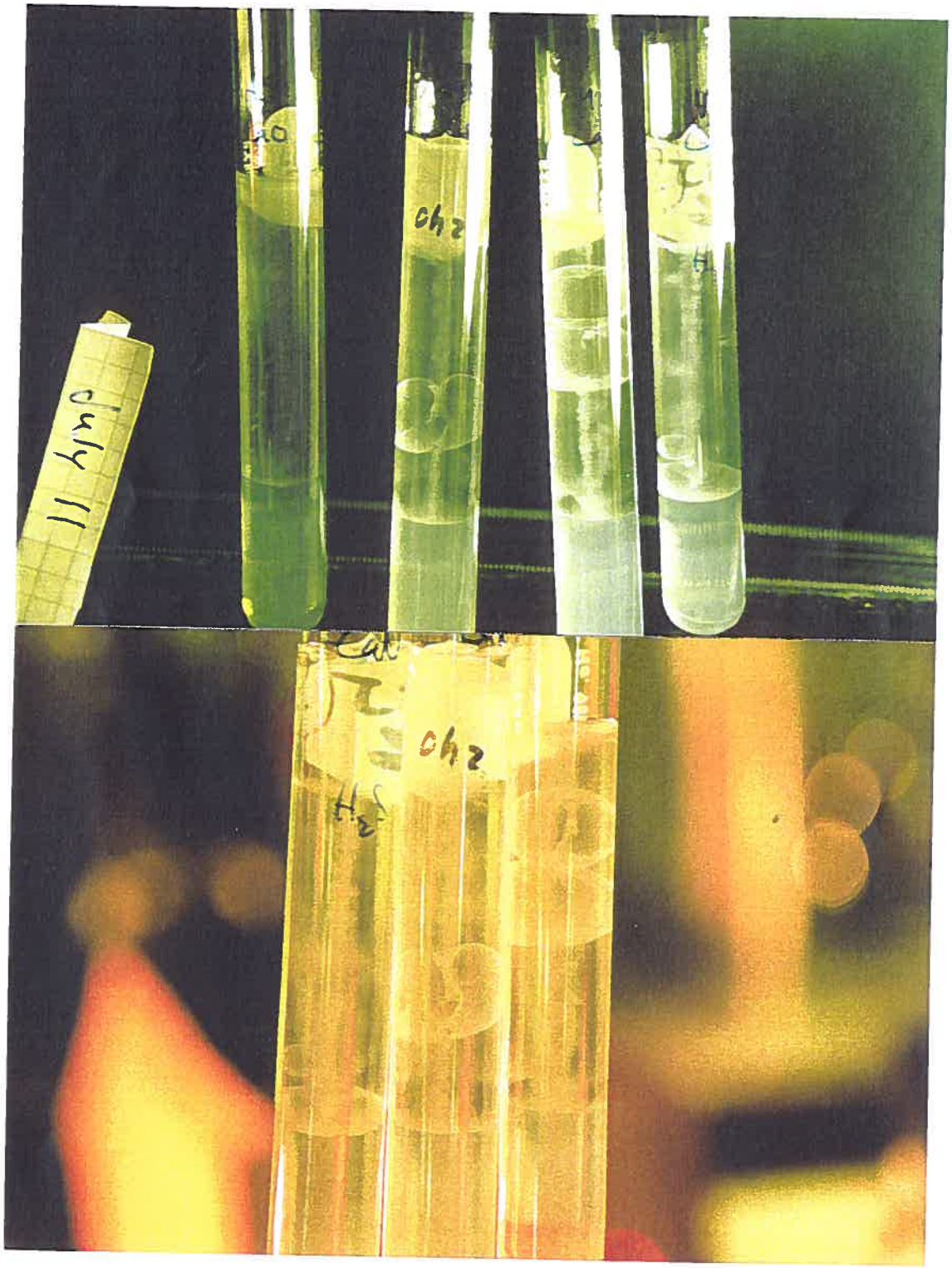

Fig 1

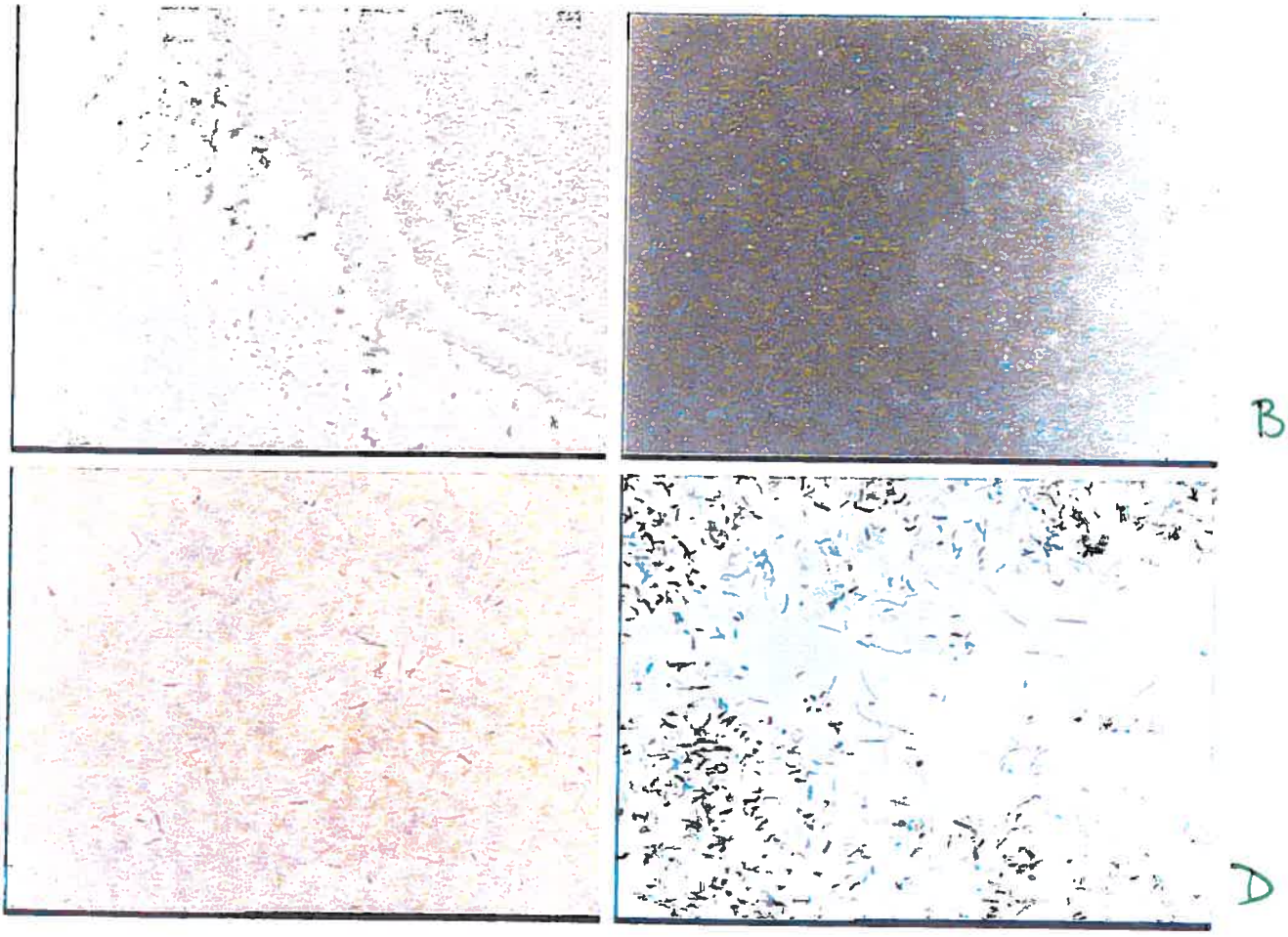

Fig 2

Microbial transformation of sulfide and 3-mercaptopropionate.

Lars Riis Damgaard
Dept. of Microbial Ecology
University of Aarhus
Denmark

Abstract. Gradient tubes and deep agar shakes were made to enrich for chemotrophs and phototrophs able to grow on sulfide or 3-mercaptopropionate (MPA) as electron donor and with O_2, NO_3- or photosynthesis as elctron acceptor. Enrichment of bacteria seemed to be possible for all the metabolisms yielding different rods, cocci and spirilla for the chemotrophic growth, purple non-sulfur bacteria for phototrophic growth on MPA - of which one isolate exhibited scotophobic response - and purple sulfur bacteria for phototrophic growth on sulfide.

Introduction.

The redox cycle of sulfur in nature includes biological as well as chemical transformations, making the study of the fate of sulfur quite complex. The sulfur redoz states commonly found on Earth are: -2 (sulfhydril and sulfide), 0 (elemental sulfur) and +6 (sulfate). Inorganic sulfur compounds can be reduced (sulfate) or oxidized (sulfide and elemental sulfur) by sulfate reducing bacteria and sulfur oxidizing bacteria, respectively. Organic sulfur compounds synthesized by living organisms enter into the biogeochemical sulfur cycling for its degradation.

Most organosulfur compounds in marine environments originate from the degradation of dimethylsulfoniopropionate (DMSP). This compound is present in many plants and algae, especially marine living forms, where it is believed to function as an osmoregulator. DMSP occurs in sediments of shallow coastal environments due to the accumulation of algae and plant detritus. DMSP may degrade to 3-mercaptopropionate (MPA) in one of two ways: by two succesive microbially mediated demethylations or by the chemical addition of H_2S to acrylate, which is an alternative microbial product from breakdown of DMSP. The H_2S used in the latter pathway may also originate from the breakdown of DMSP, but H_2S is also an abundant end-product from sulfate reduction.

H_2S has long been known to be transformed by a variety of organisms. Colorless sulfur bacteria chemolithoautotrophically or organolithotrophically oxidize H_2S with oxygen or nitrate and purple sulfur bacteria utilize H_2S as the electrondonor in anoxygenic photosynthesis.

The only report of a pure culture capable of utilizing MPA came from Visscher and Taylor (1988), who found that *Thiocapsa roseopersicina* could cleave the sulfide group of MPA and using it as electron donor in anoxygenic photosynthesis. In this organism the carbon backbone of MPA was not utilized, that is the organisms grew photolithoautotrophically. Thermodynamically, MPA could probably be converted by oxidation with both O_2, NO_3-, and SO_4-- as well as by photoheterolithotroph organisms utilizing the mercapto group as electron donor and the carbon backbone as a carbon source. None of these have been isolatede so far.

Knowledge of the organisms responsible for the degradation of MPA obtained by isolation is important in the understanding of the regulation of the sulfur cycle. The isolation of a denitrifying organism capable of utilizing MPA would provide a potential new link between the sulfur and nitrogen cycles in marine habitats.

In this study we will try to isolate some not previously described microorganisms responsible for the underlying conversions.

Materials and Methods.

Inocula.
Five different inocula were used. 1) Pin: pink material collected at Sippewissett Salt Marsh on June 13 consisting almost entirely of 20-30 µm long flagellated rods organism containing sulfur granules in the cytoplasm (Fig. 1). After collection the sample was stored at 4°C until inoculation 3 weeks later. Microscopy revealed no changes in the morphology of the cells as a consequence of storage. 2) Car: Sediment collected June 24 from Oyster Pond containing a high number of large sperical flagellated cells with cytoplasmatic granules, which was suspected to give the inoculum its purple colour. 3) Con: Sample from Sippewisset Salt Marsh collected on June 13 containing orange-coloured material. The sample was stored in a glass jar in light at room temperature for 3 weeks before inoculum was scraped from the sides of the glass. Microscopic examination prior to inoculation revealed sheets of small cocci. 4) SES: Surface mud collected at 20 m depth in Cape Cod Bay on July 2. Until inoculation the sample was stored at 4°C in the dark. 5) Wrm: Worm tube collected at 20 m depth in Cape Cod Bay on July 2.

Media.
For initial enrichments in gradient tubes and for deep agar shakes was used a mineral salts medium for purple sulfur bacteria:

per liter:	
KH_2PO_4	0.25 g
NH_4Cl	0.34 g
KCl	0.34 g
$MgSO_4 \cdot 7H_2O$	0.50 g
$MgCl_2 \cdot 6H_2O$	2.80 g
NaCl	20.0 g
$CaCl_2 \cdot 2H_2O$	0.25 g

For bacth cultures a slightly different mineral salts medium was used:

per liter:	
KH_2PO_4	0.20 g
NH_4Cl	0.25 g
NaCl	20.0 g
$MgCl_2 \cdot 6H_2O$	3.00 g
KCl	0.50 g
$CaCl_2 \cdot 2H_2O$	0.15 g

The salts solutions were autoclaved in Widdel flasks and cooled under a 80/20 N_2/ CO_2 atmosphere. Then the following sterile solutions were added:

per liter:	
$NaHCO_3$	18 mM final
6-vitamin solution	1.0 ml
Vitamin B_{12}-solution	1.0 ml
Trace elements SL12	1.0 ml

and pH was adjusted sterilely to 7.2.

Of this solution 175 ml was tapped anaerobically into a bottle and tightly sealed before 1.07 ml of 1 M sterile Na_2S or MPA was added to make1.25 mM final concentration. pH was adjusted again to 7.2 with sterile HCl by measuring pH in small aliquots after additions. Eight Phennig bottles were filled anaerobically with a total of 440 ml of this solution before another 1.57 ml of 1 M Na_2S or MPA were added to yield a final

concentration of approximately 5 mM. Of this solution, 175 ml were filled anaerobically into a bottle, which was then closed airtight.

Agar, washed 4 times in deionized water, was made up in concentrations of 1.25% and 7.5 % and autoclaved.

Gradient tubes.

While still hot after autoclaving, aliquots of 1 ml of 12.5% agar was tapped into 20 ml test tubes, which were placed in a 60°C water bath. Four ml of medium containing 5mM of sulfide or MPA was added after being preheated for 10 min. in a 40°C water bath. The test tubes were then flushed with 80/20 N_2/CO_2, closed airtight and cooled to roomtemperature in a water bath so that a solid plug of 1.5% agar containing 4mM sulfide or MPA was formed. The 1.25% agar was placed in a 60° water bath while still hot after autoclaving. The medium without sulfide or MPA was warmed up to 40°C in a water bath and mixed anaerobically with the 1.25% agar to yield a top agar with a concentration of 0.25%. Eight ml of this agar was then poured anaerobically on top of the plug in each of the test tubes to form a slush agar overlay. The test tubes were then left for three days to allow opposing gradients of oxygen and sulfide or MPA to establish through the top agar before inoculations. Nitrate gradient tubes were made by adding an anoxic 3 ml water phase containing 2 mM $NaNO_3$ after inoculation and subsequently flushing the headspace with 80/20 N_2/CO_2 before closing the tubes airtight. That means that the top agar in the nitrate gradient tubes initially contained som oxygen as they were left for 3 days under atmospheric air.

Inoculations were made by filling approximately 0.2 ml of inoculum into a Pasteur pipette which had previously been pulled in a flame. The pipette was then inserted into the agar down to the top of the agar plug and retracted while slowly dispensing the inoculum in the entire length of the top agar.

Microscopic inspection of growth was performed by pulling a Pasteur pipette in a flame, sucking out material with it from discrete locations in the tube and examining it under the microscope.

Batch enrichments.

Twenty ml of medium containing sulfide or MPA were anaerobically filled into serum bottles. The bottles had 1) the headspace flushed with atmospheric air or 2) 2 mM $NaNO_3$ the headspace flushed with 80/20 N_2/CO_2 after inoculation and were sealed airtight before they were put in the dark. The inocula were the above described and material picked from the gradient tubes.

Phototroph enrichment.

Enrichment for phototrophic bacteria that can use sulfide or MPA was performed by making deep agar shakes in 6 fold 10 times dilution series. The enrichments were left in the dark for 24 hours to eliminate any potentially harmful oxygen, before they were placed in incandescent light behind a light filter that eliminated all light with wavelengths below 840 nm. The filter was applied to give purple sulfur bacteria a selective advantage. For purification Pasteur pipettes were pulled over a flame, colonies were picked and suspended in purple sulfur bacteria medium and inoculated in new deep agar shakes..

Results.

Gradient tubes.

PIN:

Sulfide: All inocula responded with growth. Grown with oxygen PIN macroscopically at all depths showed pink clouds of cells, microscopically the cells were mostly small cocci with some short rods. Grown with NO_3^-, less growth appeared and the colour was less pink. Again, the cells were small cocci mixed with some rods and spirals.

MPA: Growth was weaker in MPA-enrichments than with sulfide, and growth was macroscopically grey with only a weak pink tone to it. Microscopically mostly cocci were observed among some rods and spirals. Grown with oxygen characteristic fat rods appeared, 5 × 10 μm without granules in the cytoplasm.

CAR:

Tubes of all treatments got growth of an agar degrading organism, which dissolved all the slush agar within 10 days leaving only a clear liquid phase with some refractile material. Microscopically the cells were long (20-30 μm) slender rods.

CON:

Very weak growth on both sulfide and MPA. The few cells consisted of cocci and rods. There were some signs of slush agar degradation, which corresponded well with the observation of cells similar to the ones found in CAR. A special cell morphology was found with enrichment with sulfide and both O_2 or NO_3^-: slender rods with a round "head" on one end. The enlargement was not refractile as typical endospores.

SES:

Sulfide: Only weak growth. With NO_3^- some very long (approximately 50 μm) slender rods, presumably gliding bacteria. Growth with oxygen produced short rods.
MPA: Almost no growth with MPA.

WRM:

Only tube with sulfide and oxygen showed a weak growth, consisting of cocci and rods. Other treatments showed close to no growth.

Batch enrichments.
No growth was visible after 10 days of growth.

Phototroph enrichments.
Good growth of colonies was observed in PIN and CAR, especially when grown on H_2S. CON, SES, and WRM had only few colonies. Of three morphologically different colonies isolated on H_2S all were large ovoid cells (Fig. 2) containing sulfur granules. Three colonies isolated on MPA from PIN and CAR were cocci, rods and spirilla, respectively, without visible intracellular sulfur granules. The spirillum was phototactic (Fig. 3).

Discussion.
This study suggests that some microorganisms are indeed able to degrade MPA in ways that has not been shown before. The gradient tubes showed heterotroph growth on both oxygen and NO_3^-, but did not create the expected result, that growth would develop as a band where opposing gradients would theoretically cross as has been shown by Nelson et al. In the present study the growth was not distributed in that manner, instead growth was diffuse in the entire length of the slush agar. This could be a result of the fact that the establishment of a gradient system is dependent on the activity of the bacteria and that the bacteria also are dependent of the establishment of the opposing gradients. If the bacteria are not growing fast enough to consume both electron acceptor (oxygen or NO_3^-) and donor (sulfide or MPA), the acceptor can diffuse all the way down through the slush agar and the donor can diffuse all the way up, thus not creating the narrow overlap intended. Nelson et al. showed that chemical oxidation of sulfide would create almost as narrow an overlap, which would be only attenuated by the biological activity. That this was the case in this study as well was supported by the observation of bands of sulfur granules in the agar. In the case of MPA such a rapid chemical oxidation is probably not possible. In any case, microbial growth was not observed to form narrow bands.

A problem with the kind of gradient tubes used in this study is that making a thin slush agar to allow bacterial movement also makes the agar susceptible to degradation.

That the batch experiment did not produce any growth was unexpected. In the case of batch with both sulfide and O_2, chemical oxidation on the expense of microbial oxidation, but the combinations with NO_3^- and sulfide or MPA should not have this problem. Still no growth was observed, although growth on the same compounds seemed possible in the gradient tubes. A possible explanation could be that the gradient tubes which were supposed to contain NO_3^- and no O_2, were loaded with enough O_2 during the preparation of the tubes (see materials and methods section) to sustain the amount of growth seen in the NO_3^- tubes. In that case the suggestions in this study, that both MPA and sulfide were oxidized with NO_3^- are maybe not correct.

Phototrophic growth was shown on both H_2S and MPA. In contrast to the finding that the purple sulfur bacteria *Thiocapsa roseopersicina* could utilize MPA, only purple non-sulfur bacteria were found to utilize MPA.

In all cases H_2S caused more growth than MPA. The precise faith of MPA in each case is unclear. The mercapto group may be cleaved of MPA and only the resulting sulfide or only the resulting propionate may be utilized by the organism.

The cells identified in the inocula PIN and CAR could not be retrieved in isolate by any of the enrichments used. The growth in gradient tubes with oxygen and H_2S showed pink growth which corresponded well with the large vacuolated cells of the inoculum, but the bacteria responsible for the colour were small rods which were not accumulating granules. This means that either a different pink organism than the most abundent in the inoculum grew up, or that the cells change their morphology as a response to growing in the dark. This could have been tested by transfering the cells to a purple sulfur bacteria enrichment system, but time didn't allow for this.

Further purification and control experiments are necessary to confirm the findings in this study.

Literature:

De Bont, J.A.M., J.P. van Dijken, and W. Harder. 1981. Dimethyl sulphoxide and dimethyl sulphide as a carbon, sulphur and energy source for growth of Hyphomicrobium. S.J. Gen. Microbiol. 127:315-323.

Kanagawa, T., and D.P. Kelly. 1985. Breakdown of dimethyl sulphide by mixed cultures and by Thiobacillus thioparus. FEMS Microbiol. Lett. 34:13-19.

Kiene, R.P., and D.G. Capone. 1988. Microbial transformations of methylated sulfur compounds in anoxic salt marsh sediments. Microb. Ecol. 15:275-291.

Kiene, R.P., R.S. Oremland, A. Catena, L.W. Miller and D.G. Capone. 1986. Metabolism of reduced methylated sulfur compounds by anaerobic sediments and a pure culture of an estuarine methanogen. Appl. Environ. Microbiol. 52:1037-1045.

Nelson, D.C., N.P. Revsbech, and Jorgensen, B.B.:

Visscher, P.T., and B.F. Taylor. 1988. Organic Thiols as Organolithotrophic Substrates for Growth of Phototrophic Bacteria. Appl. Environ. Microbiol. 59:93-96.

Zeyer, J., P. Eicher, S.G. Wakeham, and R.P. Schwarzenbach. 1987. Oxidation of dimethyl sulfide to dimethyl sulfoxide by phototrophic purple bacteria. Appl. Environ. Microbiol. 53:2026-2032.

Legend:

Fig. 1. Electronmicrograph of presumably purple sulfur bacteria. Note the two polar tufts of flagella. The cell lysed during preparation, which shows as a dark outgrowth on the side of the cell. Another artefact is the empty space between cell body and the tufts of flagella, which is probably due to shrinking of the cell body or the flagella during preparation. Cell body approx. 25 µm long.

Fig. 2. 40 × micrograph of a strain of purple sulfur bacteria.

Fig. 3. Scotophobic behavior in a strain of purple non-sulfur bacteria. The upper and lower panels represent two different levels of magnification. The panels to the left show the cells in a situation where the light diaphragm of the microscope is closed to only allow a small portion of the field of vision to be illuminated. The panels on the right show the situation approximately 1 sec. after the diaphragm was opened to the normal position. It is clear to see that the cells had accumulated in the lighted area. A few seconds later the cells were completely dispersed (Data not shown).

Fig 1.

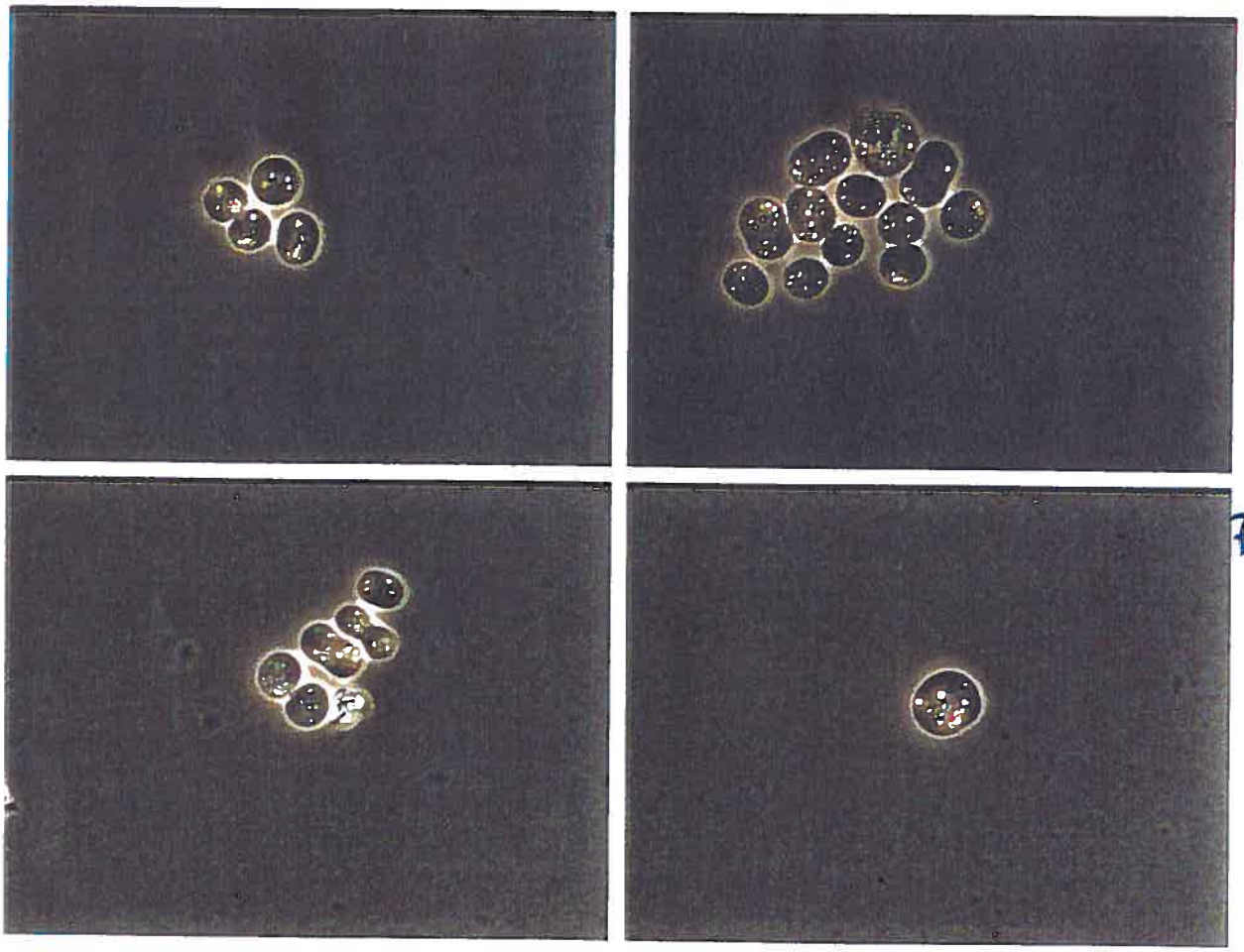

Fig. 2

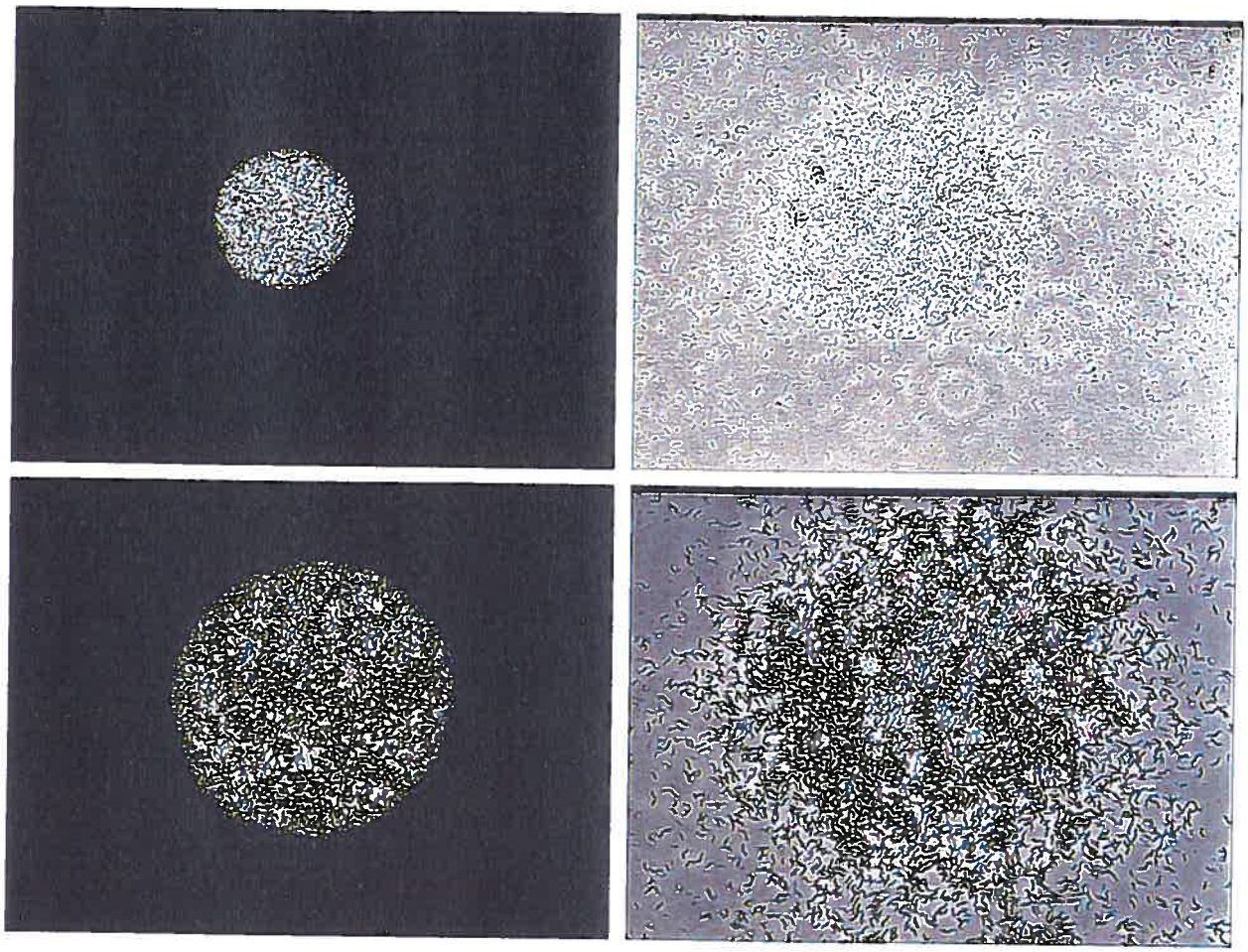

Fig. 3