NO BUG IS AN ISLAND: THE MESSY BUSINESS OF WORKING WITH COCULTURES

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Abstract

Sulfate reducers and methanogens are dominant terminal anaerobic microorganisms under conditions specific to each group. Nonetheless, both groups can generally be recovered from the same environments. Some sulfate reducers have the ability to act as fermenters, and to establish syntrophic relationships with hydrogen-consuming organisms. The potential for syntrophism among local salt-water and fresh-water sulfate reducers was explored with cocultures of locally obtained SRB enrichments and a hydrogenotrophic methanogen under sulfate-free conditions, and diverse substrates. Despite methane production in many of the cocultures, only one of the tested SRB enrichments from a marine environment yielded results suggestive of syntrophism. This observed syntrophic behavior could reflect a potential niche for sulfate reducers with consequential effects on anaerobic organic matter mineralization, even in sulfate-abundant environments.

Introduction

The study of pure cultures in the laboratory constitutes a very powerful tool for studying the metabolic capabilities of an organism, and the interaction between the organisms and potential physical and chemical parameters from the environment. However, many of these metabolic capabilities are associated with the activity of other organisms that can regulate, constraint and determine the transformation, fluxes and accumulation of particulate organic matter. Particularly in the case of anaerobic environments, a heterogeneous assembly of organisms related in a complex food web carry out the important role of converting complex organic material to simple compounds and gases that can recirculate back into the aerobic regions supporting the life (Gottschalk & Peinemann, 1992).

Two primary metabolic pathways for the remineralization of organic material are believed to dominate in anaerobic environments. In sulfate reduction, bacteria utilize the sulfate ion as terminal electron acceptor for the organic matter mineralization, and in methanogenesis, archaea utilize CO_2 as terminal electron acceptor. (Fig. 1). In nature, these two anaerobic metabolic systems should be thermodynamically unbalanced; when sulfate is present, as in marine environments, sulfate reducing bacteria (SRB) are dominant (Jorgensen, 1980) and methanogenesis is minimal. When sulfate is unavailable in the

system, methanogenesis should be the dominant metabolism (Winfrey & Zeikus, 1979). In freshwaters systems and soils methanogenesis appear as a dominating processes.

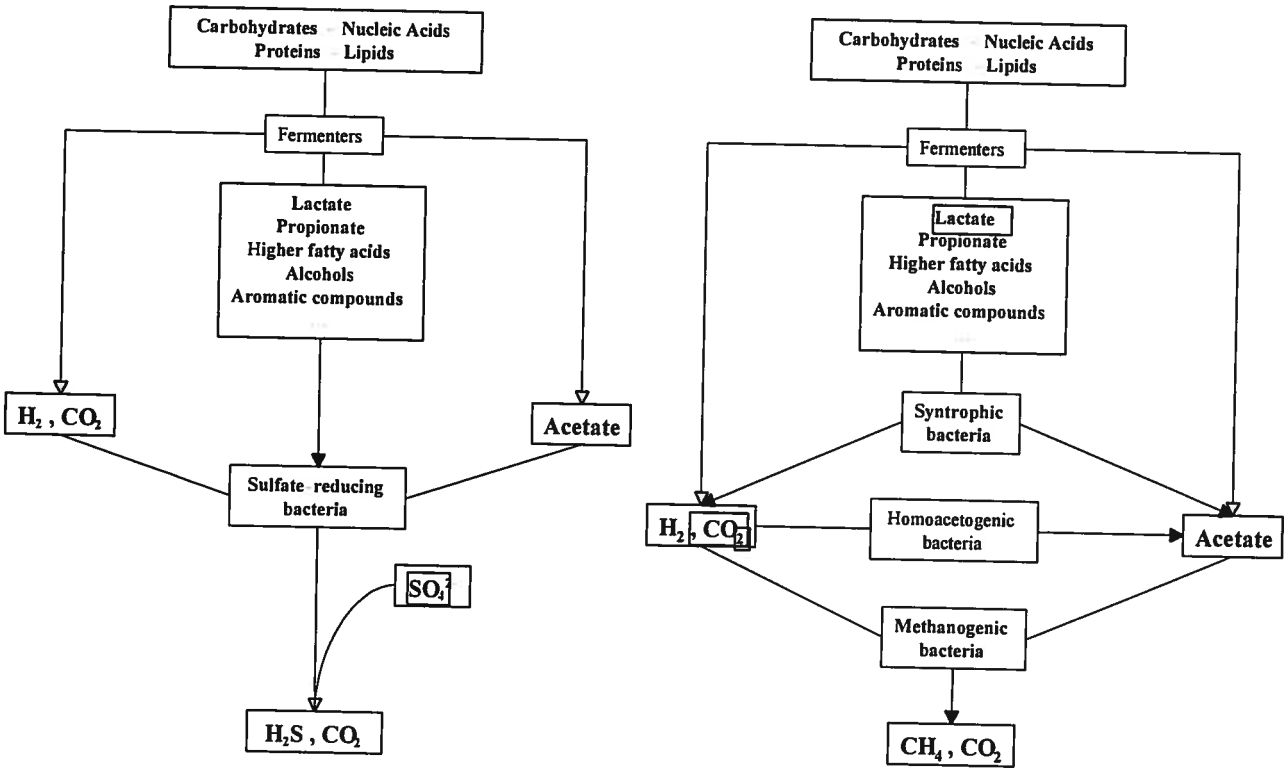

Fig. 1 Anaerobic metabolic pathways

Despite the metabolic differences between SRBs and methanogens, both groups can be recovered from enrichment cultures from the same source material. These two metabolic systems coexist under anaerobic conditions, although the activity of one or the other will reflect the specific geochemical and redox attributes of the environment. The metabolism of SRBs as a group has been reported as being very versatile with respect to utilization of substrates, from H_2 to low molecular weight fatty acids to aromatic compounds (Widdel, 1988). Consequently, the SRBs may shift from anaerobic respiration to fermentation depending on the available substrate and the presence or absence of a suitable electron acceptor, and are able to establish syntrophic relationships with H-consuming organisms such as methanogens. In the absence of sulfate, SRBs can shift from a respiratory to a fermentative mode of energy conservation.

Although a single fermentation reaction may not be energetically favorable to the SRB, the energetics of the reaction may be made more favorable if the product of the reaction (H_2) can be used by another organism, generating a more negative change in free energy (Table 1). In this way, the products of one type of organism's metabolism can be used to support the growth of the partner organism, and the two organisms can establish a syntrophic (mutual feeding) relationship.

The term "syntrophism" is used to denote those cooperations between organisms in which both partners depend entirely on each other to perform a metabolic activity and in which the mutual dependence on each other cannot be overcome by simply adding a co-substrate or any other type of nutrient. The syntrophic relationship in aerobic organisms are less important than for anaerobic organisms. The aerobic bacteria can frequently degrade complex substrate completely to CO_2 and water without the cooperation of other organisms. However in anaerobic environments, the catabolic and anabolic activities of microorganisms depend to a great extend on the cooperation of other organisms with several metabolic types participating in a complex feeding network (Schink, 1997). The syntrophic relationship is probably one way of life that the organisms use to survive in natural anaerobic environments exploiting minimal energy yields (McInerney et al., 1979).

One of the syntrophic systems demonstrated in the laboratory is that of SRBs and methanogens. SRBs have been shown to function as net H_2 producers (during fermentation) and the methanogens utilize the H_2 for the reduction of CO_2 to methane (Bryant et al, 1977; Fiebis and Gottschalk, 1983; Phelps et al, 1985).

Table 1. Changes of Gibbs free energies under standard and syntrophic conditions.

	Equations		$\Delta G_0'$ (Kj/mol)
Fermentation	Ethanol	$2\ CH_3CH_2OH + 2\ H_2O = 2CH_3COO^- + 2H^+ + 4H_2$	+ 9.6
	Lactate	$2\ CH_3CHOHCOO^- + 4\ H_2O = 2\ CH_3COO^- + 2\ CO_2 + 4\ H_2$	-3.97
	Glutamate	$20\ Glu + 24\ H_2O + 8H^+ = 20\ NH_4^+ + 20\ CO_2 + 24\ CH_3COO^- + 8$ butyrate $+ 4\ H_2$	-59
Methanogenesis	H_2/CO_2	$4\ H_2 + CO_2 = CH_4 + 2\ H_2O$	-131.0
Syntrophic relationship	Ethanol	$2\ CH_3CH_2OH + HCO_3^- = 2CH_3COO^- + H^+ + CH_4 + H_2O$	**-121.4**
	Lactate	$2\ CH_3CHOHCOO^- + H_2O = 2CH_3COO^- + H^+ + HCO_3^- + CH_4$	**-134.97**
	Glutamate	$20\ Glu + 24\ H_2O = 24\ NH_4^+ + 5\ CO_2 + 6\ CH_3COO^- + 2$ butyrate $+ CH_4 + H^+$	**-190.0**

Based on the reported metabolic versatility of sulfate reducing bacteria (SRB), the goal of this research is to explore the potential for syntrophism between a pure culture of a methanogen and locally recovered SRBs from marine and freshwater systems.

The **hypothesis** is that locally recovered SRBs should have the ability to act as H_2 producers and establish syntrophic relationships with methanogens in sulfate-depleted conditions

Methodology

Coculture is the growth of 2 or more organisms in the same physical space, usually an artificial medium of some kind. In contrast, pure culture is the growth of one specific organism in artificial media. Syntrophy is a special case of coculture in which the microbial partners each provide something for the other organism. In this case, syntrophy between SRBs and methanogens may be established when the SRB produces H_2 from its metabolic activity, and the methanogen makes it possible for the SRB to continue to grow by removing the H_2 and utilizing it for making methane. Removal of the H_2 in this manner lowers the partial pressure of the gas and makes the reaction more energetically favorable for the SRB, allowing it to continue with its metabolic activity. In our cultures, we did not add sulfate ion to the media in order to "force" the SRBs to ferment substrate rather than respire anaerobically, using sulfate as the electron acceptor. Anaerobic respiration does not generate hydrogen, and syntrophy is not possible (between these two organisms) without hydrogen production.

Rationale for selection of cultures:
The methanogen pure culture (*Methanococcus maripaludis*) was selected because it is an organism of known metabolic capability, and an obligate hydrogenotroph (and therefore could only grow when suitable products of SRB activity were present). It is a marine isolate, but since its growth is not related to sulfate concentration, the conditions present in the medium (absence of sulfate) should not directly affect its growth or metabolic activity.

The sulfate-reducing bacteria (SRB) enrichments selected for the study were used 1) to determine whether locally-derived organisms could establish syntrophic relationships with a known methanogen, and 2) they were a ready source of both marine and freshwater SRBs, having been enriched in classroom activities in the previous 1-2 weeks. The SRB syntroph partners were obtained from three locally-derived enrichments; two were obtained from material from Sippewissett marsh (marine) and one from Ceder Swamp material, a freshwater environment that readily demonstrates methanogensis. These enrichments were used with the knowledge that they most likely contained organisms that were not SRBs.

Media:
Syntroph/coculture medium:
SRB basal fresh water medium was prepared in 500 ml batches without substrate or other additions, gassed with $N2/CO_2$, stoppered and autoclaved. After autoclaving, the medium was cooled, and additions made from filter-sterilized stocks as per the recipe below. Medium was modified for the coculture of both SRB and methanogens by omitting sulfate and adding 1 ml of Se-Wo solution. From 0.5 M sterile stock solutions, substrate was added to achieve a final concentration of 10 mM. All additions to the medium were followed by gassing with sterile (filtered) N_2/CO_2 by the Hungate technique. Five ml of sterile media was dispensed into sterile Balch tube in an anaerobic hood with $N2/CO_2$ atmosphere. Tubes were capped with sterile Hungate rubber stoppers and crimped closed. Inoculations were made using sterile syringes.

Modified basal medium for syntroph culture:

1 l basal fresh water mineral medium
30 mM $NaHCO_3$
1 ml 7 vitamin solution
1 ml SL 10 (trace mineral solution)
1 ml Se-Wo solution
1 mM Na_2S
final pH 7.2 – 7.3

Headspace gas was N_2/CO_2.

Source cultures:

Sulfate-reducing bacteria were grown in enrichment cultures with media containing the following:

1 l basal marine mineral medium
30 mM Na_2SO_4
30 mM $NaHCO_3$
1 ml 7 vitamin solution
1 ml SL 10 (trace mineral solution)
1 mM Na_2S
final pH 7.2 – 7.3

SRB media were supplemented with ethanol or acetate as substrate, to a final concentration of 10 mM. Media was prepared in 50 ml Pfennig bottles. Source material for enrichments was added to the bottle aseptically, and the bottle "topped off" with sterile media so that no air bubble was present after inoculation. Incubation was in the dark at 30°C until turbidity was noted (or cells apparent under microscopic observation). Successful enrichment cultures were notable for a distinct odor of sulfide upon opening the cap. Although cellular morphology varied (as would be expected in enrichment cultures), short rods and vibrio-shaped organisms were predominant shapes. These cells were actively motile.

Methanogen cultures were grown in a medium comprised of basal fresh water mineral medium with a headspace gas of 80% H_2/20% CO_2. Five ml of media was dispensed aseptically into 22 ml Balch tubes under anaerobic conditions (80% H_2/20% CO_2), the lyophilized culture added aseptically, and the tube stoppered with a sterile Hungate stopper, and crimped closed. Incubation was in a stationary incubator at 30°C.

Culture conditions:

Cell growth was carried out in Balch tubes containing 5 ml of sterile medium, capped with Hungate stoppers and with anaerobic gas in the headspace. Medium was prepared and dispensed, amendments made where necessary (e.g. addition of inhibitor). Cultures were incubated on an enclosed shaker, with the temperature regulated to 30°C.

The potential syntrophic partnerships were composed of the methanogen, *Methanococcus maripaludis*, and one of the SRB enrichments in a suitable, sulfate-free, medium. Cultures were inoculated and maintained in strictly anaerobic conditions in Hungate tubes, which were prepared in a N2/CO_2 atmosphere, and inoculated by injection of cells with sterile syringes. Care was taken not to introduce air when taking samples.

Cell numbers:
Growth of cultured cells was determined at 0, 5, and 12 days. 100 – 200 µl of culture was withdrawn from the culture tube with a 1 ml tuberculin syringe and a sterile needle. This aliquot was centrifuged (10,000 rpm x 5 minutes), the supernatant removed (for HPLC analysis), and the cell pellet resuspended in (filtered) 2% formaldehyde in 1X phosphate buffer. 15 µl of this cell suspension was counted using a Petroff Hausser counting chamber. When possible to distinguish SRB from methanogens by cell morphology, these numbers were noted in the cell count (Methanospirillum was readily identified by its long wavy morphology as being different from the Desulfovibrio cells).

Cultures or enrichments were quantitated as to cell numbers, and inoculations made using amounts of culture that produced inoculum of approximately 1:2 methanogen to SRB, and produced a final inoculum of approximately 5-12 x 10^6 cells/ml. Initial inocula were counted to approximate cell numbers, in order to use approximately equivalent cell numbers in all treatments and controls.

Turbidity:
At intervals during the experiment (each 3 days during 11 days), tubes were visually examined for turbidity, which was used as a confirmation of cell growth. Culture tubes were compared with uninoculated media as a negative control. Turbidity was scored as 0, S (slight), or + (unequivocal growth).

HPLC:
As described above, aliquots of cell-free culture medium were prepared and placed in sterile 1.5 ml microcentrifuge tubes, and stored frozen until analysis was conducted. Samples were filtered just before analyzing on the HPLC. All samples were analyzed on an Agilent 1100 series HPLC with autosampler.

Glutamate utilization was measured by analyzing samples of culture medium for disappearance of the substrate. The glutamate was first derivatized with o-phthaldialdehyde/ethanol/2-mercaptoethanol/borate buffer (OPD), and analyzing with a 90:10 mobile phase consisting of methanol:acetate buffer/methanol/tetrahydrofuran. Glutamate eluted at approximately 2 minutes. Standard concentrations of 1 and 10 mM derivatized glutamate were prepared for standardizing the machine. The column was a Nova-Pak C18 column (standard reversed-phase column). Detection of glutamate was at 330 or 360 nm.

Acetate was measured directly from the culture fluid, using 0.0008 N H_2SO_4 as mobile phase. Standards of 1 and 10mM acetate were prepared and used for standardization of the column. Bio-rad Aminex HP70 column. For organic acids and alcohols, and sugars. Might work for lactate, similar sensitivity as for acetate, similar retention time.

Gas Chromatography (GC):
All samples were analyzed with a Shimadzu GC-8A gas chromatograph.

For analysis of bacterial methane production under culture conditions, samples were analyzed by utilizing the gas chromatograph with a Porapak N 80/100 column, and operated at an oven temperature of 75°C. Methane eluted from the column in approximately 0.25 minutes. Standards of 1, 10, 100, and 10000 ppm methane (in N_2/CO_2) were prepared and used to generate a standard curve.

Analysis of bacterial acetate formation under culture conditions was measured by using the Shimadzu GC-8A at an oven temperature of 175°C with the following packed column: 10% AT-1000 on 80/1000 Chromosorb W-AW (Alltech).

Table 2 General Experimental Design

	SRB	Methanogen	Substrate	Inhibitor
Exp	1	+	+	-
Exp	2	+	+	-
Exp	3	+	+	-
Control	+	-	+	-
Control	-	+	+	-
Control	+	+	+	+
Control	+	+	-	-

Microscopy:

Methanogens can be distinguished from Eubacteria by fluorescence microscopy. Methanogens are archaea, and contain a molecule, F420, that autofluoresces when exposed to UV light. It is possible to first observe a field under phase microscopy, then switch to UV illumination and observe only the methanogens. Eubacteria do not have F420 and do not autofluoresce. This technique was utilized for examination of cultures to ensure the presence of the methanogens.

Results

Methane production was observed after twelve days in most of the cocultures with *Methanococcus maripaludis* and the SRB enrichments (**Fig. 2**). All cocultures on glutamate produced similar amounts of methane. Methane was detected in the cocultures with lactate or ethanol as substrate only when either of the two Sippewisset SRB enrichment cultures (SRB1 or SRB2) was used as a source of syntrophic partners.

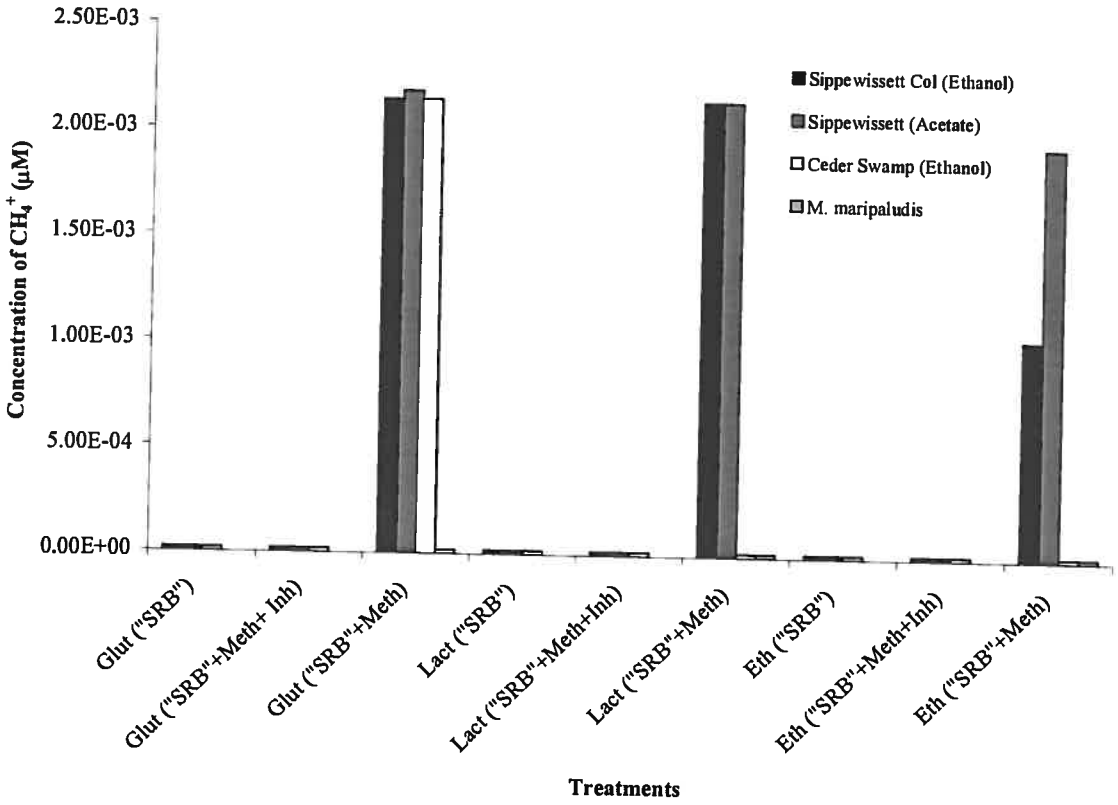

Fig. 2. Methane production as related to treatment conditions

Methanogenic cultures on all three experimental substrates contained, in addition to vibrioid and rod-shaped cells, the characteristic cocci morphotype of *Methanococcus maripaludis*. Their methanogenic character was confirmed with epifluorescent microscopy by detection of autofluorescent cells at a wavelength of 420 nm (**Fig. 3**), a diagnostic trait for methanogens. Autofluorescent cells were not detected in cultures inoculated with SRB enrichments alone, or in cocultures with BES added (**Fig. 3**), indicating that the SRB enrichments utilized did not contain considerable numbers of native methanogens.

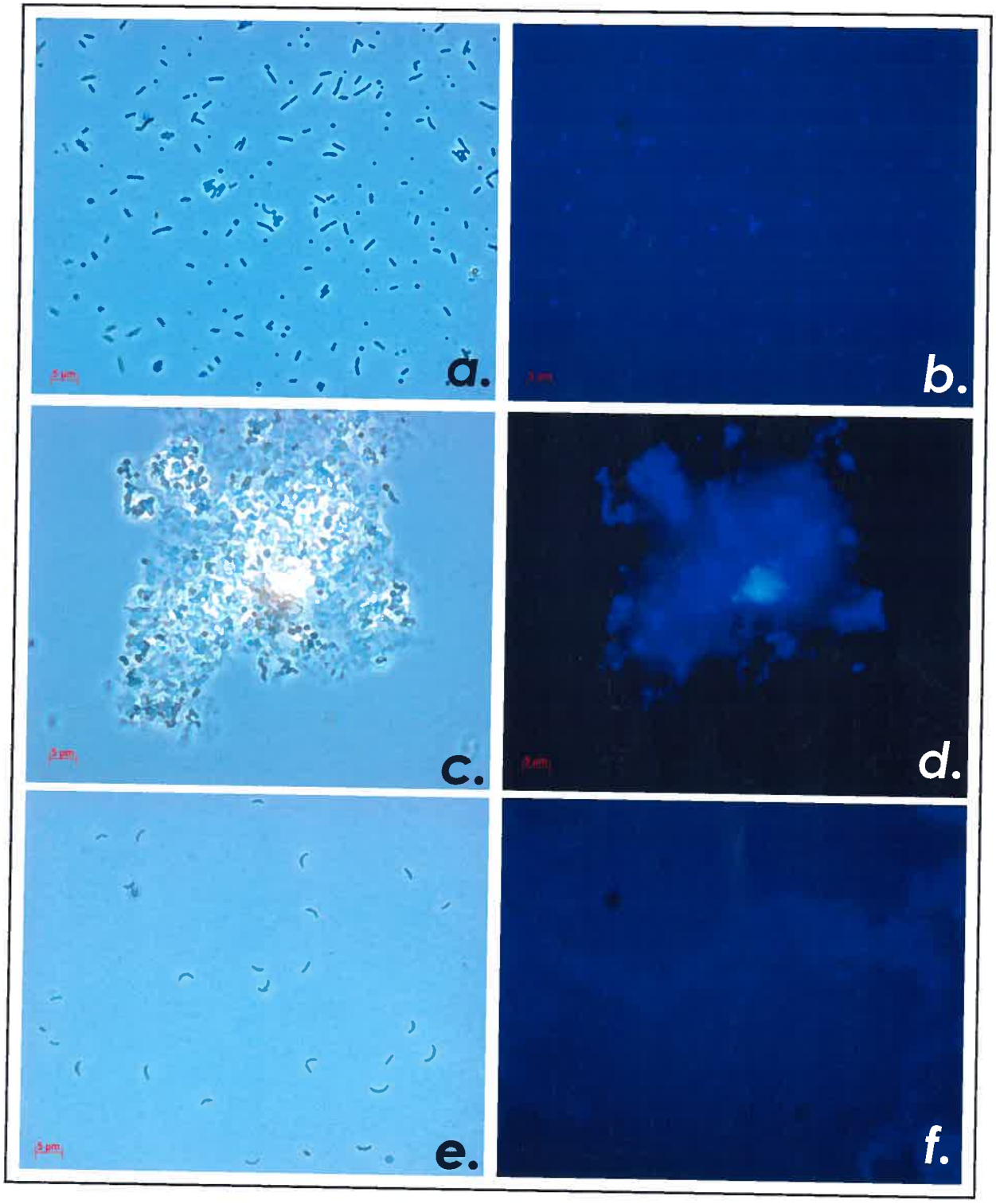

Fig. 3 – Phase contrast (left column) and epifluorescent (420 nm; right column) microphotographs from SRB2 (a,b) and SRB1 (c,d) methanogenic cocultures with *Methanococcus maripaludis* after 10 days of incubation. Methanogenic cells detected by blue autofluorescence, and present only on methanogenic cultures. Negative controls (e,f) did not present any autofluorescent cells (typical negative control shown).

Glutamate supported growth for all the cultures, including those controls where the methanogen component had been excluded, or where methanogenesis (hence hydrogen removal) was inhibited through the addition of 10 mM BES to the cocultures. Similarly, comparable number of cells were obtained with glutamate for all treatments containing SRB2 or SRB3, suggesting a minor effect of the hydrogen scavenging activity of *M. maripaludis* in these experiments. In contrast, a nearly five-fold increase in cell numbers was observed for the coculture of SRB1 and *M. maripaludis* on glutamate when compared to the cell numbers for SRB1 alone, or for the coculture plus BES on the same substrate. By day five glutamate had reached undetectable concentrations in most cultures, a surprising result considering the continued increase in cell numbers from day 5 until day 10 on the corresponding cultures **(Fig. 4)**.

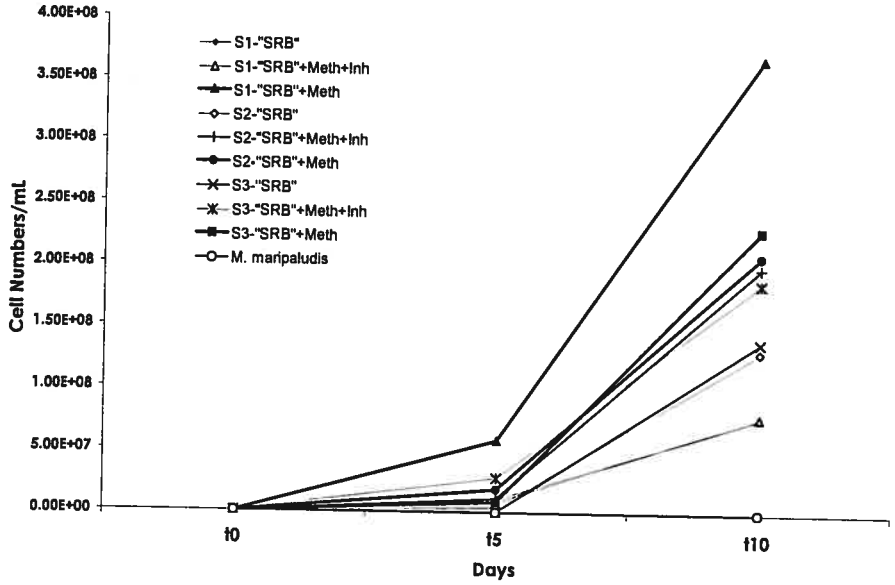

Fig. 4 Change in Cell Numbers through time on glutamate

Growth on lactate was detectable in cultures containing either the SRB1 or SRB2 enrichments **(Fig. 5)**. No growth, as determined by a considerable increase in cell numbers, was detected in lactate cultures with SRB3.

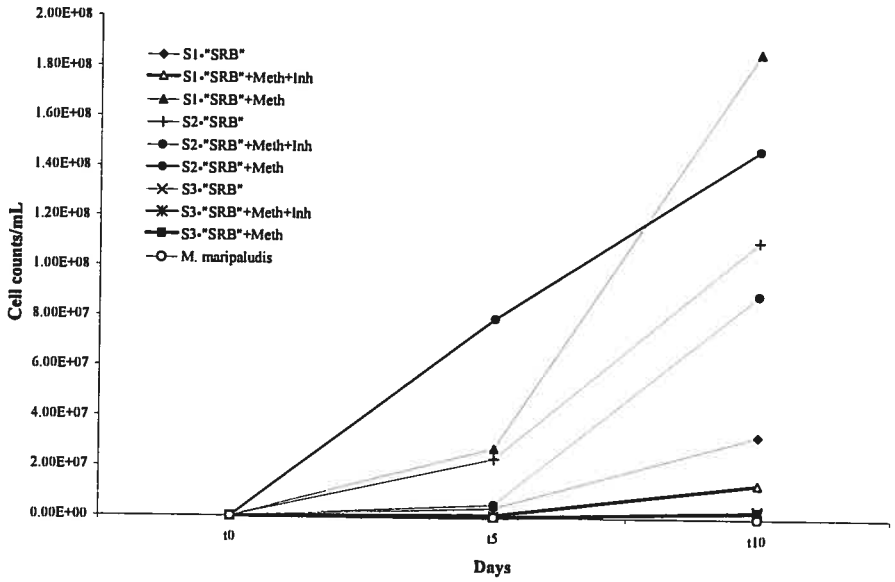

Fig. 5 Change in Cell Numbers through time on Lactate

Growth on lactate for SRB2 cultures was unaffected by active methanogenesis, whereas the methanogenic coculture on lactate containing SRB1 and *M. maripaludis* had six and fourteen times higher cell counts than the culture with SRB1 alone or the coculture with inhibitor respectively (**Fig. 6**)

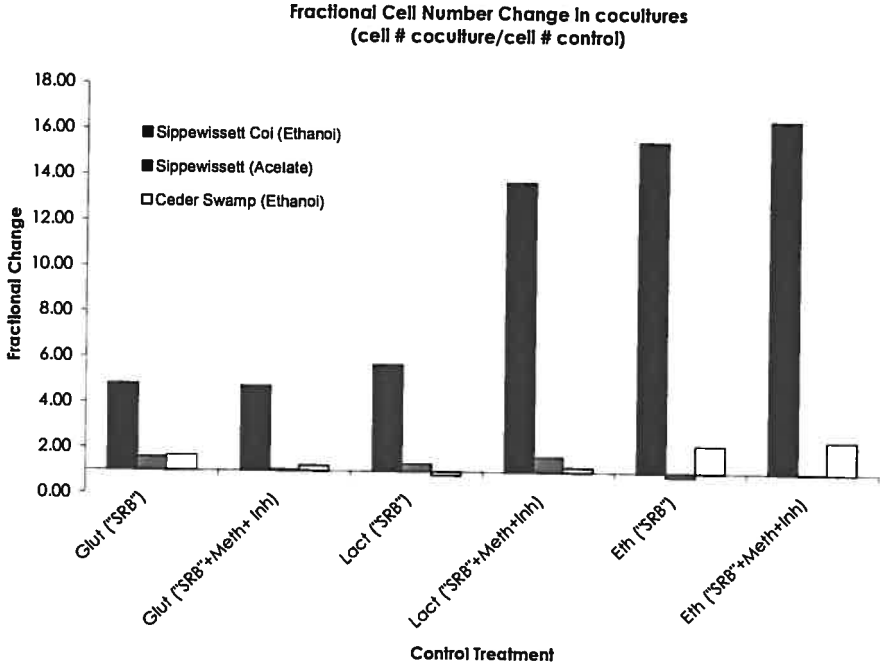

Fig. 6 Fractional Changes in Cell Numbers in Cocultures

Insignificant growth was present on cultures with SRB3, either with or without functional methanogenesis, when ethanol was provided as the energy source. Minimal amount of change in cell numbers was observed in cultures with SRB2 and ethanol as substrate. Similarly, limited growth was present in ethanol cultures with SRB1 when methanogens were absent or inhibited by BES. However, with active methanogenesis, cell numbers showed a nearly sixteen-fold increase when compared to the control cultures **(Fig. 6)**.

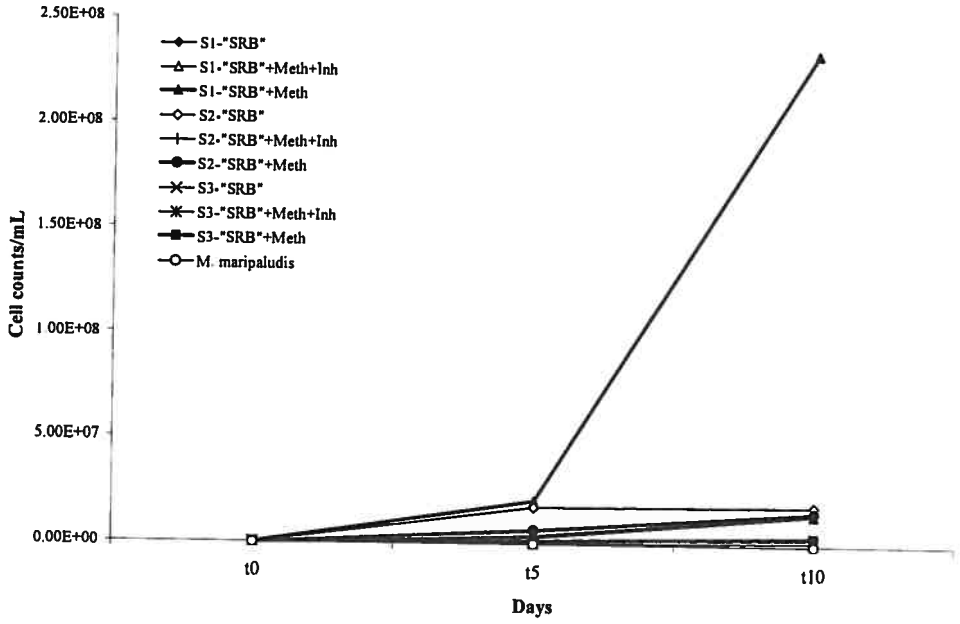

Fig. 7 Changes in Cell Numbers through time on Ethanol

The consumption patterns for substrates other than glutamate were not explored due to technical difficulties.

Discussion

The rationale for understanding syntrophic relationships lies in the advantageous thermodynamics of the combined metabolic pathways, overcoming energy limitations within the individual reactions by virtue of product removal. Growth on glutamate independent from hydrogen scavenging can be explained with a thermodynamic argument as well. According to the general equation for the fermentation of glutamate, the stoichiometric relationship between hydrogen and glutamate is 0.2 mol H_2 per each mol of glutamate fermented (Eq. 1)

$$5\ \text{Glu} + 6\ H_2O + 2\ H^+ \leftrightarrow 5\ NH_4^+ + 5\ CO_2 + 6\ CH_3COO^- + 2\ \text{butyrate} + H_2 \quad \textbf{(Eq. 1).}$$

Thus, in our experimental set up, even after complete fermentation of the initial glutamate, the hydrogen concentration would presumably remain below inhibitory levels. The limited influence of the hydrogen concentration on the fermentation of glutamate is illustrated in **Fig. 8**, where the free energy from the reaction is plotted as a function of the hydrogen partial pressure under otherwise standard conditions.

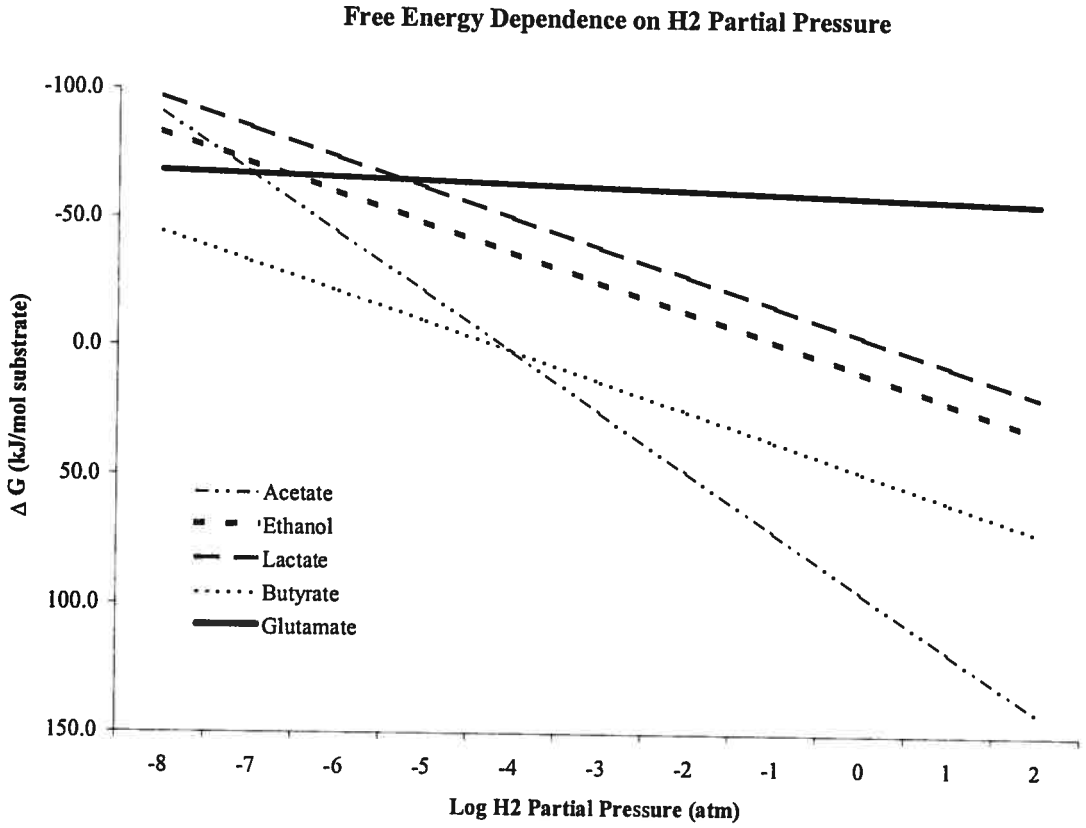

Fig. 8 Free Energy Dependence on H2 Partial Pressure

The free energy remains within the usable range for energy conservation through substrate level phosphorylation. Glutamate fermentation independent from hydrogen removal could thus account for the observed growth in cultures containing either SRB2 or SRB3. Assuming such an explanation is correct, then measuring the hydrogen content in those cultures in question from which methanogens are absent, or where methanogenesis has been inhibited, should yield amounts of hydrogen stoichiometrically equivalent to the amount of methane produced in the glutamate methanogenic cocultures involving the above-mentioned SRB enrichments. Lower amounts than expected would suggest a facilitating role of methanogenesis in the degradation of glutamate in such cultures, but which translates into such a small effect on growth numbers that it can not be detected with our methods.

The observed increase in cell numbers for SRB1 methanogenic cocultures using glutamate as substrate suggests a syntrophic relationship between the hydrogenotrophic

methanogen and a fermentative organism present in the SRB1 enrichments. This relationship however does not involve the facilitated fermentation of glutamate. Instead, it can be proposed that this syntrophy is based on the degradation of either the butyrate or the acetate produced from the initial fermentation of glutamate. As has been reported (Schink, 1997), the two latter substrates can be fermented by syntrophic systems. Measurements of the intermediates as well as the final products, i.e. acetate, butyrate, and methane, would reveal the substrate serving as the base for the syntrophic relationship. From the theoretical hydrogen production through the fermentation reactions in question, the expected amount of methane can be calculated. The following cases are possible: a) A simple fermentation of glutamate, such as that suggested by equation **1**, starting with our experimental conditions (50 μmol total glutamate) would result in approximately 10 μmol H_2, or 2.5 μmol CH_4; b) With further fermentation of the butyrate generated, the total amount of CH4 would rise to 12.5 μmol; c) If further degradation of acetate were involved, methane levels would reach 62.5 μmol; or d) 112.5 μmol if preceded by syntrophic fermentation of butyrate. Given that we were working with enrichments rather than with pure cultures, the presence of organisms capable of performing these functions cannot be dismissed a priori. The measured levels of methane in our methanogenic glutamate cultures hovered around 2.1 μmol. This quantity is close to that expected from a simple fermentation of glutamate with subsequent consumption of the hydrogen produced (case a.). Nonetheless, in order to determine which of the cases listed above is taking place, the cultures should be monitored for longer periods of time to ensure cessation of methanogenesis. Changes in cell numbers of cultures on lactate containing SRB1 hint at incipient syntrophic relationships. Similar to the effect of active methanogenesis on the growth of cocultures on glutamate, methanogenic cocultures on lactate with SRB1 showed an increase in cell numbers when compared to the corresponding methane-negative controls (see results). This observation suggests that hydrogen removal by the methanogen is allowing greater growth, presumably by maintaining thermodynamically favorable conditions for the fermentation of lactate. Nonetheless, any claim of syntrophism should be accompanied by data on substrate disappearance and methane production, measurements that were unavailable to us due to time and technical difficulties.

Growth on lactate cultures with SRB2 does not seem to be affected by the production of methane. Since fermentation of lactate is minimally thermodynamically favorable, and strongly dependent on hydrogen partial pressure (see background), we propose that the observed growth was sustained by carry-over substrate from the previous growing media. However, this does not exclude an incipient syntrophic relationship, specially considering the methane production observed on the coculture without inhibitor. One way to determine the status of the metabolic interactions would be to follow the system until methane production stops.

The effect of growth on lactate cocultures for SRB1 with active methanogenesis resembles, in dynamics if not in magnitude, that observed for the same cocultures on lactate or glutamate. A syntrophic relationship between organisms in SRB1 and *M. maripaludis* based on ethanol fermentation can thus be suspected.

From the observed results we can suspect that organisms in SRB1 have the potential to establish syntrophic relationships with hydrogenotrophic organisms for the degradation

of ethanol, lactate, and to some extent glutamate or of a secondary product from the fermentation of this last substrate. The metabolic relationships between SRB2 and *M. maripaludis* on lactate remain as yet unclear. The growth of SRB2 and SRB3 on glutamate is most likely due to a fermentation process independent of hydrogen removal. When considering the patterns of the relationships observed, two aspects are worth of consideration. SRB1 is an ethanol enrichment for SRBs using samples from marine environments. Fermentative degradation of lactate or ethanol in marine settings, where sulfate is rarely limiting, could be expected to be rare. Yet, we observed the potential for such metabolic interactions, which speaks to the high metabolic versatility of the fermentative organism involved. In contrast, enrichments from Ceder Swamp (SRB3), a fresh water environment where opportunities for syntrophy can be expected to abound, failed to establish any syntrophic association with *M. maripaludis* under our experimental conditions. One potential influencing factor is the difference in salinity between the native environment (fresh), and the experimental conditions (salt water to accommodate for the methanogen requirements). At the same time, ethanol and lactate may not be present in considerable amounts during the degradation of organic matter under anaerobic, fresh-water conditions.

Our initial interests resided in the potential for local SRBs to interact syntrophically as fermenters with hydrogenotrophic methanogens. Despite using enrichments for SRB as our source of syntrophs, the true identity of the organisms involved in the syntrophic relationship should be confirm before assigning them to the sulfate reducer group. Although our experiments may not resemble the circumstances prevailing in the corresponding environments for the organisms tested, and the results observed cannot be entirely conclusive due to the missing information about substrate disappearance dynamics, they provide a starting point for exploring active syntrophic interactions in environments hitherto considered unlikely for such metabolic activities.

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