Extremophiles (2000) 4:247–252

ORIGINAL PAPER

Oliver Rothe · Michael Thomm

A simplified method for the cultivation of extreme anaerobic Archaea based on the use of sodium sulfite as reducing agent

Received: January 4, 2000 / Accepted: April 5, 2000

Abstract The extreme sensitivity of many Archaea to oxygen is a major obstacle for their cultivation in the laboratory and the development of archaeal genetic exchange systems. The technique of Balch and Wolfe (1976) is suitable for the cultivation of anaerobic Archaea but involves time-consuming procedures such as the use of air locks and glove boxes. We describe here a procedure for the cultivation of anaerobic Archaea that is more convenient and faster and allows the preparation of liquid media without the use of an anaerobic chamber. When the reducing agent sodium sulfide (Na_2S) was replaced by sodium sulfite (Na_2SO_3), anaerobic media could be prepared without protection from oxygen outside an anaerobic chamber. Exchange of the headspace of serum bottles by appropriate gases was sufficient to maintain anaerobic conditions in the culture media. Organisms that were unable to utilize sulfite as a source for cellular sulfur were supplemented with hydrogen sulfide. H_2S was simply added to the headspace of serum bottles by a syringe. The use of H_2S as a source for sulfur minimized the precipitation of cations by sulfide. Representatives of 12 genera of anaerobic Archaea studied here were able to grow in media prepared by this procedure. For the extremely oxygen-sensitive organism *Methanococcus thermolithotrophicus*, we show that plates could be prepared outside an anaerobic chamber when sulfite was used as reducing agent. The application of this method may faciliate the cultivation and handling of extreme anaerobic Archaea considerably.

Key words Archaea · Anaerobiosis · Reducing agents · Sulfite · Plating efficiency · Anaerobic chamber · Methanogens · Hyperthermophiles

Communicated by G. Antranikian

O. Rothe · M. Thomm (✉)
Institut für Allgemeine Mikrobiologie, Am Botanischen Garten 1-9, 24118 Kiel, Germany
Tel. +49-431-880-4330; Fax +49-431-880-2194
e-mail: mthomm@ifam.uni-kiel.de

Introduction

Anaerobic Archaea are extremely sensitive even to traces of oxygen. To provide media of an adequate redox potential, efficient techniques have been developed (Balch and Wolfe 1976; Hungate 1969). For the cultivation of these organisms, generally the technique described by Balch and Wolfe (1976) is used. This procedure involves preparation of the medium without reducing agent, then removal of dissolved oxygen by boiling under a nitrogen atmosphere or by flushing the medium with nitrogen. Reducing agents, usually a cysteine–sodium sulfide mixture for the cultivation of methanogens, or sulfide or cysteine alone for the cultivation of hyperthermophiles, are added, and the flask is stoppered and transferred to an anaerobic chamber where the medium is dispensed into tubes. The tubes are sealed with rubber stoppers inside the chamber. When tubes are transferred outside the chamber, the gas phase in each tube is exchanged for the gas phase appropriate for the cultivation of the organisms, in many cases an $H_2:CO_2$ (80:20) (v/v) gas mixture, by the use of a gassing manifold.

The use of sodium sulfide as reducing agent causes several problems. First, at pH 7 or lower most of the sulfide exists as H_2S and is leaving the medium because of its volatility and low solubility. This limitation is particularly important during cultivation in fermentors where continuous flushing with gas is required. Second, the addition of sulfide anions to the media, although dissociation equilibrium occurs quickly, leads to partial precipitation of most cationic trace elements. The available concentration of cations is thus lowered. Furthermore, precipitated sulfides often hinder measurement of cell growth or make it difficult to distinguish between cells and precipitates. We describe here a simplified method for the cultivation of both methanogenic and hyperthermophilic anaerobic Archaea using sulfite as reducing agent. This method was shown to be suitable for the cultivation of all anaerobic Archaea investigated. It avoids the afore mentioned disadvantages of sulfide and renders obsolete the use of anaerobic chambers for the preparation of liquid media.

Materials and methods

Preparation of liquid media

Conventional media containing sodium sulfide as reducing agent were prepared by a modification of the standard procedure described by Balch and Wolfe (1976). Media prepared without reducing agent were flushed at room temperature for 20 min with N_2 by the use of a gassing manifold. A sodium sulfide solution was added to a final concentration of 0.5 g/l. The pH was adjusted with 25% (v/v) of H_2SO_4 through the bottle stoppers with a syringe. An aliquot was removed by a syringe and the pH measured immediately by the use of universal pH paper (duotest pH 5.0–8.0; Macherey and Nagel, Düren, FRG). The media were dispensed into serum bottles in the anaerobic chamber and sealed; depending on the organism, the gas phase in each tube was then exchanged (Table 1). The growth conditions used for the cultivation of the Archaea are also shown in Table 1.

The composition of the media used is given in the references indicated in parentheses, as follows: *Archaeoglobus fulgidus*, DSM 4304 (Stetter 1988), *Archaeoglobus profundus*, DSM 5631 (Burggraf et al. 1990b), *Desulfurococcus mobilis*, DSM 2161 (Zillig et al. 1982), *Hyperthermus butylicus*, DSM 5456 (Zillig et al. 1990, 1991), *Methanopyrus kandleri*, DSM 6324 (Kurr et al. 1991), *Methanobacterium thermoautotrophicum*, DSM 1053 (Schönheit et al. 1979), *Methanococcus thermolithotrophicus*, DSM 2095 (Huber et al. 1982), and *Methanococcus igneus*, DSM 5666 (Burggraf et al. 1990a) were cultivated in medium 3 described by Balch and Wolfe (1976); *Methanoplanus limicola*, DSM 2279, was cultivated in medium 3 described by Balch et al. (1979), but the medium contained in addition sodium acetate (1 g/l), yeast extract (2 g/l), and peptone (2 g/l) (Wildgruber et al. 1982); *Pyrococcus furiosus*, DSM 3638 and *Pyrococcus woesei*, DSM 3773 (Zillig et al. 1987), *Pyrobaculum islandicum*, DSM 4184 (Huber et al. 1987), *Pyrodictum occultum*, DSM 2709 and *Pyrodictum abyssi*, DSM 6158 (Pley et al. 1991), and *Staphylothermus marinus*, DSM 3639 (Fiala and Stetter 1986).

The media prepared by the novel procedure were inoculated with cultures grown in media prepared by the technique described by Balch and Wolfe (1976). Resazurin was added as a redox indicator at a final concentration of 0.00002%. Sodium sulfite was added aerobically as indicated in the Results section, and the pH was adjusted to the desired value (see Table 1) by addition of 50% H_2SO_4. Then, 20 ml of liquid media was dispensed into 120-ml serum bottles under aerobic conditions. The cultures were successfully transferred into sulfite media three consecutive times. Five parallel bottles were analyzed for each experiment.

Growth measurements

Microbial growth was determined by direct cell counting in a Thoma Blau Brand chamber (Omnilab-Laborzentrum, Hamburg, FRG) with a depth of 0.02 mm under a phase-contrast microscope (Zeiss standard 16).

Preparation of solid media

The dissolved compounds were supplemented with 16 g/l agar and 0.5 g/l sodium sulfite; 500 ml of media was dispensed in 1000-ml bottles, which were stoppered and sealed. The headspace was flushed with nitrogen. Immediately after autoclaving, the media were dispensed into plastic petri dishes under aerobic conditions. The plates were placed into an anaerobic pressure cylinder, and for each five plates one filled with anhydrous $CaCl_2$ was added. The cylinder (size 30 × 11 cm) (Balch et al. 1979) was then pressurized with 300 kPa H_2/CO_2 (= 80:20, v/v) and the gas atmosphere was exchanged two times. The cylinders were kept under pressure. Then, 15 ml hydrogen sulfide prepared as indicated next was added to each jar with a syringe through a rubber septum. The plates were usually

Table 1. Conditions used for the cultivation of extreme anaerobic Archaea

Organism	pH	Temperature (°C)	Gas phase
Methanopyrus kandleri	6.8	97	H_2/CO_2
Methanobacterium thermoautotrophicum	6.5	65	H_2/CO_2
Methanococcus igneus	7.0	80	H_2/CO_2
Methanococcus vannielii	7.0	37	H_2/CO_2
Methanococcus thermolithotrophicus	7.0	65	H_2/CO_2
Methanoplanus limicola	7.0	37	H_2/CO_2
Pyrococcus furiosus	6.5	95	N_2
Pyrococcus woesei	6.5	95	N_2
Archaeoglobus profundus	6.5	85	H_2/CO_2
Archaeoglobus fulgidus	6.9	85	N_2/CO_2
Thermoproteus tenax	5.5	85	N_2
Pyrobaculum islandicum	6.0	95	N_2
Pyrodictium abyssi	5.8	95	H_2/CO_2
Pyrodictium occultum	5.8	95	H_2/CO_2
Desulfurococcus mobilis	5.5	85	N_2
Staphylothermus marinus	6.5	90	N_2
Hyperthermus butylicus	7.0	95	H_2/CO_2

inoculated after 2 days of preincubation at 20°C. Inoculation of the plates was performed in an anaerobic chamber. The plates were incubated at 60°C.

Preparation of hydrogen sulfide

A saturated Na_2S solution (10–15 ml) was dispensed into a 120-ml serum bottle that was stoppered and sealed. The headspace was thoroughly evacuated. Hydrogen sulfide was generated by adding slowly 6 ml of 50% (v/v) sulfuric acid into the evacuated bottle. This exothermic reaction was accompanied by a change of color of the solution from green to yellowish white. Finally, a clear solution was obtained; 1 ml of the gas phase contained 36 μmol H_2S.

Results

The medium for the cultivation of *Methanopyrus kandleri* as described by Kurr et al. (1991) contains precipitates of metal sulfides that hamper monitoring the growth of this organism. Due to this problem and to the extreme oxygen sensitivity of this strain (Kurr et al. 1991), we searched for a reducing agent that can replace sulfide as reducing agent in this medium. Reducing agents other than sulfide, such as cysteine, 2-mercaptoethane sulfate, dithionite, ascorbic acid, sodium thioglycolate, or titanium citrate are frequently used as reductant (Atlas 1997). Addition of titanium citrate caused precipitation of components of the medium, and dithionate was not appropriate as reducing agent for the cultivation of this strain and of other methanogens most likely because of its low stability in solution (data not shown). The other redox pairs have significantly higher redox potential than the pair S^o/HS^- and therefore seem not suitable to replace sulfide.

The redox pair SO_4^{2-}/HSO_3^- has a considerably lower redox potential than the pair S^o/HS^- (−0.52 V versus −0.27 V) (Thauer et al. 1977). In addition, sulfate, the oxidation product of sulfite, is a nontoxic component of standard culture media. As sulfite has been reported to inhibit the growth of microorganisms (Bhatnagar et al. 1984), growth of *Methanopyrus kandleri* at different concentrations of sodium sulfite was analyzed. When sulfide in the original medium, which did not contain an additional source of sulfur, was replaced by sulfite, no growth occurred. This finding indicates that the organism was unable to utilize sulfite as a source of sulfur. However, when H_2S was added to the headspace of the culture bottles, growth of *M. kandleri* was observed at sulfite concentrations between 0.5 and 4.6 mM (Table 2). Optimal growth occurred in the presence of 2 ml H_2S gas phase (see Materials and methods) at sulfite concentrations between 1.5 and 3.5 mM (Table 2). Media prepared under these conditions did not show precipitates. When 3.5 or 5 ml of H_2S was added to the headspace of cultures of *M. kandleri*, growth was inhibited in part or completely (Table 2).

To investigate whether the use of sulfite as reducing agent allows a simplification of the preparation of anaerobic media, several steps of the standard preparation procedure were modified. First, the medium containing resazurin as a redox indicator but no sulfide was flushed for 20 min with nitrogen as usual. Boiling of the medium was not required. Solid sodium sulfite or a freshly prepared stock solution of sodium sulfite was added then to a final concentration of 4 mM. The medium was then dispensed into serum bottles outside the anaerobic chamber without any additional precautions to exclude oxygen. The bottles were sealed with rubber stoppers and the atmosphere exchanged three times with a $H_2:CO_2$ atmosphere (80:20, v/v) and pressurized at 300 kPa. After that step, the color of the medium had turned from blue to pink. As the next step, 2 ml of H_2S, prepared as indicated under Materials and methods, was added to the headspace by the use of a syringe. After sterilization of the medium in an autoclave, the resazurin was colorless and the medium was suitable for cultivation of *M. kandleri*.

As the use of sulfite as reducing agent makes an anaerobic chamber dispensable and saves time during the preparation of media, we studied whether this method can be generally applied for the cultivation of anaerobic Archaea. To this end, several methanogens and hyperthermophilic Archaea (Table 3) were cultivated in various media (see Table 1) containing 4 mM sodium sulfite instead of sodium sulfide as reducing agent that were prepared in the presence of air (Table 3). *Methanococcus thermolithotrophicus*, *Pyrococcus furious* and *Pyrococcus woesei*, *Thermoproteus tenax*, *Pyrodictium occultum* and *Pyrodictium abyssi*, and *Hyperthermus butylicus* could be grown

Table 2. Cells of *Methanopyrus kandleri* per milliliter obtained after 24-h incubation

Gas phase, ml H_2S	Concentration of sodium sulfite					
	0 mM	0.5 mM	1.5 mM	2.5 mM	3.5 mM	4.6 mM
0	0	0	0	0	0	0
1	0	0	4×10^6	3×10^6	0	0
2	0	35×10^6	80×10^6	70×10^6	105×10^6	2×10^6
3.5	0	70×10^6	9.5×10^6	8×10^6	2×10^6	0
5	0	0	0	0	0	0

H_2S was prepared by addition of sulfuric acid to a saturated solution of Na_2S, as indicated under Materials and methods, and was added to the headspace of 120-ml serum bottles containing 20 ml medium
Five parallel cultures were analyzed in each case

Table 3. Anaerobic Archaea cultivated successfully with the sulfite technique

Organism	With H_2S	Without H_2S
Methanopyrus kandleri	+	−
Methanobacterium thermoautotrophicum	+	−
Methanococcus igneus	+	−
Methanococcus vannielli	+	−
Methanocccus thermolithtrophicus	n.d.	+
Methanoplanus limicola	+	−
Pyrococcus furiosus	+	+
Pyrococcus woesei	n.d.	+
Archaeoglobus profundus	+	−
Archaeoglobus fulgidus	+	−
Thermoproteus tenax	n.d.	+
Pyrobaculum islandicum	+	−
Pyrodictium abyssi	n.d.	+
Pyrodictium occultum	n.d.	+
Desulfurococcus mobilis	+	−
Staphylothermus marinus	+	−
Hyperthermus butylicus	−	+

n.d., not determined

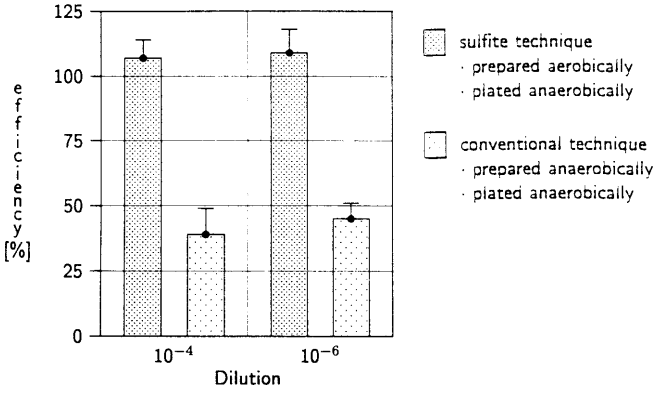

Fig. 1. Comparison of plating efficiency when solid media prepared with the sulfite or the conventional sulfide technique were inoculated inside the anaerobic chamber. Efficiency was calculated from the number of colonies formed referred to the theoretical number of plated cells. Analysis of three parallel cultures is shown

without addition of H_2S to the headspace. The media for cultivation of the two *Pyrococcus* species, *Thermoproteus*, the two *Pyrodictium* species, and *Hyperthermus* contained S^o and organic compounds such as yeast extract and/or peptone that were most likely utilized as a source for sulfur by these organisms.

It has been described that *M. thermolithotrophicus* can grow on sulfite and sulfate as the sole sulfur source (Daniels et al. 1986). As the medium used contained both sulfite and sulfate, both components could serve as the sulfur source, but no additional reducing agent was required for cultivation of this organism. Most methanogens and hyperthermophiles were grown in media not containing an additional source of sulfur, and therefore did not require the addition of H_2S for growth in the presence of sulfite as reducing agent (Table 3). This finding indicates that these Archaea are unable to utilize sulfite as the source of sulfur. However, all Archaea could be grown in liquid media dispensed aerobically in the laboratory. Their growth rates and yields were similar to the conventional method (data not shown). Therefore, the sodium sulfite procedure described here for the cultivation of *Methanopyrus kandleri* can be generally used for growing strict anaerobic Archaea in liquid media.

To test the suitability of sodium sulfite as a reducing agent for the preparation of solidified media, *Methanococcus thermolithotrophicus* was plated on solidified media prepared as described. For inoculation, exponentially growing cultures of the organism were plated inside an anaerobic chamber. The organism could be plated with an efficiency between 100% and 110% (see following). *M. thermolithotrophicus* cultures could also be plated outside an anaerobic chamber with an efficiency of 1%. Similar results were achieved with *M. thermolithotrophicus* cultures that had been plated inside the anaerobic chamber and then exposed to air at different temperatures. At 4°C and 20°C, colonies were formed after exposure to air for a time period of up to 5 min. Longer exposure or a short exposure at higher temperatures prohibited the formation of colonies.

To compare directly the plating efficiency of the newly established technique with the plating technique described by Balch and Wolfe (1976), diluted exponentially growing cultures of *M. thermolithotrophicus* were plated inside an anaerobic chamber on plates prepared using both techniques. The plates prepared by the sulfite technique were poured aerobically outside the chamber, and the plates containing sodium sulfide were poured inside the chamber. Incubation of plates inoculated in the anaerobic chamber showed that the plating efficiency with sulfite was 110% that with sulfide, between 40% and 45% (Fig. 1). As pointed out previously (Kiener and Leisinger 1983), plating efficiencies above 100% probably resulted from pairs of cells being considered as a single cell during determination of total counts; such pairs were, however, separated during plating for viable counts.

Discussion

Methods have been developed that allow the cultivation of anaerobic bacteria such as sulfate reducers and sulfur reducers without anaerobic chambers, but these procedures cannot be used for the cultivation of the extreme anaerobic methanogens and of anaerobic hyperthermophilic Archaea. We show here that anaerobic Archaea can be grown in the presence of sodium sulfite as reducing agent. Using this compound as reducing agent, glove boxes are not required for the preparation of media for the cultivation of a wide variety of methanogens and hyperthermophilic Archaea (see Table 3). Alternative reducing agents or sulfur sources (e.g., L-cysteine) can be used for the cultivation of hyperthermophilic archaea. The method described here can be in particular recommended for cultivation of methanogens.

Due to its chemical properties, sulfite has several advantages compared to sulfide, the reagent commonly used as reducing agent for the cultivation of methanogens (Balch and Wolfe 1976). First, its redox potential is lower; second, it does not cause precipitation of cations; and finally, in contrast to sulfide, it is not volatile at pH 7 or lower pH values. We found that all Archaea investigated could be grown at sulfite concentrations of 4 mM. Bhatnagar et al. (1984) reported inhibition of growth of two *Methanobacterium* species at sulfite concentrations of 5 mM. We observed also inhibition of growth of *Methanopyrus kandleri* at sulfite concentrations ≥4.6 mM (see Table 2). The major reason for inhibition of growth observed by Bhatnagar et al. seems to be the simultaneous presence of 20 mM 2-mercaptoethanol in the culture medium; 2-mercaptoethanol at high concentrations has been found to be greatly inhibitory to the growth of several methanogens in the presence of sulfite (Daniels et al. 1986).

In contrast to our results, Daniels et al. reported inhibition of growth of *Methanococcus thermolithotrophicus* and of two additional methanogens at a sulfite concentration of 2 mM. The reason for this discrepancy is unclear, but most likely differences in the preparation procedure are responsible. Daniels and coworkers added sulfite after sterilization to a medium made anaerobic by repeated evacuation and flushing with H_2:CO_2 and used a standing incubation technique for 10–12 h before cultures were grown while shaking. We added sulfite before autoclaving to an aerobically dispensed medium, and our cultures were shaken vigorously from the beginning.

Most of the Archaea grown in the presence of sulfite required addition of H_2S to the headspace of the cultures (Table 3). Both *Methanococcus thermolithotrophicus* and *Methanobacterium thermoautotrophicum* have been reported to utilize sulfite as single source of sulfur (Daniels et al. 1986), but only the former species could be cultivated by our procedure in the absence of H_2S (Table 3). Therefore, under the conditions employed here, *M. thermoautotrophicum* seems to require H_2S as a source for cellular sulfur or as an additional reducing agent. The media used for cultivation of all other organisms that grew in the absence of H_2S contained additional sulfur compounds or elemental sulfur (Table 3). Therefore, it is unclear whether these organisms can utilize sulfite as a source of sulfur. However, our data show clearly that sulfite can replace sulfide as reducing agent for the cultivation of anaerobic Archaea.

The addition of H_2S to the headspace, which is necessary for the cultivation of many Archaea in media not containing an additional source of sulfur (Table 3), has the advantage that it leads to reduced precipitation of cations in culture media because only small amounts of the sulfide exist as S^{2-} at pH 7 or lower. The concentration of added H_2S is critical. At high concentrations, growth of *Methanopyrus kandleri* was inhibited (Table 2). Possibly, H_2S at high concentrations may react with the heterodisulfide complex of 7-mercaptoheptanoyl-threonine phosphate with coenzyme M and thus inhibit methanogenesis, but additional work is required to clarify the reason for this inhibition.

The major advantage of the use of sulfite as reducing agent is that liquid media for extreme anaerobes can be prepared without the use of an anaerobic chamber. The application of this novel procedure reduced the time required for preparation of anaerobic liquid media by at least a factor of 2. This technique can even be applied to the preparation of solidified media for *Methanococcus thermolithotrophicus*. Plating of methanogens on solidified media in petri dishes with incubation in a pressurized cylinder and a procedure for plating and incubation of methanogens in serum bottles have been described (Tumbula et al. 1995), but these procedures require aseptic dispensation of media in bottles and pouring of plates inside an anaerobic chamber.

Although inoculation of the solidified media must still be carried out inside an anaerobic chamber, the sulfite technique described here allows the preparation and storage of plates outside the glove box. As a consequence, the time required for the preparation of the solidified media was reduced by a factor of 3 because time-consuming procedures such as pouring of plates and assembling of anaerobic jars can be done in the laboratory, and steps like preequilibration of plastic petri dishes in the glove box and the use of air locks are redundant. Even after exposure to air the plates remained at a redox potential below −110 mV for hours, as indicated by the unchanged color of resazurin. In addition, it was possible to remove plates from the anaerobic cylinder and to inspect growth of colonies on inoculated plates under aerobic conditions. After exchange of the gas phase in the cylinder, cultivation was continued successfully, and cultures remained viable as indicated by subsequent growth of transferred colonies in liquid media.

Methanococci are among the most oxygen-sensitive methanogens. Growth of these organisms on solidified media with the conventional technique requires high skill and extreme care in handling the anaerobic chamber (Kiener and Leisinger 1983). Our finding that the plating efficiencies for *M. thermolithotrophicus* with the sulfite technique were higher than those obtained with the conventional sulfide technique demonstrates the potential value of this novel procedure for plating of extreme anaerobic Archaea.

Acknowledgment This work was supported in part by the Fonds der Chemischen Industrie.

References

Atlas RM (1997) Handbook of microbiological media. CRC Press, Boca Raton

Balch WE, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethane-sulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl Environ Microbiol 32:781–791

Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260–296

Bhatnagar L, Henriquet M, Zeikus J, Aubert JP (1984) Utilization of mercapto-2-ethanol as medium reductant for determination of the

metabolic response of methanogens towards inorganic sulfur compounds. FEMS Microbiol Lett 22:155–158

Burggraf S, Fricke H, Neuner A, Kristjansson J, Rouvier P, Mandelco L, Woese CR, Stetter KO (1990a) *Methanococcus igneus* sp. nov., a novel thermophilic methanogen from a shallow hydrothermal system. Syst Appl Microbiol 13:263–269

Burggraf S, Jannasch HW, Nicolaus B, Stetter KO (1990b) *Archaeoglobus profundus* sp. nov. represents a new species within the sulfate-reducing archaebacteria. Syst Appl Microbiol 13:24–28

Daniels L, Belay N, Rajagopal BS (1986) Assimilatory reduction of sulfate and sulfite by methanogenic bacteria. Appl Environ Microbiol 51:703–709

Fiala G, Stetter KO (1986) *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch Microbiol 145:56–61

Huber H, Thomm M, König H, Thies G, Stetter KO (1982) *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. Arch Microbiol 132:47–50

Huber R, Kristjansson J, Stetter KO (1987) *Pyrobaculum* gen. nov., a new genus of neutrophilic, rod-shaped archaebacteria from continental solfataras growing optimally at 100°C. Arch Microbiol 149:95–101

Hungate RE (1969) A roll tube method for cultivation of strict anaerobes. In: Norris JR, Ribbons WD (eds) Methods in microbiology, vol 3B. Academic Press, New York, pp 117–132

Kiener A, Leisinger T (1983) Oxygen sensitivity of methanogenic bacteria. Syst Appl Microbiol 4:305–312

Kurr M, Huber R, König H, Jannasch HW, Fricke H, Trincone A, Kristjansson JK, Stetter KO (1991) *Methanopyrus kandleri*, gen. and sp. nov., represents a novel group of hyperthermophilic methanogens, growing at 110°C. Arch Microbiol 156:239–247

Pley U, Schipka J, Gambacorta A, Jannasch HW, Fricke H, Rachel R, Stetter KO (1991) *Pyrodictium abyssi* sp. nov. represents a novel heterotrophic marine archaeal hyperthermophilic growing at 110°C. Syst Appl Microbiol 14:245–253

Schönheit P, Moll J, Thauer RK (1979) Nickel, cobalt and molybdenum requirement for growth of *Methanobacterium thermoautotrophicum*. Arch Microbiol 123:105–107

Stetter KO (1988) *Archaeoglobus fulgidus* gen. nov., sp. nov.: a new taxon of extremely thermophilic archaebacteria. Syst Appl Microbiol 10:172–173

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180

Tumbula DL, Bowen TL, Whitman WB (1995) Growth of methanogens on solidified medium. In: Robb FT, Place AR, Sowers KR, Schreier HJ, DasSarma S, Fleischmann EM (eds) Archaea: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview, NY, pp 49–55

Wildgruber G, Thomm M, König H, Ober K, Ricchiuto T, Stetter KO (1982) *Methanoplanus limicola*, a plate-shaped methanogen representing a novel family, the *Methanoplanaceae*. Arch Microbiol 132:47–50

Zillig W, Stetter KO, Prangishvilli D, Schäfer W, Wunderl S, Janekovic D, Holz I, Palm P (1982) *Desulfurococcaceae*, the second family of the extremely thermophilic, anaerobic, sulfur-respiring *Thermoproteales*. Zentralbl Bakteriol Hyg 1 Abt Orig C 3:304–317

Zillig W, Holz I, Klenk HP, Trent J, Wunderl S, Janekovic D, Imsel E, Haas B (1987) *Pyrococcus woesi*, sp. nov., an ultrathermophilic marine archaebacterium representing a novel order, *Thermococcales*. Syst Appl Microbiol 9:62–70

Zillig W, Holz I, Janekovic D, Klenk HP, Emsel E, Trent J, Wunderl S, Forjaz V, Couthino R, Ferreira T (1990) *Hyperthermus butylicus*, a hyperthermophilic sulfur-reducing archaebacterium that ferments peptides. J Bacteriol 172:3959–3965

Zillig W, Holz I, Wunderl S (1991) *Hyperthermus butylicus* gen. nov., sp. nov., a hyperthermophilic, anaerobic, peptide-fermenting, facultatively H_2S-generating archaebacterium. Int J Syst Bacteriol 41:169–170