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Biocatalytic transformations of hydrothermal fluids

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The occurrence of copious animal populations at deep-sea vents indicates an effective microbial chemosynthetic biocatalysis of hydrothermal fluids on their emission into oxygenated ambient seawater. The large metabolic and physiological diversity of microbes found at these sites, including anaerobic and aerobic hyperthermophiles, reflects an even higher variety of biocatalytic or enzymatic reactions that greatly influence deep-sea hydrothermal geochemistry.

1. Introduction

The extent of biocatalysis in geochemical transformations is still an enigmatic issue. Although it is well understood that processes such as microbial photosynthesis, sulphate reduction, denitrification, etc., do not occur in the absence of the responsible organisms, geochemists generally are often unclear as to the effectiveness of biocatalytic transformations, where they occur, where they cannot occur, and how can they be predicted. This dilemma is due, in part, to an incompatibility between quantitative approaches in geochemical and microbiological studies. The unexpected discovery of high chemosynthetic bacterial production of biomass near deep-sea vents has resulted in studies that led to the following clarifying remarks for geochemists on biocatalytic transformation of hydrothermal emissions.

The observations contain that certain reduced inorganic compounds may serve as biocatalytically utilizable 'sources of energy' wherever they occur under conditions that allow the production of enzymes through microbial growth. During the Earth's history, biochemical–physiological evolution has resulted in large numbers of biogeochemical processes that are mediated by a large microbial and, thereby, biocatalytic diversity. Many microbes contributed to determining the composition of the Earth's atmosphere, hydrosphere and upper crust during the period of about two billion years before higher forms of life appeared, including the onset of oxygen formation by cyanobacteria (Cloud 1976; Schidlowski 1984; Cohen *et al.* 1984). While today 'green plants' are limited to photosynthesis and 'animals' to heterotrophy (see later), all of their primordial forms have died out. In contrast, many of the early microbes are probably identical, at least phenotypically, with those that we find today wherever conditions are similar to those of the early Earth, especially in hydrothermal marine and terrestrial environments. The fact that microbes live in microenvironments and can survive long periods of time in a dormant stage—possibly millions of years (Cano & Borucki 1995)—without genetic change, may turn a few cm^3 of ancient reheated smoker wall material into a microbial 'jurassic park'.

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2. Definitions

Organic catalysts or enzymes are complex proteins with characteristic metal-containing subunits or co-enzymes. Imprinted as 'genes' on DNA (the reproducing genetic storage for each species), individual enzymes can be identified in known organisms or even on strands of DNA extracted from water or sediment samples (Pace *et al.* 1986; Muyzer *et al.* 1993; Burggraf *et al.* 1994). Biocatalytic processes are principally autocatalytic as enzymes are continuously produced during growth and multiplication of the organism.

'Lithotrophic' microbes are able to use electrons from reduced inorganic compounds in oxidations of the electron acceptor with a gain of energy, e.g. the methanogenic bacteria:

$$\tfrac{1}{4}CO_2(aq) + H_2(aq) \longrightarrow \tfrac{1}{4}CH_4(aq) + \tfrac{1}{2}H_2O \quad (\Delta G^\circ = -48). \tag{2.1}$$

Since the free energies (ΔG° in kJ mol^{-1} of the electron donor) are calculated for standard conditions with respect to temperature and pressure at pH 7, they are useful for comparative purposes only. They cannot be applied directly to the natural environment, especially to the unknown microenvironment of the metabolically active cell.

Most lithotrophic microbes are also 'autotrophic', i.e. they are able to couple the energy gained from lithotrophic oxidations to the reduction of CO_2 to organic carbon:

$$\tfrac{1}{3}CO_2(aq) + H_2(aq) \longrightarrow \tfrac{1}{6}[CH_2O] + \tfrac{1}{6}CH_4(aq) + \tfrac{1}{2}H_2O \quad (\Delta G^\circ = -39). \tag{2.2}$$

In contrast, the anaerobic lithotrophic oxidation of H_2S is endergonic (requiring free energy) and requires light as an external source of energy as in pigmented bacteria that conduct an anoxic photosynthesis ('photosystem I', Pagan & Cohen 1982):

$$\tfrac{1}{2}CO_2(aq) + H_2(aq) \longrightarrow \tfrac{1}{2}[CH_2O] + S^0 + \tfrac{1}{2}H_2O \quad (\Delta G^\circ = +26). \tag{2.3}$$

When 'photosystem II' evolved within the photosynthetic cyanobacteria, the electron donor H_2S was replaced by H_2O:

$$CO_2(aq) + H_2O \longrightarrow [CH_2O] + O_2(aq) \quad (\Delta G^\circ = +487). \tag{2.4}$$

This enabled these early photosynthetic organisms to spread much further in the environment but also resulted in the production of free oxygen (instead of sulphur or sulphate) as a byproduct. Because of the formation of the toxic peroxide, oxygen acts as a 'poison' to all anaerobic life forms. Subsequently, after the evolution of H_2O_2-detoxifying biocatalysts, free oxygen became a highly efficient metabolic oxidant:

$$H_2(aq) + 2O_2(aq) \longrightarrow HSO_4^- + H^+ \quad (\Delta G^\circ = -801), \tag{2.5}$$

empowering aerobic lithoautotrophic bacteria to metabolize independently of light:

$$CO_2(aq) + H_2S(aq) + H_2O + O_2(aq) \longrightarrow [CH_2O] + HSO_4^- + H^+ \quad (\Delta G^\circ = -314), \tag{2.6}$$

and widely available in the early atmosphere and hydrosphere. The resulting oxidized forms of sulphur became, in turn, readily available electron acceptors for other microbes such as the autotrophic and heterotrophic sulphate and sulphur reducing bacteria.

Aerobic biocatalysis can also use H_2:

$$H_2(aq) + \tfrac{1}{2}O_2(aq) \longrightarrow H_2O \quad (\Delta G^\circ = -263), \tag{2.7}$$

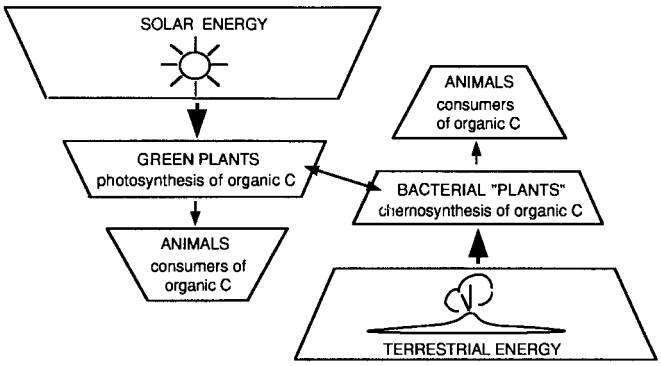

Figure 1. Analogy between the photo- and chemosynthetic food chains (plants = Calvin–Bensen cycle enzymes reducing inorganic to organic carbon).

or NH_3, NO_2^-, CH_4, Fe^{2+}, Mn^{2+} and possible other reduced metals as electron donors. In other words, specific microbes potentially chemosynthesize and grow wherever relevant reduced inorganic compounds reach free oxygen, particularly in the marine sediment–water interface.

Organic, i.e. reduced, carbon is also an electron donor readily available for biocatalytic oxidations. This 'heterotrophic' decomposition of organic matter depends on even more complex biocatalytic systems in a variety that is as large as the number of possible organic compounds produced by plants, animals or microbes themselves. Without showing the coupling to biosynthesis, one example is the aerobic oxidation of hexose (glucolysis):

$$C_6H_{12}O_6 + 6O_2(aq) \longrightarrow 6CO_2(aq) + 6H_2O \quad (\Delta G^\circ = -2920), \tag{2.8}$$

or the much less energy-efficient anaerobic oxidation (fermentation) of acetate and ethanol to butyrate:

$$CH_3COO^- + CH_3CH_2OH \longrightarrow CH_3CH_2CH_2COO^- + H_2O \quad (\Delta G^\circ = -38.7). \tag{2.9}$$

Many of the enzymatic reactions are reversible, i.e. they occur in 'anabolic' biosynthesis as well as in 'catabolic' respiration.

3. Aerobic chemosynthesis

Pfeffer (1897), impressed by Winogradsky's discovery of microbial 'chemo-autotrophy' in *Beggiatoa* (1887), coined the term 'chemosynthesis' in analogy to photosynthesis. It is now often used in place of chemolithoautotrophy. The process is driven by energy derived from biocatalytically mediated chemical oxidations and transferred to the Calvin–Bensen cycle enzymes that synthesize organic carbon from CO_2. Because this system is also found in green plants, the aerobic chemosynthetic bacteria can be described as 'plants' that fix CO_2 in the dark (figure 1). Until the discovery of deep-sea 'oases', it had always been imagined that chemosynthesis could serve as the food chain for whole animal communities.

While many chemosynthetic bacteria are living as symbionts in the most prominent vent animals, the population of free-living microorganisms, invisible to the naked eye, is probably even more extensive. This is indicated by scanning electronmicrographs of bacterial mats covering basalt lava surfaces (Jannasch & Wirsen 1981; Jannasch 1985). The emissions of dense bacterial, often flocculent, suspensions from 'warm'

vents (less than approximately 40 °C) indicate productive growth chambers and mat formation in the layers of porous lava below the sea floor where entrained oxygenated seawater mixes with hydrothermal fluid. Here the aerobically chemolithoautotrophic genus *Thiomicrospira* appears to be prevalent (Ruby *et al.* 1981; Muyzer *et al.* 1995).

Large masses of white material were also observed to occur just after new volcanic activity at Juan de Fuca and 9° N East Pacific Rise vent sites (Haymon *et al.* 1993). From examining samples that we obtained we believe that the bulk of this material constitutes mineral encrusted sheaths of *Leptothrix*-like aerobic metal-oxidizing bacteria. They might have grown in shallow subseafloor lava pockets and been dislodged during the new volcanic activity. The sheaths did not contain cells any longer, which is not unusual for this type of organism that autolyses readily while the sheaths stay behind (D. Emerson, personal communication). Both the enzymatic activity and concentration of organic carbon were low relative to the amount of sample material. Juniper *et al.* (1995) report similar observations. This supports the assumption that populations of these normally slow-growing organisms led to a subsurface accumulation of metabolic products, such as the iron and silica-encrusted sheaths, over some period of time.

The chemosynthetic carbon fixation in bacterial suspensions or in microbial mats can be quantitatively determined by the uptake of $^{14}CO_2$ (Wirsen *et al.* 1993; Jannasch 1995) and showed that the organisms are mesophilic (optimal activity between 25–35 °C) and hardly affected by the *in situ* pressure of 260 atm (Ruby & Jannasch 1982). In contrast to the occurrence of anaerobic hyperthermophilic bacteria at hydrothermal vents, no comparable aerobic chemosynthetic organisms have yet been found (Wirsen *et al.* 1993). At sediment-covered vent sites, chemosynthetic activity takes place in yellow (cytochrome-containing) *Beggiatoa* mats that may be several cm thick (Nelson *et al.* 1989).

The visibly highest productivity of biomass is carried out by chemosynthetic bacteria—so far uncultured—that live in symbiosis with novel invertebrates, especially the large white clams (*Calyptogena*) and vestimentiferan tube worms (*Riftia*). It is remarkable that the chemosynthetically based food chain (figure 1) gives rise to animal populations that are more dense and productive, though locally restricted, than any comparable populations maintained by photosynthesis. At cold seeps and other benthic environments where methane is vented, methane-oxidizing symbionts occur in mussels and smaller vestimentiferans.

The argument has been raised, for example, in readers' comments to a paper by Jannasch & Mottl (1985), that the aerobic chemosynthesis is not entirely independent of photosynthesis since it requires free oxygen. In other words, the production is not 'primary' as in photosynthesis, but secondary.

4. Anaerobic chemosynthesis and high-temperature biocatalysis

This is not true for the strictly anaerobic chemosynthesis which was probably one of the earliest biocatalyses in the Earth's history. The exergonic (free energy producing) anaerobic reduction of CO_2 in hydrothermal fluid (equations (2.1) and (2.2)) requires hydrogen. This biocatalysis was first observed in a *Methanococcus* isolated from a deep-sea vent site by Jones *et al.* (1983) and growing at 90 °C. Its formation of methane, as dependent on temperature and pressure, was later studied by Miller *et al.* (1988) and the purification and properties of some of the biocatalysts involved by Shah & Clark (1990). The methanogenic new genus, *Methanopyrus* (Huber *et al.*

Table 1. *Energy-yielding reactions catalysed by some lithoautotrophic marine hyperthermophilic vent isolates*

electron donor	electron acceptor	reaction	organisms
H_2	CO_2	$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O$	*Methanopyrus*, *Methanococcus*
H_2	S^0	$H_2 + S^0 \longrightarrow H_2$	*Pyrodictium*[a]
H_2	SO_4^{2-}, $S_2O_3^{2-}$	$H_2 + H_2SO_4 \longrightarrow H_2 + 4H_2O$	*Archaeoglobus*[a]
S^{2-}; S^0	O_2	$H_2 + \frac{1}{2}O_2 \longrightarrow H_2O$, $2S^0 + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4$	*Aquifex Sulfolobus*[b]

[a]Facultatively heterotrophic
[b]Not yet isolated from marine hydrothermal sources.

1989; Kurr *et al.* 1991) grew at temperatures up to 110 °C. This hyperthermophilic anaerobic chemosynthesis represents a truly 'primary' production which is, however, not catalysed by the Calvin–Bensen enzyme system but by a variety of new metabolic pathways summarized by Schönheit & Schäfer (1995).

Over the years hydrothermal vents yielded a large number of other anaerobic autotrophic and heterotrophic types of microbes (table 1) containing biocatalysts that are active at temperatures between 80 and 110 °C (Stetter *et al.* 1990; Ply *et al.* 1991; Jannasch *et al.* 1992). So far the hydrostatic pressures have little affect on their activity and most of the hyperthermophilic microbes described from deep-sea vents also occur in shallow marine hot springs. Nelson *et al.* (1992) observed a stabilizing influence of pressure on some biocatalytic activities. How protein and nucleic acids are protected from denaturation at these high temperatures is not yet fully understood. Most hyperthermophilic microbes belong to the kingdom of the archaea. What is an archaeum?

When Woese *et al.* (1990) based modern phylogeny, the evolutionary relationships between organisms, on the particular nucleotide sequences of the 16S ribosomal RNA, three domains emerged (figure 2): the *Bacteria*, the—also microbial—*Archaea*, and the *Eucaria* (all higher forms of life with nuclei-containing cells). Members of the archaea include many of those organisms that live under 'extreme' conditions such as temperatures of up to 110 °C, pH as low as 2 or as high as 11, and concentrated brines. These 'extremophiles' were thought to have lived under conditions of the early Earth.

Originally it appeared that all hyperthermophilic microbes had to be placed into the first 'kingdom' of the archaeal domain, the Crenarchaeota, with only two genera, *Thermococcus* and *Methanococcus*, in the Euryarchaeota (figure 2). Then two hyperthermophilic genera were found that clearly belonged to the bacterial domain, *Thermotoga* and *Aquifex*. The fact that hyperthermophiles occupy the lower branches in both the bacterial and the archaeal domains (figure 2), has been interpreted to the effect that life may have originated within a high temperature regime (Woese *et al.* 1990; Woese 1991). In fact, the discovery of mid ocean hydrothermal ridges has rejuvenated interest in theoretical and experimental studies on 'the origin of life' (Holm 1992). The first hyperthermophilic organism isolated from a deep-sea vent,

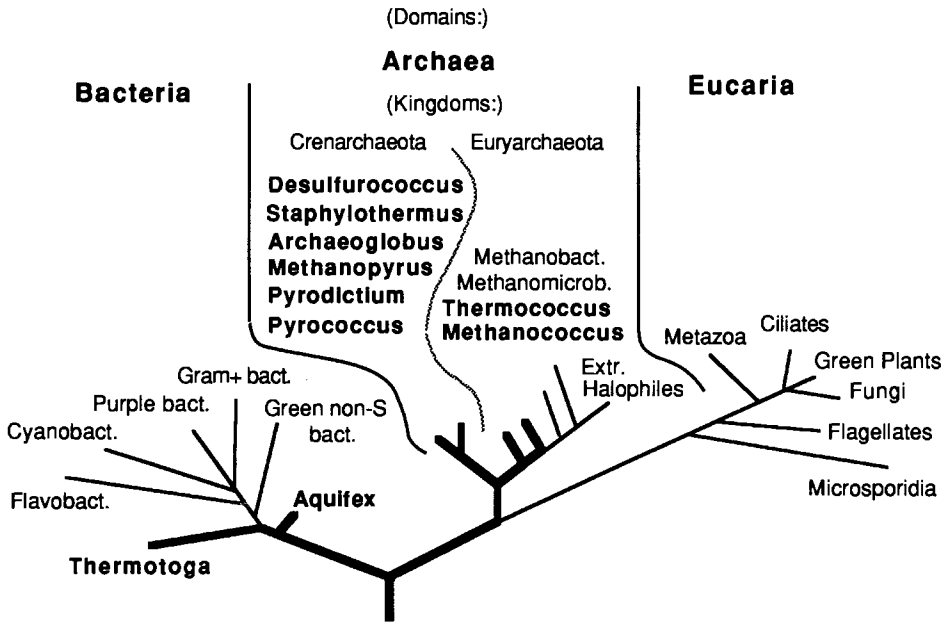

Figure 2. The major marine genera of hyperthermophilic archaea and bacteria, isolated from hydrothermal vents, superimposed (in bold) on the 16S rRNA-based phylogenetic tree as proposed by Woese *et al.* 1990 (modified from Jannasch 1995).

the previously mentioned *Methanococcus* (Jones *et al.* 1983), recently also became the first archaeum of which the entire genome has been sequenced. This involved the decoding of 1.7×10^6 base pairs and substantiating the uniqueness of the archaeal domain (news item 1996 *Science* **271**, 1061).

Aerobic hyperthermophiles were recently also discovered in both microbial domains. One of them is the bacterial genus *Aquifex* (the 'water maker', equation (2.7) and table 1, Huber *et al.* 1992). The facultatively aerobic hyperthermophilic archaeal *Pyrobaculum* (Völkl *et al.* 1993) is able to catalyse the reduction of nitrate to dinitrogen (denitrification) if oxygen is absent. More and more cases emerge indicating that physiological characteristics of phenotypes are not as easily predictable from their positioning on the phylogenetic tree as anticipated. A number of biocatalysts active at high temperatures have been identified and purified (figure 3, table 2). Some of them are of high commercial value, especially the DNA cleaving polymerases that are used in molecular biology.

During one of our earlier dive series at the sediment-covered Guaymas Basin spreading centre in the Gulf of California, Jørgensen *et al.* (1990) observed in sediment cores sulphate reducing activity with maxima around 90 °C. From collected material a new sulphate reducing archaeum was isolated (*Archaeoglobus*) that grew at temperatures of up to 90 °C (Burggraf *et al.* 1990). As electron donors, this organism uses hydrogen for autotrophic and acetate (or other organic substrates) for heterotrophic metabolism (table 1). Besides sulphate, electron acceptors can be sulphite and thiosulphate. Speculations on the existence of substantial and metabolically active populations of microbes in deeper parts of the globe's crust (Deming & Baross 1993) must also be based on an anaerobic chemosynthetic catalysis at extremely high temperatures, largely involving methane production.

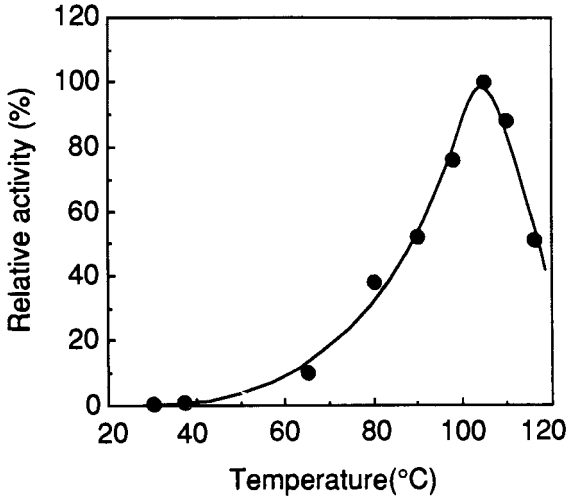

Figure 3. Temperature–activity plot of a protease purified from *Desulfurococcus* strain SY:200ng enzyme tested in 0.1 M HEPES buffer and 1% Azocasein for 10 min above and 30 min below 60 °C (from Hanzawa *et al.* 1996).

Table 2. *Examples of purified hyperthermophilic biocatalysts (enzymes) (from Adams (1993))*

enzyme	catalytic activity	temp. (°C)	organism
protease	peptide hydrolysis	100	*Pyrococcus*
amylase	starch hydrolysis	100	*Pyrococcus*
sulphide	sulphur reduction	> 95	*Pyrococcus*
hydrogenase	hydrogen generation	> 95	*Pyrococcus*
α-galactidase	galactomannan hydrolysis	> 90	*Thermotoga*
xylanase	xylan hydrolysis	105	*Thermotoga*
glucose isomerase	glucose isomeration	105	*Thermotoga*
amylopollulanase	starch degradation	118	*Thermococcus*
DNA polymerase[a]	DNA synthesis	> 95	*Thermococcus*

[a]NE Biolabs Catalog 1995

5. Discussion

From a geochemical point of view it remains enigmatic that many of the various microbial processes have been identified by bacterial isolations only and, therefore, have to be considered as 'potential'. How important such transformations may actually be in a natural setting depends on conditions that are conducive for growth of the responsible organisms, i.e. production of the biocatalyst. In other cases activities were measured but the organisms not yet isolated. For instance, in geothermal Guaymas Basin sediments, rates of sulphate reduction were 19–61 μM SO_4^{2-} day^{-1} (Jørgensen *et al.* 1992) which is unusually high for normal deep-sea conditions and resemble rates that are found in coastal waters (Jørgensen 1983). The maximum temperature of this biocatalysis is 110 °C (optimum at 103–106 °C). This approaches the lower temperature limit for thermolytic sulphate reduction (Krouse *et al.* 1988) and

Table 3. *Pyrite formation as hypothetical source of energy for biocatalytical formation of organic carbon as proposed by Wächtershäuser et al. (1988, 1990)*

reaction	free energy (ΔG° in kJ mol^{-1})
(I) $FeS + H_2S$ (aqueous) $\rightarrow FeS_2$ (crystalline) $+ H_2$	$\Delta G^0 = -41.9$
(II) CO_2 (aqueous) $+ H_2 \rightarrow HCOOH$	$\Delta G^0 = +30.2$
(III) $FeS + H_2S + CO_2 \rightarrow FeS_2 + H_2O + HCOOH$	$\Delta G^0 = -11.7$

might become 'important for an interpretation of the formation of sulphide deposits and their sulphur isotope distribution' (Jørgensen *et al.* 1992). The isolation of the sulphate reducer growing optimally at 106 °C has not yet been successful.

A quantitative estimate of the overall chemosynthesis of organic carbon at deep-sea vents can be made by using the figure for total annual seawater percolation through the oceanic crust at tectonic spreading centres, expressed as sulphate entrainment, of 1.2×10^8 tonnes (Edmond *et al.* 1982). Three quarters of this amount are estimated to be deposited as polymetallic sulphides, and one quarter emitted as dissolved sulphide by diffusive and hot venting. Assuming further that half of this deep-sea sulphide emission is used for chemosynthesis (the stoichiometric C:S proportion being 1:1 and the molecular weight proportion 1:3), the annual production of organic carbon of 5×10^6 tonnes would just amount to 0.03% of the 18×10^9 tons produced by oceanic photosynthesis (Woodwell *et al.* 1978). Since, however, only approximately 1% of this photosynthetic organic matter reaches the deep-sea (Honjo & Manganini 1993), it can be estimated that about 3% of all organic carbon found in the deep-sea derives from chemosynthesis at hydrothermal vents. The assumption that half of the emitting sulphide is used for chemosynthesis may be too generous, but is based on the predominant occurrence of diffuse flow at hydrothermal vent sites (Shultz *et al.* 1992; Rona & Trivett 1992; Ginster *et al.* 1994) where the conditions for biocatalysis are most favourable. The visible animal biomass is, most likely, much smaller than that of the non-visible bacteria in suspension and in mats covering all solid surfaces within vent regions (Jannasch & Wirsen 1981).

As well as the oxidation of sulphur by microbial metabolism, the reduction of oxidized sulphur compounds to sulphide can also be biocatalyzed by a number of microbes as indicated in figure 4. Elemental sulphur is actively reduced by almost all anaerobic hyperthermophilic archaea, partly as a detoxification reaction. The accumulation of molecular or ionic hydrogen during fermentative microbial metabolism quickly reaches inhibitory concentrations. The reduction of elemental sulphur (added in the laboratory to the growth media for this purpose) eliminates hydrogen by the formation of hydrogen sulphide. Sulphate is reduced by *Archaeoglobus* and thiosulphate by *Thermotoga* (Ravot *et al.* 1995) as well as by *Archaeoglobus* (Burggraf *et al.* 1990).

Considering the tremendous amount of polymetallic sulphides available at mid-ocean ridges, Wächtershäuser's suggestion (1988, 1990) that an energetically feasible biogeochemical mechanism for the earliest appearance of life on Earth might result from oxidation of primary precipitates of pyrrhotite (FeS) to form pyrite (FeS_2) seems reasonable. In table 3 the overall reaction III would be favoured by the insolubility

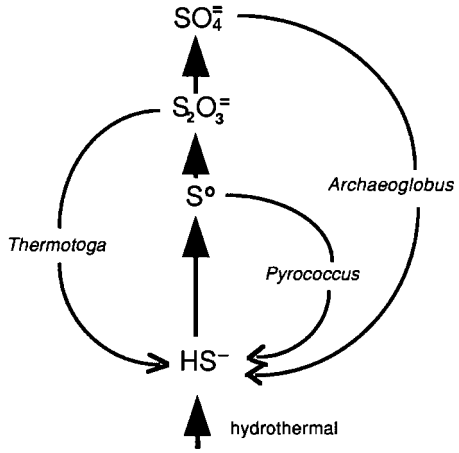

Figure 4. Hydrothermal and biocatalytic origin of sulphide in Guaymas Basin geothermal sediment (modified and updated from Jannasch 1995).

of pyrite at a low pH. Only reaction I, which is enhanced by elevated temperatures, has so far been verified experimentally (Drobner *et al.* 1990).

Modern approaches to the *in situ* identification of organisms as carriers and producers of specific biocatalysts have been developed through the use of 'molecular probes' (Pace *et al.* 1986; Muyzer *et al.* 1993; Burggraf *et al.* 1994): particular nucleotide sequences from pure culture isolates are compared to matching sequences within DNA extractions from samples such as sediment, mat material, smoker wall scrapings, etc. For biotechnological purposes, genes of certain commercially useful biocatalysts or enzymes (table 2), such as highly thermostable lipases, proteases (figure 3) or amylases, may be extracted and PCR-amplified. Aiming at geochemically more relevant enzymes, such approaches could be combined with microbiological studies in order to make quantitative estimates of biocatalytic contributions to geochemical transformations. Hydrothermal vent environments will provide a rich source of such materials.

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