

## **Isolation of Extremely Thermophilic Sulfate Reducers: Evidence for a Novel Branch of Archaeobacteria**

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Extremely thermophilic archaeobacteria are known to be metabolizers of elemental sulfur and the methanogens. A novel group of extremely thermophilic archaeobacteria is described, which consists of sulfate-respiring organisms that contain pure factor 420 and that have been isolated from marine hydrothermal systems in Italy. They possess a third type of archaeobacterial RNA polymerase structure previously unknown, indicating an exceptional phylogenetic position. Most likely, this group represents a third major branch within the archaeobacteria. The existence of sulfate reducers at extremely high temperatures could explain hydrogen sulfide formation in hot sulfate-containing environments, such as submarine hydrothermal systems and deep oil wells.

TWO MAIN SUBDIVISIONS OF Archaeobacteria are distinguished: one comprising the methanogenic bacteria and the extreme halophiles, and a second consisting of the sulfur-metabolizing extreme thermophiles (1). These branches can be recognized on the basis of a comparison

of RNA polymerase structures (2), 16S ribosomal RNA (rRNA) sequences (1), and metabolic properties. Members of the group of archaeobacterial sulfur metabolizers are able to grow by either oxidizing or reducing elemental sulfur, depending on the genera (3, 4). Energy conservation by dissimilatory

**Table 1.** Substrates for growth of isolate VC-16. Portions of 20-ml anaerobic sulfate- or thiosulfate-containing mineral medium (MGG) (7) were supplemented with possible substrates (2 g/liter) and then inoculated (1% inoculation) with strain VC-16. Incubation was usually at 85° and 65°C in the case of glucose and pyruvate, respectively. Substrate utilization was determined after four transfers in sequence into the same culture medium (1% inoculation), each followed by an incubation period. Acids were added as sodium salts.

Class of nutrients		
Intermediates in anaerobic degradation	Sugar	Complex substrates
Molecular hydrogen,* formate, formamide, L(+)- and D(-)-lactate, pyruvate	Glucose	Yeast extract, casamino acids,* beef extract, peptone, cell homogenates of <i>E. coli</i> , <i>Lactobacillus bavaricus</i> and <i>Methanothermus fervidus</i>

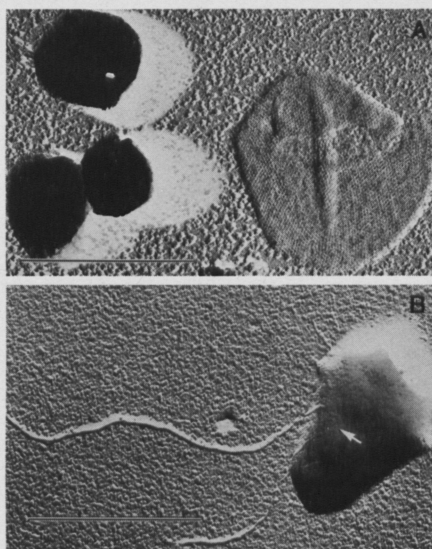
\*Growth only in thiosulfate-containing medium.

sulfate reduction has been observed up to now exclusively within some eubacteria (5). Eubacterial sulfate reducers are mesophiles or moderate thermophiles and play an important role in the global sulfur cycle (5). We describe here a novel group of extremely thermophilic sulfate reducers that belong to the archaeobacterial kingdom.

To study microbial life at extremely high temperatures, we collected 45 anaerobic samples (6) of hot sediments from marine hydrothermal systems in the vicinities of Vulcano and Stufe di Nerone, Italy. The original temperatures of the sediments ranged between 70° and 100°C. In the laboratory, oxygen-free marine mineral medium supplemented with 0.1% (w/v) yeast extract (Difco) was inoculated with portions of each sample (7, 8) and then incubated at 85°C (gas phase: N<sub>2</sub>/CO<sub>2</sub> = 80/20). After 1 week we observed coccoid bacterial cells in 28 of the 45 cultures that we attempted to form. They showed a strong blue-green fluorescence under the ultraviolet (UV) microscope at 420 nm characteristic of methanogenic bacteria (8).

Surprisingly, however, only traces of methane (about 10<sup>-4</sup> times that produced by a methanogen culture of comparable cell density) were detected. The novel organisms were cloned by plating on culture medium solidified by 1.5% agar (Oxoid). The plates were incubated anaerobically at 70°C (8). Isolate VC-16 from Vulcano was the first

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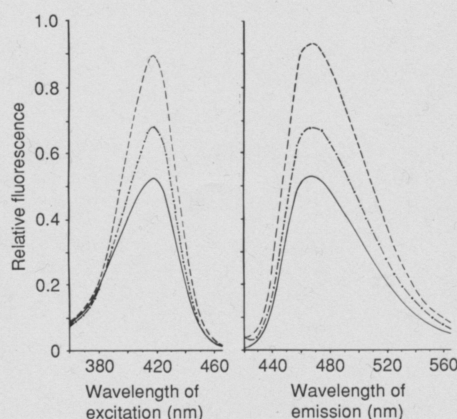
**Fig. 1.** Electron micrographs of isolate VC-16, platinum-shadowed. (A) Cells and empty envelope, (B) cell with two flagella protruding from a single origin (arrow). Scale bars, 1 µm.

strain obtained in pure culture and was therefore studied in detail.

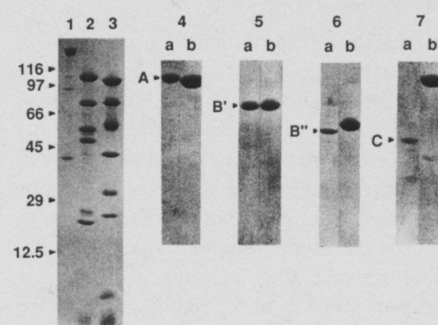
Cultures of VC-16 consisted of irregular spherical cells with envelopes composed of subunits in hexagonal array (Fig. 1A). The cells were motile and possessed flagella protruding from a common location (Fig. 1B, arrow). The new organisms grew within a temperature range between 64° and 92°C, with an optimum at approximately 83°C (corresponding to a doubling time of about 4 hours). Growth depended on the presence of sulfate, which was supplied in the culture medium (14 µmol/ml). Large amounts of H<sub>2</sub>S (up to 6 µmol/ml) and CO<sub>2</sub> were formed during growth, indicating that the new organisms carried out dissimilatory sulfate reduction (5). In addition, methane (up to 0.01 µmol/ml) was formed. Sulfate could be replaced by thiosulfate (14 µmol/ml) and by sulfite (1 µmol/ml), but not by elemental sulfur. Molecular hydrogen and some simple organic molecules served as substrates (Table 1). In contrast to eubacterial sulfate reducers (5), complex organic substrates and even glucose were used (Table 1). As expected, crude oil (Höhenrain; Preussag, Hannover) did not support growth. However, it was not inhibitory when present with substrates. This finding suggests that the new organisms could be responsible for the formation of H<sub>2</sub>S in geothermally heated oil wells ("sour oil") when suitable substrates and sulfate-containing water are present. In batch culture with L-(+)-lactate as the substrate, 140-g cells (wet weight) were obtained in a 300-liter, enamel-protected fermentor (Pfaudler, West Germany).

The isolate VC-16 was recognized as an archaeobacterium on the basis of (i) its 16S

rRNA sequence (1, 9), (ii) the existence of an adenosine diphosphate-ribosylable protein in the crude extract (10, 11), (iii) the presence of phytanyl ether lipids (12, 13), (iv) a cell wall consisting of glycoprotein instead of murein (14), and (v) the presence of an RNA polymerase with eight subunits resistant to 200 µg/ml of rifampicin and streptolydigin (2). DNA from isolate VC-16 had a content of guanosine plus cytosine of 46 mol% (4, 15). Spectra of the enriched compound that fluoresced blue-green in the UV light showed the same excitation and emission maxima (419 and 467.5 nm, respectively) as pure factor 420 (F<sub>420</sub>) from *Methanobacterium thermoautotrophicum* and as enriched F<sub>420</sub> from *Methanococcus thermolithotrophicus* (Fig. 2) (16). This finding suggests that the unknown compound was an 8-OH-5-deazaflavin similar to F<sub>420</sub> in methanogenic bacteria (17). Cell extracts of VC-16 also contained substances showing the same ultraviolet/visual spectrophotometry spectrum as methanopterin and 5,10-methenyltetrahydro methanopterin (18, 19). In contrast to methanogens, no substance showing the spectrum of factor 430 (F<sub>430</sub>) (18) could be detected. Moreover, coenzyme M (2-mercaptoethanesulfonic acid) as determined by the bioassay described by Balch and Wolfe (20) was not evident. The formation of methane without coenzyme M and F<sub>430</sub> could be analogous to the "mini methane production" described for some eubacteria (21). The essential enzymes of the dissimilatory sulfate reduction pathway, namely, adenosine triphosphate sulfurylase, adenylyl-sulfate reductase, and bisulfite re-



**Fig. 2.** Excitation and emission spectra of the fluorescent compound of the new isolate VC-16 (---) and of F<sub>420</sub> from *M. thermolithotrophicus* (- · - ·), and *M. thermoautotrophicum* (—). The fluorescent compound of isolate VC-16 and F<sub>420</sub> from *M. thermolithotrophicus* was extracted from the cells with acetone and was purified by chromatography on a QAE-Sephadex A 25 column (16). The excitation and emission spectra were measured in a Cary 118 C spectrophotometer and a Hitachi-Perkin-Elmer fluorescence spectrophotometer MPF-44 A, respectively (buffer: 50 mM tris, pH 7.5, and 1M NaCl).



**Fig. 3.** SDS-polyacrylamide gel electrophoresis and immunoblotting (Western blots) of DNA-dependent RNA polymerases. RNA polymerases of 1, *Escherichia coli*; 2 and 4a to 7a, *M. thermolithotrophicus*; 3 and 4b to 7b, isolate VC-16. Coomassie staining of 1 to 3. The positions of molecular weight standards in the polyacrylamide gel are on the left side. *Escherichia coli* RNA polymerase was obtained from Boehringer Mannheim. RNA polymerases of *M. thermolithotrophicus* and of isolate VC-16 were purified by standard methods (23). Western blots of components of RNA polymerases of *M. thermolithotrophicus* and of isolate VC-16 were challenged (2, 26) with antibodies directed against single components A, B', B'', and C (lanes 4 to 7, respectively) of the RNA polymerase of *M. thermoautotrophicum* (2). Bound antibodies were visualized with peroxidase-coupled antibodies to immunoglobulin G.

ductase exist in cell extracts of VC-16 and are under investigation (22).

The phylogenetic position of the newly isolated sulfate reducers within the archaeobacteria was investigated by comparing DNA-dependent RNA polymerases. Within archaeobacteria, only two distinct types of RNA polymerase structures are known that parallel the two phylogenetic branches: the BAC-type of the S<sup>0</sup>-metabolizing and the AB'B'C-type of the methanogenic-halophilic archaeobacteria. The basis of this difference is the occurrence of two polypeptides, B' and B'', in the AB'B'C-type, which show homology to component B of the other type (2, 23). The RNA polymerase purified from VC-16 exhibited eight identifiable components, each having a different molecular weight (Fig. 3, lane 3). Structural homologies between the RNA polymerases of the novel sulfate reducer and other archaeobacteria were revealed by immunological cross-reactions. Antibodies directed against the single components of *M. thermoautotrophicum* RNA polymerase were challenged with isolated components of the enzymes of VC-16 (Fig. 3, lanes 4b to 7b) and, as a control, of *M. thermolithotrophicus* (Fig. 3, lanes 4a to 7a). The three heaviest components of the VC-16 enzyme showed serological cross-reaction with components A, B', and B'' of the methanogen enzyme, so far indicating homology to the AB'B'C structure. Antibodies directed against the fourth largest component (C) of the enzyme of methano-

genic bacteria (Fig. 3, lane 7a) exhibited a strong cross-reaction with the heaviest component (A) of the VC-16 RNA polymerase (Fig. 3, lane 7b). This cross-reaction demonstrates that the largest component of the RNA polymerase of the sulfate reducer contained structural elements of components A and C of the methanogen enzyme. The new sulfate reducer thus exhibits a previously unknown third type of RNA polymerase (A + C)B'B'' within the archaeobacteria, which is phylogenetically removed from those of the two accepted archaeobacterial branches. This finding provides evidence for the existence of a third phylogenetic branch within the archaeobacterial kingdom.

On the basis of their phylogenetic uniqueness, archaeobacterial sulfate reducers may have existed since ancient times. Early Archaean ocean waters were generally poor in sulfate and were thus unfavorable for the presence of sulfate reducers that were thought to have originated later (24). This theory is in line with the lack of significant biogenic sulfur isotope fractionation within such sediments (24). However, there should

have been reasonable quantities of sulfate of magmatic origin present locally within Archaean hydrothermal systems (25). Therefore, sulfate reduction by extremely thermophilic archaeobacteria could have existed since early Archaean times and may be an ancient type of metabolism.

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