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Isolate B12, which harbours a virus-like element, represents a new species of the archaebacterial genus *Sulfolobus*, *Sulfolobus shibatae*, sp. nov.

Dennis Grogan[1,2], Peter Palm, and Wolfram Zillig

Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, W-8033 Martinsried, Federal Republic of Germany

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Abstract. The *Sulfolobus* isolate B12 and its endogenous virus-like element SSV1 have provided a fruitful system for detailed analysis of certain aspects of archaebacterial molecular biology, especially those concerning gene expression. In the course of clarifying this isolate's taxonomic position, we determined DNA base composition, ability to grow autotrophically, nucleotide sequence of 16*S* ribosomal RNA, and level of total genomic homology to other *Sulfolobus* strains. Although the results generally demonstrate a similarity to *S. solfataricus*, DNA-DNA hybridisation and 16*S* rRNA sequence data indicate that isolate B12 in fact represents a distinct species.

Key words: Sulfolobus isolate B12 – Archaebacterial taxonomy – G + C content – Sulphur oxidation – DNA-DNA hybridisation – 16S rRNA sequence

The genus *Sulfolobus*, described by Brock et al. (1972) occurs naturally in acidic geothermal springs, water holes or mud holes containing elemental sulphur, and stems phylogenetically from the extremely thermophilic branch of the archaebacteria (Klenk et al. 1986). It currently consists of two validly described species, *S. acidocaldarius* (Brock et al. 1972) and *S. solfataricus* (Zillig et al. 1980), which differ with respect to the antigenic determinants and apparent molecular-weights of components of their DNA-dependent RNA polymerases (Zillig et al. 1980). Similar isolates which were previously designated "Sulfolobus" have more recently been found able to grow autotrophically by means of sulphur reduction in addition to sulphur oxidation and have for this and other reasons been assigned to new genera (Segerer et al. 1986; Zillig et al. 1986). *Sulfolobus* isolates have provided favorable subjects for study of molecular-biological and biochemical aspects of extremely thermophilic archaebacteria. An isolate from Japan, designated B12, has proven particularly interesting for its UV-inducible amplification of a 15.5-kb plasmid (Yeats et al. 1982) and the subsequent release of virus-like particles containing this circular DNA (Martin et al. 1984). Analysis of this system has provided considerable information about archaebacterial gene expression, promoter and terminator structure, and site-specific recombination (Reiter et al. 1988a, b, 1989; Zillig et al. 1988a, b). However, proper taxonomy of isolate B12, which was initially considered to be a strain of *S. acidocaldarius* (Yeats et al. 1982) and later, of *S. solfataricus* (Reiter et al. 1987) has remained problematic based upon the information currently available. For example, the ability to grow autotrophically via sulphur oxidation has historically been an important criterion of the genus (Brock 1974), yet isolate B12 had been presumed to be obligately heterotrophic (Zillig et al. 1988b). Isolate B12 yields a pattern of chromosomal restriction fragments quite different from those of *S. solfataricus* or *S. acidocaldarius* yet resembles these species with respect to a wide array of physiological characteristics (Grogan 1989). rRNA-DNA hybridisation values (Klenk et al. 1986), and the surface contours of the *S*-layer cell envelopes (Prüschenk et al. 1987) agree in depicting isolate B12 as more closely related to *S. solfataricus* than to *S. acidocaldarius*. The metabolic and genomic characterisation presented here indeed place isolate B12 within the genus *Sulfolobus*, but as a third species, distinct from both *S. acidocaldarius* and *S. solfataricus*.

Materials and methods

Isolation and cultivation

Samples were taken from a series of geothermal pools and bubbling "mud pots" located in Beppu, Kiushu Island, Japan, in August of

[1] *Present address:* California Institute of Technology, Jet Propulsion Laboratory 125-112, 4800 Oak Grove Drive, Pasadena, CA 91109, USA

Offprint requests to: D. Grogan

Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen, Mascheroder Weg 1B, D-3300 Braunschweig, FRG; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

1979. One sample having an initial temperature of approximately 87°C and pH of 3.7 was partially neutralised and later used to inoculate the complex medium of Brock et al. (1972) at 80°C. Streaking the resulting culture on a starch plate yielded a plasmid-bearing clone, designated B12 (Yeats et al. 1982). Preparation of the cultures used in this study [strains B12, *Sulfolobus acidocaldarius* 98-3 (Brock et al. 1972, received as DSM 639), and *S solfataricus* P1 and P2 (Zillig et al. 1980)] has been described elsewhere (Grogan 1989). Total genomic DNA was purified by repeated phenol-extraction and isopycnic centrifugation in the presence of ethidium bromide (Zillig et al. 1980).

Oxidation of sulphur

Inocula (0.5 ml each), grown to late exponential or early stationary phase using 0.1% dextrin-10 (Serva, Heidelberg, FRG) as sole carbon source were added, in 100-ml serum bottles, to approximately 50 mg sulphur powder in 30 ml mineral mixture (Brock et al. 1972) modified as follows: the $(NH_4)_2SO_4$ concentration was reduced to approximately 0.2 mM, 9 mM NH_4Cl was added, 1 mM sodium citrate was included as supplementary pH-buffer, and the initial pH was 4.0. The gas phase in the closed bottles was supplemented with 3% CO_2 gas, and the cultures were shaken at 78°C. Aliquots to be analysed (1 ml) were withdrawn with a needle through the stopper: small volumes (approx. 0.1 ml) of 1 M Na_2CO_3 were similarly added as needed to prevent the medium pH from falling significantly below a value of pH 3.0. The concentration of cells not attached to sulphur particles in the samples was determined by counting in a gridded chamber: the concentration of sulphate was measured turbidometrically by centrifuging the culture samples to remove cells and sulphur particles and adding aliquots of the resulting supernatants to 200 mM $BaCl_2$, 2% dextrin, and 50 mM sodium acetate, pH 5.5. After 2 min, the turbidity (apparent OD_{600} in a Uvikon 820 spectrophotometer (Kontron), 1-cm cuvette) was measured and compared to a standard curve.

Guanosine-plus-cytosine content

Total genomic DNAs were analysed by nuclease-digestion and chromatography (method I) and by thermal denaturation (method II). The former was carried out as described by Zillig et al. (1980), except that the DNA was heat-denatured before addition of nuclease P1, and that the digestions were performed at 56°C. For the latter method, purified DNAs were dialysed against a common chamber containing 40 mM NaCl, 4 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 7.0), and 0.2 mM EDTA (total sodium concentration of 42 mM); temperature of the denaturation midpoint was determined as outlined by Johnson (1985). Mole-percent G + C was calculated by comparison to coliphage DNA analysed in parallel: phage T7 DNA, assumed to contain 26.0% A, 26.6% T, 23.6% C, and 23.8% G (Volkin et al. 1958), and phage T5 DNA, assumed to contain 39.2% G + C (Volkin et al. 1958; this work, method I).

DNA-DNA hybridisation

Hybridisation in solution was measured as outlined by Johnson (1985). Genomic DNAs in TE buffer, pH 8.0, at a concentration of 0.5 mg ml^{-1} were sonicated to give a uniform length-distribution (0.5 to 2.0 kbp, as judged by electrophoresis in agarose). Aliquots of these preparations, diluted to 20 µg ml^{-1} in TE, were used as templates for the synthesis of α-[^{32}P] dATP-labelled DNA (Feinberg and Vogelstein 1983). Less than approximately 5 ng of each labelled DNA was hybridised with 4 to 5 µg of each unlabelled ("driver") DNA in 1 M NaCl, 20% formamide (pH 7, total volume of 20 µl) at 55–56°C for a period of 50 to 100 h. The duplex fraction was purified from each mixture by chromatography on hydroxylapatite, and radioactivity was determined by counting Cerenkov radiation (Johnson 1985). Each series of hybridisations for a given probe included duplicate controls for 0% (sonicated, duplex T7 DNA) and 100% (single-stranded driver DNA identical to probe) homology.

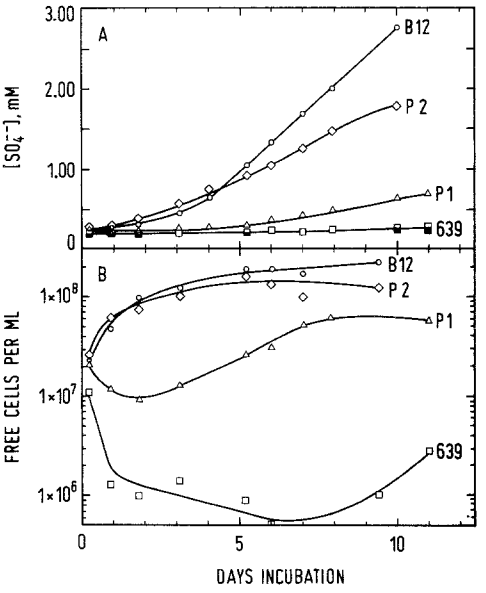

Fig. 1 A, B. Autotrophic growth by sulphur oxidation. Cells grown heterotrophically in synthetic medium were incubated aerobically with CO_2 as sole carbon source and elemental sulphur as sole energy source, as described in Methods. **A** concentration of sulphate in the medium as a function of time. **B** concentration of free cells (i.e., not adsorbed to sulphur) in the medium as a function of time. *Symbols: open circles*, strain B12; *squares*, strain DSM 639; *triangles*, strain P1; *diamonds*, strain P2; *solid squares* in panel **A**, uninoculated control

16S rRNA gene sequence

The gene of the 16*S* rRNA was isolated as described previously (Reiter et al. 1987). A 1.9 kb (EcoRV/BamHI) fragment was subcloned into M13tg131. DNA sequences were determined from M13 DNA by the dideoxy chain termination method (Sanger et al. 1977, 1980). Overlapping fragments were produced by progressive Bal31 exonuclease digestion (Poncz et al. 1982). Sequence editing and aligning was done with the aid of computer programs UWGCG (Devereux et al. 1984) and Staden (1980).

Results and discussion

Though its plasmid and associated particle distinguish isolate B12 from other known *Sulfolobus* strains, the virus-like nature of the particle argues aginst its use as a taxonomic criterion. In the absence of obvious morphological (Yeats et al. 1982) and physiological (Grogan 1989) traits distinguishing isolate B12 from other *Sulfolobus* strains, the reported lack of autotrophic capability was re-examined. In each of two independent trials, one of which used washed inocula, strains B12 and P2 grew soon after transfer to autotrophic conditions and produced millimolar concentrations of sulphate; Fig. 1 shows one of these experiments. We could not conclude

Table 1. Base compositions of *Sulfolobus* DNAs[a]

Source	Method I		Method II		Overall mean[b]	Literature values
	n	mean ± SD	*n*	mean ± SD		
S. acidocaldarius	2	37.0 ± 0.3	1	37.6	37.2	61[c], 70[d], 39±2[e], 36[f]
S. solfataricus	3	36.1 ± 2.2	2	33.7 ± 0.5	35.1	39[d], 36±1[e], 34[f]
B12	5	35.2 ± 2.5	4	34.0 ± 0.4	34.6	

[a] Mole % G + C in total genomic DNA, as described in Methods
[b] Unweighted average of all indicated determinations
[c] Brock et al. (1972)
[d] DeRosa et al. (1975)
[e] Zillig et al. (1980)
[f] Huber et al. (1989)

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   1  AATCCGGTTGATCCTGCCGGACCCGACCGCTATCGGGGTGGGGCTAAGCCATGGGAGTCG
  61  TACGCTCCCGGGCAAGGGAGCGTGGCGGACGGCTGAGTAACACGTGGCTAACCTACCCTG
 121  AGGAGGGAGATAACCCCGGGAAACTGGGGATAATCTCCCATAGGCGAGGAGTCCTGGAAC
 181  GGTTCCTCGCTGAAAGGTTCATGGGCTATTTCCCGCTCATGAGCGCCTCAGGATGGGGCT
 241  GCGGCCCATCAGGTAGTTGGGGGGGTAAGGGCCCCCCAAGCCTATAACGGGTAGGGGCCG
 301  TGAGAGCGGGAGCCCCCAGTTGGGCACTGAGACAAGGGCCCAGGCCTACGGGGCGCACCA
 361  GGCGCGAAACGTCCCCAATGCGCGGAAGCGTGAGGGCGCCACCCCGAGTGCTCCCGTAAG
 421  GGAGCTTTTCCCCGCTCTACAAAGGCGGGGGAATAAGCGGGGGGCAAGTCTGGTGTCAGC
 481  CGCCGCGGTAATACCAGCCCCGCGAGTGGTCGGGACTCTTACTGGGCCTAAAGCGCCCGT
 541  AGCCGGCCCGACAAGTCACTCCTTAAAGACCTCGGCTCAACCGGGGGAATGGGGGTGATA
 601  CTGTCGGGCTAGGGGGCGGGAGAGGCCAGCGGTACTCCCGGAGTAGGGGCGAAATCCTCA
 661  GATCTCGGGAGGACCACCAGTGGCGAAAGCGGCTGGCTAGAACGCGCCCGACGGTGAGGG
 721  GCGAAAGCCGGGGCAGCAAAAGGGATTAGATACCCCTGTAGTCCCGGCTGTAAACGATGC
 781  AGGCTAGGTGTCACATGGGCTTAGAGCCCATGTGGTGCCGCAGGGAAGCCGTTAAGCCTG
 841  CCGCCTGGGGAGTACGGTCGCAAGACTGAAACTTAAAGGAATTGGCGGGGGAGCACCACA
 901  AGGGGTGGAACCTGCGGCTCAATTGGAGTCAACGCCTGGAATCTTACTAGGGGAGACCGC
 961  AGGATGACGGCCAGGCTAACGACCTTGCCTGACTCGCGGAGAGGAGGTGCATGGCCGTCG
1021  CCAGCTCGTGTTGTGAAATGTCCTGTTAAGTCAGGCAACGAGCGAGACCCCCACCATTAG
1081  TTGGTATCCTGGTCTCCGGGCCGGGACCACACTAGTGGGACTGCCGGCGTAAGCCGGAGG
1141  AAGGAGGGGGCCACGGCAGGTCAGCATGCCCCGAAACCCCTGGGCCGCACGCGGGTTACA
1201  ATGGCAGGGACAGCGGGATTCCGACCCCGAAAGGGGAAGGTAATCCCTTAAACCCTGCCG
1261  CAGTTGGGATCGAGGGCTGAAACTCGCCCTCGTGAACGAGGAATCCCTAGTAACCGCAGA
1321  TCAACAATCTGCGGTGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCGCTCCACCCG
1381  AGTAGGAGAGGGGTGAGGCCCCTTGCCTTTAGGTGGGGGGTCGAGCTTCTCTCCTGCAAG
1441  GGGGGAGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGCTGGATCACCTCA
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Fig. 2. Complete nucleotide sequence of the gene of 16*S* rRNA of *Sulfolobus shibatae*

from our data, however, whether the *S. acidocaldarius* type strain (DSM 639), which was characterised as a relatively weak sulphur-oxidiser soon after its isolation (Shivvers and Brock 1973), has in fact lost its autotrophic capability, as recently suggested (Huber et al. 1989) or merely requires a long adjustment period or other special conditions to commence sulphur oxidation. Authenticity of the autotrophic cultures of Fig. 1 was confirmed by subsequent transfer to heterotrophic medium; the control yielded no growth, whereas all others, including DSM

Table 2. Table of S_{AB} values

	Shib	Ssol	Saci	Pocc	Tcel	Hcut	Mvan	Scer	Ecol
Sulfolobus shibatae	1.00	0.83	0.47	0.38	0.35	0.20	0.18	0.07	0.10
Sulfolobus solfataricus	0.83	1.00	0.39	0.34	0.34	0.20	0.19	0.07	0.08
Sulfolobus acidocaldarius	0.47	0.39	1.00	0.45	0.36	0.21	0.18	0.07	0.08
Pyrodictium occultum	0.38	0.34	0.45	1.00	0.46	0.27	0.21	0.07	0.11
Thermococcus celer	0.35	0.34	0.36	0.46	1.00	0.17	0.16	0.07	0.12
Halobacterium cutirubrum	0.20	0.20	0.21	0.27	0.17	1.00	0.24	0.09	0.07
Methanococcus vannielii	0.18	0.19	0.18	0.21	0.16	0.24	1.00	0.06	0.08
Saccharomyces cerevisiae	0.07	0.07	0.07	0.07	0.07	0.09	0.06	1.00	0.04
Escherichia coli	0.10	0.08	0.08	0.11	0.12	0.07	0.08	0.04	1.00

SAB values were calculated from the oligonucleotide catalog of *Sulfolobus solfataricus* (Woese et al. 1984) and oligonucleotide catalogs derived from the total sequences of *E. coli* (Carbon et al. 1979), *Pyrodictium occultum* (Kaine et al. 1989), *Saccharomyces cerevisiae* (Rubtsov et al. 1980), *Sulfolobus acidocaldarius* (Olsen et al. 1985), *Thermococcus celer* (Achenbach-Richter et al. 1988), *Halobacterium cutirubrum* (Hui and Dennis 1985) and *Methanococcus vannielii* (Jarsch and Böck 1985). Oligonucleotides equal or greater than six were used for calculations

639, yielded rapid growth consisting only of the designated strain, as indicated by close comparison of genomic restriction-fragment patterns (not shown).

We determined the G + C contents of total DNA from isolate B12, *S. acidocaldarius* and *S. solfataricus* (Table 1). The results for *S. solfataricus* and B12 were the same (35 mol%) within experimental error, and slightly below that of *S. acidocaldarius*. It should be noted that early estimates of G + C content for *S. acidocaldarius* DNA are spuriously high (Table 1). These were based entirely on buoyant density determinations, which may have been perturbed by some property peculiar to the *S. acidocaldarius* DNA preparations used (Brock et al. 1972; DeRosa et al. 1975).

We determined the total sequence of the 16*S* rRNA of isolate B12, shown in Fig. 2. Comparison of this sequence to those of other *Sulfolobus* strains had to account for several factors: i) only a T_1 RNase catalogue was available in one case (Woese et al. 1984), ii) the strain kept by T. A. Langworthy (which originated from an isolate of T. D. Brock) yielded an RNA polymerase component pattern identical with that of *S. solfataricus* (Zillig et al. 1980), so that the T_1 RNase catalogue of the 16*S* rRNA of this strain (Woese et al. 1984) must be appointed to *S. solfataricus* rather than *S. acidocaldarius*, iii) conversely, the strain from our laboratory from which the total sequence of "*S. solfataricus*" 16*S* rRNA had been determined (Olsen et al. 1985) proved to be *S. acidocaldarius* rather than *S. solfataricus* by its RNA polymerase component pattern (not shown).

The 16*S* rRNA of strain B12 was 90.5% identical to that of this *S. acidocaldarius* strain. This is a relatively low value, as compared in 85% overall sequence identity between this latter *Sulfolobus* strain and *Pyrodictium occultum*, a strict anaerobe growing optimally at about 105° C (Woese and Olsen 1986). Lacking a total sequence of authentic *S. solfataricus* 16*S* rRNA, we derived a T_1 RNase-catalogue from the B12 sequence and calculated the corresponding S_{AB} values between this catalogue and those of *S. acidocaldarius* (Olsen et al. 1985) and the *S. solfataricus* of Langworthy (Woese et al. 1984). As shown in Table 2, isolate B12 is more closely related to *S. solfataricus* (S_{AB} = 0.83) by this criterion than to

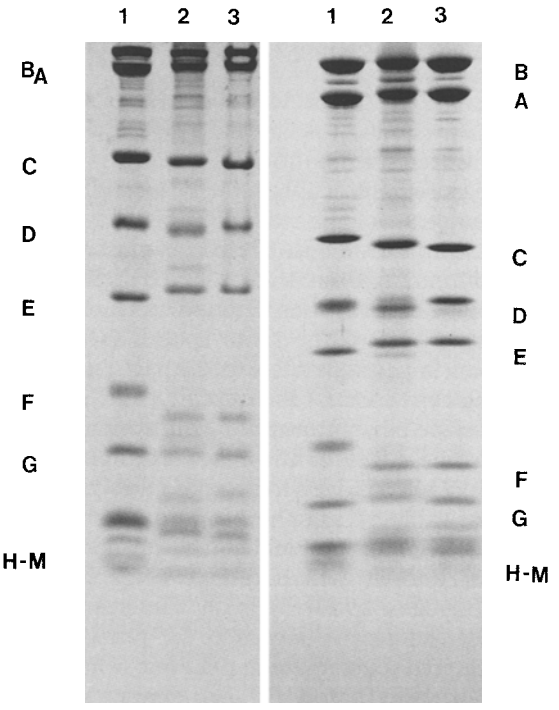

Fig. 3. SDS PAGE component patterns of DNA-dependent RNA polymerases of *S. acidocaldarius* (lanes 1), *S. solfataricus*, strain of Langworthy (lanes 2) and *S. shibatae* (lanes 3). Left three lanes Schägger gels (Schägger and von Jagow 1987), right three lanes 5–25% exponential gradient gels according to Mirault and Scherrer (1971). Designations of components on the margins

S. acidocaldarius (S_{AB} = 0.47), in close agreement with previous rRNA-DNA hybridisation data (Klenk et al. 1986) and DNA-DNA hybridisation using other strains (see below).

Such a relationship was also indicated by SDS-PAGE patterns of the DNA-dependent RNA polymerase components (Zillig et al. 1980). As shown in Fig. 3, the patterns of apparent molecular weights of the RNA polymerase subunits from isolate B12 and that from the *S. solfataricus* of Langworthy share many more similarit-

Table 3. DNA-DNA hybridisation among *Sulfolobus* isolates[a]

Source of probe DNA[b]	Source of driver DNA[b]						
	P1	P2	MT4[c]	B12	639	B6/2[c]	L10[c]
P1	(100 ± 2)[d]	95 ± 8	86 ± 4	25 ± 4	2 ± 1	8 ± 1	<1
P2	96 ± 2	(100 ± 2)	91 ± 7	21 ± 4	2 ± 1	6 ± 3	<1
MT4[c]	89 ± 4	89 ± 14	(100 ± 1)	19 ± 5	<1	nd[d]	<1
B12	26 ± 7	22 ± 3	20 ± 2	(100 ± 1)	2 ± 1	15 ± 3	<1
DSM 639	<1	3 ± 1	<1	<1	(100 ± 1)	12 ± 4	<1
B 6/2[c]	<1	3 ± 1	nd	3 ± 1	<1	(100 ± 4)	nd
L10[c]	<1	<1	<1	<1	<1	nd[de]	(100 ± 1)

[a] Values are relative homology, in percent (see Methods)
[b] References to organisms not described in Method: *S. solfataricus* MT4, DeRosa et al. (1974); *Sulfolobus* sp. B6/2, Zillig et al. (1988b); *Desulfurolobus ambivalens* L10, Zillig et al. (1986)
[c] Data in this row or column are averages of two determinations; all others are averages of four determinations, which included two probe preparations
[d] Uncertainty is expressed as total range of values for the homologous controls and as standard deviation in the heterologous cases
[e] nd, not determined

ies than either shares with the RNA polymerase of *S. acidocaldarius*.

Finally, the species affiliation of strain B12 was examined by measurement of DNA-DNA hybridisation among *Sulfolobus* strains. The results (Table 3) confirm that, among the strains tested, isolate B12 is most closely related to *S. solfataricus*, but the observed homology (20–25%) is well below that considered to indicate affiliation of two organisms to the same species (Johnson 1984). In addition to setting isolate B12 apart from the two known *Sulfolobus* species, the quantitative hybridisation results provide new information concerning intrageneric relationships. They confirm, for example, that strains P1, P2, and MT4 indeed form an unambiguously coherent species (Table 3), in spite of the differences in restriction fragment patterns, biochemical properties of purified *S*-layers, and growth parameters which have been observed (Grogan 1989). It is also interesting to note that another isolate, included here for purposes of comparison, exhibited no significant genomic homology to any other strain tested (Table 3). Though not yet adequately characterised, this isolate (B6/2) has been affiliated with *S. acidocaldarius* by the criteria of rRNA-DNA hybridisation and outer contour of the *S*-layer cell wall (Klenk et al. 1986; Prüschenk et al. 1987). Our result thus reinforces other lines of evidence (Bohlool and Brock 1973; Stetter 1986) that the genus *Sulfolobus*, as currently defined, encompasses a rich variety of organisms in nature, which has to date been only superficially explored.

In summary, this study has shown that isolate B12, though sharing many properties of *S. solfataricus*, in fact represents a separate species by the criterion of overall genomic homology. We name this species *Sulfolobus shibatae*. It also perhaps raises the question whether *S. acidocaldarius* should remain affiliated to the same genus. Our results confirm the coherence of strains P1, P2 and MT4 as a species (*S. solfataricus*), and suggest that the ability to grow autotrophically via sulphur oxidation remains a valid characteristic of this genus.

Description of Sulfolobus shibatae, sp. nov.

[Morphological and physiological data are those of Martin et al. (1984) and Grogan (1989)].

Sulfolobus shibatae, Grogan, Palm and Zillig (spec. nov.) shi.ba.tae M. L. gen. of the family name of Dr. Masaru Shibata who enabled us to obtain the samples from which the organism was isolated.

Cells coccoid but may have several flattened-to-deeply indented faces; occur singly. Overall diameter 0.7 to 1.5 μm. Cells devoid of peptidoglycan, surrounded by regular protein layer; periodic hexagonal structure visible in electron microscope. Colonies are pale tan, translucent, smooth, and convex with entire margins. Aerobic. Weakly motile, depending on culture conditions. Growth inhibited by phosphate concentrations above about 10 mM. In 0.2% yeast extract, pH 3, optimal growth temperature approx. 81°C, maximal approx. 86°C. D-arabinose, D-galactose, D-glucose, D-mannose, lactose, lactulose, maltose, sucrose, raffinose, starch, and tryptone also utilized. Facultatively chemolithoautotrophic growth by sulphur oxidation. DNA composition 35 mol% G + C. DNA-dependent RNA polymerase component pattern similar to that of *S. solfataricus* and different from that of *S. acidocaldarius*. According to DNA-DNA cross-hybridisation and S_{AB} value phylogenetically close to *S. solfataricus*. Isolated from acidic geothermal spring. The type strain is B12 (DSM 5389).

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