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The Hyperthermophilic Archaeon *Sulfolobus*:
from Exploration to Exploitation

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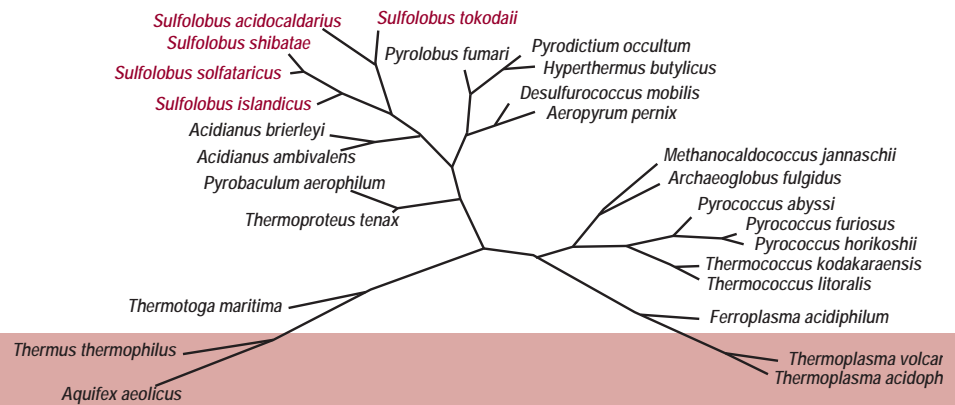[4] Chemical Engineering, Sheffield University, UK
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ABSTRACT

In the early 1970s, *Sulfolobus* was first isolated by Thomas Brock and co-workers from sulfur-rich acidic hot springs in Yellowstone National Park. *Sulfolobus* became one of the model organisms of *Archaea* in general, and of Crenarchaea in particular. Many of its unusual physiological characteristics have been investigated, and several of its thermostable enzymes have been studied in considerable detail. For fundamental reasons, and because of its potential for industrial applications, *Sulfolobus* has been selected for a genome sequence project. The recent completion of the *Sulfolobus solfataricus* genome has set the stage for a series of post-genome research lines that will be reviewed here. Comparative genomics aims to unravel the cell's metabolic potential (enzymes, pathways, and regulation) and compare it to other organisms. This *in silico* analysis of *Sulfolobus* has revealed several unique metabolic features and confirmed that the major control processes (transcription, translation, and replication) resemble eukaryal much more than bacterial equivalents. Currently, several research groups use a functional genomics approach—a genome-based, high-throughput analysis of the complete *Sulfolobus* cell—at the level of RNA (transcriptomics), protein (proteomics), and metabolic intermediates and products (metabolomics). Apart from analyzing the cell's response to different cultivation conditions, the comparison of different genotypes (wild type and mutants) would be very interesting for both basic science and applied reasons. Much effort has recently been put into the establishment of tools that allow genetic engineering of *Sulfolobus*. The future challenge is to integrate available knowledge in order to understand the relevant mechanisms that enable *Sulfolobus* to thrive in its extreme habitats. Moreover, such a Systems Biology approach is essential as a basis for the directed engineering and industrial exploitation of this unique microorganism.

1.0 INTRODUCTION

Until the late 1960s it was generally accepted that life was impossible beyond a temperature of 55-60°C. In 1969, however, the temperature limit was extended to 75°C when a microorganism was isolated from thermal springs in Yellowstone National Park by Thomas D. Brock and colleagues (Brock and Freeze 1969). This organism was characterized as a gram-negative bacterium called *Thermus aquaticus*. Subsequently, Brock's group isolated a remarkable thermo-acidophilic microorganism that they named *Sulfolobus acidocaldarius* from Congress Pool, a thermal feature at Yellowstone's Norris Geyser Basin with an average temperature of 80°C and average pH of 3.0 (Brock et al. 1972). *Sulfolobus*-like strains were later found in several acidic hot pools in El Salvador and Italy at temperatures of 65-90°C and pH levels of 1.7-3.0. Based on morphological analysis, *Sulfolobus* was initially considered to be a thermo-acidophilic bacterium with some remarkable properties (Shivvers and Brock 1973). However, the molecular classification introduced by Carl Woese and colleagues (Woese 1987; Pace 1997) indicated that *Sulfolobus* does not belong to *Bacteria*, but rather to a newly discovered prokaryotic Domain: *Archaea* (**Figure 1A, next page**). Brock's pioneering work set the stage for further exploration of a broad range of thermal ecosystems worldwide. This has resulted in an ever-growing collection of archaeal and bacterial thermophiles that grow optimally at 60-80°C, and hyperthermophiles (mainly archaea and some bacteria) with optimal growth temperatures >80°C that have been isolated from terrestrial solfataric fields such as those in Yellowstone, Italy, Iceland, Japan, and New Zealand, as well as from marine ecosystems ranging from shallow vents like the beaches of Vulcano Island, Italy, to deep-sea smokers in the Pacific and Atlantic Oceans (Stetter 1999; Huber et al. 2000; Rothschild and Mancinelli 2001).
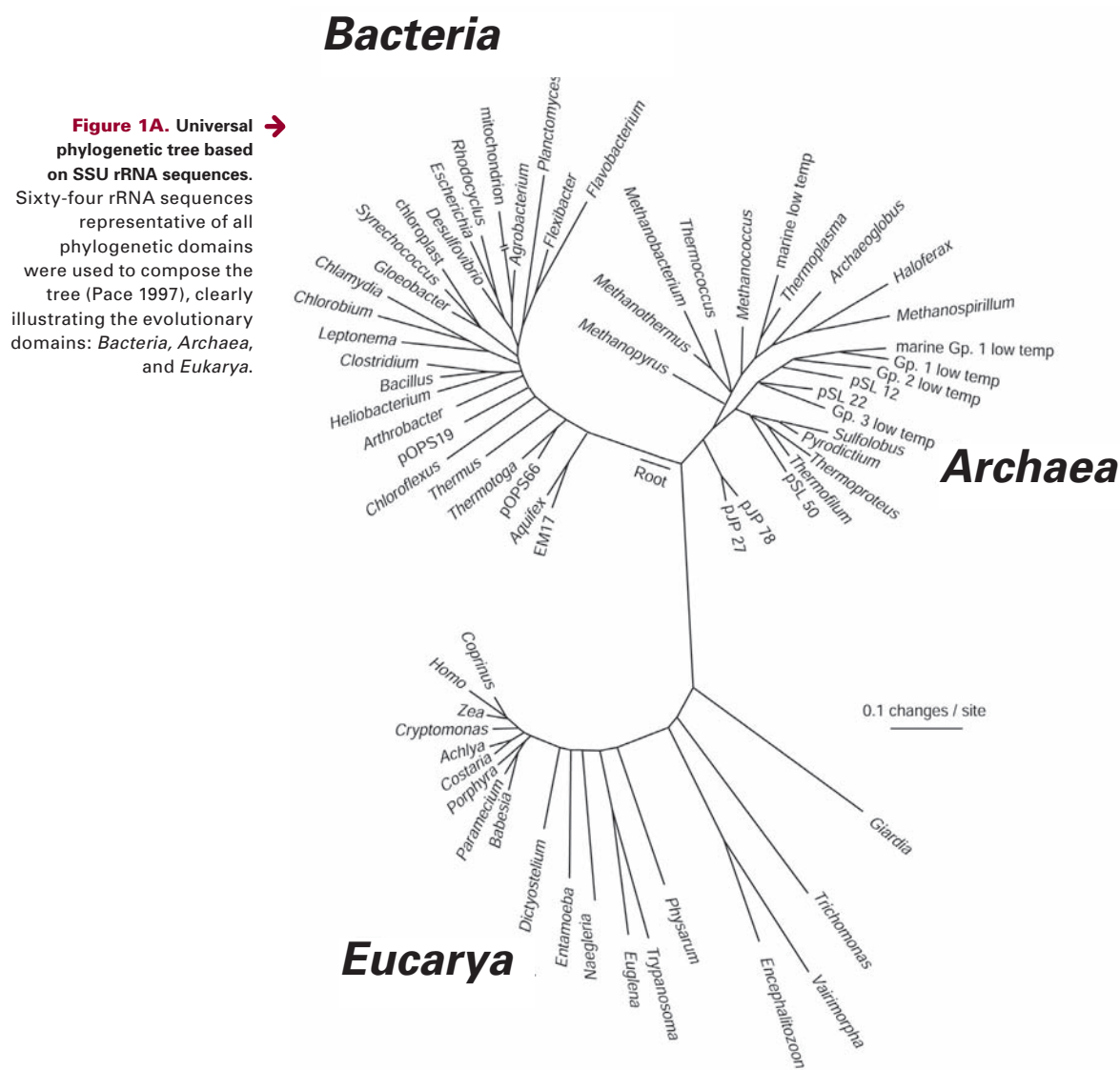
2.0 *SULFOLOBUS* AS MODEL ARCHAEON

The Domain *Archaea* has two principal Kingdoms—Crenarchaeota, home to *Sulfolobus*, *Pyrobaculum*, and *Pyrodictium;* and Euryarchaea, which includes *Pyrococcus*, *Methanococcus*, and *Halobacterium* (**Figure 1A**). Although the majority of the archaea were initially isolated from extreme environments—high temperature, high salt concentration, extreme pH—it has become clear that archaea also thrive in non-extreme environments. Archaea are abundant in many ecosystems ranging from soils to oceans (Pace 1997). Since its original discovery in Yellowstone, species of the genus *Sulfolobus* have been isolated from various solfataric fields, such as *S. solfataricus* in Pisciarelli, Italy (Zillig et al. 1980); a species provisionally called *Sulfolobus islandicus* in Sogasel, Iceland (Zillig et al. 1994); and *Sulfolobus tokodaii* from Japan's Beppu Hot Springs (Suzuki et al. 2002). *Sulfolobus* species are generally aerobic, and heterotrophic growth has been reported, during which a range of carbohydrates, yeast extract, and peptide mixtures are oxidized to CO_2 (Grogan 1989; Schönheit and Schäfer 1995). In addition, both autotrophic—oxidation of $S_2O_3^{2-}$, $S_4O_6^{2-}$, $S°$ and S^{2-} to sulphuric acid, and of H_2 to water—and heterotrophic growth has been described for *S. acidocaldarius* (Shivvers and Brock 1973; Schönheit and Schäfer 1995), and it has been suggested that anaerobic respiration (e.g., reduction of NO) by certain Sulfolobales might be possible (She et al. 2001). Other closely related genera (**Figure 1B**) that have been relatively well-characterized include *Acidianus* (order Sulfolobales, obligatory chemolithoautotroph, aerobic S^0 oxidation to sulphuric acid, anaerobic S^0 reduction coupled to H_2 oxidation); *Hyperthermus* (order Desulfurococcales, anaerobic, amino acid fermentation); and *Aeropyrum* (order Desulfurococcales, aerobe, heterotroph on starch and peptides). *Aeropyrum pernix* was the first Crenarchaeote for which the complete genome was sequenced (Kawarabayasi et al. 1999). *Sulfolobus* species are considered model archaea because of their global abundance (Delong and Pace, 2001); their relatively easy cultivation on a variety of carbon sources (Grogan 1989); and their possession of mobile genetic elements, such as insertion sequences (IS elements), viruses, small plasmids, and large conjugative plasmids (Zillig et al. 1998). These genetic elements are instrumental in the ongoing development of genetic tools (see below).

2.1 Genomics

In the mid-1990s, *S. solfataricus* P2 was selected for complete genomic sequencing because of the anticipated insight into the evolution of the eukaryotic cell (see below); the molecular basis of thermo-acidophilic growth; and metabolic enzymes, pathways and their regulation. In 2001 the *S. solfataricus* genome was completed (She et al.), followed by completion of the *S. tokodaii* genome

(Kawarabayasi et al. 2001). Currently the genome sequencing projects of *S. acidocaldarius*, *S. islandicus*, *Hyperthermus butylicus*, and *Acidianus* sp. are ongoing (R. Garrett, personal communication). Genome analysis of *S. solfataricus* and several other archaea confirmed (i) the monophyletic position of *Archaea*, with respect to *Bacteria* and *Eukarya* (**Figure 1A**); (ii) the bacterial-like condensed chromosomal organisation: clustering of genes

Figure 1A. Universal phylogenetic tree based on SSU rRNA sequences. Sixty-four rRNA sequences representative of all phylogenetic domains were used to compose the tree (Pace 1997), clearly illustrating the evolutionary domains: *Bacteria, Archaea*, and *Eukarya*.

as polycistronic units (operons), and few interrupted genes (introns); and (iii) the eukaryal-like systems that drive the flow of genetic information such as transcription, translation, replication, and DNA repair (reviewed by Makarova and Koonin 2003).

2.2 Mobile Genetic Elements

One of the most striking observations of the overall comparative analyses of multiple genomes has been their unexpected plasticity. In the case of many Sulfolobales, mobile genetic elements are anticipated to play an important role in the ever-changing genome composition. The relatively large genome of *S. solfataricus* (2.99 Mbp) contains an unprecedented high number of IS elements and repeats (in total >200; She et al. 2001), some of which have been demonstrated to actively move through the genome (Martusewitsch et al. 2000). Interestingly, the *S. tokodaii* genome (2.69 Mbp) contains much less (Kawarabayasi et al. 2001), and the *S. acidocaldarius* genome (2.76 Mbp) apparently does not contain a single one (R. Garrett, personal communication).

The best characterized archaeal virus is SSV1 isolated from *Sulfolobus shibatae* (Yeats et al. 1982; Reiter et al.

1987; Schleper et al. 1992). Upon infection of *S. shibatae*, as well as *S. solfataricus*, the viral genome was generally found to be integrated into a specific tRNA^{Arg} gene of the host chromosome, indicating that site-specific integration is involved in establishing the lysogenic state (Schleper et al. 1992). The pRN family of small plasmids from the Sulfolobales includes pSSVx from *S. islandicus* REY 15/4, a hybrid of a plasmid and a fusellovirus. In the presence of the helper virus, SSV2, it can spread as a virus satellite (Arnold et al. 1999). Most conjugative plasmids have been isolated from a collection of *S. islandicus* strains. As well as spreading through *S. islandicus* cultures, these plasmids have also been reported to be capable of replicating in *S. solfataricus* (e.g., pNOB, pING, pSOG; Prangishvili et al. 1998; Stedman et al. 2000). In addition, exchange of marker genes has been well documented for *S. acidocaldarius* (Schmidt et al. 1999; Reily and Grogan 2001), suggesting a high frequency of recombination, or Hfr-like, system as present in some conjugative bacteria. In line with this, a 30 kb fragment of the *S. tokodaii* chromosome strongly resembles conjugative plasmids like pSOG (Erauso, unpublished); the recently completed *S. acidocaldarius* genome sequence is expected to reveal similar features.
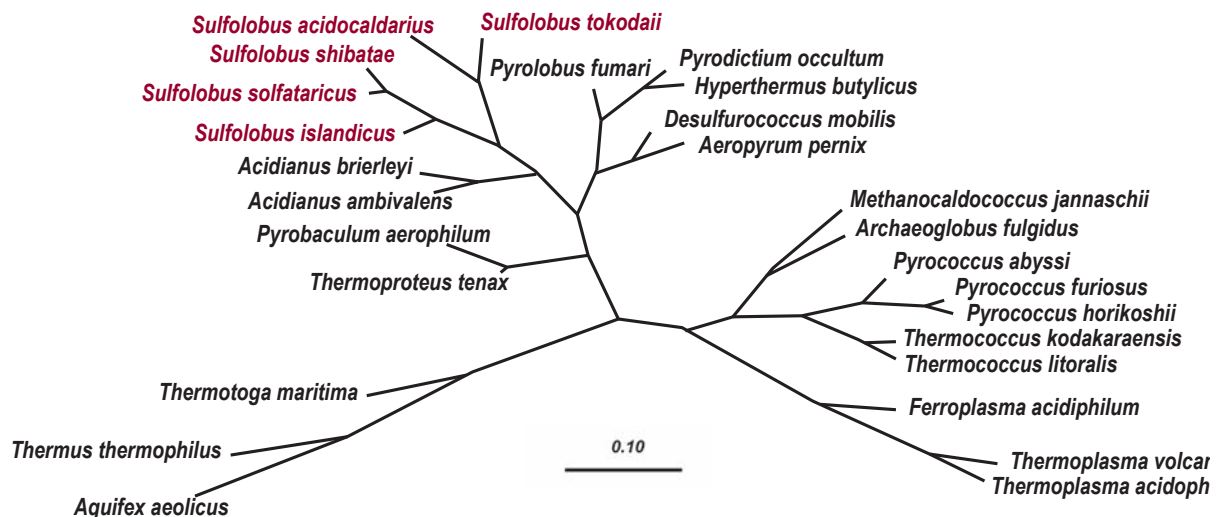
↑ **Figure 1B. Phylogenetic tree based on archaeal and bacterial 16S rRNA sequences**. Alignment and phylogenetic analysis were performed with the ARB software (Ludwig et al. 2004), and the tree was constructed using the neighbour-joining method (Saitou et al. 1987). The reference bar indicates the branch length that represents 10% diversity.

2.3 Post-genomics challenges

After the genome sequence of *S. solfataricus* was established, comparative genomics analyses were performed to predict relevant physiological functions for many genes (She et al. 2001). As in all studied genomes, many hypothetical genes were found for which a function could not reliably be predicted *in silico*. Hence, the main challenge of the post-genome era is to integrate classical approaches (physiology, biochemistry, and molecular genetics) with genomics-based, high-throughput approaches (comparative, functional, and structural genomics). In the case of *Sulfolobus* genomes the obvious goals are to (i) identify missing links in central metabolic pathways (degradation and biosynthesis of carbohydrates hexose and pentose, amino acids, nucleotides, and vitamins); (ii) elucidate functions of hypothetical proteins (e.g., those conserved in archaea and/or eukarya); and (iii) unravel global regulatory circuits, such as the control of RNA and protein turnover (transcription/exosome, translation/proteasome).

We present here an overview of currently ongoing developments of genetic tools and of high-throughput functional genomics technologies for *Sulfolobus*. These developments could offer significant insight into the cell biology of the model archaeon *Sulfolobus* in the near future.

3.0 MATERIALS AND METHODS

3.1 Bioinformatics

Using the completed genome of *S. solfataricus*, standard bioinformatics tools have been applied for identification of the enzymes that constitute its central metabolic pathways. An example is described in some detail below—the unravelling of the genes that encode key enzymes of the glycolytic pathway. Relevant websites include: BLAST and COG at the National Center for Biotechnology Information (**http://www.ncbi.nlm.nih.gov/**); context analysis using STRING (**http://string.embl.de**); and pathway analysis at Kyoto Encyclopedia of Genes and Genomes. (**http://www.genome.ad.jp/kegg/**).
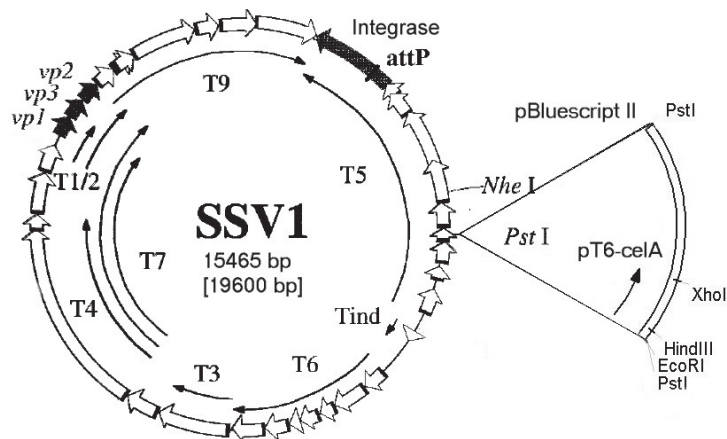
⬆ **Figure 2.** Map of plasmid pSBS04, a derivative of pKMSD48 (see Table 1), for the homologous production of CelA2 cellulase from *S. solfataricus*.

3.2 Molecular Genetics

All molecular biology procedures were performed using standard protocols (Sambrook et al. 1989). *Escherichia coli* XL1-Blue and XL10-Gold chemically competent cells (Stratagene) were used in all cloning steps. PCR reactions were performed with *Pfu TURBO®* polymerase (Stratagene).

A cellulase expression cassette was constructed by PCR amplification of ORF Sso1949 from *Sulfolobus solfataricus* P2 genomic DNA using primers 5'-ATGAAT<u>AAGCTT</u>TATATTGTGCTTCCGGTAA-3' (sense, *Hin*dIII site underlined) and 5'-AAGGTC<u>CTCGAG</u>TCTTATATTGTTTAGAGG-3' (anti-sense, *Xho*I site underlined). The T6 promoter from SSV1, that gives rise to high rates of *in vitro* transcription (Bell et al. 1999), was chosen to drive efficient transcription of the cellulase gene. A 203 bp fragment was PCR-amplified using primer pair 5'-TA T<u>GAATTC</u>ATCTATTTGTTTTGCT-3' (sense, *Eco*RI site underlined) and 5'-GGTTTG<u>AAGCTT</u>ATTCAT TTTTTGAACCCTCTA-3' (anti-sense, *Hin*dIII site underlined) from SSV1 DNA. Both PCR products were digested with *Hin*dIII, ligated and PCR amplified with flanking primers, and cloned into *Eco*RI and *Xho*I digested pBluescriptII KS+ (Stratagene). The resulting plasmid was linearized with *Pst*I, treated with alkaline phosphatase, and cloned into a unique *Pst*I site at position 3953 in the

Table 1. Overview of published genetic tools available for the genus *Sulfolobus*

Vector name (size)	Selectable property / phenotypic marker	Archaeal replicon	*E. coli* replicon	Hosts	Features	References
pCSV1 (6.11 kb)	none	pGT5	pUC19	*P. furiosus* *S. acidocaldarius*	low copy number	Aagaard 1996
pAG21 (6.5 kb)	*Sso adh*, butanol and benzylalcohol tolerance	pGT5	pBR322	*P. furiosus* *S. acidocaldarius*	low copy number	Aravalli 1997
pBS-KK (48 kb)	none (conjugation) blue/white screening	pNOB8	pBluescript	*S. solfataricus* P1 derivative PH1 *lacS*⁻	Severe growth retardation, recombination into host genome	Elferink 1996
pEXSs (6.35 kb)	*hph*$_{mut}$ Hygromycin^R	SSV1ori (4938-6617)	pGEM5Zf(-)	*S. solfataricus* Gθ derivative GθW Δ*lacS*	low copy number sometimes high background	Cannio 1998 Cannio 2001 Contursi 2002 Bartolucci 2003
pKMSD48 (18.5 kb)	none (self spreading) plaque assay screening	SSV1	pBluescriptII	*S. solfataricus* P1, P2 *S. solfataricus* Gθ	Single integration site and present as plasmid copy (5-40 copies/cell)	Stedman 1999
pMJ03 (21.8 kb)	*pyrEF*, self spreading Uracil auxotrophy complementation blue/white screening	SSV1	pBluescriptII	*S. solfataricus* P1 derivatives PH1 *lacS*⁻ *pyrEF*⁻, *lacS*⁻	Single integration site and present as plasmid copy with varying copy number	Martusewitch 2000 Jonusheit 2003
Knockout vector	*lacS* Lactose utilization complementation	none	pNEB193, pUC19	*S.solfataricus* 98-2 derivatives *lacS*⁻, Δ*lacS* PBL2002, PBL2025	Homologous recombination suicide vector	Worthington 2003 Schelert 2004

SSV1 genome (**Figure 2**). Restriction analysis of this 19.6-kb shuttle vector, designated pSBS04, revealed that the orientation of the expression cassette corresponded to that of the T5 transcript (Reiter et al. 1987; **Figure 2**). pSBS04 was transformed to *S. solfataricus* P1 by electroporation as previously described (Schleper et al. 1992).

3.3 Biochemistry

S. solfataricus P1 cells were grown aerobically in medium 182, as recommended by the German Culture Collection (DSMZ), supplemented with 0.1% yeast extract and 0.2% D-glucose. At stationary phase (OD$_{600}$ ~ 1.2), the cells were spun down (10 min, 5000 rpm) and the supernatants filtered (0.22 µm). Next, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.5 mM to inactivate serine proteases. As such, cellulase activity was preserved for several months at 4°C. Attempts to concentrate activity using 10 kDa non-cellulose-containing membranes (Pall) or S-sepharose cation exchange chromatography (Pharmacia) were unsuccessful. Activity assays were performed in 50 mM citric acid buffer (pH 3.5) supplemented with 5 g/L carboxymethylcellulose (CMC). The reaction was initiated by addition of 50 µL of supernatant to 700 µL of preheated assay buffer at 78°C. After 20 minutes, the mixture was rapidly cooled on ice and neutralized by the addition of 35 µL of 0.5 M NaOH. As an internal control, 15 µL of 25 mM D-glucose was then added, before reducing sugar analysis

using 3,5-dinitrosalicylic acid, or DNS (Miller 1959). All measurements were performed in duplicate. Values were corrected for spontaneous CMC hydrolysis and remaining D-glucose concentrations in each supernatant. One unit of CM-cellulase activity was defined as the amount of the enzyme that liberated one μmole of reducing sugar per minute.

4.0 RESULTS AND DISCUSSION

4.1 Vector Development for *Sulfolobus solfataricus*

One primary reason why *S. solfataricus* has become a model archaeon has been its ability to grow aerobically on a wide variety of heterotrophic substrates like peptides and sugars, in both rich and chemically defined media, in liquid media, as well as on plates (Grogan 1989). Moreover, *Sulfolobus* isolates from all over the world appeared to possess a wide array of extrachromosomal genetic elements (Zillig et al. 1998; Rice et al. 2001). These findings have encouraged researchers to develop tools for genetic manipulation.

Currently available genetic tools for *Sulfolobus* are summarized in **Table 1**. Many attempts have been made to generate shuttle vectors that replicate stably in both *E. coli* and *Sulfolobus,* while maintaining a relatively high copy number in both organisms. Hybrid vectors were constructed containing the replicase gene from euryarchaeal rolling circle plasmid pGT5 (Erauso et al. 1996), which could be transformed to *S. acidocaldarius* by electroporation, and maintained by applying butanol or benzyl alcohol tolerance selection (Aagaard et al. 1996; Aravalli and Garrett 1997). In the absence of selectable markers, the highly efficient self-spreading capabilities of conjugative plasmid pNOB8 (Schleper et al. 1995) and *S. shibatae* fusellovirus SSV1 (Reiter et al. 1987) were exploited to generate a population in which the majority of the cells contained the recombinant plasmid (Elferink et al. 1996; Stedman et al. 1999). A thermostable hygromycin phosphotransferase mutant gene enabled selection of *S. solfataricus* Gθ transformants containing plasmid pEXSs, a small shuttle vector replicating from a 1.7 kb SSV1 origin (Cannio et al. 1998, 2001). Most success, however, has been obtained by using β-galactosidase (*lacS*) deficient strains derived from spontaneous insertion element

disruption (Schleper et al. 1994; Worthington et al. 2003) or large chromosomal deletions at this locus (Bartolucci et al. 2003; Schelert et al. 2004). Phenotypically, β-galactosidase complementation allows blue/white activity screening using chromogenic compound 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-β-Gal), but has in some cases allowed selection for lactose utilization as a sole carbon and energy source (Bartolucci et al. 2003; Worthington et al. 2003).

The latter property has led to a successful homologous recombination system for *S. solfataricus* strain 98-2, in which a specific gene can be disrupted by a suicide vector (Worthington et al. 2003; Schelert et al. 2004). For the first time in hyperthermophilic crenarchaeota, this system has enabled researchers to construct knock-out mutants and to study gene function *in vivo*. Significant progress has also been made with the construction of viral shuttle vector pMJ03. This SSV1-derived vector, which includes *lacS* for blue/white screening, also contains genes coding for orotidine-5'-monophosphate pyrophosphorylase (*pyrE*) and orotidine-5'-monophosphate decarboxylase (*pyrF*), which serve as a selectable marker complementing uracil auxotrophic *Sulfolobus* strains (Martusewitsch et al. 2000; Jonuscheit et al. 2003). This selection marker appears to be crucial for the stability of the shuttle vector; without selective pressure, rearrangement of the shuttle vector was frequently observed during prolonged cultivation (Jonuscheit et al. 2003).

4.2 Thermoacidophile Cell Factory

4.2.1 Cellulase production. Apart from fundamental studies like the identification of physiological functions, *Sulfolobus* vectors can also be employed for biotechnological purposes such as the overproduction of enzymes. Industrially relevant enzymes include extracellular proteases and polysaccharide hydrolases, such as glucoamylases, cellulases, mannanases and xylanases; however, it appeared to be difficult to produce this class of enzymes from *Sulfolobus* in common production hosts like *E. coli*, *Bacillus subtilis*, *Lactococcus lactis*, *Pichia pastoris*, and *Aspergillus niger*. In part this might be due to the fact that these enzymes are only capable of folding correctly at very low pH, as encountered in *Sulfolobus*' natural

extracellular environment. For that reason, it was decided to use an SSV1 based *E. coli/S. solfataricus* shuttle vector (Stedman et al. 1999) to overproduce one of three putative cellulases (Sso1949, CelA2) encoded in the *S. solfataricus* P2 genome. Based on sequence similarity they belong to family 12 of glycoside hydrolases (Henrissat 1991). Sequence comparison reveals that Sso1354 (CelA1) and Sso1949 (CelA2) are highly similar (91%) at the amino acid level (**Figure 3A, next page**). Genomic context analysis revealed a recent duplication event leading to both paralogous sequences (6-kb fragment containing eight genes: including a cellulase, an unidentified glycoside hydrolase, an N-acyl-L-amino acid amido hydrolase, and a dolichol-phosphate mannosyltransferase; **Figure 3B**). CelA1 and CelA2 share 46% sequence similarity to PF0854 (EglA), an extracellular cellulase from the euryarchaeon *Pyrococcus furiosus* (Bauer et al. 1999), and roughly 35% to TM1524 (TmCelA) and TM1525 (TmCelB) derived from the hyperthermophilic bacterium *Thermotoga maritima* (Liebl et al. 1996). Interestingly, the latter two were found in an operon as a pair of cellulases of which only one (TmCelB) contained a signal sequence for membrane translocation. A third cellulase gene from *Sulfolobus*, Sso2534 (CelS), is more distinct from CelA1 and CelA2 (37% similar). Transcripts of the celS gene were recently found in *S. solfataricus* MT4, along with corresponding cellulase activity in culture supernatants (Limauro et al. 2001). All archaeal cellulase sequences appear to contain more or less conserved signal sequences, followed by a linker region that is rich in serine and threonine residues. These residues are most likely O-glycosylated *in vivo*, similar to S-layer-bound proteins from *Sulfolobus* (Elferink et al. 2001). This indicates that they could be partially bound to the outer surface layer of the cell. Despite this, we could easily detect cellulase activity in supernatants of *S. solfataricus* P1 cultures grown on 0.1% yeast extract and 0.2% sucrose (data not shown). In *S. solfataricus* P1 transformants containing the cellulase overexpression vector, pSBS04 (**Figure 2**), a 25% increase in cellulase activity was detected. Upon changing the media from sucrose to glucose we observed a dramatic decrease in background cellulase activity (**Figure 3C**). The diagram clearly distinguishes cellulase activity of the expression construct driven by a viral promoter from the wild type control and empty vector. Apparently, endogenous cellulase production is subject to catabolite repression, as has been suggested previously (Haseltine et al. 1996; Limauro et al. 2001).

This study clearly shows the usefulness of vectors for hyperthermophiles in the post-genomic era. Several research groups are continuing their efforts to construct convenient genetic systems for hyperthermophilic archaea including: (i) small, stable, high-copy number shuttle vectors; (ii) strong, thermostable selection markers; and (iii) inducible promoters.

4.3 Bioinformatics & Biochemistry

4.3.1 Prediction and verification of glycolytic pathway.
Archaea have been shown to have a highly flexible metabolism (Schönheit and Schäfer 1995). In this respect, the best documented case concerns the high variability of the archaeal glycolytic pathways (reviewed by Verhees et al. 2003). The glycolytic pathway in the genus *Sulfolobus* has originally been described as a "non-phosphorylating" variant of the Entner-Doudoroff (ED) pathway (De Rosa et al. 1984). The key enzyme of this pathway, the KDG aldolase, acts on the non-phosphorylated intermediate 2-keto-3-deoxy-gluconate (KDG) and has been studied in detail (Buchanan et al. 1999). Recently, it has been shown that the KDG-aldolase displays broader substrate specificity, since the enzyme also was found to display activity towards 2-keto-3-deoxy-galactonate (KDGal). Apparently, the lack of facial selectivity in the aldolase-catalyzed reaction is unusual and permits the same enzyme to be used for the cleavage of KDG and KDGal, yielding both glyceraldehyde and pyruvate. (Lamble et al. 2003).

In an attempt to identify missing links of the *Sulfolobus* glycolysis, we have used a comparative genomic approach to analyze the conserved functional organization of genes anticipated to be involved in the ED pathways. This approach resulted in the detection of a glycolytic ED gene cluster that is present in both *S. solfataricus* and *S. tokodaii* and partially conserved in *Halobacterium* sp. and *Thermoproteus tenax*—two other archaea that have been shown to utilize a modified ED pathway (**Figure 4**).

A
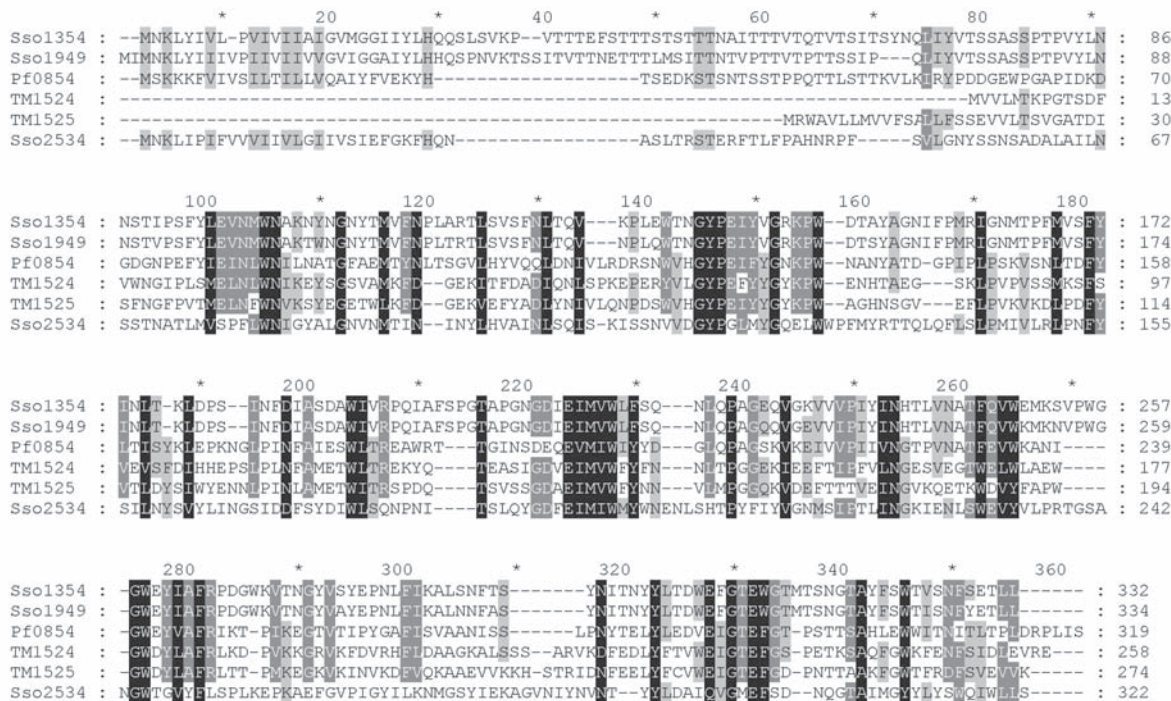
↑ **Figure 3A.** **Alignment showing closest homologues as found by BLASTP analysis using Sso1949 (CelA2) in all completed genomes.**
Sso1354: CelA1; Sso1949: CelA2; PF0854: EglA (Bauer et al. 1999); TM1524: CelA intracellular cellulase; TM1525: CelB extracellular
cellulase (Liebl et al. 1996); Sso2534: CelS (Limauro et al. 2001). Alignment was created by Clustal X 1.81 using a BLOSUM62
substitution matrix.

B
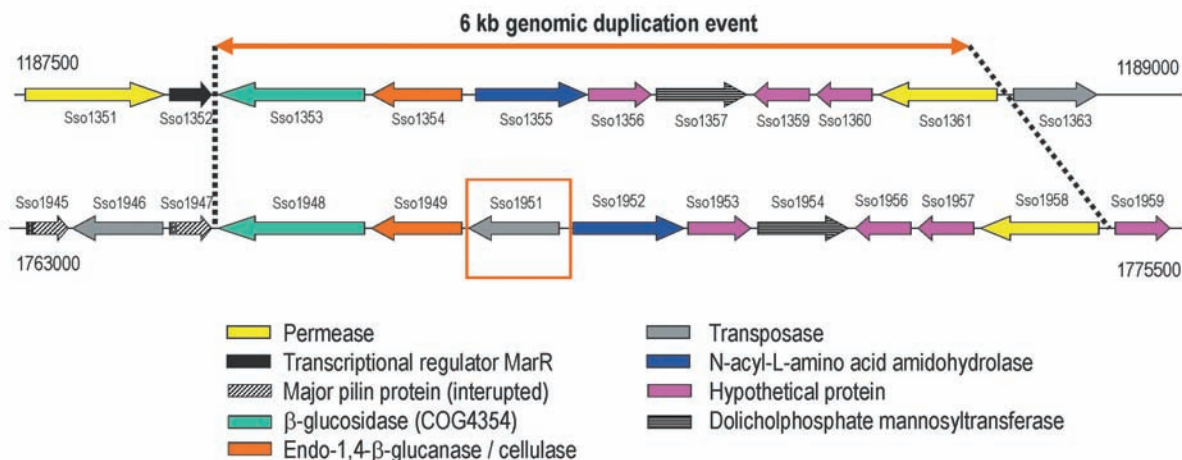
↑ **Figure 3B.** Cellulase gene cluster organization, evidence for a 6-kb genomic duplication event. (See also Figure 3C at right)
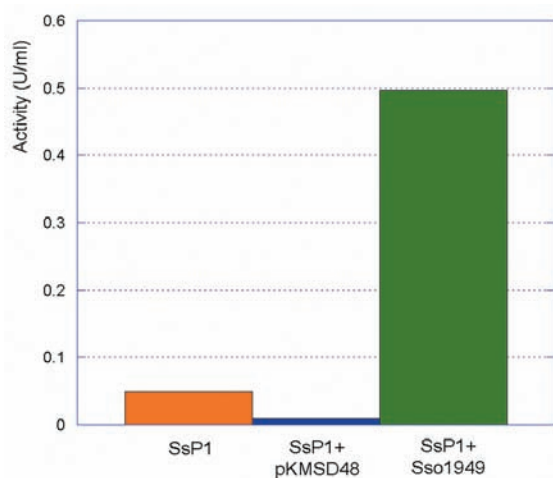
↑ **Figure 3C. Cellulase activity profiles in supernatants of wild type and recombinant *Sulfolobus solfataricus* P1 clones.** Activities were measured at 78°C using CMC as a substrate and DNS for detection of reducing sugars. *S. solfataricus* P1+pKMSD48: empty SSV1-pBluescriptII shuttle vector transformant (Stedman et al. 1999); *S. solfataricus* P1+Sso1949: SSV1-pBluescriptII shuttle vector with a SSV1 T6 promoter-CelA2 expression cassette.

Apart from the characterized KDG aldolase, the *Sulfolobus* operon contained genes predicted to encode a sugar kinase (COG0524); a GAPN-like glyceraldehyde-3-phosphate dehydrogenase (COG1012; Brunner et al. 1998); and a protein that belongs to the mandelate racemase/muconate lactonizing enzyme family, a diverse protein family that includes several types of dehydratases (COG4948; Babbitt et al. 1995). BLAST analysis revealed that the latter gene resembles the well-characterized DgoA protein, the galactonate dehydratase from *E. coli*. This suggests that the *Sulfolobus* gene might encode a dehydratase that, because of its genomic context, is anticipated to be more related to the modified ED pathway. The conserved clustering of the kinases and GAPN with the KDG

aldolase was surprising, since glycolysis in *Sulfolobus* has been reported to proceed via non-phosphorylated intermediates at the C6-level. Only at the C3-level has the glyceraldehyde been suggested to be phosphorylated to phosphoglycerate.

All four genes from the *Sulfolobus* cluster as well as the counterparts from *T. tenax* (**Figure 4**) have been functionally expressed in *E. coli* using the T7 expression system. The subsequent biochemical characterization indicates that the *Sulfolobus* cluster encodes (i) a gluconate dehydratase; (ii) a KDG kinase; (iii) a broad-specificity enzyme catalyzing the aldol-cleavage of KD(P)Glu (Ahmed et al., in press); and (iv) a NADP+-dependent glyceraldehyde-3-phosphate dehydrogenase. Altogether, this strongly suggests that the ED pathway in archaea is "semi-phosphorylated" as has first been described for *Halobacterium* (reviewed by Verhees et al. 2003). Ongoing functional genomics analysis (see below) is expected to address the remaining uncertainties concerning the archaeal central metabolic pathways, including glycolysis and gluconeogenesis, as well as metabolism of pentoses, amino acids, vitamins, and nucleotides.

4.4 Proteome and Transcriptome Analysis

Traditionally, two-dimensional gel electrophoresis (2-DGE) for separation of complex mixtures of proteins has been the preferred method for proteomics. This technique separates proteins orthogonally, on the basis of their charge and size. Proteins on a 2-D gel can be visualized and quantified using Coomassie blue, silver, or fluorescent stains (**Figure 5A**). Proteins are extracted from individual
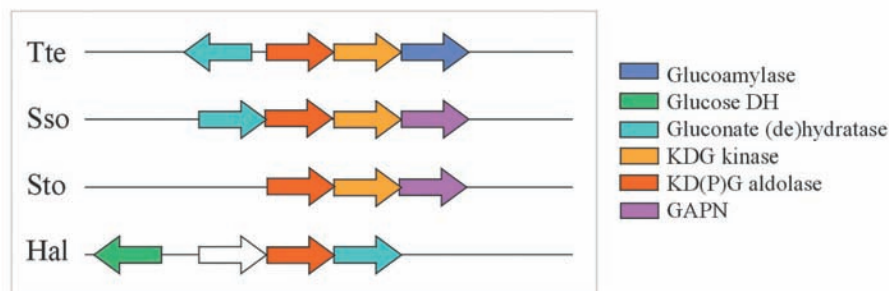
↑ **Figure 4.** Gene clusters encoding glycolytic enzymes of the variant ED pathway.

spots and trypsin digested before the actual analysis. Mass spectrometry has become the preferred method for protein identification, since the introduction of soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) in the 1990s, especially when operated in the tandem mode (Aebersold and Mann 2003). In this mode, peptides with a particular mass are selected for fragmentation in the second mass spectrometry (MS) stage. In this way, the intact mass and the sequence information generated in the second stage can be combined for database searching. Generated mass spectra can be matched to the annotated genome. Thus, each expressed protein can be correlated to an open reading frame (ORF) in the genome. In a recent study (Snijders et al., submitted), crude cell extracts of *S. solfataricus* grown on glucose were analyzed using 2-DGE. Subsequently, proteins corresponding to 349 ORFs were identified using LC-ESI-MS/MS and database searching (Mass Spectrometry protein sequence DataBase, or MSDB: (**http://csc-fserve.hh.med.ic.ac.uk/msdb.html**). Among them was a complete set of tricarboxylic acid (TCA) cycle proteins, glycolytic proteins, and a range of proteins involved in amino acid, cofactor, and lipid metabolism.

In recent years, protein and peptide separation on the basis of high-performance liquid chromatography (HPLC) and capillary HPLC (CHPLC) has become increasingly popular. This approach provides a powerful tool for high-throughput protein separation and identification, especially when coupled online with ESI tandem mass spectrometry (Link et al. 1999). Quantitation in LC-based proteomics relies on the introduction of stable isotopes. This is usually achieved by the introduction of ^{13}C or ^{15}N species in the medium (metabolic labeling), or by *in vivo* coupling of labeled linkers (e.g., ICAT; Washburn et al. 2002). This is currently being implemented in the ongoing *Sulfolobus* project.

Whole-genome DNA microarray analysis is a recently developed tool for high-throughput detection of gene expression at the RNA level. Based on the genome sequences of *S. solfataricus* (2.99 Mbp; She et al. 2001) and *S. acidocaldarius* (2.2 Mbp; R. Garrett, personal

↑ **Figure 5 A**. Example of 2D gel of S. solfataricus P2 cell free extract. **B**. Dual whole-genome DNA microarray of *S. solfataricus* and *S. acidocaldarius* on which cDNA of *S. solfataricus* has been hybridised.

communication), internal fragments of genes (~100-800 bp) were PCR-amplified, checked by agarose gel electrophoresis, and linked to glass slides (Andersson et al. 2002). At present, a total of approximately 4400 genes from the two species have been printed in triplicate on the arrays (**Figure 5B**), and high sensitivity and resolution has been demonstrated in a series of pilot experiments. The microarray system is currently being used in a variety of applications, including mapping of replication origins, analysis of cell-cycle-specific gene expression, global mRNA half-life determinations, and delineation of metabolic pathways.

5.0 OUTLOOK: INTEGRATED APPROACH IN FUNDAMENTAL AND APPLIED RESEARCH

The intention of this overview of *Sulfolobus* research is to describe ongoing developments at different levels. Obviously, *Sulfolobus* would not have gained the status of "model archaeon" without a long list of classical studies (physiology, biochemistry, and molecular genetics) that have been performed over the last three decades in

numerous research laboratories. At present, important developments in biochemical and molecular genetics analyses are under way, allowing novel approaches for analysis *in vitro* (heterologous expression, directed and random mutagenesis, study of protein/protein and protein/DNA interactions), and *in vivo* (chromosomal knockouts, *in trans* over-expression, phenotype characterization). Classical research has provided the basis on which modern genomics-related approaches have been built (comparative, functional, and structural genomics). Now the stage is set for yet another challenge in the study of archaea, i.e. the integration of classical and modern technologies to acquire knowledge in the functioning of (i) metabolic networks: a mosaic of bacterial, eukaryal, and unique archaeal features; (ii) information processing, generally consisting of the core of eukaryal complexes; and (iii) the regulatory circuits of these systems. Many breakthroughs are expected in the near future, resulting in a gain of insight into the evolution and functioning of archaeal-like key systems in Eukaryotes. Moreover, understanding essential details of the archaeal cell will contribute to its future application as an industrial "cell factory."

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