Extragenic Pleiotropic Mutations That Repress Glycosyl Hydrolase Expression in the Hyperthermophilic Archaeon *Sulfolobus solfataricus*

Cynthia Haseltine, Rafael Montalvo-Rodriguez, Audrey Carl, Elisabetta Bini and Paul Blum

George Beadle Center for Genetics, School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588-0666

Manuscript received March 14, 1999
Accepted for publication May 13, 1999

ABSTRACT

The hyperthermophilic archaeon *Sulfolobus solfataricus* employs a catabolite repression-like regulatory system to control enzymes involved in carbon and energy metabolism. To better understand the basis of this system, spontaneous glycosyl hydrolase mutants were isolated using a genetic screen for mutations, which reduced expression of the *lacS* gene. The specific activities of three glycosyl hydrolases, including an α-glucosidase (*malA*), a β-glycosidase (*lacS*), and the major secreted α-amylase, were measured in the mutant strains using enzyme activity assays, Western blot analysis, and Northern blot analysis. On the basis of these results the mutants were divided into two classes. Group I mutants exhibited a pleiotropic defect in glycosyl hydrolase expression, while a single group II mutant was altered only in *lacS* expression. PCR, Southern blot analysis, comparative heterologous expression in *Escherichia coli*, and DNA sequence analysis excluded *cis*-acting mutations as the explanation for reduced *lacS* expression in group I mutants. In contrast *lacS* and flanking sequences were deleted in the group II mutant. Revertants were isolated from group I mutants using a *lacS*-specific screen and selection. These revertants were pleiotropic and restored glycosyl hydrolase activity either partially or completely to wild-type levels as indicated by enzyme assays and Western blots. The *lacS* mutation in the group II mutant, however, was nonrevertible. The existence of group I mutants and their revertants reveals the presence of a *trans*-acting transcriptional regulatory system for glycosyl hydrolase expression.

SMALL subunit (16S) ribosomal RNA sequence comparisons have identified a unique lineage or grouping of prokaryotic organisms called archaea (Woese *et al.* 1990). They are as distantly related to bacterial prokaryotes as they are to eukaryotes and the three groups have therefore been assigned to distinct phylogenetic taxons termed domains (Woese *et al.* 1990). Members of the archaeal domain, particularly the crenarchaeal subdivision, dominate acidic hot springs and both aerobic and anaerobic forms can be readily cultured from these environments. Many of these organisms reside within the order Sulfolobales, which includes the genus Sulfolobus (Brock *et al.* 1972; DeRosa *et al.* 1975). Members of this genus are obligate aerobes, which conduct both lithoautotrophic (Brock *et al.* 1972; Kandler and Stetter 1981) and chemoheterotrophic metabolism (DeRosa *et al.* 1975; Grogan 1989). As these organisms can be readily cultured aerobically in liquid and solid media, genetic studies have begun in *Sulfolobus acidocaldarius* (Grogan 1996; Jacobs and Grogan 1997), and several plasmid-based vectors have been developed (Elferink *et al.* 1996; Arvalli and Garret 1997; Cannio *et al.* 1998). *S. solfataricus* is also the subject of a genome project (Sensen *et al.* 1998). The combination of genetic methodologies and genomics strategies holds strong promise for the ability to conduct functional genomics in this member of the archaea.

Bacterial prokaryotes and eukaryotes employ transcriptional regulatory mechanisms to coordinate expression of genes involved in carbohydrate utilization. These systems of genes generally are subject to a process termed the glucose effect of which catabolite repression (CR) is one of the key components and results from the fact that carbon substrates, including their metabolic intermediates, attenuate gene expression (Magasanik and Neidhardt 1987). Among the gram-negative bacteria, coordination of gene expression entails the action of cAMP and catabolite repressing protein (CRP) acting to balance availability of low- and high-quality carbon resources (Saier *et al.* 1996). In certain gram-positive bacteria, cAMP and CRP are absent and CcpA, a negative-acting transcription factor, is used to affect promoter activity of target genes (Henkin *et al.* 1991; Deutscher *et al.* 1995). Coordinated expression in eukaryotes of genes involved in carbon catabolism also is accomplished by *trans*-acting transcription factors including the *Aspergillus nidulans* protein CREA and the human protein CREB (Cubero and Scazzocchio 1994; Roesler *et al.* 1995). In all of these organisms CR is mediated at the level of transcription initiation.

In *S. solfataricus*, starch utilization necessitates the inducible synthesis and secretion of a highly stable α-amylase (Haseltine *et al.* 1996); however, the gene for this α-amylase is as yet undescribed. The resulting

Corresponding author: Paul Blum, E234 Beadle Ctr., University of Nebraska, Lincoln, NE 68588-0666. E-mail: pblum@biocomp.unl.edu

hydrolytic products, including dextrins and maltodextrins, are further hydrolyzed by the action of a cell-associated α-glucosidase encoded by *malA* (Rolfsmeier and Blum 1995). Expression of *malA* is modestly affected by carbon source type while levels of the α-amylase are strongly influenced (Haseltine *et al.* 1996; Rolfsmeier *et al.* 1998). The variation in levels of these glycosyl hydrolases, which occurs in response to carbon source type, represents one of the hallmarks of CR (Magasanik and Neidhardt 1987). Similar observations in *S. solfataricus* led to the proposal of the existence of an analogous system (Haseltine *et al.* 1996). It is unknown whether the key features that distinguish bacterial and eukaryotic CR systems, such as global transcriptional gene regulation, signal molecules, or *trans*-acting regulatory factors, are present in the archaea. If, in fact, the catabolite repression-like system of *S. solfataricus* operates at the transcriptional level, the mechanism must accommodate distinctly eukaryotic-type transcription components. These include conserved promoter sequences (Reiter *et al.* 1990; Hain *et al.* 1992), TATA binding protein (TBP) homologs (Marsh *et al.* 1994; Rowlands *et al.* 1994; Qureshi *et al.* 1995), TFIIB homologs (Gohl *et al.* 1995; Qureshi *et al.* 1995; Qureshi and Jackson 1998), and an RNA Polymerase II homolog (Klenk *et al.* 1992).

Isolation and characterization of *Escherichia coli* CR regulatory mutants greatly clarified the CR mechanism in this organism. Such mutants exhibited a pleiotropic defect in CR gene expression where the affected genes had a reduced or uninducible level of expression. The CR regulatory mutations, in fact, occurred in *cya*, encoding adenylate cyclase, and *crp*, encoding the catabolite repression protein and clearly established how coordinate gene expression was accomplished (Magasanik and Neidhardt 1987; Saier *et al.* 1996). The *S. solfataricus lacS* gene encodes a β-glycosidase with broad substrate specificity including activity against β-galactosides and their chemical analogs such as the colorimetric indicator 5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside (X-gal; Cubellis *et al.* 1990; Grogan 1991). Like *E. coli*, *S. solfataricus* forms blue colonies upon exposure to X-gal. Disruption of *lacS* by insertion element (IS) transposition results in the formation of colorless colonies on X-gal (Schleper *et al.* 1994). To better understand the catabolite repression-like response of *S. solfataricus*, mutants that had reduced expression of *lacS* were isolated, characterized, and subjected to reversion analysis in an effort to determine if this organism coordinates glycosyl hydrolase gene expression.

MATERIALS AND METHODS

Archaeal strains and cultivation: *S. solfataricus* strain 98/2 (Rolfsmeier and Blum 1995) was grown at 80° at pH 3.0 as described previously (Rolfsmeier and Blum 1995). The medium used contained 20 mm ammonium sulfate, 4 mm dibasic potassium phosphate, 4 mm magnesium sulfate, 1 mm calcium chloride, 0.2 mm iron chloride, 18 mm manganese chloride, 0.02 mm sodium borohydride, 1.5 μm zinc sulfate, 0.74 μm copper chloride, 0.25 μm sodium molybdate, 0.37 μm vanadium sulfate, and 0.13 μm colbalt sulfate (basal salts). Sucrose was added at a final concentration of 0.2% (w/v) and yeast extract was added at a final concentration of 0.1% (w/v). Lactose and starch were used at final concentrations of 0.2% (w/v). Unless otherwise indicated, cells were grown in sucrose minimal medium with added yeast extract. Growth in liquid culture was monitored spectrophotometrically at a wavelength of 540 nm. A solid medium was prepared using 0.6% (w/v) gelrite (Kelco), and basal salts containing either 0.2% (w/v) tryptone or 0.1% (w/v) glucose, 0.1% (w/v) casamino acids and 0.1% (w/v) yeast extract, adjusted to pH 3.0 with sulfuric acid. Magnesium chloride was added at a final concentration of 8.0 mm to solidify the medium. Plates were incubated at 80° in plastic containers with sufficient hydration to prevent dessication. Growth was monitored daily and extra water was drained from the plates. Colonies reached a diameter of 2 mm in 6 days. The efficiency of plating was typically 10%. A solution of X-gal was prepared at a 10 mg/ml concentration in dimethyl formamide. The solution was applied to colonies as an aerosol consisting of ~0.25 ml per plate. Treated colonies were incubated at 80° to allow for development of blue color. Colony color was scored after overnight incubation.

Molecular biology methods: Restriction digestion and ligation of DNA were performed as described previously (Blum *et al.* 1992). Plasmid transformation was performed using DH5α cells as described (Hanahan 1983). Isolation of plasmid DNA was performed by the alkalai lysis procedure (Birnboim and Doly 1979). DNA sequence analysis was as described (Rockabrand and Blum 1995) and DNA alignment and analysis was performed using the fragment assembly programs of the Wisconsin Genetics Computer Group software package version 9.0. All manipulations of *E. coli* were as described previously (Rockabrand *et al.* 1995). PCR was performed using Pfu DNA polymerase (Stratagene, La Jolla, CA) under conditions suggested by the manufacturer and PCR amplicons were resolved by chromatography in agarose gels.

Enzyme assays: Assays for the α-glucosidase (*malA*) and the β-glycosidase (*lacS*) used cell extracts prepared by sonicating cells resuspended in 100 mm sodium acetate, pH 4.5, or 10 mm Tris hydrochloride, pH 7.0, respectively. The hydrolysis of *p*-nitrophenyl-α-glucopyranoside (α-PNPG) was used to measure the α-glucosidase (*malA*) as described previously (Rolfsmeier and Blum 1995). Hydrolysis of *p*-nitrophenyl-β-d-glucopyranoside (β-PNPG) was used to measure the β-glycosidase (*lacS*) using the identical procedure as employed for the α-glucosidase (*malA*). A unit of activity is defined as the amount of enzyme required to liberate 1 μmol PNP/min/mg protein. Measurement of secreted α-amylase enzyme activities was determined as described (Haseltine *et al.* 1996) using cell-free culture supernatants concentrated by ultrafiltration and diafiltered as necessary. The α-amylase activity assay determines the production of sugar-reducing ends (Park and Johnson 1949) and was performed by adjusting the samples to 25 mm sodium acetate, pH 3.0, 0.5 mm calcium chloride, and 0.5% (w/v) starch and incubating for 30 min at 80°. The reaction was terminated by addition of sodium carbonate to a final concentration of 0.04 m. The sample was then adjusted to 15.4 mm potassium cyanide, 0.24 mm potassium ferricyanide, and 72.3 mm sodium carbonate and heated at 100° for 10 min. One unit of activity is defined as the amount of enzyme that produced 1 nmol of reducing ends in 1 min. Glucose was used as a reducing end standard. All samples were assayed in duplicate and the averages of the sample results are reported.

Protein purification and antibody production: Recombinant enzyme purification used transformants of *E. coli* strain DH5α (GIBCO-BRL, Gaithersburg, MD) harboring either the *malA* (α-glucosidase) expression plasmid pBN56 (Rolfsmeier *et al.* 1998) or the *lacS* (β-glycosidase) expression plasmid pBN55. Preparation of cell mass for recombinant enzyme purification was as described (Rolfsmeier *et al.* 1998). Cells were harvested by centrifugation, resuspended in 30 mm morpholine propanesulfonic acid, pH 8.0 (MOPS buffer), and lysed by sonication at 4°. The resulting lysates were clarified by centrifugation (3000 × *g* for 30 min) and then heated at 85° for 30 min and clarified by centrifugation two successive times. The heat-treated supernatants were concentrated by ultrafiltration and applied to a Mono Q FPLC column (Pharmacia, Piscataway, NJ) previously equilibrated with MOPS buffer. The recombinant enzymes were eluted with linear gradients of sodium chloride. Active fractions for each enzyme were identified by enzyme assay, pooled, concentrated by ultrafiltration, and dialyzed into 100 mm sodium phosphate buffer, pH 6.0. The dialyzed samples were applied at a flow rate of 0.5 ml/min to a Superdex 200 HR 10/30 FPLC column (Pharmacia) previously equilibrated with 100 mm sodium phosphate, pH 6.0. Active fractions were again pooled and concentrated by ultrafiltration. The purified enzymes were hydrolyzed using cyanogen bromide in 70% formic acid as described (Gross 1967; Matsudaira 1990). The resulting peptides were used to prepare mouse anti-α-glucosidase antibodies and rabbit anti-β-glycosidase as described previously (Blum *et al.* 1992; Rockabrand *et al.* 1998).

Protein electrophoresis and Western blot analysis: Proteins were resolved by SDS PAGE under reducing conditions using unstained low- and high-molecular-weight markers (BioRad, Richmond, CA). Prior to electrophoresis, samples were adjusted to 2% (w/v) SDS and 3 mm β-mercaptoethanol and boiled for 10 min. SDS polyacrylamide gels were stained with Coomassie Blue R250 to visualize protein. Chemiluminescent Western blot analysis using the Tropix system was performed as described (Rockabrand *et al.* 1998). The α-glucosidase (*malA*) and β-glycosidase (*lacS*) protein standards were prepared as described above for use in the preparation of antibodies with some modification. The recombinant *E. coli* extracts were subjected to only one heat treatment at 85° for 1 hr followed by clarification at 14,000 × *g* for 5 min at room temperature. The relative abundance of the two proteins in these extracts was determined by comparison to purified samples.

Isolation and DNA sequence analysis of *lacS* and flanking regions: The *S. solfataricus* library was constructed using genomic DNA prepared as described previously (Rolfsmeier *et al.* 1998). Genomic DNA was partially digested with *Sau*3AI and then fractionated by electrophoresis, and DNA of a size ranging between 3 and 5 kb was ligated into the *Bam*HI site of pUC19 (New England Biolabs, Beverly, MA) and transformed into *E. coli* strain DH5α with selection for ampicillin resistance. Two thousand individual colonies were picked and propagated in 96-well microtiter plates in rich medium containing ampicillin. The *S. solfataricus lacS* gene (β-glycosidase) was identified by screening these isolates that had been preheated at 80° for 1 hr for the ability to hydrolyze β-PNPG at 80°. One such isolate was identified using this method and its recombinant plasmid was called pBN55.

The *lacS* locus from the Car1 mutant (see results) was PCR amplified and cloned into pUC19 using the forward primer 5′ CGCGGATCCGATCAATACTAGGAGGAGTAGCA TATAATTAC 3′, which includes an added *Bam*HI site, and the reverse primer 5′ CGGGGTACCCCCAAAAGGTACAAAA TAAATAATATAGAA 3′, which includes an added *Kpn*I site. The primers were complementary to positions 54–84 and 1783–1814, respectively, in the published sequence (GenBank accession no. M34696). The insert of pBN55 and the PCR amplicon of the *lacS* gene from Car1 were subcloned for sequencing. The plasmids were digested with *Eco*RI resulting in three fragments, the first of which consisted of the 725-bp 5′ end of the insert and the pUC19 vector. It was religated to itself to generate the 5′ end subclone. The remaining two fragments produced by the *Eco*RI digestion were 491 and 552 bp and represented the central and 3′ portions of the insert, respectively. They were each ligated into the *Eco*RI site of pUC19 to generate the middle and 3′ end subclones. The inserts of all three subclones were then sequenced. The wild-type *lacS* sequence has been deposited in GenBank under accession no. AF133096. Regions lying 3′ to *lacS* were determined by restriction analysis of recombinant phage λ-7F8 recovered from an *S. solfataricus* genomic library (Rolfsmeier *et al.* 1998). A 2.5-kb *Eco*RI-*Sac*I fragment representing sequences located 2 kb 3′ to *lac*S was subcloned into phage M13 mp18, creating phage M13-S2, and portions of the insert subjected to DNA sequence analysis. An open reading frame was identified (Figure 4A, ORF 3). This sequence has been deposited in GenBank under accession no. AF148510.

Northern blot analysis: *S. solfataricus* total RNA was extracted and fractionated by electrophoresis as described previously (Rolfsmeier *et al.* 1998). The RNA was electrophoretically transferred to Hybond N+ (Amersham, Arlington Heights, IL) membranes and cross-linked by shortwave UV irradiation. RNA riboprobes were generated using the riboprobe buffer kit (Promega, Madison, WI) and the manufacturer's protocol. Riboprobe templates were a 2081-bp fragment encoding the *malA* region comprising positions 141 to 2265 bp relative to the *malA* start codon (Rolfsmeier *et al.* 1998) and a 493-bp *Eco*RI *lacS* fragment including positions 544 to 1037 relative to the *lacS* start codon. The DNA fragments used to generate the riboprobes were cloned into plasmid pT7T3 18U (Pharmacia). Northern hybridizations were performed at 55° with 50% formamide as described (Rolfsmeier *et al.* 1998). Washed membranes were used to prepare autoradiograms with Kodak X-Omat film. Molecular weight standards were RNA Transcripts (United States Biochemical, Cleveland).

Southern blot analysis: Southern blot analysis was performed essentially as described (Sambrook *et al.* 1989). Genomic DNA was isolated from *S. solfataricus* as described previously (Yeats *et al.* 1982). Genomic DNA restriction digests were transferred electrophoretically to Hybond N (Amersham) overnight in 25 mm sodium phosphate buffer, pH 6.4, at 250 mA in a water-cooled chamber. Blots were probed using stringent conditions at 42° with 50% (v/v) formamide, 5× SSPE, 5× Denhardt's reagent, 0.5% (w/v) SDS, and 200 ng/ml yeast tRNA as described (Sambrook *et al.* 1989). Probes used for Southern blot analysis were radiolabeled using random hexanucleotide primers and Klenow enzyme as described by the manufacturer (Boehringer Mannheim, Indianapolis).

RESULTS

Isolation and characterization of glycosyl hydrolase mutants: It has been shown previously that wild-type *S. solfataricus* strain P2 colonies develop a dark blue color when exposed to aerosols of the chromogenic substrate X-gal (Schleper *et al.* 1994). This color results from the activity of the *S. solfataricus* β-glycosidase that is encoded by *lacS*. Five mutants of *S. solfataricus* strain 98/2 with reduced *lacS* function were recovered as white colonies among 4 × 10^5 colonies screened. These isolates

TABLE 1

Levels of glycosyl hydrolases in the *S. solfataricus* mutant isolates

Strain[a]	Glycosyl hydrolase activities		
	β-Glycosidase (μmol PNP[b]/min/mg)	α-Glucosidase (μmol PNP/min/mg)	α-Amylase (units/ml)
Wild type	31.7 ± 0.3	12.7 ± 0.9	1.15 ± 0.03
Mutant 1 (Car1)	1.0 ± 0.1 (32)[c]	12.3 ± 1.7 (1)	0.19 ± 0.02 (6)
Mutant 2 (Car2)	1.1 ± 0.6 (29)	25.6 ± 1.8 (2)	0.17 ± 0.01 (7)
Mutant 4 (*lacS100*)	<0.001	14.3 ± 3.7 (1)	1.13 ± 0.02 (1)
Mutant 5 (Car5)	0.9 ± 0.1 (35)	10.0 ± 0.8 (1)	0.18 ± 0.02 (6)

[a] Names in parentheses refer to phenotypic and genotypic designations.
[b] PNP is para-nitrophenol.
[c] Values in parentheses indicate the fold change in specific activity relative to wild type rounded to the nearest integer.

initially were designated mutants 1–5; however, mutant isolate 3 exhibited a severe growth defect preventing its purification and was subsequently eliminated. Surprisingly, the frequency of recovery of the white-colony phenotype was 100- to 1000-fold lower than previously reported for this organism (Schleper *et al.* 1994).

Three glycosyl hydrolases were characterized in each of the mutant isolates: the α-amylase (Haseltine *et al.* 1996), the α-glucosidase encoded by *malA* (Rolfsmeier and Blum 1995; Rolfsmeier *et al.* 1998), and the β-glycosidase (Grogan 1991) encoded by *lacS* (Cubellis *et al.* 1990). Levels of each of the three enzymes were determined from cells in the midexponential phase of growth in a minimal sucrose medium with added yeast extract (Table 1). The cell-associated activities, α-glucosidase (*malA*) and β-glycosidase (*lacS*), were determined as specific activities while activity levels of the secreted α-amylase were normalized to total cell protein present at the time of assay. Levels of β-glycosidase (*lacS*) were greatly reduced in all four mutants as expected; however, mutants 1, 2, and 5 had 3% of wild-type levels, whereas mutant 4 had undetectable levels. A twofold increase in α-glucosidase (*malA*) was observed in mutant 2, while the other mutants were not significantly different from wild type. Levels of the secreted α-amylase were significantly reduced in mutants 1, 2, and 5 and were ~16% of wild-type levels. In contrast, mutant 4 exhibited no significant alteration in this activity. Because mutants 1, 2, and 5 were pleiotropically defective, they were renamed Car for catabolite repression and were designated Car1, Car2, and Car5, respectively. Mutant 4, altered only in levels of the β-glycosidase (*lacS*) and in light of the results presented below, was designated *lacS100*. Because the reduction in β-glycosidase activity (*lacS*) was so pronounced for all of the mutants, they were tested for utilization of β-linked sugars such as cellobiose, lactose, and salicin as sole carbon and energy sources in liquid batch culture. None of the mutants grew under these conditions. In contrast, the wild type grew on each of these sugars with generation times of 8, 17, and 9 hr, respectively.

Reduction in enzyme activities results from alterations in enzyme levels: Western blot analysis of levels of the α-glucosidase (*malA*) and the β-glycosidase (*lacS*) was performed to test for changes in enzyme abundance rather than enzyme activity (Figure 1). All of the mu-

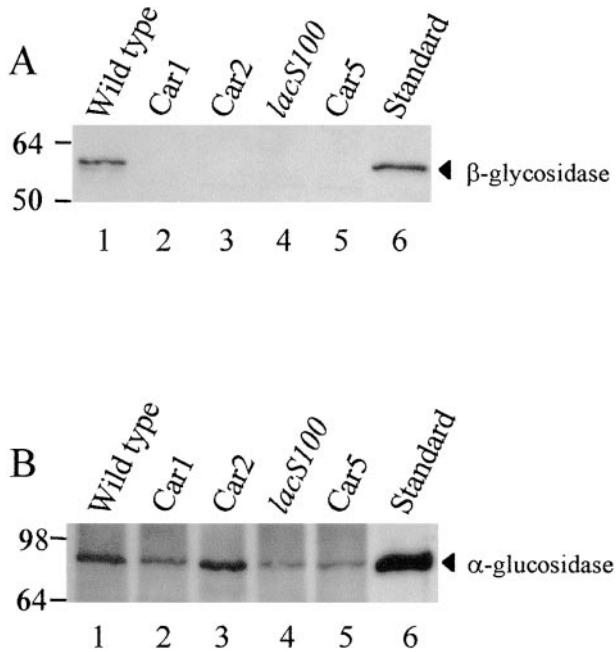

Figure 1.—Western blot analysis of β-glycosidase and α-glucosidase in Car mutant isolates. (A) Levels of LacS protein. Cell extracts were loaded in 40 μg amounts per lane. Lanes: 1, wild-type *S. solfataricus* extract; 2, Car1; 3, Car2; 4, *lacS100*; 5, Car5; 6, recombinant β-glycosidase (10 ng). (B) Levels of MalA protein. Cell extracts were loaded in 30 μg amounts per lane. Lanes: 1, wild-type *S. solfataricus* extract; 2, Car1; 3, Car2; 4, *lacS100*; 5, Car5; 6, recombinant α-glucosidase (9 ng). Right-hand arrows indicate the positions of the β-glycosidase and α-glucosidase; molecular mass markers in kilodaltons are shown on the left side.

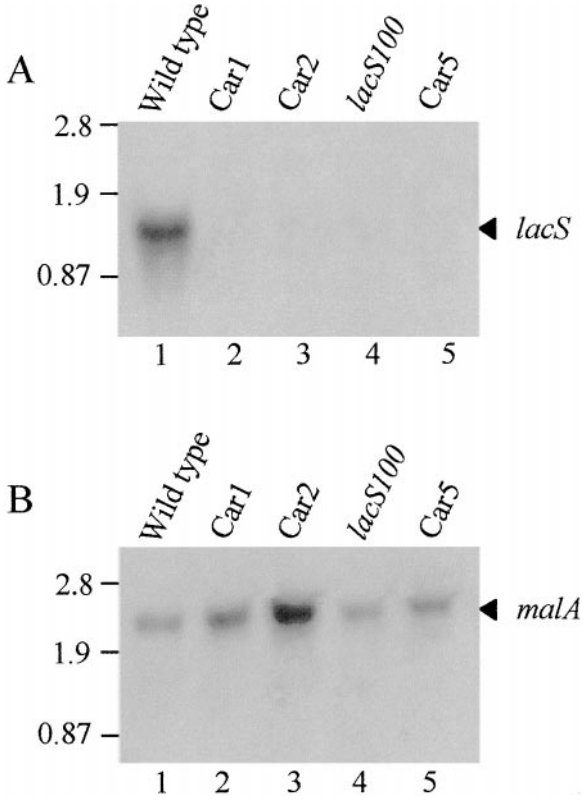

Figure 2.—Northen blot analysis of *lacS* and *malA* in Car mutant isolates. Total cellular *S. solfataricus* RNA was loaded in 5 μg amounts per lane. (A) Levels of *lacS* mRNA. Lanes: 1, wild-type *S. solfataricus*; 2, Car1; 3, Car2; 4, *lacS100*; 5, Car5. (B) Levels of *malA* mRNA. The blot used for Figure 2A was reprobed to measure *malA* mRNA. Lane order is as for Figure 2A. Right-hand arrow indicates the position of *lacS* and *malA* mRNAs; molecular weight markers in kilobases are shown on the left side.

tants had undetectable levels of LacS protein (Figure 1A). In contrast, no significant alteration was observed in levels of MalA protein (Figure 1B). Car2, however, had slightly elevated levels of the α-glucosidase (*malA*). The lack of detectable levels of LacS protein was consistent with the large reduction in enzyme activity detected in cell extracts (Table 1). These results indicate that allosteric control over enzyme activity or other forms of regulation operating at the post-translational level were not significant factors in the observed mutant phenotypes.

Reduction in enzyme levels results from decreased mRNA abundance: Northern blot analysis was conducted to determine whether the observed reductions in LacS protein resulted from corresponding changes in levels of *lacS* transcript (Figure 2). Analysis of *malA* was used for comparison. Riboprobes complementary to *lacS* and *malA* mRNA were used to successively probe a blot of total *S. solfataricus* RNA derived from cells in the exponential phase of growth. *lacS* mRNA was undetectable in the mutant strains (Figure 2A). In contrast, the levels of *malA* mRNA were similar for Car1,

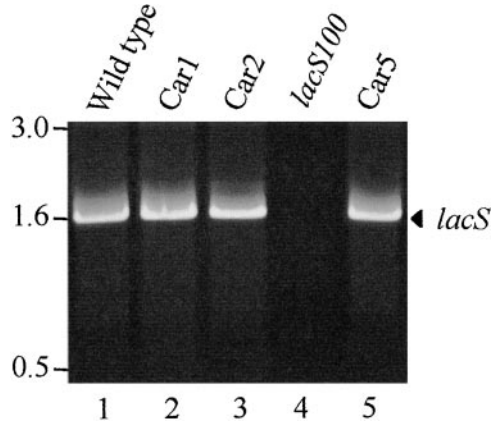

Figure 3.—PCR analysis of the *lacS* locus in Car mutant isolates. Lanes: 1, wild-type *S. solfataricus*; 2, Car1; 3, Car2; 4, *lacS100*; 5, Car5. Molecular weight markers in kilobases are shown.

lacS100, and Car5 and the wild-type strain, whereas Car2 exhibited slightly elevated levels. The reduced levels of *lacS* mRNA suggest that either mRNA synthesis or mRNA degradation rather than protein stability or turnover is the primary target of the mutations in these mutants.

One possible explanation for the pleiotropy of Car mutants is linkage of the genes in an operon. However, sequence analysis of the 1.32-kb 5′ and 2.07-kb 3′ to *lacS* (C. Haseltine, unpublished results; Cubellis *et al.* 1990; Prisco *et al.* 1995) revealed no sequences with homology to known α-amylases. Further, Northern blot analysis presented here excludes the presence of a large polycistronic mRNA encoding MalA and LacS. Instead, the apparent size of the transcript was close to the size of the corresponding open reading frame as expected for a monocistronic mRNA. These results indicate that the *Car* mutations act on physically unlinked loci.

Genomic alterations in *lacS*: The results presented above indicated that Car1, Car2, and Car5 could be grouped together (group I) but separately from *lacS100* (group II). Previous Lac$^-$ mutants resulted from transposition of an IS into the *lacS* gene (Schleper *et al.* 1994). It seemed, therefore, that IS element transposition could explain the properties of the group II mutant (*lacS100*) but not the group I mutants. To resolve these differences in greater detail, PCR analysis of *lacS* was conducted on the wild-type strain and the four mutants using the forward primer 5′ GCTTAAATAATATTAAT CATAAATAAAGTC 3′ (F2S) complementary to positions −31 to −1 relative to the *lacS* start codon and a reverse primer 5′ GCAATCTAATGAAAATGAGATTA GAATAAG 3′ (R2S) complementary to sequences spanning positions located 44 to 74 bp 3′ to the end of the *lacS* stop codon (Figure 3). A 1.6-kb amplicon was detected in the wild type and group I mutants while no amplification product was evident with the group II mutant (*lacS100*). This result excluded the possible exis-

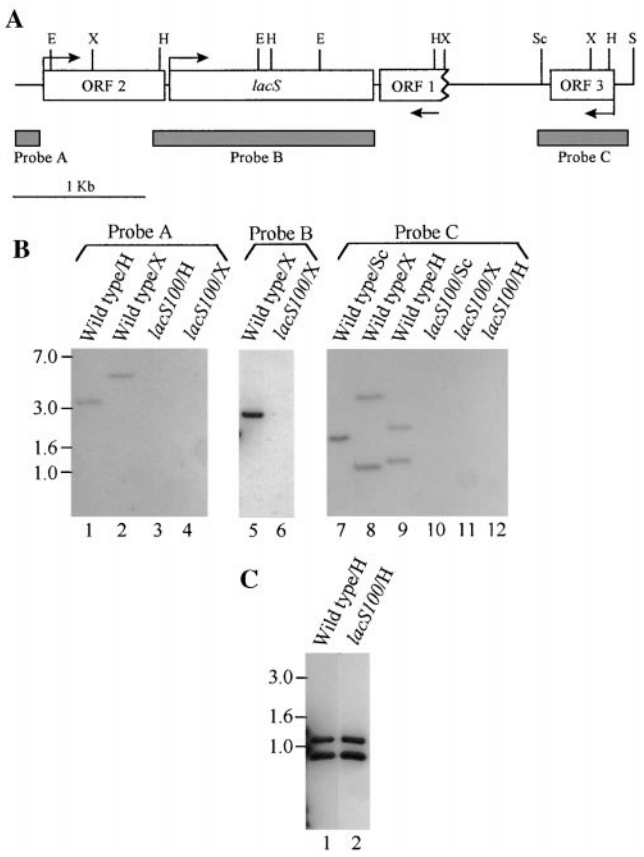

Figure 4.—Southern blot analysis of the *lacS* locus in *lacS100*. (A) Schematic of the *lacS* locus. The *lacS* and flanking open reading frames are indicated in boxes. The location and size of the various probes are shown as hatched boxes. The direction of transcription on the basis of either apparent reading frame or primer extension analysis is indicated by the arrows. Key restriction sites are indicated by single letters: B, *Bam*HI; E, *Eco*RI; X, *Xba*I; H, *Hin*dIII; Sc, *Sca*I; S, *Sac*I. (B) Autoradiogram of Southern blot of *lacS* locus. Lanes 1, 2, 5, 7, 8, and 9 contain genomic digests of wild-type *S. solfataricus* DNA; lanes 3, 4, 6, 10, 11, and 12 contain genomic digests of *lacS100* DNA. Restriction enzyme treatment of genomic DNAs are indicated and are labeled as for Figure 4A. Molecular weight markers in kilobases are shown on the left side. (C) Autoradiogram of Southern blot of *malA* locus. Lane 1, wild type; lane 2, *lacS100*. Genomic DNA were digested with *Hin*dIII.

tence of an insertion element in *lacS* in all four of the mutants. Instead, the results suggested that the group II mutant might be deleted for *lacS*.

To test the hypothesis that the group II mutant was deleted for *lacS*, Southern blot analysis was used to characterize the *lacS* and *malA* regions in this mutant (Figure 4). Three probes derived from the *lacS* region were used (Figure 4A). Probe A was a 191-bp PCR amplicon complementary to positions 3–193 as described (Prisco *et al.* 1995) and lying 1157 bp 5′ to the *lacS* coding region. Probe B was the entire 1760-bp *lacS* coding sequence including 175 bp 5′ to the *lacS* start codon. Probe C was a 650 *Sac*I-*Sca*I fragment subcloned from phage M13-S2 (see materials and methods) and located 1420 bp 3′ to the *lacS* stop codon. Probe A detected fragments of 3.5 and 5.8 kb in *Hin*dIII and *Xba*I genomic digests, respectively, in the wild type but not the *lacS100* mutant. Probe B detected a 2.76-kb band in *Xba*I genomic digests of the wild type but not *lacS100*. Probe C detected fragments of 1.7 kb in *Sca*I genomic digests, 4.0 and 1.13 kb in *Xba*I genomic digests, and 2.0 and 1.2 kb in *Hin*dIII genomic digests of wild-type DNA but no fragments in *lacS100* DNA (Figure 4B). Southern blot analysis of the *malA* locus was conducted to verify that the alteration in *lacS100* was specific to the *lacS* region. The *malA* probe was a 2.1 genomic *Kpn*I-*Pst*I fragment previously cloned into pLITMUS 29 (Rolfsmeier *et al.* 1998) encoding the entire *malA* open reading frame. Southern blot analysis of *Hin*dIII digests of genomic DNA from wild type and the group II mutant exhibited two strongly hybridizing bands of 1.3 and 0.8 kb. These results indicated that the *lacS100* was deleted for *lacS* and regions lying both up- and downstream but was unaffected at the *malA* locus. *lacS100* was therefore designated Δ*lacS100*.

The *lacS* allele in group I mutants: Gross alterations of the *lacS* gene could be excluded by PCR analysis in group I mutants. To test whether more subtle alterations might have occurred, such as substitution mutations, Car1 was selected for more detailed analysis. The *lacS* gene was cloned from Car1 and expressed in *E. coli* and the resulting level of thermostable β-glycosidase activity was compared to that produced using the wild-type *lacS* allele. The *lacS* gene was amplified by PCR and cloned into pUC19 under the control of P_{lac}. The resulting plasmids were transformed into DH5α and cell extracts were prepared. The specific activity of thermostable β-glycosidase for the wild-type strain was 10.1 μmol PNP/min/mg protein, and for Car1 was 9.2 μmoles PNP/min/mg protein. This result indicates that *lacS* is normal in Car1. The DNA sequence of the entire *lacS* transcribed region, including sequences lying 175 bp 5′ to the *lacS* start codon and 126 bp 3′ to the *lacS* stop codon, which includes the putative lacS transcription terminator (Cubellis *et al.* 1990), was unaltered in Car1. These results indicate that the mutation affecting *lacS* expression in Car1 is extragenic.

Reversion analyses: To determine whether the pleiotropic phenotype of group I mutants resulted from a single mutation, reversion analysis was conducted on several of the group I mutants. Two procedures were employed. Colonies grown on nonselective medium were sprayed with an X-gal solution; spontaneous blue revertants were obtained at frequencies of $\sim 10^{-3}$ for all three group I mutants. For example, the frequency of reversion of Car1 was 6.6×10^{-4} (21 revertants recovered from 32,000 colonies screened). In the second approach, revertants able to utilize lactose were selected after growth in liquid medium. Reverted cultures were streaked on solid medium for purification. All revertants were called Scr for suppressor of catabolic repression.

TABLE 2

Levels of glycosyl hydrolases in revertants of group I mutants

Strain	Glycosyl hydrolase activities		
	β-Glycosidase (μmol PNP/min/mg)	α-Glucosidase (μmol PNP/min/mg)	α-Amylase (units/ml)
Carl	1.5 ± 0.1	17.0 ± 1.7	0.19 ± 0.02
Scr-1B1	6.4 ± 0.1	21.4 ± 1.2	0.60 ± 0.07
Scr-1B2	3.6 ± 0.2	15.3 ± 0.2	0.57 ± 0.04
Scr-1B9	7.9 ± 0.4	16.7 ± 0.3	0.65 ± 0.03
Scr-1B10	8.1 ± 0.7	18.6 ± 0.1	0.78 ± 0.04
Scr-1L1	123.7 ± 4.5	16.7 ± 1.4	1.00 ± 0.03
Wild type	31.7 ± 0.3	12.7 ± 0.9	1.21 ± 0.01

For the Car1 mutant, revertants obtained on Xgal were labeled Scr-1B1 and those from lactose selection, Scr-1L1, etc.

β-Glycosidase (*lacS*) activities were elevated in both classes of group I revertants relative to the levels of this enzyme in their respective parental strains (Table 2). Only partial restoration of β-glycosidase activity was observed in revertants recovered from plates screened for colonies exhibiting increased color after application of X-gal. This class of revertants (Scr-1B1 through Scr-1B10) exhibited β-glycosidase levels ~20% of wild type and four- to sixfold greater than the parent strain Car1. In contrast, a revertant such as Scr-1L1, which had regained the ability to utilize lactose, exhibited greater levels of β-glycosidase activity relative to wild type. Both classes of revertants also exhibited significant increases in α-amylase levels (Table 2), demonstrating that the reversion event was pleiotropic.

Western blot analysis of Scr-1L1 confirmed that the increase in β-glycosidase activity resulted from increased levels of LacS and was not a result of an increase in the activity or level of some other enzyme with overlapping substrate specificity (Figure 5). Phenotypic analysis of the revertants for their abilities to utilize lactose or starch as sole carbon and energy sources was also examined (Table 3). Despite the sixfold increase in β-glycosidase activity, Scr-1B1 remained unable to grow in a lactose minimal medium. Scr-1L1, however, exhibited wild-type generation times in a lactose-minimal medium. This indicates that an amount of β-glycosidase in excess of 20% of wild-type levels is necessary for growth on lactose as the sole carbon and energy source. Surprisingly, Scr-1L1 grew even faster than wild type on a complex medium. The ability to utilize starch as a sole carbon and energy source was also examined in these strains. Group I mutants, such as Car1 with 16% of wild-type α-amylase levels, were severely limited in their ability to grow in a starch minimal medium. Restoration of α-amylase levels in Scr-1B1 to 50% of wild-type levels, however, greatly increased its ability to grow in a starch minimal medium. This result further demonstrates the pleiotropic nature of both the extragenic mutations affecting glycosyl hydrolase activity and their revertant derivatives.

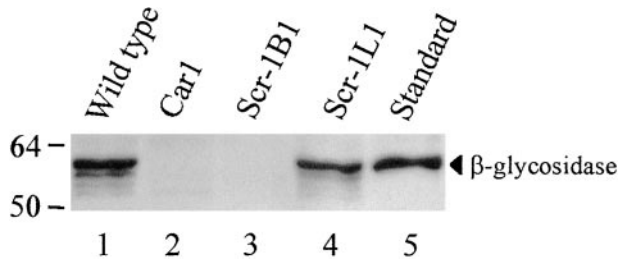

Figure 5.—Western blot analysis of LacS protein levels in Scr revertants. Total protein (40 μg) was loaded in each lane. Lanes: 1, wild-type *S. solfataricus*; 2, Car1; 3, Scr-1B1; 4, Scr-1L1; 5, 10 ng of recombinant β-glycosidase.

DISCUSSION

The existence of the group I mutant class indicates that glycosyl hydrolase production in *S. solfataricus* requires a *trans*-acting factor for wild-type levels of expression. Because coordinate gene expression is a common characteristic of catabolite repression systems, the results presented here provide further support for the existence of a catabolite repression-like system in *S. solfataricus*. For at least the *lacS* gene (β-glycosidase), the

TABLE 3

Growth of group I mutant revertants on selected carbon sources

	Generation time (hr)		
	Starch	Lactose	SYE[a]
Carl	20	NG[b]	7.5
Scr-1B1	12	NG	7.5
Scr-1L1	10	17	5.0
Wild type	9	17	7.5

[a] Sucrose with added yeast extract.
[b] No growth (NG) was observed after 1 wk of incubation.

mechanism of control occurs at the level of transcription; the mutations result in undetectable levels of *lacS* mRNA. The mechanism of reduction in *lacS* mRNA levels is unknown. It may result from events that act at the level of mRNA synthesis or mRNA degradation. The *trans*-acting factor must recognize elements inherent to all target genes, whether they be their promoters or their transcripts.

Low but significant levels of β-glycosidase (*lacS*) activity, however, were evident in the group I mutants. Because β-glycosidase activity in the group II mutant was undetectable and this *lacS* allele was shown to be a complete deletion of the gene, there are apparently no other enzymes in *S. solfataricus* produced under the conditions used that hydrolyze β-PNPG. The residual β-glycosidase activity detected in group I mutants must therefore result from low levels of *lacS* expression. Car2 exhibited moderately elevated levels of α-glucosidase activity, protein, and mRNA relative to wild type and the other Car isolates. This suggests that Car2 is likely to be a different mutation than Car1 and Car5. This result may indicate that the *Car* locus controls expression of all three of the glycosyl hydrolases, including *malA* (α-glucosidase), which were examined in this work. Until the *Car* locus is characterized at the molecular level, it remains possible that there are multiple genes resulting in the Car phenotype that influence glycosyl hydrolase expression in *S. solfataricus*. The Scr-1L1 revertant pleiotropically affected α-amylase and β-glycosidase (*lacS*) production as was observed for the other Scr isolates. However, it exhibited significantly higher β-glycosidase activities relative to wild type. The apparent differences observed between the various Car isolates and their revertants exclude a simple regulatory mechanism involving negative- or positive-acting control of gene expression. Instead these results indicate a more complex regulatory mechanism either involving multiple regulatory elements or complex interactions between a single element and the basal transcription components.

The mutant classes affecting *lacS* expression could be divided into intragenic and extragenic classes. Unlike previously described *lacS* mutants in *S. solfataricus* strain P2 (Schleper *et al.* 1994), none resulted from disruption of *lacS* by insertion element (ISC1217). A second distinguishing feature of the work described here concerns the frequency of occurrence of mutants with reduced *lacS* expression. In *S. solfataricus* strain P2, *lacS* mutants were found at frequencies of 1–0.1% (Schleper *et al.* 1994), two to three orders of magnitude greater than was observed in the work presented here. Because strain 98/2 of *S. solfataricus* (Rolfsmeier and Blum 1995) was used in the present work and its *lacS* sequence contains the putative sequence targeted by the insertion element ISC1217, it seems that strain 98/2 must either lack this element or contain only nonfunctional copies. The lack of insertion-element-mediated *lacS* disruption was, in fact, key to the recovery of the extragenic group I regulatory mutant class. These pleiotropic mutants might have otherwise been obscured by the overwhelming frequency of transposition-mediated events in strain P2. The apparent difference in mutant types recovered from these two strains of *S. solfataricus* may be related to their geographic origins. Strain P2 was isolated from Italy (DeRosa *et al.* 1975) while strain 98/2 was isolated from the United States (Rolfsmeier and Blum 1995).

The efficiency of plating of *S. solfataricus* in the present work is ~10%. Consequently the observed *lacS* forward and *lacS* reversion frequencies should be corrected by 10-fold. Thus the forward event occurred at a frequency of $\sim 10^{-6}$ and the reversion event at a frequency of $\sim 10^{-4}$. The forward mutation rate and the phenotypes of these mutants are consistent with either altered-function or loss-of-function mutations. The reversion rate, however, is significantly greater than would be expected of an intragenic event and is more consistent with a much larger genetic target. One possibility is the occurrence of tRNA suppressors. Such suppressors are known to occur at high frequencies in prokaryotic. In addition tRNA mutant suppressors generally exhibit only partial suppression of the defect caused by the initial forward mutation. Partial restoration of expression of *lacS* and production of the α-amylase is consistent with this hypothesis. Future efforts concerning the *S. solfataricus* catabolite repression-like system are focused on plasmid-mediated genetic analysis of the Car mutants and their revertants.

J. Austin, A. Clark, T. Hill, and the other members of the Blum lab are gratefully acknowledged for their comments and support. This work was supported by grant MCB-9604000 to P.B. from the National Science Foundation.

LITERATURE CITED

Arvalli, R. N., and R. A. Garret, 1997 Shuttle vectors for hyperthermophilic archaea. Extremophiles **1:** 183–191.

Birnboim, H. C., and J. Doly, 1979 A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:** 1513–1523.

Blum, P., J. Bauernfiend, J. Ory and J. Krska, 1992 Physiological consequences of DnaK and DnaJ overproduction in *Escherichia coli*. J. Bacteriol. **174:** 7436–7444.

Brock, T. D., K. M. Brock, R. T. Belly and R. L. Weiss, 1972 *Sulfolobus*: a genus of sulfur oxidizing bacteria living at low pH and high temperature. Arch. Microbiol. **84:** 54–68.

Cannio, R., P. Contursi, M. Rossi and S. Bartolucci, 1998 An autonomously replicating transforming vector for *Sulfolobus solfataricus*. J. Bacteriol. **180:** 3237–3240.

Cubellis, M. V., C. Rozzo, P. Montecucchi and M. Rossi, 1990 Isolation and sequencing of a new β-galactosidase-encoding archaebacterial gene. Gene **94:** 89–94.

Cubero, B., and C. Scazzocchio, 1994 Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. EMBO J. **13:** 407–415.

DeRosa, M., A. Gambacorta and J. D. Bu'lock, 1975 Extremely thermophilic acidophilic bacteria convergent with *Sulfolobus acidocaldarius*. J. Gen. Microbiol. **86:** 156–164.

Deutscher, J., E. Kuster, U. Bergstedt, V. Charrier and W. Hillen, 1995 Protein kinase-dependent Hpr/CcpA interaction

links glycolytic activity to carbon catabolite repression in gram-positive bacteria. Mol. Microbiol. **15:** 1049–1053.

Elferink, M. G. L., C. Schleper and W. Zillig, 1996 Transformation of the extremely thermoacidophilic archaeon *Sulfolobus solfataricus* via a self-spreading vector. FEMS Microbiol. Lett. **137:** 31–35.

Gohl, H. P., B. Grondahl and M. Thomm, 1995 Promoter recognition in archaea is mediated by transcription factors: identification of transcription factor aTFB from *Methanococcus thermolithotrophicus* as archaeal TATA-binding protein. Nucleic Acids. Res. **23:** 3837–3841.

Grogan, D. W., 1989 Phenotypic characterization of the archaebacterial genus *Sulfolobus*: comparison of five wild-type strains. J. Bacteriol. **171:** 6710–6719.

Grogan, D. W., 1991 Evidence that β-galactosidase of *Sulfolobus solfataricus* is only one of several activities of a thermostable β-D-glycosidase. J. Bacteriol. **57:** 1644–1649.

Grogan, D. W., 1996 Exchange of genetic markers at extremely high temperatures in the archaeon *Sulfolobus acidocaldarius*. J. Bacteriol. **178:** 3207–3211.

Gross, E., 1967 The cyanogen bromide reaction. Methods Enzymol. **11:** 238–255.

Hain J., W.-D. Reiter, U. Hudepohl and W. Zillig, 1992 Elements of an archaeal promoter defined by mutational analysis. Nucleic Acids Res. **20:** 5423–5428.

Hanahan, D., 1983 Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:** 557–580.

Haseltine, C., M. Rolfsmeier and P. Blum, 1996 The glucose effect and regulation of α-amylase synthesis in the hyperthermophilic archaeon *Sulfolobus solfataricus*. J. Bacteriol. **178:** 945–950.

Henkin, T. M., F. J. Grundy, W. L. Nicholson and G. H. Chambliss, 1991 Catabolite repression of an amylase gene expression in *Bacillus subtilis* involves a trans-acting gene product homologous to *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:** 575–584.

Jacobs, K. L., and D. W. Grogan, 1997 Rates of spontaneous mutation in an archaeon from geothermal environments. J. Bacteriol. **179:** 3298–3303.

Kandler, O., and K. O. Stetter, 1981 Evidence for autotrophic CO_2 assimilation in *Sulfolobus brierleyi* via a reductive carboxylic acid pathway. Zentbl. Bakteriol. Hyg. Abt. Orig. C. **2:** 111–121.

Klenk, H.-P., P. Palm, F. Lottspeich and W. Zillig, 1992 Component H of the DNA-dependent RNA polymerase of archaea is homologous to a subunit shared by the three eucaryal nuclear RNA polymerases. Proc. Natl. Acad. Sci. USA **89:** 407–410.

Magasanik, B., and F. C. Neidhardt, 1987 Regulation of carbon and nitrogen utilization, pp. 1318–1325 in *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*, edited by F. C. Neidhardt, J. L. Ingrahm, K. Brooks-Low, B. Magasanick, M. Schaechter *et al.* American Society for Microbiology Press, Washington.

Marsh, T. L., C. I. Reich, R. B. Whitelock and G. J. Olsen, 1994 Transcription factor IID in the Archaea: sequences in the *Thermococus celer* genome would encode a product closely related to the TATA-binding protein of eukaryotes. Proc. Natl. Acad. Sci. USA **91:** 4180–4184.

Matsudaira, P., 1990 Limited N-terminal sequence analysis. Methods Enzymol. **182:** 602–613.

Park, J. T., and M. J. Johnson, 1949 A submicro determination of glucose. J. Biol. Chem. **181:** 149–151.

Prisco, A., M. Moracci, M. Rossi and M. Ciaramella, 1995 A gene encoding a putative membrane protein homologous to the major facilitator superfamily of transporters maps upstream of the β-glycosidase gene in the archaeon *Sulfolobus solfataricus*. J. Bacteriol. **177:** 1614–1619.

Qureshi, S. A., and S. P. Jackson, 1998 Sequence-specific DNA binding by the *S. shibatae* TFIIB homolog, TFB, and its effect on promoter strength. Mol. Cell **1:** 389–400.

Qureshi, S. A., P. Baumann, T. Rowlands, B. Khoo and S. P. Jackson, 1995 Cloning and functional analysis of the TATA binding protein from *Sulfolobus shibatae*. Nucleic Acids Res. **23:** 1775–1781.

Reiter, W.-D., U. Hudepohl and W. Zillig, 1990 Mutational analysis of an archaebacterial promoter: essential role of a TATA box for transcription efficiency and start-site selection in vitro. Proc. Natl. Acad. Sci. USA **87:** 9509–9513.

Rockabrand, D., and P. Blum, 1995 Multicopy plasmid suppression of stationary phase chaperone toxicity in *Escherichia coli* by phosphogluconate dehydratase and the N-terminus of DnaK. Mol. Gen. Genet. **249:** 498–506.

Rockabrand, D., T. Arthur, G. Korinek, K. Livers and P. Blum, 1995 An essential role for the *Escherichia coli* DnaK protein in starvation-induced thermotolerance, H_2O_2 resistance and reductive division. J. Bacteriol. **177:** 3695–3703.

Rockabrand, D., K. Livers, T. Austin, R. Kaiser, D. Jensen *et al.*, 1998 Roles of DnaK and RpoS in starvation-induced thermotolerance of *Escherichia coli*. J. Bacteriol. **180:** 846–854.

Roesler, W. J., J. G. Grahm, R. Kolen, D. J. Klemm and P. J. McFie, 1995 The cAMP response element binding protein synergizes with other transcription factors to mediate cAMP responsiveness. J. Biol. Chem. **270:** 8225–8232.

Rolfsmeier, M., and P. Blum, 1995 Purification and characterization of a Maltase from the extremely thermophilic crenarchaeote *Solfataricus solfataricus*. J. Bacteriol. **177:** 482–485.

Rolfsmeier, M., C. Haseltine, E. Bini, A. Clark and P. Blum, 1998 Molecular characterization of the α-glucosidase gene (*malA*) from the hyperthermophilic archaeon *Sulfolobus solfataricus*. J. Bacteriol. **180:** 1287–1295.

Rowlands, T., P. Baumann and S. P. Jackson, 1994 The TATA-binding protein: a general transcription factor in eukaryotes and archaebacteria. Science **264:** 1326–1329.

Saier, M. H., Jr., T. M. Ramseier and J. Reizer, 1996 Regulation of carbon utilization, pp. 1325–1343 in *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology*, edited by F. C. Neidhardt, R. Curtiss III, J. L. Ingrahm, E. C. C. Lin, K. Brooks-Low *et al.* American Society for Microbiology Press, Washington, DC.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 6. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schleper, C., R. Roder, T. Singer and W. Zillig, 1994 An insertion element of the extremely thermophilic archaeon *Sulfolobus solfataricus* transposes into the endogenous β-galactosidase gene. Mol. Gen. Genet. **243:** 91–96.

Sensen, C. W., R. L. Charlebois, C. Chow, I. G. Clausen, B. Curtis *et al.*, 1998 Completing the sequence of the *Sulfolobus solfataricus* P2 genome. Extremophiles **2:** 305–312.

Woese, C., O. Kandler and M. L. Wheelis, 1990 Towards a natural system of organisms: proposal for the domains archaea, bacteria and eucarya. Proc. Natl. Acad. Sci. USA **87:** 4576–4579.

Yeats, S., P. McWilliams and W. Zillig, 1982 A plasmid in the archaebacterium *Sulfolobus acidocaldarius*. EMBO J. **1:** 1035–1038.

Communicating editor: C. J. Daniels