Regulation of expression of copper responsive genes in *Sulfolobus solfataricus*

by

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ABSTRACT OF THE DISSERTATION

Regulation of expression of copper responsive genes in *Sulfolobus solfataricus*

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Copper is an essential micronutrient, but toxic in excess. Cells must maintain their internal level of copper within a narrow range of concentrations. This is accomplished mainly by copper efflux mediated by ATP-driven copper transporters that are induced at the level of transcription. *Sulfolobus solfataricus* has the ability to adapt to fluctuations of copper levels in its environment. Two *Sulfolobus* strains P2 and 98/2, showed different sensitivity to copper To better understand the molecular mechanism behind the organismal response to copper, the expression of the cluster of genes *copRTA*, which encodes the copper-responsive transcriptional regulator CopR, the copper-binding protein CopT, and CopA, has been investigated. The expression of the *copR* and *copA* transcripts was monitored by quantitative real-time RT-PCR. The data showed that only *copA* was induced by copper. By comparing the patterns of *copA* expression and cellular copper accumulation, as determined by Inductively Coupled Plasma Optical Emission spectrometry, it was concluded that the level of *copA* depends on the internal fluctuations of copper. To investigate the

role of CopR, a mutant carrying a disruption of the *copR* gene was created. The mutant strain was uncapable of growth in the presence of excess copper, and under the same conditions no transcription of *copA* was observed. These data suggested that CopR positively regulates transcription of *copA*. The knockout mutant strain PBL2070, its parent strain and the mutant complemented with a wild type copy of *copR*, were compared with respect to their physiological and transcriptional response to copper. Results confirmed that, under copper stress, CopR, activates the transcription of *copA*, and its presence restores the wild type phenotype resistant to copper. Finally, a reporter system based on the thermostable β-glucuronidase of *S. solfataricus* was developed to study the promoter of regulated genes. This system was applied to the analysis of the *copTA* promoter region fused to the *S. solfataricus* β-glucuronidase reporter. GUS activity assays showed that after copper addition, the activity of GusB was consistent with the transcriptional changes observed for *copA* under similar conditions. The results provide the basis for a model of the molecular mechanisms of copper homeostasis in *Sulfolobus*.

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Chapter 1

Introduction

Sulfolobus solfataricus

Archaea together with Eukaryotes and Bacteria compose the three domains of life. The archaeal prokaryotes are a group constituted of organisms originally isolated from extreme environments (high salt, pressure, extremes of temperature and/or pH), but abundant also in soil and aquatic mesophilic habitats as revealed by environmental surveys (Brochier-Armanet *et al* 2008; Fuhrman *et al* 1992; Schleper *et al* 2005). Members of the genus *Sulfolobus*, which are part of the *Crenarchaeota* phylum, inhabit hot springs and geothermal mud pots. They can be found in different locations all over the world including Japan, Iceland, Italy, Kamchatka and USA, in Yellowstone National Park hot springs. The name itself means lobed sulfur-oxidizing microorganism (*Sulfo*- sulfur, *lobus*-lobe), since most *Sulfolobus* strains oxidize sulfide to molecular sulfur and to sulfate (Kletzin *et al* 2004). They are generally spherical cells with lobes, but some can be irregularly shaped (Brock *et al* 1972). *Sulfolobus* species are named for the area where they were first isolated. The most studied member of this genus is *Sulfolobus solfataricus* P2 which was originally isolated from the surrounding area of the Solfatara, a shallow volcanic crater at Pozzuoli near Naples in Italy. *S. solfataricus* is an acidophile and a hyperthermophile growing optimally at pH 3 (range from 2-4) and at 80°C (range from 75 to 85°C). Even though it grows at low pH, its internal pH is about 6.5, maintained by the generation of a large proton gradient across the membrane (Anemuller and Schafer

1990). It grows aerobically and is a chemoorganotroph capable of growth with sucrose, maltose, lactose and other sugars (She *et al* 2001). *Sulfolobus*, like all archaea, does not possess a peptidoglycan wall and their cells stain Gram negative. *Sulfolobus* cells have flagella, governed by a clockwise nonreversible motion (Grogan 1989). In *S. solfataricus* the main metabolic pathways are the citric acid cycle, a glycolytic pathway and a pentose phosphate pathway. *S. solfataricus* is able to synthesize all twenty amino acids and its genome encodes homologue of sequences involved in protein translocation like the signal recognition particle (SRP) and SRP receptor protein as well as homologues of *SecE* and *SecY*, part of the protein secretion pore (She *et al* 2001).

Studies with *S. solfataricus* have addressed issues in chromatin-binding proteins, replication, cell cycle, repair, transcription, translation among other themes. Regarding chromatin and chromatin structure, two main chromatin-binding proteins have been identified and characterized in *S. solfataricus*: Sso7d and Sso10b. Sso7d is a 7 kD DNA binding protein that creates structural distortion, acts as an ATP dependant chaperone and undergoes methylation in its lysine residues (Edmondson and Shriver 2001; Gao *et al.* 1998; Napoli *et al.* 2002); and Sso10b (also called ALBA) is a homodimer that induces negative supercoiling, protects nucleic acids from digestion by nucleases and is acetylated in one of its lysine residues (White and Bell 2002).

Replication studies in *S solfataricus* have revealed similarities to that of eukaryotes elucidating the fundamental DNA replication processes in both domains. Wang and coworkers reported recent evidence on the interaction among the archaeal

multiple Cdc6 proteins which regulate DNA replication in *S. solfataricus* as well as in eukaryotes (Wang *et al* 2007). *S. solfataricus* cell cycle's replication and post replication phases have been shown to be longer (157 and 255 minutes, respectively) than those of *E. coli*, along with evidence that showed that chromosome replication starts shortly after cell division (Bernander and Poplawski 1997).

Investigations on DNA repair have demonstrated that the *Sulfolobus* XPF–PCNA complex (involved in DNA repair) is strikingly similar to that of human XPF–ERCC1 (White 2003), thus suggesting function conservation across domains. A recent structural study on the *S. solfataricus* HflX-type GTPase (Wu *et al* 2009) has shed some light on the functional role of this family of GTPases whose function is unknown to date. Perhaps more important is the progress attained with transcriptional studies in *Sulfolobus*.

Enzymes from thermophilic microorganisms are often sought by the biotechnology industries for their ability to catalyze chemical reactions at high temperatures. Thermophilic enzymes are more stable than their mesophilic counterparts, prolonging the shelf life of commercial products. But most importantly, overall industrial enzymatic processes occur more rapidly at high temperatures. The application of thermophilic cells for fermentation processes is more cost effective as well. When using mesophilic organisms, an extra cooling step most be incorporated during the fermentation process, adding an extra 10% of energy cost to the overall microbial fermentation (Brock 1985). If a thermophilic microorganism is used instead, no cooling step would be needed resulting in savings in energy cost. Some *Sulfolobus* species have been used in laboratory bioleaching studies (*Sulfolobus*

acidocaldarius) that proved that leaching occurs more rapidly when thermophilic microorganisms are used and others, like *Sulfolobus metallicus*, are routinely used in agitated tank bioleaching reactors (Bode *et al* 2008). Finally, one example of a thermostable enzyme derived from *S. solfataricus* is the α-amylase, a commercially important enzyme used in the production of trehalose from soluble starch (Miura *et al* 1999).

Besides its potential applications in biotechnology *S. solfataricus* is the model organism of the *Crenarchaea*, which has provided much of the information currently available on the physiology and metabolic processes of hyperthermophilic archaea. In regards to culturing, it is easily grown on both liquid and solid media, allowing formation of lawns and isolation of single colonies as well. It grows aerobically making it advantageous over anaerobic archaea used in similar studies. Moreover, the genomes of *S. solfataricus* 98/2 (http://genome.ornl.gov/microbial/ssol_98/) and P2 have already been completely sequenced (She *et al* 2001).

Transcription in Archaea

For genetic material to be translated into proteins, DNA has to be transcribed first to RNA. The process of transcription starts with one of the two DNA strands called the template, because it provides the order of the sequence of nucleotides in the RNA transcript. The other strand (coding strand) has the same exact sequence as the newly formed messenger RNA (mRNA). It is in the template DNA sequence that transcription starting points are found. The transcriptional system in archaea, closely resembles the eukaryotic RNA polymerase II apparatus, but also includes some bacterial features (Wan *et al* 2004). In Archaea the transcription start points are defined by the TATA box element and by the B-recognition element (BRE). The TATA element is an A/T rich sequence located approximately 25 bases upstream of the transcription start site. Saturation mutagenesis studies of the TATA box of the archaeal *bop* gene promoter from *Halobacterium* sp. strain S9 demonstrated the requirement of a TATA sequence for promoter activity (Baliga and DasSarma 1999). BRE element is a seven base pair sequence upstream of the TATA box that defines the orientation of the transcription pre initiation complex (Bell *et al* 1999). Mutagenesis experiments of the BRE element from *Sulfolobus shibatae* viral T6 promoter has confirmed that its sequence contribute directly to determining promoter strength (Qureshi and Jackson 1998). There are other elements that affect promoter activity. The initiator element (IE), containing a pyrimidine–purine dinucleotide sequences near the transcription start site, and the promoter proximal element (PPE), comprising A/T-rich sequences that are located between -12 and -1 base pairs from the transcription start site, are necessary for full promoter activity of the of the

16S/23S gene of *S. shibatae* (Hain *et al* 1992). A recent whole genome analysis of protein binding sites modified to select only those sequences that transcription initiator factors bind to in *Methanocaldococcus jannaschii* provided evidence that IE sequences are important and are predicted transcriptional start sites (Li *et al* 2008).

The TATA box binding protein (TBP) is responsible for organizing the transcription initiation complex. It recognizes, binds and bends the TATA sequence for subsequent recruitment of transcription factor B (TFB) to the transcription start site (Rowlands *et al* 1994). The crystal structure of the TBP from *Methanococcus jannaschii* has recently been solved by Horikoshi's group and indeed it showed a highly conserved sequence, for DNA binding, and a group specific conserved sequence, for interaction with TFB (Adachi *et al* 2008). Immediately following the formation of TBP-TATA complex, TFB comes into play. TFB is divided into a C-terminal globular domain and an N-terminal zinc ribbon domain that also contains B-finger sequences (Littlefield *et al* 1999, Zhu *et al* 1996). TFB binds the TBP-TATA complex through its C-terminal domain by sequence-specific recognition of BRE (Bell *et al* 1999, Bushnell *et al* 2004). Then TFB recruits RNA polymerase (RNAP) by the interactions between its N-terminal zinc ribbon domain and RNAP subunit A', which acts as the dock domain of RNAP (Werner and Weinzierl 2005). The B-finger sequence is positioned in the active site of RNAP stimulating catalysis (Renfrow *et al* 2004). A third archaeal transcription factor, transcription factor E (TFE), stimulates transcription by stabilizing the pre-initiation complex by interacting with RNAP subunits F and E (Naji *et al* 2007). TFE could also have a role in the transition from the closed to open initiation complex (Forget *et al* 2004).

The structure of the complete RNA polymerase of *S. solfataricus* has been solved (Hirata et al., 2008), demonstrating a remarkable structural similarity to the eukaryotic enzyme. The archaeal RNAP has homologues of all 12 subunits, therefore can be used as a simple model for analyses of the eukaryotic transcriptional machinery. The subunits can be divided in three groups: catalysis (A'/A", B'/B"), assembly (L, N, D and P), and auxiliary functions (F, E, H, and K) (Fig 1.1). The catalytic subunits, also called the large

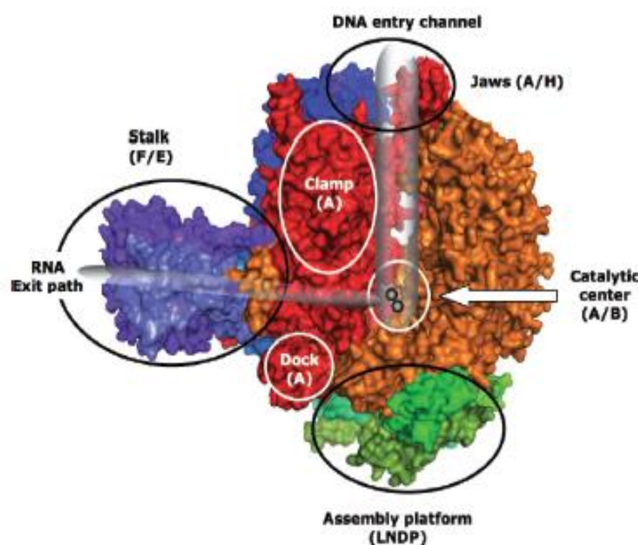

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Fig. 1.1 Structural organization of archaeal RNA polymerase. The RNAP subunits are coloured-coded according to function; large subunits are highlighted in red/orange, assembly platform subunits in greens and auxiliary subunits in blues, and the two magnesium ions in the active site are shown as metallic spheres. The major DNA entry channel and the exit path of the RNA transcript are highlighted as grey-transparent cylinders.

subunits, comprise more than 70% of the actual mass of the RNAP (Korkhin *et al* 2009). Far-Western analysis of several RNAP subunit complexes from *Pyrococcus furiosus* revealed that even though the large subunit A is an essential part of the clamp and of the active center of RNAP, it is not required for recruitment of RNAP to the transcription start site. In contrast, the data presented suggested that the large subunit B has a major role in RNAP recruitment (Goede *et al* 2006). Of particular interest is a recent study by Naji and coworkers. Using a ΔP RNAP in transcription assays they showed that the enzyme was not capable of forming the open complex,

but was capable of transcription elongation, adding more evidence that subunit P is necessary for assembly of the open complex but not for catalytic purposes. A ΔH RNAP was used in a gel shift assay and was recruited by the pre initiation complex, but it had little to none transcriptional activity, indicating a catalytic role for subunit H during transcription elongation (Thomm *et al* 2009).

Gene expression is regulated at many different stages. During transcription the most crucial step or the primary target for regulation is at the initiation step. This holds true for all three domains of life. Transcriptional regulators can be classified as positive or negative regulators, depending on their effect on transcription, and there are instances where a transcriptional regulator can act as both. The galactose-mediated induction of gene expression in yeast is one of the earliest model system used to study transcriptional regulation in eukaryotes. Studies on this system have shown that the role of the positive transcriptional regulator Gal4 is to recruit the transcriptional machinery to the promoter region (Traven *et al* 2006). Two examples of negative transcriptional regulation that are very well documented are the lactose repressor (LacI) and the purine repressor (PurR). The lactose repressor is, under normal conditions, bound to its operator region preventing transcription of genes in the *lac* operon. Repression is then relieved by an inducible molecule, decreasing the affinity of the protein for DNA (Gilbert and Müller-Hill 1966). In comparison, the purine repressor is not bound to the promoter region, but its affinity for DNA increases when an inducible molecule binds to it consequently repressing transcription by binding to its operator region (Schumacher *et al* 1995). Amongst prokaryotic transcriptional regulators the TetR family and the IclR family are two of

the most studied so far. Members of the TetR family are two-domain proteins that possess an HTH DNA binding motif and a C-terminal regulatory domain (Ramos *et al* 2005) and most of them are involved in the regulation of multi drug resistance efflux transporters. Recently the crystal structures of two of its representatives have been solved (AcrR and CmeR) providing more insight into the mechanisms of transcriptional regulation (Routh *et al* 2009). IclR members are also two-domain proteins that regulate glyoxylate shunt, degradation of aromatics, inactivation of quorum sensing signals and sporulation (Molina-Henares *et al* 2006). The Ic1R family comprises repressors, activators and proteins that act as both. There are some examples of transcriptional regulators in archaea that have been examined either *in vitro* or *in vivo*. Possibly, the first study of transcription regulated by metals in archaea was carried out by Bell and co-workers (Bell et al., 1999) on the metal responsive repressor MDR1 of *Archaeoglobus fulgidus*. The metal dependent repressor MDR1 of *Archaeoglobus fulgidus* is a negative regulator that binds to its own promoter region blocking recruitment of RNAP *in vitro*. When metal ions are removed from the growth medium, MDR1 is released from its promoter allowing transcription to be initiated by RNAP (Bell *et al* 1999). Most of the characterized archaeal transcriptional regulators are part of the Lrp/AsnC (leucine responsive regulatory protein) family that consists of proteins with an N-terminal HTH DNA binding motif and a C-terminal RAM (regulation of amino acid metabolism) domain. A member of the Lrp family of proteins of *S. solfataricus*, LrpB, was shown to bind in a cooperative manner to three conserved sequences in its promoter region by DNAse I footprinting and electrophoretic mobility shift assays, providing negative

autoregulation (Peeters *et al* 2004; Peeters *et al*, 2009). Other identified negative regulators in archaea include the *Methanococcus maripaludis* nitrogen regulator (NrpR), the *Pyrococcus furiosus* LrpA, and the maltose binding TrmB from *Thermococcus litoralis* (Geiduschek and Ouhammouch 2005). In some instances, the presence of a positive transcriptional regulator is required for the formation of the pre-initiation complex. Examples of positive transcriptional regulators in Archaea include LysM and Ptr2. *LysM* is part of the lysine biosynthesis operon. *In vitro* studies using cell extracts from *S. solfataricus* grown on medium devoid of lysine showed that LysM binds upstream of BRE and the TATA box, together with the observation that the *lysWXJK* cluster was induced *in vivo* under the same conditions, suggested an activation role for LysM (Brinkman *et al*., 2002). However, the in vitro experiment showed no change in the transcription of the gene cluster after binding of LysM, possibly because additional factors were required for induction. Ptr2 from *Methanococcus jannaschii* is a member of the Lrp/AsnC family of regulators. It has been shown to bind to multiple sequences of TATA boxes increasing transcription by facilitating the binding of TBP to the promoter region. More specifically, the *in vitro* studies of Ptr2 have shown that the transcriptional regulator activates transcription of the *rb2* (rubredoxin 2) gene by binding to an upstream activating site (UAS) and facilitating the recruitment of TBP and subsequently of RNAP to the respective promoter region (Ouhammouch *et al* 2003, 2005). Another positive transcriptional regulator that has been identified is Bat activates the promoters of at least four different transcription units, including the bacterioopsin gene (Baliga *et al* 2001). A limited number of archaeal regulated systems have been fully investigated,

and of these only a few have been corroborated by *in vivo* data, all in euryarchaea. In fact, molecular studies of transcription in archaea have been conducted almost exclusively using *in vitro* transcription systems with cell-free extracts or purified components (Thomm and Stetter, 1985; Thomm *et al*, 1989; Hudepohl *et al*, 1990; Reiter *et al*, 1990), and conducting structural and functional studies employing purified or recombinant factors of transcription Nieuwlandt *et al*, 1991; Hausner and Thomm, 1993). Efficient genetic tools exist for euryarchaeota species: *Methanosarcina acetivorans* (Pritchett *et al*, 2004; Zhang *et al*, 2000), *Haloferax* volcani (Bitan-Banin *et al*, 2003), *Pyrococcus* spp. (Lucas *et al*, 2002), which have been critical to the production of *in vivo* data in support of physiology and biochemical evidence (Allers *et al*, 2005). In contrast, the progress of molecular studies in *Sulfolobus*, and crenarchaeota in general, is hampered by the scarcity of genetic tools, such as selectable markers, reporter genes, and genetically tractable strains.

Genetic systems in Sulfolobus

Research on archaeal, and especially crenarchaeal, microorganisms has been developing somewhat slower than their bacterial counterparts mainly due to a lack of genetic tools. Thus, the discovery, production and improvement of genetic tools for archaea are a top priority. Fortunately, over the past few years we have seen an increase in the repertoire of vectors, markers and strains available for research on archaeal microorganisms. *Sulfolobus solfataricus* is one of the few representatives of the Archaea in which a *bona fide* genetic system has been established to study the molecular mechanisms of regulated transcription.

To develop a genetic system, vectors, recipient strains, and selectable markers are required, and transformation methods need to be optimized. Transformation in *Sulfolobus* is done by electroporation (Berkner and Lipps 2008). Most of the time, the electroporation is carried out using a Genepulser (Bio-Rad) with 1 mm cuvettes at at 1.5 kV, 400 Ω and 25 μF. Several laboratories have tried variations of this protocol achieving successful transformation results. Kurosawa and Grogan carried out a study altering the electroporation conditions and found out that the best parameters to work with are electroporation at 1,250 V, 1,000 Ω and 25 μF (Kurosawa and Grogan 2005). Transformation efficiencies for *Sulfolobus* are generally low, ranging from 4.2×10^2 to 1.1×10^6 transformants per microgram of DNA with the SSV1 virus (Schleper et al. 1992) and from 1×10^2 to 6×10^4 transformants per microgram of DNA with the pRN1 based vectors (Berkner *et al* 2007). A selection is then needed to isolate the transformant strains.

When creating a genetic system that could be widely used, a broad host range replicon is also needed. Of the three types of genetic elements used to transform *Sulfolobus*, SSV1 virus-based type vector was the first and is still used by different laboratories. It is able to replicate in *S. solfataricus* P1 and P2 (Stedman *et al* 2003) and in *S. islandicus* (Arnold *et al* 1999). The plasmid-virus hybrid pSSVx is another genetic element used to transform *Sulfolobus* and is able to replicate in *S. solfataricus* P1, P2 and Gθ (Aucelli *et al* 2006). The third and final genetic element is the pRN1 vector. It was originally isolated from the crenarchaeote *Sulfolobus islandicus* (Keeling *et al* 1996). It is a 5350 bp multi copy plasmid, which is now been predominantly used for transformations in *Sulfolobus*. Replication of pRN1 and its derivatives has been reported in *S. solfataricus* P1 and P2, and in *S. acidocaldarius* (Berkner *et al* 2007, Lipps 2009, Schelert *et al* 2006). Interestingly, a member of the pRN1 plasmid family (pDL10) was found in the crenarcheote *Acidianus ambivalens* (Kletzin *et al* 1999), illustrating the broad host range of this replicon. Taking all of this information into account, it seems that plasmid-based vectors have a wider host range as compare to their viral counterparts, making them the most suitable vecors. The development of shuttle vectors, which are the central piece of genetic complementation and protein expression studies, for *Sulfolobus* has fallen behind the developments in other extremophiles like methanogens and halophiles. So far only a few shuttle vectors have been developed and even fewer have been applied to genetics analysis by the *Sulfolobus* community. Of the earliest *Sulfolobus-Escherichia coli* vectors described pDM1 was the first one. It was made from an intron from *Desulfurococcus mobilis* combined with the plasmid pUC18

(Aagaard *et al* 1995). The same group developed the vector pAG21 mentioned herein before, with improved stability and lower copy number (Aravalli and Garret 1997). The vector pEXSs (also mentioned before) has part of the genome of the SSV1 virus cloned into the plasmid pGEM5Zf(-) (Cannio *et al* 1998). None of these vectors have been successfully applied in further studies.

Vector pMJ03 was the first to be used successfully by different researchers. It is comprised of the plasmid pBluescript and the complete genome of the virus SSV1. The *pyrEF* genes from *S. solfataricus* P2 were added as selectable marker along with a copy of the *lacS* gene as a phenotypic marker (Jonuscheit *et al* 2003). This vector has been used in complementation experiments for example on studies of the genes and proteins involved in bindosome assembly in *S. solfataricus* (Zolghadr *et al* 2007), as well as in protein expression studies like the over expression of the ABCE1 protein from *S. solfataricus* (Barthelme *et al* 2007). A series of shuttle vectors based on the plasmid pRN1 that contain the *pyrEF* genes for uracil selection and the *lacS* gene for lactose selection has been developed (Berkner *et al* 2007). The pRN1 plasmid part was combined with a deletion derivative of pBluescript. Berkner and coworkers showed that expression of proteins is possible with these plasmids in both *S. solfataricus* and *S. acidocaldarius*. Many other shuttle vectors based on the pRN1 plasmid have been developed recently like pHZ2 and pHZ2lacS (Deng *et al* 2009) and without a doubt many others will soon be published.

The two selection systems used in *Sulfolobus* are antibiotic resistance and the use of auxotrophic mutants. So far, only two shuttle vectors have been described that use antibiotic selection: pAG21 and pEXSs. Aravalli and Garret reported the

construction of a shuttle vector that could be maintained in both archaea and bacteria (Aravalli and Garret 1997). Their primary construct was produced by combining portions of the archaeal plasmid pGT5 and the bacterial plasmid pUC19, and was named pAG1. A second construct, pAG2, was generated by the introduction of the Rom/Rop gene from plasmid pBR322 into pAG1. Then the alcohol dehydrogenase gene from *S. solfataricus* was cloned into pAG2, conferring resistance to butanol and benzyl alcohol. Thus vector pAG21 was produced. The pEXSs vector was constructed by the insertion of the autonomously replicating sequence of the virus particle SSV1 into the *E. coli* plasmid pGEM5Zf(-) (Cannio *et al* 1998). Of special interest is the selectable marker, the gene coding for hygromycin phosphotransferase from *E. coli*, which was made thermostable by error-prone PCR. With this thermostabilized protein, *S. solfataricus* transformants can withstand hygromycin B concentrations of up to 150 μg/ml. Even though the findings of these two laboratories are very interesting and should have immediate applications, there have been some problems concerning reproducibility. Many other researchers have tried to no avail to obtain successful transformations using antibiotic selection.

Due to the unreliability of antibiotic selection in *Sulfolobus*, auxotrophic mutants have been the preferred choice of selection after transformation of *Sulfolobus* cells. The two kinds of selection that are more widely used are uracil and lactose. Uracil auxotrophs strains show mutations in their *pyrE* or *pyrF* gene sequences. The two coding enzymes, orotatephosphoribosyl transferase and orotidine-5'-monophosphate decarboxylase, catalyze the last steps in the synthesis of uracil. For transformation purposes, the intact *pyrEF* genes are supplied as selectable marker genes in the

shuttle vectors and are used along with uracil free medium (Grogan and Gunsalus 1993). Recently Lipps and coworkers developed multicopy, non-integrative, plasmid based *Sulfolobus–E. coli* shuttle vectors that have the *pyrEF* genes as selectable markers that are suitable for the use in protein expression and reporter gene studies (Berkner *et al* 2007). Lactose selection is achieved through mutants lacking the *lacS* gene that codes for a β-glycosidase that degrades lactose. *LacS* mutants are unable to grow in medium that have lactose as the sole carbon source. This selection has been applied in *S. solfataricus* to develop knockout systems (Worthington *et al* 2003 and Schelert *et al* 2004) and shuttle vectors (Berkner *et al* 2007).

Several promoters have been studied and used for expression purposes in *Sulfolobus*. The promoter of the α-subunit of the thermosome, *tf55α*, has a very strong basal activity. It has been used recently in the development of shuttle vectors (Jonuscheit *et al* 2003) and in the production of recombinant and tagged proteins from *S. solfataricus* (Albers *et al* 2006). The promoter of the arabinose binding protein, *araS*, has also been shown to have strong transcriptional activity (Albers *et al* 2006, Lubelska *et al* 2006). A new promising promoter from *Sulfolobus shibatae*, the viral T6 promoter, has been characterized recently (Qureshi 2006).

Recipient strains commonly used among the *Sulfolobus* genus have poor genetic stability. This represent a major problem since the outcome of a transformation will drastically be changed if parts of the genome in the recipient strain can be deleted or inverted. Looking at the sequenced genome of *S. solfataricus* P2 we find a very high number of mobile genetic elements (She *et al* 2001), which is not a desirable trait when choosing an appropriate recipient strain. Spontaneous mutation frequencies are

also high among members of *Sulfolobus*, ranging from 10^{-4} to 10^{-8} (Berkner and Lipps 2008, Martusewitsch *et al* 2000). Restriction and/or modification activity is another factor to take into account when choosing a suitable recipient strain because it can interfere with the establishment and maintenance of a vector in the recipient strain. Fortunately, most *Sulfolobus* strains have little or no restriction/modification activity (Albers and Driessen 2008, Grogan 2003, Söllner *et al* 2006). And those that do can still be used after methylation of the transformed DNA (Grogan 2003). Because of their innate differences, not all *Sulfolobus* strains are genetically tractable, and tools and methodologies developed for selected strains are not applicable to others. *S. solfataricus* strain 98/2 and its derivatives, which are used for the work described in this dissertation, are the only strains for which targeted disruption of functional genes was successfully achieved by integration of a truncated homolog within the chromosomal alleles. The truncated genes were interrupted by the *lacS* gene that served as selectable marker.

The *S. solfataricus* strain 98/2 is more amenable to genetic manipulations than the strain P2, and has been used to study molecular and biochemical aspects of different proteins like ATPase (Hochstein and Stan-Lotter 1992), α-glucosidase (Rolfsmeier and Blum 1995) and dioxigenase (Chae *et al* 2007). Even though they have virtually identical genomes, their behaviors under specific conditions are different. *S. solfataricus* P2 was isolated in Pisciarelli Solfatara in Italy, while 98/2 was isolated from Yellowstone National Park in USA, that can explain their different environmental responses. PBL2025, a derivative strain of *S. solfataricus* 98/2 with a 58-kb deletion spanning open reading frames SSO3004 to SSO3050 (Haseltine *et al*

1999) is currently been used to create gene disruption mutants (Schelert *et al* 2004 and 2006), and as recipient for non-integrative shuttle vectors (Berkner *et al* 2007), through lactose auxotrophy.

Reverse genetics seek to find the phenotype produced from a specific genetic sequence, and the first step to achieve this is to engineer a change or disruption in the DNA. Methods for targeted gene disruption/ gene deletion are preferred to random mutagenesis. A method to inactivate specific genes in *Sulfolobus* was developed by Blum and coworkers. This system utilizes lactose selection and the spontaneous *lacS* deletion mutant *S. solfataricus* PBL2025 as recipient strain (Schelert *et al* 2004). To obtain desirable disrupted genes, the *lacS* gene from *S. solfataricus* and its promoter is introduced between the genes to be deleted. By homologous recombination the targeted gene is disrupted and the *lacS* gene is integrated into the host chromosome making the transformed mutant able to grow on lactose. After transformation, selection is carried out first in liquid medium (with lactose as the sole source of carbon) before plating on solid medium. The high applicability of the knockout system can be appreciated in different studies done on the α-amylase (Worthington *et al* 2003), the mercuric reductase (Schelert *et al* 2004, Schelert et al., 2006), the editing domain of the threonyl-tRNA synthetase (Korencic *et al* 2004) and the structural flagellin gene (Szabo *et al* 2007).

Often genetic systems rely on reporter genes to quantitatively measure the results of a transformation for either protein expression assays or for promoter studies. Among the most common reporter genes are green fluorescent protein (GFP) and the luciferase enzyme. So far no work has been published in *Sulfolobus* that uses either

one of these reporter genes. Instead, the only reporter gene used so far is the *lacS* gene that shows both β-glycosidase and β-galactosidase activity. The activity of the glycosidase is detected after addition of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) which is converted to a blue colored product and can be detected easily on Gelrite plates. In a recent study *cis*-acting elements controlling arabinose-responsive expression in archaea were characterized using *lacS* as a reporter gene (Peng *et al* 2009). But before this report other laboratories have also used *lacS* successfully as a reporter gene (Aucelli *et al* 2006, Jonuscheit *et al* 2003).

Genetic systems in *Sulfolobus* are still in the early steps of development, leaving a lot of room not only for improvement of the existing ones, but also for the creation of newer and better ones. A clear and universal transformation protocol, along with broader range vectors, more efficient reporter genes and promoters (with low basal activity and/or repressible) will allow the progress desired by many researchers working with crenarchaeal microorganisms.

Heavy metal metabolism: copper transport

Metals have an essential role as trace elements in many biological systems. Approximately more than half of all proteins contain metal ions either as a catalytic cofactor or as a structural component (Degtyarenko 2000). Copper is one of these essential trace elements found in all living organisms in the oxidized Cu(II) and reduced Cu(I) states. It serves as an important catalytic cofactor for proteins that carry out fundamental biological functions that are required for growth and development. Some of these proteins are: Cu/Zn superoxide dismutase, cytochrome c oxydase, lysyl oxydase, dopamine ß-hydroxylase, clotting factors V and VII, ceruloplasmin, metallothionein, hephaestin, tyrosinase and prion protein among others (Peña *et al* 1999).

The functional importance of copper relies on the ability of copper to cycle between a stable oxidized state, Cu(II), and an unstable reduced state, Cu(I), which is used by copper containing enzymes (also called cuproenzymes) in redox reactions (Camakaris *et al* 1999). However, when copper is present in excessive amounts, this same ability may lead to the generation of reactive oxygen species (ROS), like superoxide radicals and hydroxyl radicals, which are responsible for lipid peroxidation in membranes, direct oxidation of proteins, and cleavage of DNA and RNA molecules (Tapiero *et al* 2003).

The main sources of copper are seeds, grains, nuts, beans, shellfish and liver (Tapiero *et al* 2003). The average intake of copper by human adults is about 1 mg/day, whereas the total amount of copper in a human adult body is about 110 mg/day (Linder *et al* 1998), so the majority of the copper in the body is recycled

back and forth between tissues. Because of this just a slight change of copper levels can cause toxicity in humans, which is also true for microorganisms. Malfunction in copper homeostasis has been correlated to inflammation, infection and cancer problems. Some copper related diseases include: 1) aceruloplasminemia, which is caused by a decrease in the production or action of ceruloplasmin; 2) prion diseases, in which copper stimulates endocytosis of the prion protein (PrPC) and facilitates its refolding into the abnormal form (PrPSc); 3) Alzheimer's disease, copper is involved in the formation of the ß-amyloid protein that aggregates in the brain of Alzheimer's patients; 4) Menkes and Wilson diseases (Camakaris *et* al 1999; Cox and Moore 2002; Tapiero *et al* 2003).

P-type ATPases are a large family of cation-translocating pumps. They are classified into five groups according to substrate specificity. One of these subfamilies transports soft Lewis acids, and is referred to as soft metal transporting P-type ATPases (Banci *et al* 2002; Rensing *et al* 2000). Copper transporting ATPases are among this group, like the Menkes and Wilson proteins. The Menkes and Wilson proteins which are encoded by the ATP7A and ATP7B genes, respectively, are the most studied P-type ATPases. Menkes disease is an X-linked disorder which impairs transport of dietary copper from the intestine, resulting in a generalized copper deficiency in most tissues. Patients with Menkes disease have neurodegeneration, developmental delay, connective tissue abnormalities, seizure, kinky twists of the hair, hypothermia, and usually death in infancy (Cox and Moore 2002). Wilson disease is an autosomal recessive disorder wich results in the accumulation of excess copper in the liver with other tissues accumulating oxidative

damages. Patients with Wilson disease present brain disorders and liver disease (Tapiero *et al* 2003). The soft metal pumps are much conserved throughout all three kingdoms of life, suggesting a very important role in cell biology.

Prokaryotes have developed strategies to utilize and maintain metals within physiological ranges. Genetic determinants of copper homeostasis have been described for several bacterial species. In particular, the various components of the *Enterococcus irae* and *E. coli* copper-homeostasis systems, their regulation, and interactions have been thoroughly studied (Rensing and Grass 2003; Solioz and Stoyanov 2003). The *Enterococcus hirae* copper transport system is currently the best understood and most extensively studied and reviewed copper homeostatic system (Magnani and Solioz 2005). The cop operon of *E. hirae* consists of two CPx-type copper transporting ATPases, CopA and CopB, a chaperone, CopZ, and a repressor, CopY (Odermatt *et al* 1993; Odermatt & Solioz 1995). In short, Cu(II) is converted to Cu(I) at the cell surface by a yet to be characterized reductase and then is imported into the cell by CopA. The copper ions that enter the cells are sequestered by CopZ, mediated by its interaction with CopA (Multhaup *et al* 2001). CopZ then delivers the copper ions to CopY (Cobine *et al* 2002), which is then released from the promoter region allowing transcription of the four *cop* genes. CopB export excess copper from the cell. At the time it was described it was a novel induction mechanism whereby the inducer is delivered to the repressor by a protein.

Maintenance of copper homeostasis in Archaea, like in Bacteria and Eukarya, is achieved through three regulated stages: uptake, intracellular sequestration and transportation, and efflux. Mechanisms of cellular copper uptake have not been well

defined, although it has been proposed that P-Type ATPases might be involved. The intracellular sequestration and transport of copper is carried out by metallothioneine and by other copper binding proteins (Kim *et al* 1995; Tanaka *et al* 2004). One of these copper chaperones has been recently identified and named TRASH for its predicted role in trafficking, resistance and sensing of heavy metals. TRASH is encoded by prokaryotic as well as eukaryotic genomes and is present in cation-transporting ATPases, transcriptional regulators and is also present as a stand alone molecule (Ettema *et al* 2003). TRASH contains a distinct and conserved cysteine signature (CxxC), which is found in other metal binding proteins. Copper efflux is mainly carried out by P-type ATPases (Camakaris *et al* 1999; Banci *et al* 2002; Cox and Moore 2002; Rensing *et al* 2000). Structural studies of individual functional domains of the copper-transporting ATPase CopA in *Archeoglobus fulgidus,* including the characterization of the actuator domain (Sazinsky et al 2006a), ATP binding domain (Sazinsky et al 2006b), metal binding domains (Mandal and Arguello 2003) and copper binding site (Mandal et al 2004) have provided useful insights into the activities and functions of the ATPase. In the extreme acidophilic archaeon "*Ferroplasma acidarmanus*" strain Fer1, copper-dependent expression of the copper binding protein CopZ and the copper transporting ATPase CopB (a Cu(I) transporter, despite its designation), respectively, were reported (Baker-Austin *et al* 2005). An interesting mechanism for copper detoxification has been observed in *Sulfolobus metallicus*, which is based on sequestration by organic phosphate, possibly followed by active efflux of the metal-phosphate complex (Remonsellez *et al* 2006). In the presence of copper, an exopolyphosphatase protein (PPX) is

activated, which hydrolyzes inorganic polyphosphate (P_i), and the accumulated P_i is removed from the cell through the inorganic phosphate transport system (Pit), accompanied by Cu(I) transport (Remonsellez *et al* 2006). This is thought to be mediated by a symporter.

S. solfataricus has two putative genes coding for cation-transporting ATPases, Sso2896 and Sso2651. The putative cation-transporting ATPase Sso2651 has a predicted structure similar to that of *E. coli*'s CopA P-type ATPase (Camakaris *et al* 1999; Rensing *et al* 2000). In common with other P-type ATPases Sso2651 has conserved ATP binding domain (GDGIN), phosphorylation domain (DKTGT), phosphatase domain (TGE), a conserved sequence found only in soft metal P-type ATPases (SEHPI), and a membrane embedded metal binding sequence (CPC) in one of its eight transmembrane segments. In contrast to other P-type ATPases, Sso2651 has only a single cytosolic CxxC metal binding domain (MBD) (Banci *et al* 2002; Camakaris *et al* 1999; Rensing *et al* 2000).

Objectives

The goal of this thesis is to contribute to the understanding of the molecular mechanisms behind copper homeostasis, focusing on the events controlling the transcription of the genes involved in this process in *Sulfolobus*. Homeostasis of copper is a fundamental process. Despite their fundamental role in virtually all living beings, our knowledge of the copper homeostatic systems and their functioning in archaea is still very limited.

The hyperthermophilic archaeon *Sulfolobus solfataricus* will be employed as model system, because of its ability to effectively respond to metal stress, which is key to

survival in its harsh original environment: acidic, rich in metal ions (Simbahan et al., 2005), and strongly oxidative (Limauro et al., 2006); because there is great interest in the elucidation of fundamental physiological processes occurring at high temperatures; and finally, *Sulfolobus* is a simple system, can be easily grown in defined minimal medium with accurate control of copper concentration, newly developed tools are available for genetics studies, and has a completely sequenced genome.

Therefore, the study of the expression of copper responsive genes in *S. solfataricus* will contribute to a better understanding of the underexplored mechanisms of copper homeostasis and resistance in archaea.

The stated goal will be accomplished through the following specific objectives:

1. Monitoring the response to excess copper in *Sulfolobus*, and identifying the responsible genes (Chapter 2).

2. Knocking out the gene encoding CopR, to test the hypothesis that CopR is a copper responsive regulator of transcription, controlling the expression of the copper binding protein CopT and the copper transporter CopA (Chapter 3).

3. Developing a reporter gene system based on the *Sulfolobus* thermostable β-glucuronidase to be applied to the *in vivo* analysis of promoter gene expression, using fusions between the promoter of copper responsive genes and the reporter *gusB* (Chapter 4).

Chapter 2

Response to excess copper in the hyperthermophile *Sulfolobus solfataricus* strain 98/2

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Introduction

Copper is a transition metal and an important trace element because of the essential role it plays in a range of biological processes. In contrast, the occurrence of copper levels beyond the physiological range causes serious damage to all molecular components. Studies on yeast have led to the proposition that virtually no free copper ions are present in the cell under normal conditions (Rae *et al* 1999). The response of cells to copper excess/deficiency is accomplished through the interplay of copper-binding proteins, copper-responsive regulators, transporters for the efflux and uptake of copper and copper-requiring enzymes. Genetic determinants of copper homeostasis have been described for several bacterial species (Chintalapati *et al* 2008; Espariz *et al* 2007; Magnani *et al* 2008; Sitthisak *et al* 2007; Smaldone and Helmann 2007). In particular, the various components of the *Enterococcus irae* and *Escherichia coli* copper-homeostasis systems, their regulation, and interactions have been thoroughly studied (Rensing and Grass 2003; Solioz and Stoyanov 2003). Many sequenced archaeal genomes encode homologs of Cu (I) and Cu (II) transporting ATPases (Bini 2008). However, investigations of the response in archaea to changes of copper levels are still limited. Structural studies of individual functional domains of the Cu (I)-transporting ATPase CopA in *Archeoglobus fulgidus* have provided useful insights into its activities and functions (Cattoni *et al*

2008; Mandal *et al* 2004; Rice *et al* 2006; Sazinsky *et al* 2006a; Sazinsky *et al* 2006b). *A. fulgidus* also possesses a Cu(II)-transporting ATPase, CopB, that has been biochemically characterized (Mana-Capelli *et al* 2003). The transcriptional analysis of a cop locus responsible for survival in the presence of copper has been reported in the extreme acidophilic archaeon ‘‘*Ferroplasma acidarmanus*” strain Fer1, where cotranscription of genes encoding the copper-binding protein CopZ and the putative copper-transporting ATPase CopB was shown to increase in response to Cu (II) (Baker-Austin *et al* 2005). An interesting mechanism for copper detoxification has been described in *Sulfolobus metallicus*, which is based on sequestration by organic phosphate, possibly followed by active efflux of the metal-phosphate complex (Remonsellez *et al* 2006). The *Sulfolobus solfataricus* genome encodes a cop locus, which includes the three open reading frames (ORFs) Sso2651, Sso2652, and Sso10823, encoding the CopA ATPase, a copper-responsive regulator, and a putative copper-binding protein, respectively (She *et al* 2001). Cotranscription of Sso2651 and Sso10823 has been reported to specifically increase in the presence of copper, while the copper-responsive regulator binds sequences surrounding the putative copA promoter in *S. solfataricus* strain P2 (Ettema *et al* 2006). Inspection of the *S. solfataricus* genome reveals the presence of a second *cop* locus, including the gene *copB*, its putative regulator *copR* and *copC*. This cluster of genes is encoded by a genomic region distant from the *copRTA* operon. CopB has been reported to have a catalytic domain phosphatase activity stimulated by copper (Deigweiher *et al* 2004). In this study, the response of *S. solfataricus* to copper has been further investigated in the strain 98/2. The selection of the genetically tractable strain 98/2 (Worthington

et al 2003) will expand the scope of analyses aimed to the elucidation of archaeal interactions with copper. To gain better insights into the *Sulfolobus* response to copper levels, the transcription of the three genes of the copRTA operon has been examined under different conditions and in a time course experiment, and the changes in the amount of copper associated with the cells have been monitored over time. To determine whether CopB played a role in copper homeostasis, we analyzed its expression in the presence of copper excess or in copper depleted cultures. Based on the data obtained, a preliminary model for the maintenance of copper homeostasis in *Sulfolbus* is proposed.

Materials and methods

Growth conditions. *Sulfolobus solfataricus* strains 98/2 or P2 (DSM 1617) were cultured at 80 °C in a defined standard medium (SM) as described in (Worthington *et al* 2003), the medium was supplemented with 0.2% sucrose as the carbon and energy source. Batch cultures were inoculated to obtain a density corresponding to an OD540 of about 0.025, with aliquots withdrawn from mid-log phase cultures. Growth was monitored at a wavelength of 540 nm on a Beckman DU-520 spectrophotometer (Beckman Coulter, USA). All the experiments were carried out on the strain 98/2, unless otherwise stated.

RNA extraction. Total RNA was isolated from *S. solfataricus* cultures in their exponential phase of growth (OD540 = 0.3–0.6). Before centrifugation at 3500g for 15 min, cells were mixed with two volumes of RNA Protect (Qiagen, USA). RNA was extracted from the cell pellets using the RNAeasy Mini kit (Qiagen, USA) and

treated with DNase (Ambion, USA), as recommended by the manufacturer. DNA contamination was excluded by PCR using primers targeting the 16S rRNA gene. The quantity and quality of the RNA obtained was evaluated both spectrophotometrically on a NanoDrop ND- 1000 spectrophotometer (NanoDrop, USA) and by agarose gel electrophoresis (Sambrook *et al* 1989).

Reverse-transcription PCR (RT-PCR) and real-time quantitative RTPCR (qRT-PCR) analyses. For RT-PCR analysis, total RNA (0.5 μg) was analyzed in 25-μl reactions using the Enhanced Avian HS RT-PCR Kit (Sigma–Aldrich, USA). The amplification products were separated on a 1.2% agarose gel by electrophoresis and the gel images were acquired using a GelLogic 440 Imaging System (Eastman Kodak, USA). Specific transcripts were quantified by qRT-PCR using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, USA) and the real-time detection system iCycler iQ (Bio-Rad, USA). Reactions, in triplicate, were assembled according to the protocol of the manufacturer, and using 0.2 μg of total RNA in a 25-μl reaction. Primers were designed using the OligoPerfect Designer software (Invitrogen, USA) to have a composition that was suitable for use in both RT-PCR and qRT-PCR (Table 1). Specificity of each pair of primers was confirmed by sequencing. The efficiency of the PCR amplifications was determined from the slopes of the dilution curves of the target RNA. The cycle threshold (Ct) values obtained were used in the ‘‘$2^{-\Delta\Delta Ct}$ Method” to calculate the relative changes in gene expression (Livak and Schmittgen 2001). Expression of the target RNAs of interest was normalized to the level of the Sso0067 transcript, detected using the primers q0067-F and q0067-R (Table 2.1). Sso0067 encodes a ribosomal protein and its

expression is not affected by copper exposure, as determined by microarray analysis (unpublished).

Analysis of copper content. Cell samples were harvested from exponentially growing *S. solfataricus* cultures. Cell pellets were washed with 10 mM EDTA to remove the copper adsorbed to the cell wall, then rinsed with SM without added trace metals (Zn, Cu, Mo, V, and Co). Before centrifugation at 3500 g for 15 min, aliquots were removed from each sample for determination of protein concentration using the BCA Protein Assay (Pierce, USA). Cell pellets were resuspended in 50% nitric acid, and digested for 16 h at 25°C. The total copper content was analyzed using a Vista Pro radial view Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Varian Inc., USA). Controls included non-inoculated SM medium and untreated cultures.

Table 2.1 Oligonucleotides used in chapter 2.

ID	Sequence (5'-3')	Target
q2651-F	GAATAGTTGGGATGCATTGT	*copA*
q2651-R	ACTACCCCCTTAACGTTTTC	"
q2652-F	TTTATTGCCTTCGCCATTTC	*copR*
q2652-R	GTTGCGTGCAAATTTTTCCT	"
2652-F	TGCAATTCTTGCTTGTCTGG	*cop* operon (paired with q2651-R)
q10823-F	ATGATAATCGATCCGGTTTG	*copT*
q10823-R	ATTCCTTAAATACTCTTCCGGA	"
q0067-F	TACCAATTGTCGCTTTTGCT	Reference transcript
q0067-R	CAAATCACCATCTGGAGGAA	"
q2896-F	CGGTGTTGCTGGAATATCCT	*copB*
q2896-R	CTGGTTCAGATTCGCCAGTT	"
2897-F	GGAAATGGCTACTCCTTGTCC	*copR2C*
11412-R	CCTACCATGTATCCGAGGTCA	"

Results and discussion

Physiological response to copper

To establish the optimal concentration of copper to be used in this study, cells were exposed to $CuCl_2$ at concentrations ranging from 0 mM to 2.5 mM. The MIC, defined as the lowest concentration that completely inhibits cell growth immediately after exposure, was determined to be 1.5 mM (Fig. 2.1A). In response to copper concentrations that were equal or greater than 1.5 mM $CuCl_2$, a lag phase of variable duration was observed. The duration of the lag phase was directly proportional to the metal concentration, and growth resumed thereafter, indicating a slow adaptation to levels of copper that were above the MIC. To rule out the possibility that this effect was due to the appearance of copper-resistant mutants, cells adapted to 2 mM copper-containing medium were subcultured in fresh medium in the absence of the metal. After 4 cycles of cell divisions, the cells were re-inoculated into fresh medium containing 2 mM copper. A lag phase was observed again, indicating that the response to copper concentrations above the MIC was the result of physiological adaptation (unpublished). To limit the manifestation of extensive stress responses that would overshadow the response to copper, only the sublethal concentration of 0.75 mM copper was used for transcript measurements. The level of tolerance to copper observed in *S. solfataricus* 98/2 was within the range observed for most microorganisms. It was previously observed that the carbon source affected the copper sensitivity of microbes (Miller *et al* 1992), probably because of the binding of copper to the thiol groups of some amino acids and other molecules. Therefore, all experiments were carried out using cells grown on 0.2% sucrose as the sole carbon

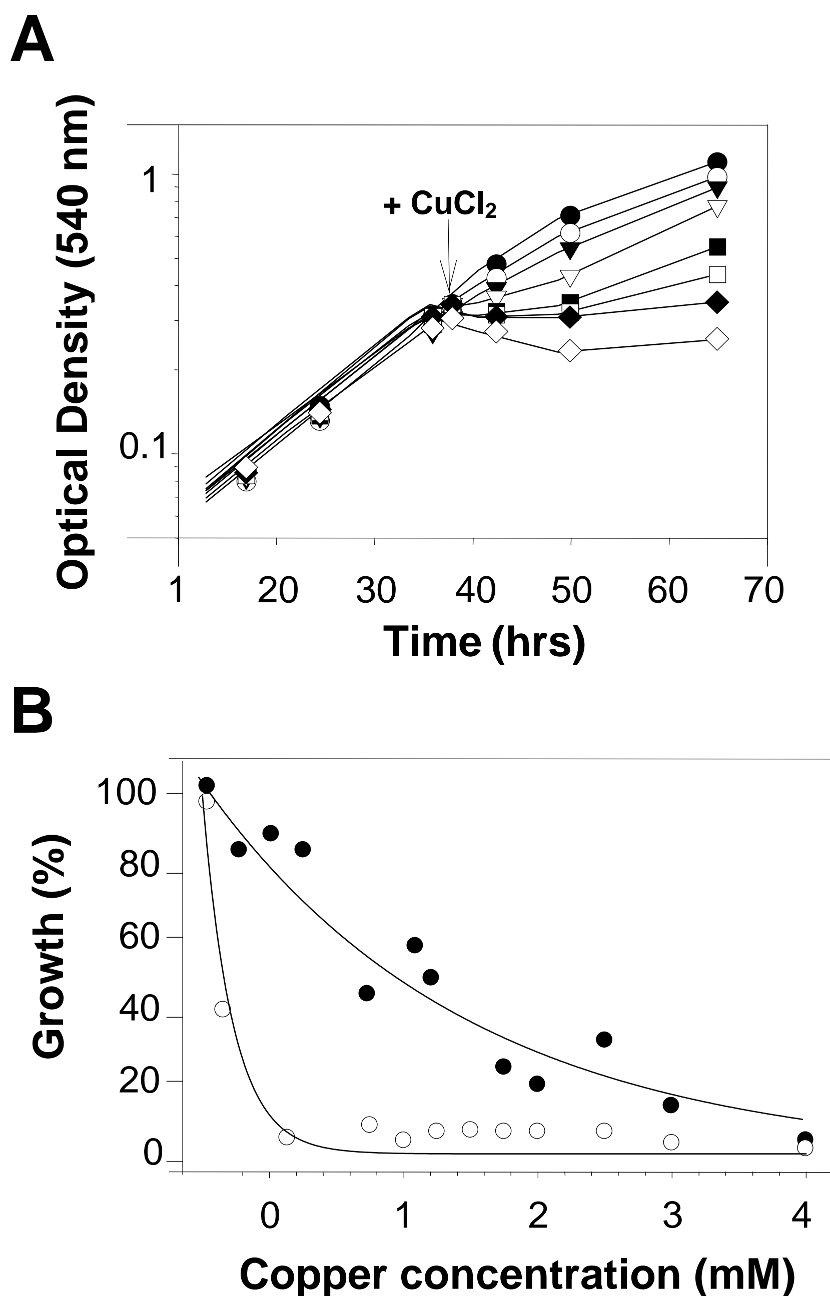


Fig. 2.1 Effect of $CuCl_2$ on growth of *S. solfataricus*. (A) An exponentially growing culture of strain 98/2 was used as inoculum for 8 subcultures. Copper was added at the time indicated by the arrow, at the following final concentrations: (●), 0 mM; (○), 0.75 mM; (▼), 1 mM; (▽), 1.25 mM; (■), 1.5 mM; (□), 1.75 mM; (♦), 2 mM; (◊), 2.5 mM. (B) Growth of P2 (○) or 98/2 (●). Copper was added at the time of inoculum and cell growth is expressed as percentage of the untreated control. Best fit curves were obtained by nonlinear regression applied to both sets of data.

source. Interestingly, the *S. solfataricus* strain P2 showed a higher copper sensitivity than the strain 98/2, displaying a growth rate on 0.2 mM copper comparable to that observed for strain 98/2 grown in the presence of 2.5 mM copper (Fig. 2.1B). The two strains were originally isolated in Italy and USA, respectively. It was speculated that their physical separation might have led to different functionality of the copper responsive system. Thus, genetic diversity either at the level of the transcriptional regulator CopR or within the region of the putative copTA promoter was hypothesized to be responsible for the different copper sensitivities of the two strains. However, the sequencing of the segment of the *cop* operon that includes *copR*, *copT*, and their intergenic space in the strain 98/2 (GenBank Accession No. EU544670), showed that this region was identical to the corresponding sequence in strain P2, indicating that other factors probably contribute to these differences.

Cotranscription of copRTA

The cop operon consists of three genes oriented in the same direction, represented by ORF's Sso2652, Sso10823, and Sso2651. With the possible exception of the Cu (I) transporter CopA, and the Cu (II) transporter CopB, there are no unique identifiers for functional homologs of copper responsive sequences in prokaryotes. Herein, it is proposed the designation ''CopR" for the product of ORF Sso2652, in agreement with the nomenclature applied to metal responsive transcriptional regulators: ArsR, NikR, MerR (Pennella and Giedroc 2005), and CopR (Cantini *et al* 2009; Mills *et al* 1994). ''CopT" is suggested for Sso10823, which corresponds to a stand alone TRASH domain (Ettema *et al* 2003). In an attempt to elucidate the molecular mechanisms behind the regulation of the *copRTA* operon (Fig. 2A), RT-

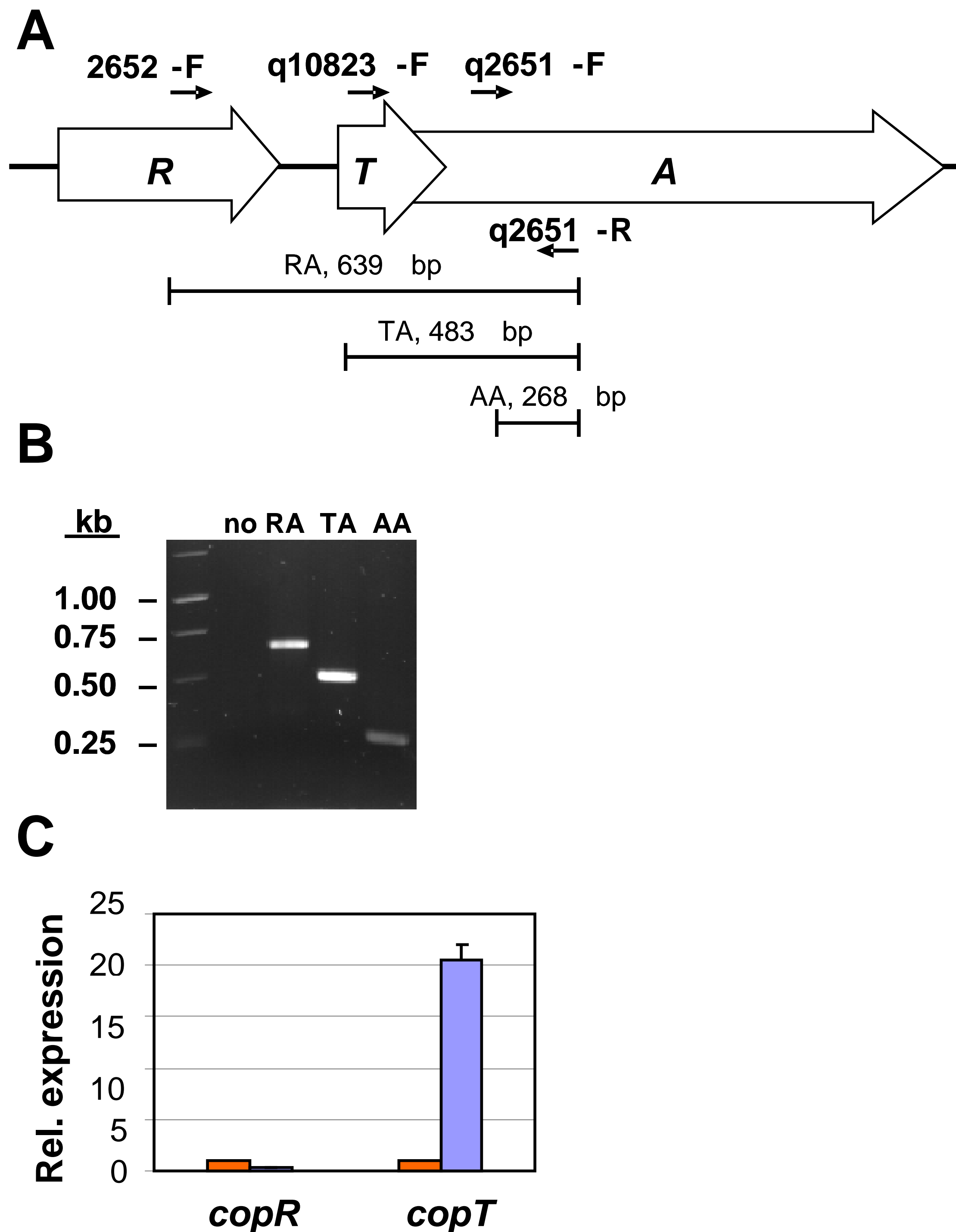


Fig. 2.2 Co-transcription of copR, copT, and copA. (A) Diagram of the cop operon and location of the primers. (B) Agarose gel electrophoresis of the amplicons corresponding to the regions encompassing copR/copA (RA), copT/copA (TA) and targeting copA (AA), (no) negative control without reverse transcriptase. (C) Expression of copR and copT in the presence of copper (blue bars) relative to untreated controls (orange bars). Results are reported as means ± standard errors.

PCR was used to test whether the genes *copR*, *copT*, and *copA* were cotranscribed. Using the primers pair 2652-F and q2651-R, designed to amplify across the genes *copR* and *copA*, a unique band of the expected size was obtained (Fig. 2.2A and B), sequencing of the amplicon confirmed its specificity and indicated the cotranscription of all the three genes. Cotranscription of the copper-binding protein and the ATPase-coding genes was previously reported to be detected by primer extension (Ettema *et al* 2006), but the cotranscription of the *copR* gene was not addressed in that analysis. By specifically targeting the region encompassing the three genes, the regulator-encoding copR was also shown to be a part of the transcript. This is an interesting finding because it provides additional insight into the regulation of copper homeostasis in *Sulfolobus*, as discussed later in this section. In the next step, the induction of individual genes was investigated by qRT-PCR. The primer pairs used for the detection of *copR* and *copT* are listed in Table 2.1. In cells treated with copper, the levels of copT mRNA exceeded the amounts of the same transcript in untreated cultures, displaying a greater than 20-fold induction after 120 min, thus behaving as copA (see section *Transcription of copA occurs transiently in response to copper*), however, the level of copR expression was unaffected by copper (Fig. 2.2C). This trend agreed with the data obtained by primer extension (Ettema *et al* 2006), and, moreover, this method provided an accurate quantitation of the fold induction. The fact that the genes of the operon *copRTA* are cotranscribed, that copR is constitutively expressed (Fig. 2.2C), and that *copTA* transcription is considerably affected by excess copper, suggests a model where, in the absence of excess copper, copT and copA are transcribed together with copR, constitutively,

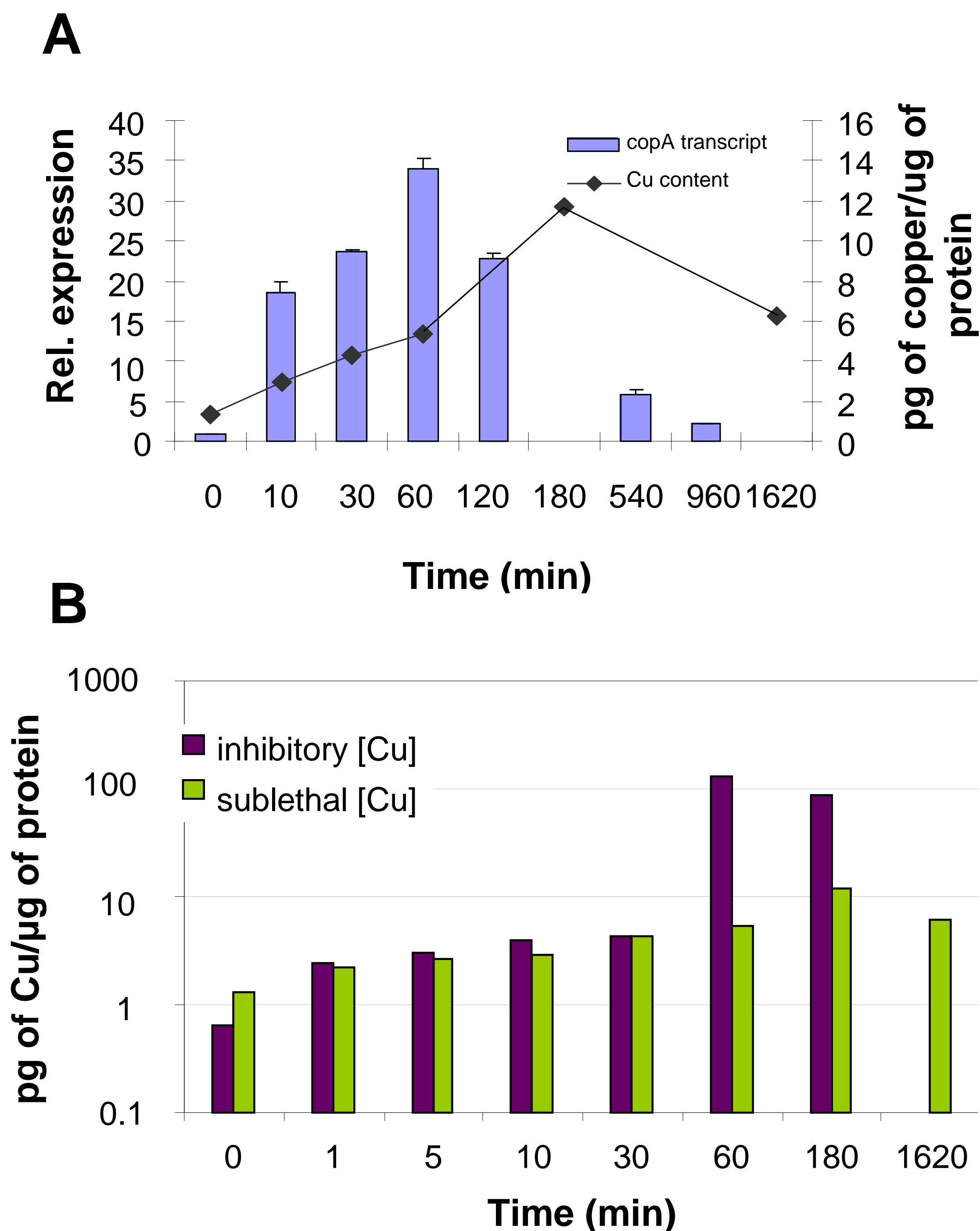


Fig. 2.3 Time course analysis of copper content and copA induction in response to copper. (A) Total RNA was isolated at different times after treatment of the cultures with 0.75 mM copper. Samples were not collected at 180 and 1620 min. Results are expressed as means ± standard errors. Bars represent the normalized ratio between amounts of transcript in copper treated and untreated cultures. For comparison, changes in copper content (♦) after exposure to 0.75 mM copper are superimposed. (B) Copper content in cultures treated with 0.75 mM (purple bars) or 1.25 mM copper (green bars).

from the copR promoter, whereas the supplementary transcription of *copTA* in the presence of copper results from the induction of a second promoter upstream of copTA promoter. The constitutive expression of *copTA* probably provides a constant and low-level supply of the proteins CopT and CopA that maintain homeostasis, allowing the cell to adjust to small fluctuations in copper levels under normal conditions.

Transcription of copA occurs transiently in response to copper

To better understand the regulation of cell response, the expression of copA was monitored in a time course experiment. Total RNA samples, isolated at different times after treating cell cultures with 0.75 mM $CuCl_2$ were subjected to qRT-PCR analysis using primers targeting the copA transcript (Table 2.1). *Sulfolobus* responded to exposure to a sub-lethal copper excess by the transient active transcription of the *copA* gene. The *copA* transcript reached a peak 1 h after treatment, corresponding to approximately 35-fold the uninduced level, thereafter, the transcript level decreased until a steady-state level of 2–3-fold induction is reached in the following 16 h (Fig. 2.3A). Moreover, in cells cultured for several generations in the presence of 0.75 mM copper, the amount of copA transcript was maintained at 2–3-fold the uninduced level (unpublished), indicating that its rate of expression was maintained constant during long-term exposures, provided the concentration of copper did not change. The above observation can be explained as follows. A high rate of CopA synthesis is necessary to reestablish homeostasis during early copper exposure. Thereafter, the drop in intracellular concentration of copper causes a decrease in copA transcription, which leads to the establishment of a

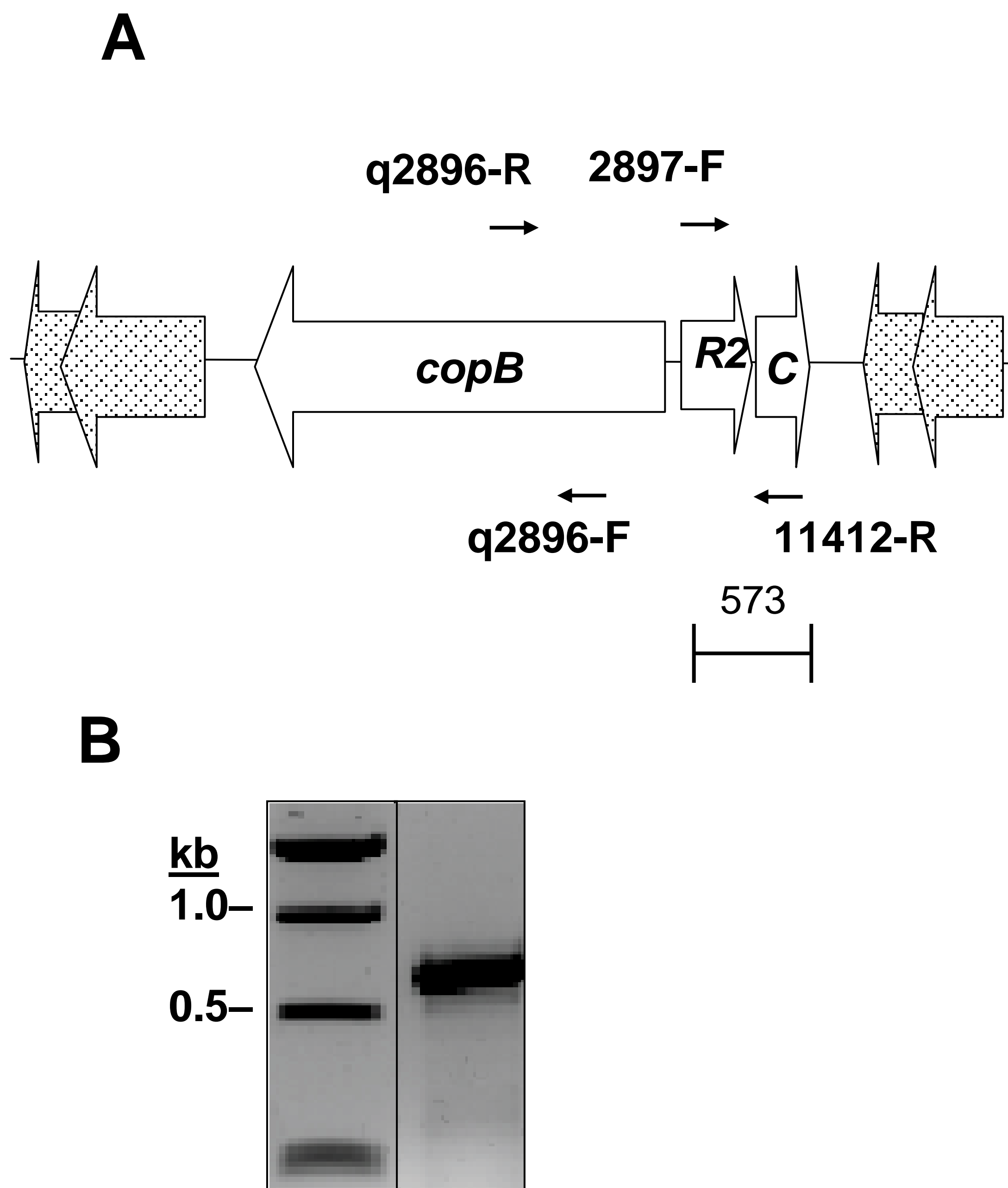


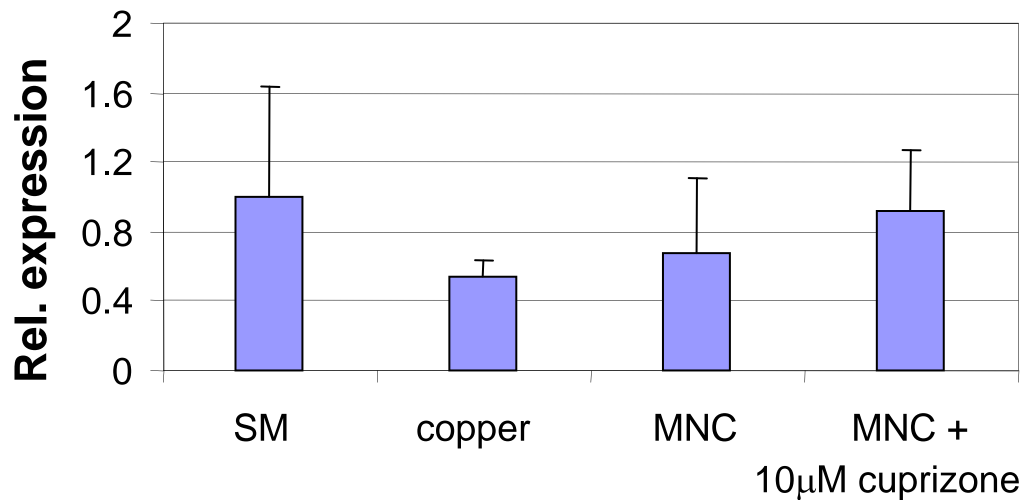
Fig. 2.4 Organization of a second *cop* operon in *S. solfataricus* and co-transcription of *copR* and *copC*. (A) Schematic of the gene cluster including *copB*, *copR2* (R2) and *copC* (C). Textured arrows on either side of the cluster represent transposase genes. (B) Agarose gel electrophoresis of the amplicon obtained by RT-PCR analysis of total RNA without copper added and using the pair of primers as indicated in Fig. 2.4A.

rate of CopA synthesis sufficient to maintain the internal equilibrium at the new cytoplasmic copper concentration. It has been previously reported (Ettema *et al* 2006) that treating *S. solfataricus* P2 with 5 mM copper causes an accumulation of the *copA* transcript over a 2-h period. This might be explained by a higher demand for CopA, extended over time, in the presence of copper concentrations above the MIC. This observation is also consistent with the behavior of *S. solfataricus* 98/2 for copper levels above 0.75 mM (Fig. 2.1A). Furthermore, the fact that copA is transiently induced after copper challenge and that its transcription subsequently declines but is maintained at a low basal induced level for the duration of exposure might indicate an accumulation of the CopA transporter. The persistence of a stable CopA protein would reduce the demand for further copA transcription and translation. To test if the expression of CopA is specifically induced by copper, different metals were tested: arsenic (1 mM), cadmium (1 mM), chromium (1mM), nickel (0.5 mM), vanadium (1.44 mM), and zinc (50 mM). It was observed that *copA* transcript increases specifically in response to copper and to a lesser extent to zinc. However, the amount of zinc used largely exceeded the amounts seen in natural environments, thus the effect observed is likely due to non-specific induction (data not shown).

Determination of copper content

To test if the pattern of expression of copA depended on internal fluctuations of copper, the copper associated with the cells was analyzed in a time course experiment by ICP-OES spectrometry, and was observed to slowly increase during the first 3 h of monitoring. This might be explained with the sequestration and

A



B

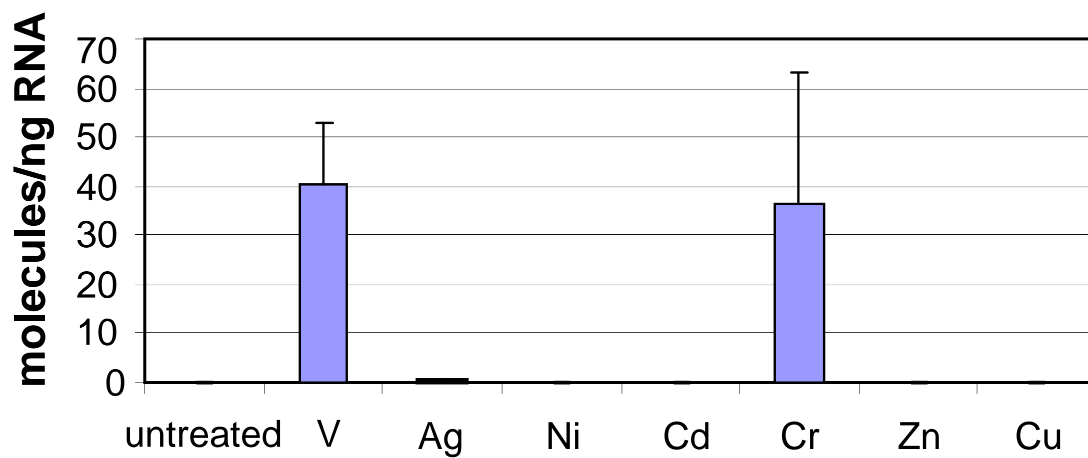


Fig. 2.5 Expression of *copB* in copper depleted media and in the presence of different metals. (A) Expression of *copB* in the absence of copper relative to an untreated control. (B) Expression of *copB* in the presence of: vanadium (V), silver (Ag), nickel (Ni), cadmium (Cd), chromium (Cr), zinc (Zn) and copper (Cu), represented as molecules per nanogram of total RNA. Results are reported as means ± standard errors.

consequent accumulation of intracellular excess copper by CopT. Alternatively, although the cell pellets were washed with EDTA, these measurements might reflect some residual copper adsorption to the cell surface. After prolonged exposure, the amount of copper returned to the level measured 1 h after treatment (Fig. 2.3B), probably due to efflux mediated by CopA. The amount of copper in a 0.75 mM solution is 47.6 x 10^3 μg/L. However, the copper associated with the cells in a similar medium, 30 min after treatment, is three orders of magnitude lower, being approximately 11.6 lg/L (or 183 nM). These data suggest that active efflux has a major role in maintainance of copper homeostasis, and that sequestration also contributes to it, possibly by fine tuning the system. Cells treated with inhibitory copper concentrations showed a similar pattern of copper accumulation, except for a large increase 60 min after exposure (Fig. 2.3B). Bioremediation studies have established that dead cells, i.e. algae, have a higher capacity of binding metal ions (Kadukova and Vircikova 2005). Therefore the large increase in copper content may indicate cell death.

Transcription of copB and copRC

The *Sulfolobus* genome possesses a second cluster of genes, located 234.8 Kbp from *copRTA*. It includes three open reading frames (ORFs), Sso2897, Sso2896, Sso11412, encoding a putative transcriptional regulator with a metal binding domain, CopR2, a P-type ATPase CopB, and the copper binding protein CopC, respectively. The organization of the genes is shown in Fig. 2.4A. Regulator and ATPase are transcribed in opposite directions. To test whether the genes encoding the regulator CopR2 and the copper binding protein CopC are co-transcribed, we used the pair of

primers 2897-F and 11412-R to perform an RT-PCR experiment, obtaining a band slightly lower than 600 bp, consistently with the expect size of 573 bp in case of co-transcription (Fig. 2.4B). CopC possesses a heavy metal binding domain and displays high sequence similarity to proteins characterized as copper chaperones or copper binding proteins (Altschul *et al* 1997). CopC and CopT might be copper chaperones with different affinity for copper. To investigate the effect of copper on the transcription of the ATPase CopB, *Sulfolobus* cells were exposed to 0.75 mM $CuCl_2$ for 2 hrs and total RNA was isolated. Level of expression was determined by qPCR using the primers q2896-F and q2896-R. Since transcription of *copB* was not affected by the presence of copper, we speculated that it might have a role in the uptake of copper. To test this hypothesis, the expression of copB was investigated using qRT-PCR, in total RNA samples from cells grown on copper depleted media (Fig. 2.5A). Transcription of *copB* was not affected by the lack of copper in the medium, suggesting that *copB* may not be involved in the uptake of copper either. CopB is a putative soft-metal transporting P-type ATPase. This group of proteins can transport different metals like cadmium, silver and zinc among others (Banci *et al* 2002; Rensing *et al* 2000). To see if copB might have a role in the transport of another metal, the *copB* transcript was studied on total RNA extracted from cell cultures exposed to sublethal concentrations of different metals such as vanadium (1.4mM), silver (1μM), nickel (0.8mM), cadmium (2mM), chromium (0.15mM) and zinc (10mM). Only sublethal concentrations were chosen to limit extensive stress responses that would cloud the response of the respective metal. The metals were chosen based on the affinity to other metals presented by P-type transporting

ATPases and because of the physiochemical similarities to copper. The level of expression of *copB* was not significantly affected by the presence of other metals (Fig. 2.5B). Discarding, at least for now, the possibility of a role for *copB* in copper homeostasis.

Chapter 3

***CopR* is a positive transcriptional regulator in *Sulfolobus solfataricus* strain 98/2**

To be submitted to the Journal of Bacteriology

Introduction

The transcriptional system in *Sulfolobus*, like in other archaea, closely resembles the eukaryotic RNA pol II apparatus, including the structure of its complete RNA polymerase (RNAP) (Hirata *et al* 2008) the architecture of the promoter (Hain *et al* 1992; Soppa *et al* 1999; Qureshi *et al* 1998), the presence of homologs of the eucaryal TATA binding protein (TBP) and TFIIB (Qureshi *et al* 1995; Geiduschek and Ouhammouch 2005). Interestingly, no homologs of eukaryotic TBP-associated factors (TAFs), or other accessory factors, were discovered in archaeal genomes (Bell *et al* 1999). Archaeal transcription is mostly regulated by factors belonging to bacterial families of regulators, as revealed by bioinformatics methods (Aravind and Koonin 1999; Bell 2005). Transcription regulators shared by archaea and bacteria may have evolved before the separation between the two domains. Most of these factors are characterized by the winged HTH motif, considered one of the oldest DNA binding domains (Perez-Rueda *et al* 2004). On the other hand, these regulators may have been acquired by horizontal transfer after the archaeal bacterial separation (Koonin *et al* 1997; Aravind and Koonin 1999). The subunit composition of the archaeal RNAP is very much like the eukaryal one, harboring homologs of all RNAPII subunits (Grohmann *et al* 2009). Very recently the crystal structure of *S. solfataricus* RNAP has been determined (Hirata *et al* 2008). During the initiation of transcription archaeal RNAP interacts with two transcription factors: TATA-binding

protein (TBP) and transcription factor B (TFB). TBP binds to the TATA element of archaeal promoters. TFB then binds to TBP-TATA complex by recognition of BRE elements and subsequently RNAP is recruited to form the pre-initiation complex (Bartlett *et al* 2004). Different mechanisms of action were described for various repressors (Peeters *et al* 2004; Vierke *et al* 2003; Napoli *et al* 1999; Bell 2005), but still little evidence is available regarding the workings of transcriptional activators. Exceptions are the euryarchaeal activator Ptr2, reported in *Methanococcus* (Ouhammouch *et al* 2003), the activation of viral promoters in *Sulfolobus islandicus* by Sta1 (Kessler *et al* 2006), Ss-LrpB which activates at least three unlinked genes (Peeters *et al* 2009), and regulators with both activities (Bose and Metcalf 2008). A common denominator that emerges from these studies is that the vast majority of regulators act as repressors.

The *Sulfolobus solfataricus* genome contains gene sequences for two metal-transporting ATPases: Sso2651 and Sso2896, encoding CopA and CopB, respectively (Villafane *et al* 2009). Both ATPases display high sequence homology and the typical architecture of both prokaryotic and eukaryotic copper-transporting ATPases (Bini 2008). The *cop* locus of *S.solfataricus* includes the three open reading frames (ORFs) Sso2651, Sso2652, and Sso10823, encoding the CopA ATPase, a copper-responsive regulator, and a putative copper-binding protein, respectively (Villafane *et al* 2009; Ettema *et al* 2006). It was previously shown that the whole operon is cotranscribed at low levels from the copR promoter under all conditions, whereas increased transcription from the copTA promoter occurs in the presence of excess copper (Villafane *et al* 2009), while a constitutively expressed

copper-responsive regulator binds sequences surrounding the putative *copA* promoter (Ettema *et al* 2006). *In vitro* assays showed that the regulator binds extensive regions up and downstream the putative TATA-box of the *copA* promoter, but no specific binding to sequences with a conserved consensus was demonstrated (Ettema *et al* 2006).

In vivo studies of regulated transcription in archaea are rare due to the lack of genetic systems in these organisms. With few exceptions, cell processes responsive to metals are regulated in all three domains of life and copper transport is one of the processes regulated by metal responsive transcriptional regulators. Although copper transport is well studied in Bacteria (Rensing and Grass 2003; Solioz and Stoyanov 2003), the mechanisms of copper import and export are still poorly understood in Archaea. The present study is aimed to demonstrate that CopR is responsible for the transcription of *copTA*, and to determine whether it acts as a negative or a positive regulator of transcription in copper stressed cells of *S. solfataricus*. To unravel the role of CopR, a genetic approach was used in which a mutant carrying a disruption of the copR gene was created. Knockout mutant, parent strain and mutant complemented with a wild type copy of copR, were compared respect to their physiological and transcriptional response to copper.

Materials and methods

Growth conditions. *S. solfataricus* strains 98/2, PBL2025, a *ΔlacS* mutant (Haseltine *et al* 1999), PBL2065, a *ΔlacS*, *ΔmalA* mutant, and derivative strains constructed as described in this work (PBL2050 and PBL2070) were cultured at

80°C in a defined standard medium (SM) as described previously (Villafane *et al* 2009) and supplementing the medium with 0.2% sucrose as the carbon and energy source. Batch cultures were inoculated to obtain a density corresponding to an OD_{540} of about 0.025, with aliquots withdrawn from mid-log phase cultures. Growth was monitored at a wavelength of 540 nm on a Beckman DU-520 spectrophotometer (Beckman Coulter, USA).

DNA isolation. DNA extraction was carried out using the following protocol. *S. solfataricus* cells were harvested by centrifugation at 5000 rpm for 15 minutes, washed with doubled distilled water (ddH_2O), and resuspended in 500 µl of TEN buffer (10mM Tris-Cl pH 8.0, 1 mM EDTA, 100 mM sodium chloride). Cells were lysed by the addition of 50 µl of 25% (w/v) N-lauryl sarcosine sodium salt with gentle mixing. After cell lysis, the DNA was extracted twice with equal volumes of Tris saturated phenol (pH 8), and twice with equal volumes of chloroform/isoamyl alcohol (24:1). The DNA was then precipitated with two volumes of cold 100% ethanol, washed with 1 ml of cold 70% ethanol, and resuspended in 50 µl of ddH_2O. Two µl of RNAse A (20 mg/ml) were added and incubated at 37°C for 30 minutes. The quantity and quality of the DNA obtained was evaluated both spectrophotometrically on a NanoDrop ND- 1000 spectrophotometer (NanoDrop, USA) and by agarose gel electrophoresis (Sambrook *et al* 1989).

Generation of knockout construct. Overlap extension PCR was used to combine three PCR fragments to create the knockout construct (Horton *et al* 1990; Taylor and Logan 1995). The three PCR products were obtained using three different primer sets (Table 3.1): BamF and b were used for the amplification of the upstream region of

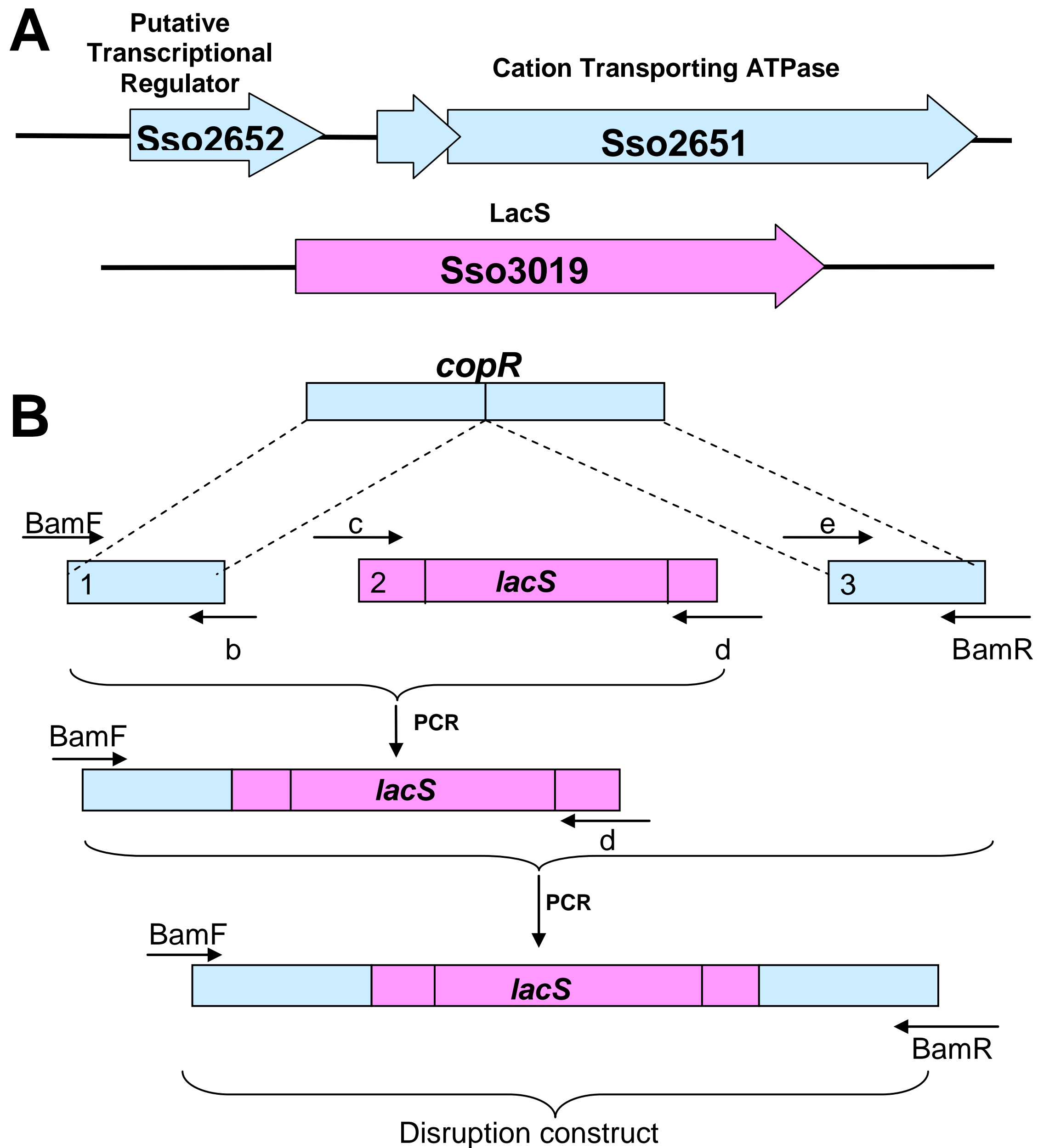


Fig. 3.1. Strategy for the assembly of *copR* knockout construct. (A) Sso2652, encoding a putative transcriptional regulator, is located upstream of the genes encoding a copper binding protein (not labeled) and the cation transporting ATPase, Sso2651. The *lacS* gene, Sso3019 and flanking regions, was used for the disruption of the regulator. (B) The two halves of the copR gene were separately amplified by PCR and combined with the *lacS* gene and flanking regions by overlap extension PCR to generate the disruption construct.

copR (fragment 1), c and d were used for *lacS* (fragment 2), and e and BamR were used for the downstream region of *copR* (fragment 3). Primers c and b are complementary to each other and were used to join together fragments 1 and 2 using overlap extension PCR. Similarly, primers d and e are complementary and were used to obtain the final fragment that consisted of the the lacS gene (1767 bp) and its flanking regions inserted within the target gene Sso2652 (Fig. 3.1).

General molecular biology methods. PCR reactions were set up using 250 ng of genomic DNA in 25-µl reactions with the JumpStart AccuTaq LA DNA polymerase (Sigma-Aldrich, USA), and following the protocol recommended by the manufacturer. Amplification products were separated on a 0.8% agarose gel by electrophoresis and the gel images were acquired using a GelLogic 440 Imaging System (Eastman Kodak, USA). Where necessary, PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, USA) or a Qiaex II Gel Extraction Kit (Qiagen, USA). Cloning, selection, plasmid isolation and sequencing were performed according to standard procedures (Sambrook *et al* 1989).

RNA isolation and analysis. Total RNA was isolated from *S. solfataricus* cultures in their exponential phase of growth (OD540 = 0.3–0.6), as described previously (Villafane *et al* 2009). The quantity and quality of the RNA obtained was evaluated both spectrophotometrically on a NanoDrop ND- 1000 spectrophotometer (NanoDrop, USA) and by agarose gel electrophoresis (Sambrook *et al* 1989). For RT-PCR analysis, total RNA (0.5 µg) was analyzed in 25-µl reactions using the Enhanced Avian HS RT-PCR Kit (Sigma–Aldrich, USA). The amplification products were separated on a 1.2% agarose gel by electrophoresis and the gel images

were acquired using a GelLogic 440 Imaging System (Eastman Kodak, USA). Specific transcripts were quantified by qRT-PCR using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, USA) and the real-time detection system iCycler iQ (Bio-Rad, USA), using the primers q2651-F/R and q0067-F/R as previously described (Villafane *et al* 2009).

Preparation of electrocompetent Sulfolobus *cells and electroporation*. Mid-logarithmic cultures (OD_{540} 0.3-0.5) of *S. solfataricus* (8 ml)were centrifuged at 5000 rpm in an Eppendorf 5810R centrifuge (Brinkmann Instruments Inc., USA) for 10 minutes in two separate 50 ml falcon tubes. Pellet was resuspended with 1 ml of 20 mM sucrose, followed by addition of 4 ml of 20 mM sucrose and centrifugation at 5000 rpm for 10 minutes. Pellet was resuspended with 1 ml of 20 mM sucrose. The contents of both falcon tubes were combined, followed by addition of 3 ml of 20 mM sucrose and centrifugation at 5000 rpm for 10 minutes. Pellet was washed once more and resuspended in 800 µl of 20 mM sucrose. Fifty µl aliquots of electro competent cells were incubated at 50°C for 15 minutes for both experimental and control samples. After incubation, experimental samples were mixed with 1 µg of plasmid DNA and the contents of both experimental and control samples were transferred to a sterile disposable 1 mm electroporation cuvettes (Bio-Rad, USA) and incubated for an additional 3 minutes at 50°C. Electroporation was performed with a Gene Pulser apparatus (Bio-Rad, USA) with 2 kV, 25 µF and 100 Ohms as the parameters. Milliseconds were recorded and electroporated cells were resuspended in 500 µl of preheated 1x Brock salts and transferred to 25 ml screw cap tubes with 8 ml of 1x Brock salts + 0.2% tryptone. After 8 hours of outgrowth at 80°C, cells were

centrifuged at 5000 rpm in an Eppendorf 5810R centrifuge (Brinkmann Instruments Inc., USA) for 10 minutes, pellets were resuspended in 1 ml of 1x Brock salts and transferred to 8 ml of 1x Brock salts + 0.2% selective carbon source. Tubes were incubated at 80°C for seven days and growth was followed and recorded. Finally,

Table 3.1. Oligonucleotides used in this work

ID	Sequence (5'-3')	Purpose
BamF	TTGGATCCTATAATAGCTATAATTCTAGG	Knockout construct
b	AATTATTATGAAACATAACAAGCCTTTTTGATAGTTCAGTGAC	"
c	GTCACTGAACTATCAAAAAGGCTTGTTATGTTTCATAATAATTTTATTG	"
d	GGCAGTGCTCCTACTGATATTACTTTCCAATTAGGCTAACATTAAG	"
e	CTTAATGTTAGCCTAATTGGAAAGTAATATCAGTAGGAGCACTGCC	"
BamR	GCGGATCCATGTAAGTGCAAGCCATTGTTGCG	"
q2651-F	GAATAGTTGGGATGCATTGT	qRT-PCR (*copA*)
q2651-R	ACTACCCCCTTAACGTTTTC	"
q0067-F	TACCAATTGTCGCTTTTGCT	qRT-PCR (Reference)
q0067-R	CAAATCACCATCTGGAGGAA	"
M13-pUC	GGCCAGGGTTTTCCCAGTCACGAC	Sequencing
M13Rev-pUC	AGCGGATAACAATTTCACACAGGA	"
LacS-F	TGTTATGTTTCATAATAATTTTATTG	"
LacS-R	CTTTCCAATTAGGCTAACATTAAG	"
copRcomp-F	TAGAGGTACCAGTTGGATGGATATTAG	"
copRcomp-R	AATTGGTACCTATATATTGTTGATTAC	"
2652His-F	GAACCATATGGAAAAGTTGACAGATTTAG	presence of pJcopR2
2652His-R	TCTACTCGAGATGTAAGTGCAAGCCATTGTTGCG	"

cells were plated on solid selective medium and single colonies were isolated, grown and the disruption validated by PCR.

Results and discussion

Creation of the copR *disruption mutants PBL2050 and PBL2070*

The *cop* operon of *S. solfataricus* encodes three genes transcribed in the same direction named *copR*, *copT* and *copA*. *CopA* codes for a Cu (I)-transporting ATPase and *copT* encodes a hypothetical metal binding protein containing a stand alone TRASH domain (Ettema *et al* 2003; Villafane *et al* 2009). Based on sequence similarity, CopR is annotated as metal responsive transcriptional regulator belonging to the Lrp/AsnC family. This family of proteins usually consists of an N-terminal DNA-binding domain and the C-domain, which is involved in dimerization, further assembly and/or metal binding (Yokoyama *et al* 2006). The CopR N-terminus contains a winged Helix-Turn-Helix (HTH) motif similar to the one found in the MarR domain (Alekshun *et al* 2001, Gajiwala and Burley 2001), while the C-terminus has a TRASH domain containing two conserved motifs involved in metal binding (Ettema *et al* 2003) (Fig. 3.2). To demonstrate that CopR is responsible for the transcription of *copTA*, and to determine whether it acts as a negative or a positive regulator of transcription, we created a knock-out mutant carrying a disruption of the *copR* gene. A linear copy of *copR* truncated by the *lacS* gene (ORF Sso3019), was used to transform *Sulfolobus* strain PBL2025 carrying a *lacS* deletion (Fig. 3.1A) and therefore unable to grow on lactose, using the technique developed

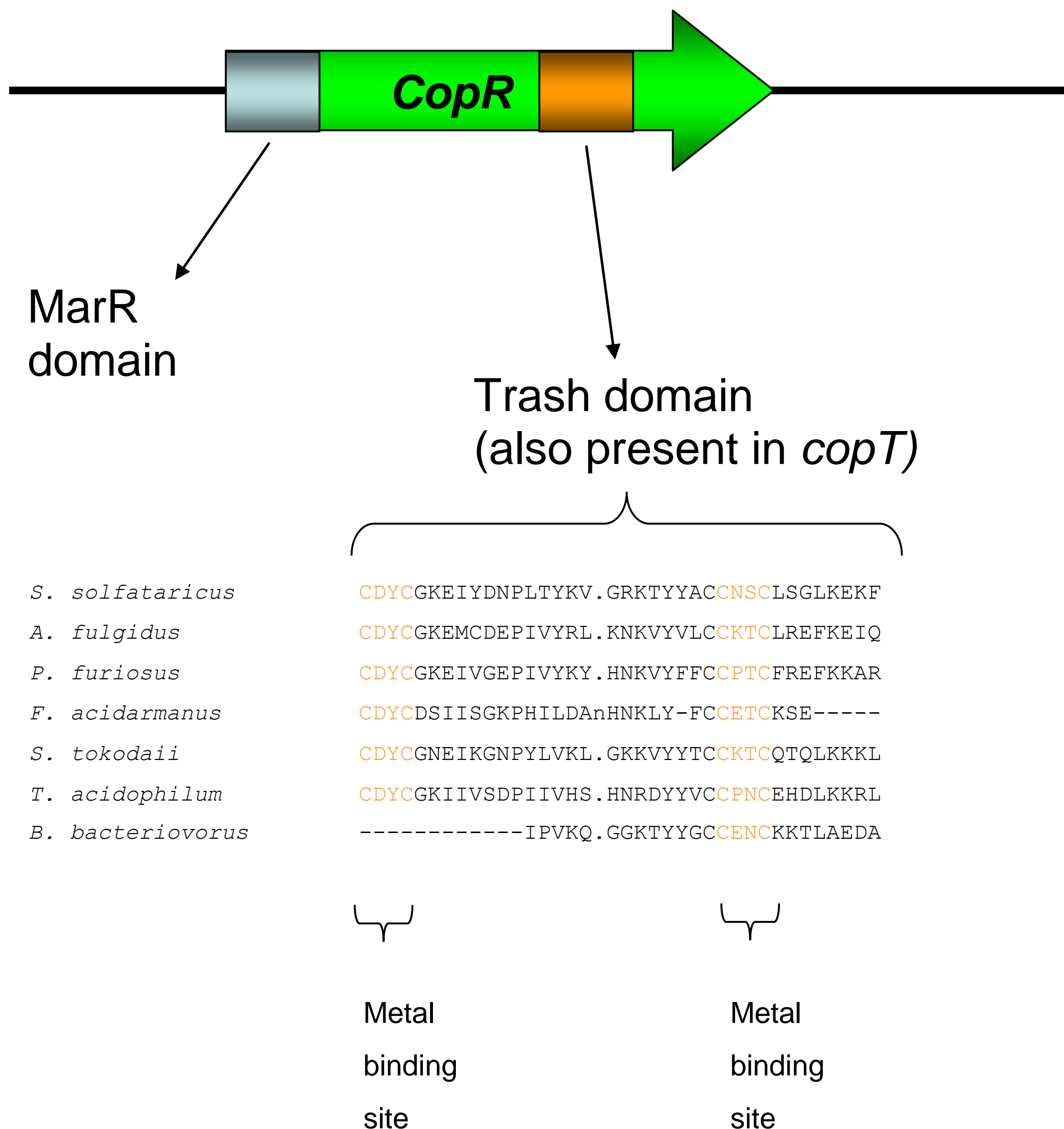
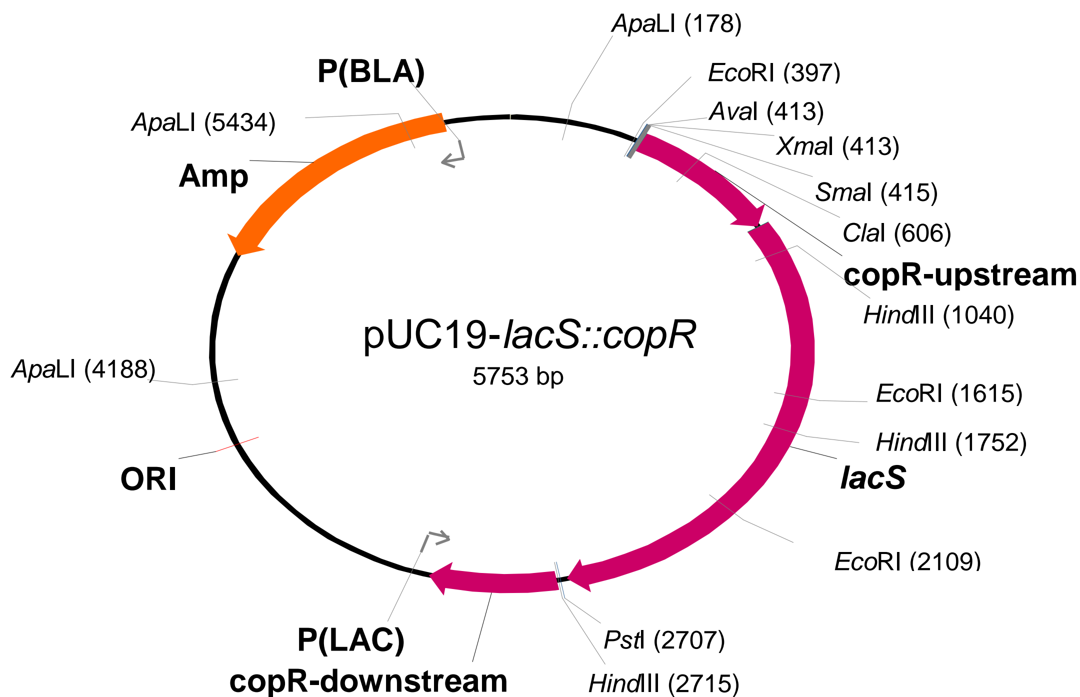


Fig. 3.2. Domain features of the putative transcriptional regulator Sso 2652 (*copR*). The TRASH (trafficking, resistance and sensing of heavy metals) domain contains two conserved metal binding sites. This domain is also present in CopT, that is a stand-alone TRASH module and may act as a copper binding protein. The N-terminus contains a Helix-Turn-Helix motif similar to the MarR domain (PFam). MarR domains are characterized by a winged HTH structure, present in transcriptional repressors acting as dimers.

by Worthington et al. (2003). To disrupt *copR*, the *S. solfataricus lacS* gene (Sso3019), was chosen to be used as a selectable marker in the *lacS* mutant strain PBL2025 (Fig. 3.1A) (see section *Cloning of lacS::copR construct in S. solfataricus PBL2025*). Three separate PCR reactions were peformed to amplify the first half of the regulator, the second half of the regulator and the *lacS* gene. For the first half of the regulator a forward primer with a BamHI restriction site (BamF) and a reverse primer complementary to the upstream region of *lacS* (b) were used. A forward primer complementary to the first half of *copR* (c) and a reverse primer complementary to the second half of *copR* (d) were used for amplification of *lacS*. For the second half of the regulator a forward primer complementary to the downstream region of *lacS* (e) and a reverse primer with a BamHI restriction site (BamR) were used (Fig 3.1B). Overlap extension PCR was performed to combine the first half of *copR* and *lacS* using BamF and d primers. The product of this reaction was used with the second half of *copR*, along with primers a and BamR in overlap extension PCR to create the disrupted *lacS::*copR construct. The size of the construct and specificity was corroborated by agarose gel electrophoresis. To clone the created disrupted gene, the entire *lacS::copR* construct was inserted into the unique BamHI site in the small, high copy vector pUC19 (Fig. 3.3A) and subsequently used to transform *E. coli* DH5α. Screening for presence of the insert was conducted using the diagnostic sites BamHI and XmnI.

Finally, the recombinant plasmid was validated through sequencing. The plasmid was used as template for the preparation of a linear fragment containing the disrupted

gene. The fragment, obtained by PCR amplification was electroporated into *S.*



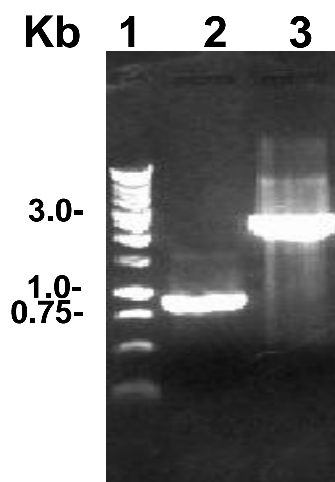


Fig. 3.3 pUC19 *lacS::copR* shuttle vector and amplification of *copR* in parent and knockout strain. (A) pUC19 *lacS::copR* plasmid map showing the disrupted gene. (B) Agarose gel electrophoresis of the amplicons corresponding to the *copR* region of PBL2025 (lane 2) and of PBL2050 (lane 3).

solfataricus strain PBL2025. Strain PBL2025 has a large deletion spanning from ORF Sso3004 to ORF Sso3050 (Schelert *et al* 2006), therefore is not able to grow on lactose. However, a successful replacement of *copR* with its disrupted copy would allow electroporated cells to grow on lactose and therefore for selection of recombinants. This *copR* disruption mutant strain was named PBL2050. To validate the gene disruption, the *copR* regions of both the parent strain PBL2025 and of PBL2050 were PCR amplified using primers BamF and BamR. For PBL2025 and PBL2050, expected bands of approximately 0.9Kb and 2.6Kb, respectively, were obtained (Fig. 3.3B). The PCR reaction did not produce any additional bands, demonstrating the purity of the mutant strain. Attempts to complement strain PBL2050 with a wild type copy of *copR*, and using copper resistance as selection, were not successful. Thus, a second *copR* knockout mutant was created using the same disruption construct described above. This second mutant, named PBL2070, was generated using *S. solfataricus* strain PBL2065, carrying a deletion of both *lacS*, and *malA*, and uncapable of growing on lactose or maltose. The avalability of the second selectable marker makes possible the selection of complemented strains.

PBL2050 is sensitive to copper and copA induction is abolished

To better understand the role of CopR in the physiological response to copper, strains PBL2025 and PBL2050 were exposed to excess $CuCl_2$. To prevent general stress responses, other than copper related stress, a sublethal concentration of 0.75mM $CuCl_2$, which was previously defined (Villafane *et al* 2009), was used. The addition of copper had no effect on PBL2025 growth, which behaved exactly as its parent strain 98/2. Instead, PBL2050 growth was inhibited by the addition of copper,

suggesting that *copR* has a direct role in copper homeostasis. To further investigate the role of *copR*, the expression of *copA* was measured using qRT-PCR, in total RNA samples from cell cultures exposed to copper during log phase. *CopA* is constitutively expressed at basal levels in untreated cells of both strains PBL2025 and PBL2050 (Fig. 3.4B). After copper treatment, the level of *copA* transcript increases sharply in PBL2025. However, in the disruption mutant PBL2050 the expression of the ATPase gene remains low. These results are consistent with the growth behavior of both strains and suggest that *copR* is an activator of transcription, since its disruption prevented the accumulation of the *copA* transcript. This is in agreement with previous studies of the *S. solfataricus cop* operon by DNA-protection assays that showed that *copR* binds to the *copA* promoter (Ettema *et al* 2006). Interestingly, most archaeal-metal responsive transcriptional regulators and its bacterial homologues (Magnani and Solioz 2005, Reyes *et* al 2006, Studholme and Pau 2003) are repressors of transcription, while most eukaryotic homologues are activators of transcription (Eide 1998, Winge 1998).

PBL2050 accumulates copper faster than wild type

To investigate the effects of *copR* disruption on cell growth, the copper associated with the cells was analyzed in a time course experiment by ICP-OES spectrometry. In the parent strain PBL2025, treated with 0.75 mM copper, the metal was observed to slowly increase and be maintained at a low level for the time of monitoring; similar to what was observed in wild type (Villafane et al 2009). When the *copR* disruption mutant was treated with copper, it showed a much faster accumulation of the metal compared to the wild type, that continued until cells death

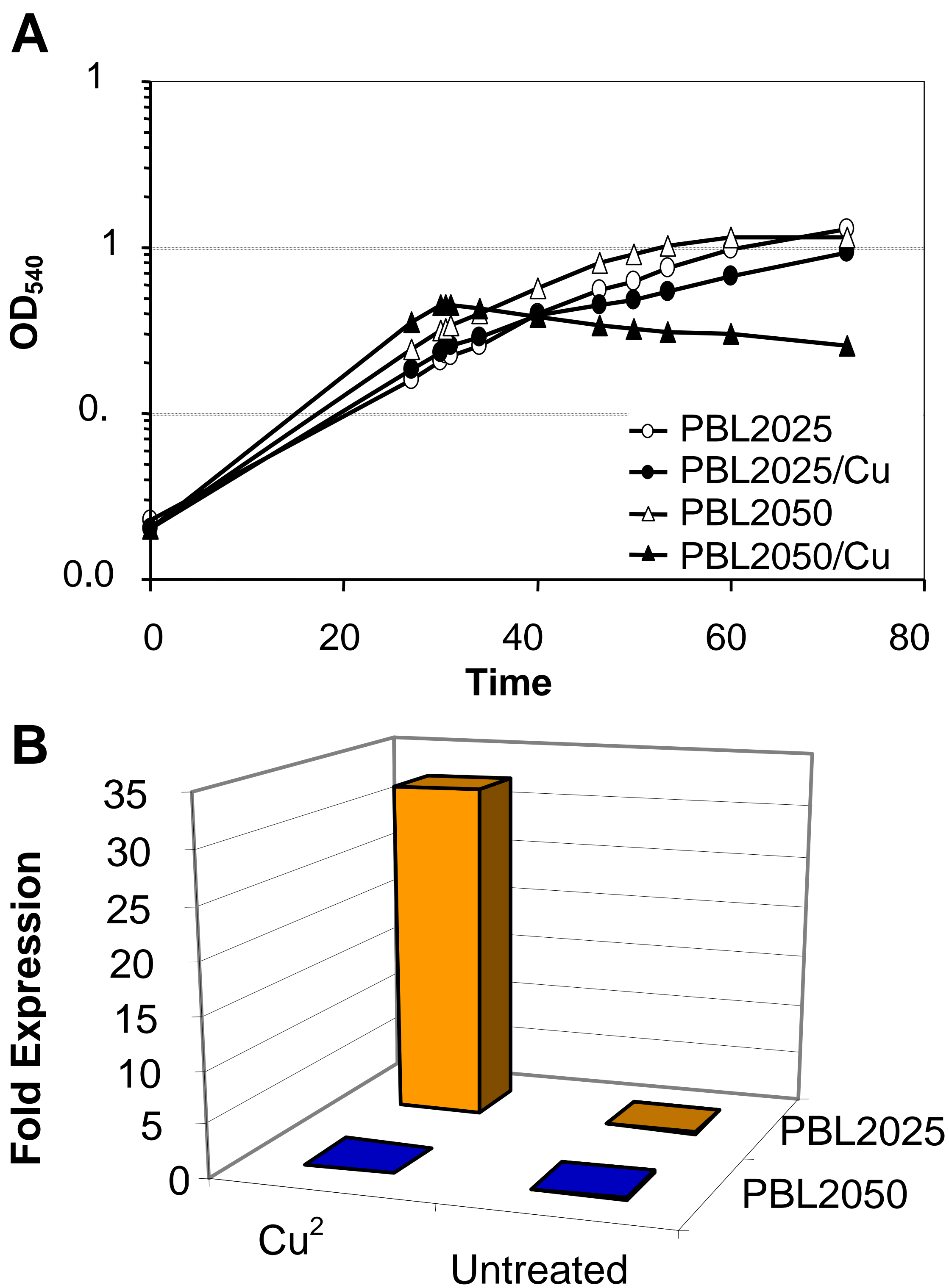


Fig. 3.4. Effect of $CuCl_2$ on growth of parent and knockout mutant strains and on *copA* induction. (A) Growth curves of PBL2025 and PBL2050 cultures. Copper was added to cell cultures while in log phase. (B) Expression of *copA* in PBL2025 (orange bars) and PBL2050 (blue bars) before and after treatment with 0.75mM $CuCl_2$.

(Fig. 3.5). This observation cannot be explained with binding to the cells surface, but it is consistent with the lack of a functional CopA.

Complementation of copR with a wild type copy of copR

To confirm the role of CopR as a positive regulator of transcription, the characterization of strain PBL2050 complemented with a functional copy of *copR* was planned. However, strain PBL2050 possesses already a copy of the *lacS* gene in its disrupted *copR* gene, and at the time *lacS* was the only selectable marker available in this strain of *Sulfolobus* (Berkner *et al* 2007, Ciaramella *et al* 2002). Therefore *S. solfataricus* PBL2065, a *ΔlacS, ΔmalA* mutant, was used for generating the *copR* knockout PBL2070, a strain with the same genetic background as PBL2050, and with a deletion of the α- glucosidase gene (*malA)* necessary for growth on maltose (see methods). PBL2070 was transformed with pJmalA-copR, a derivative of pJmalA, which was obtained by replacing the *lacS* gene of pJlacS (Berkner *et al* 2007) with *malA*. pJcopR2 carries a wild type copy of *copR,* including its own promoter, inserted at the unique *Kpn*I site of pJmalA (Fig. 3.6A). After selection on maltose, to confirm the presence of the plasmid, total DNA was isolated from putative transformants and controls and the *copR* region of both were tested by PCR

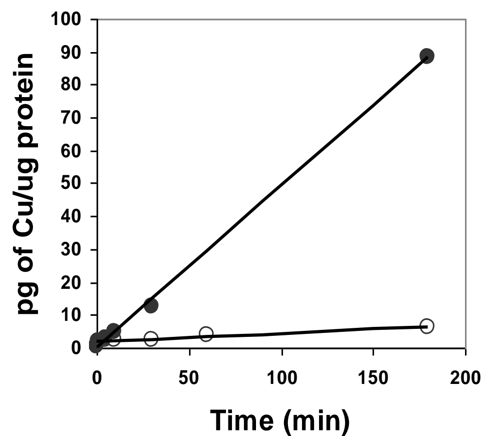


Figure 3.5. Determination of total copper content in *CopR*⁻ mutant and its parent PBL2025. Cu content was normalized to total protein content.

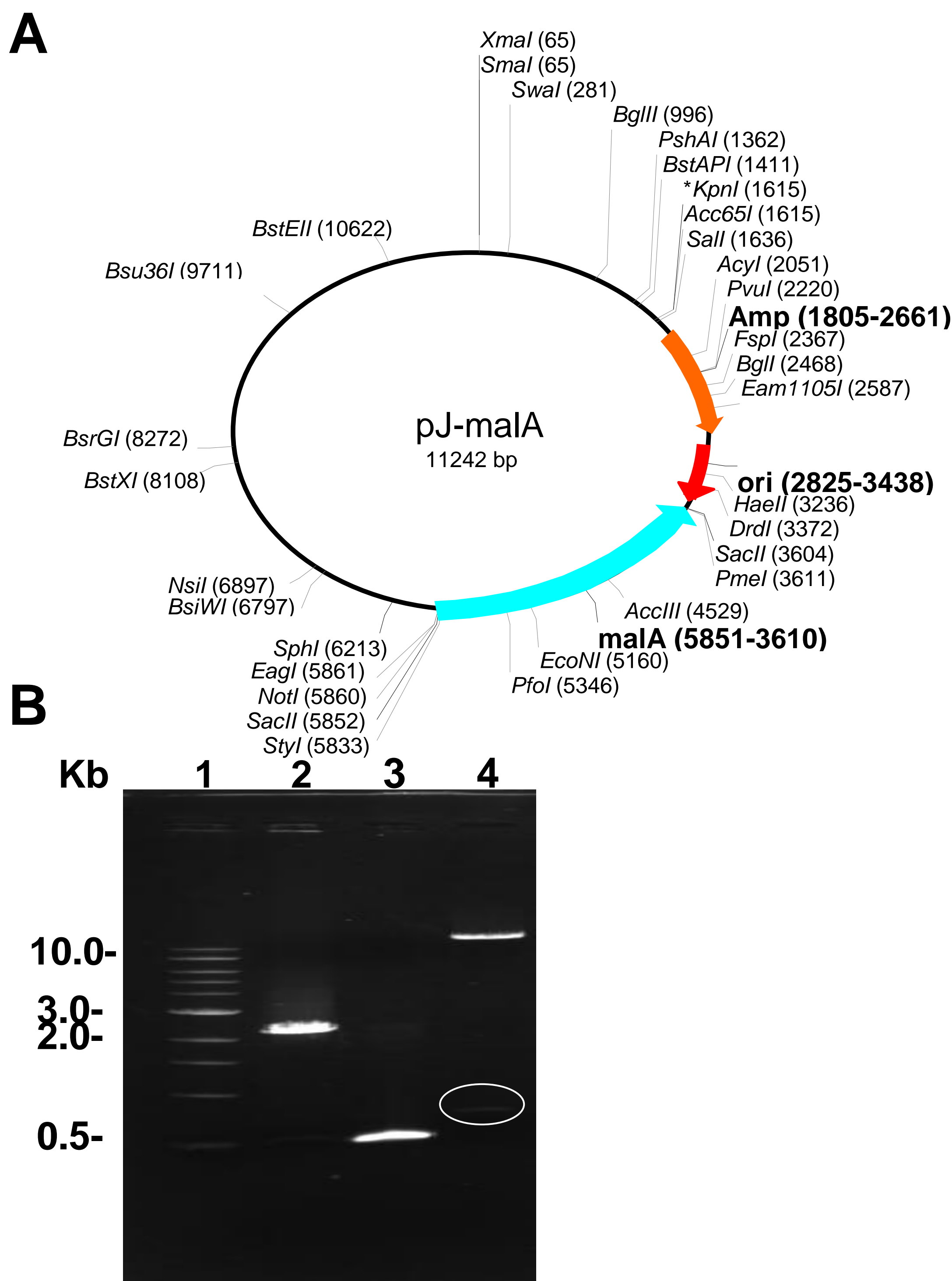


Fig. 3.6. pJ-malA shuttle vector and amplification of *copR* in knockout and transformed strains. (A) pJ-malA plasmid map showing the location of the malA and ampicillin resistance genes. (B) Agarose gel electrophoresis of the amplicons corresponding to the *copR* region of PBL2070 (lane 2) and PBL2070/pJmalA-copR (lane 3). Lane 4 is the restriction digestion of the plasmid from *E.coli* DH5α transformed with DNA extracted from PBL2070/pJmalA-copR. The faint band in the circle corresponds to the insert *copR* and upstream region.

amplification using primers 2652His-F and 2652His-R. A band of the expected size was obtained (Fig. 3.6B) for non-electroporated control cultures (2262 bp) and for electroporated PBL2070 cultures (492 bp). Further controls to confirm the presence of pJmalA-copR in transformants cells consisted of retransformation of *E.coli* DH5α with DNA extracted from PBL2070/pJmalA-copR, followed by plasmid isolation and restriction digestions. Digests were separated by gel electrophoresis, that showed two fragments (Fig. 3.6B), one of approximately 11000 bp (pJ-malA) and another of approximately 700 bp, consistent with the size (695 bp) of copR and upstream region. All of this proves a successful transformation of PBL2070 with the plasmid pJmalA-copR.

Copper resistant phenotype is restored and copA *transcript is upregulated in PBL2070/pJmalA-copR*

To demonstrate that CopR is a transcriptional activator, strain PBL2070/pJmalA-copR, was examined to test if the wild-type phenotype was restored, with respect to copper resistance and ability to regulate copA expression. PBL2070 and PBL2070/pJmalA-copR cells were exposed to 0.75mM $CuCl_2$ (Fig 3.7A). As expected, PBL2070 stopped growing after addition of copper, due to the disruption of the *copR* gene. The same effect was observed in PBL2050 which has the same genetic background. The addition of copper had no effect on PBL2070/pJmalA-copR growth in sucrose, as can be observed comparing to its growth curve without addition of copper, providing conclusive proof that *copR* is indeed the positive transcriptional factor regulating transcription of *copTA* under copper stress in *S. solfataricus*. The physiological response to copper of PBL2070/pJmalA-copR was

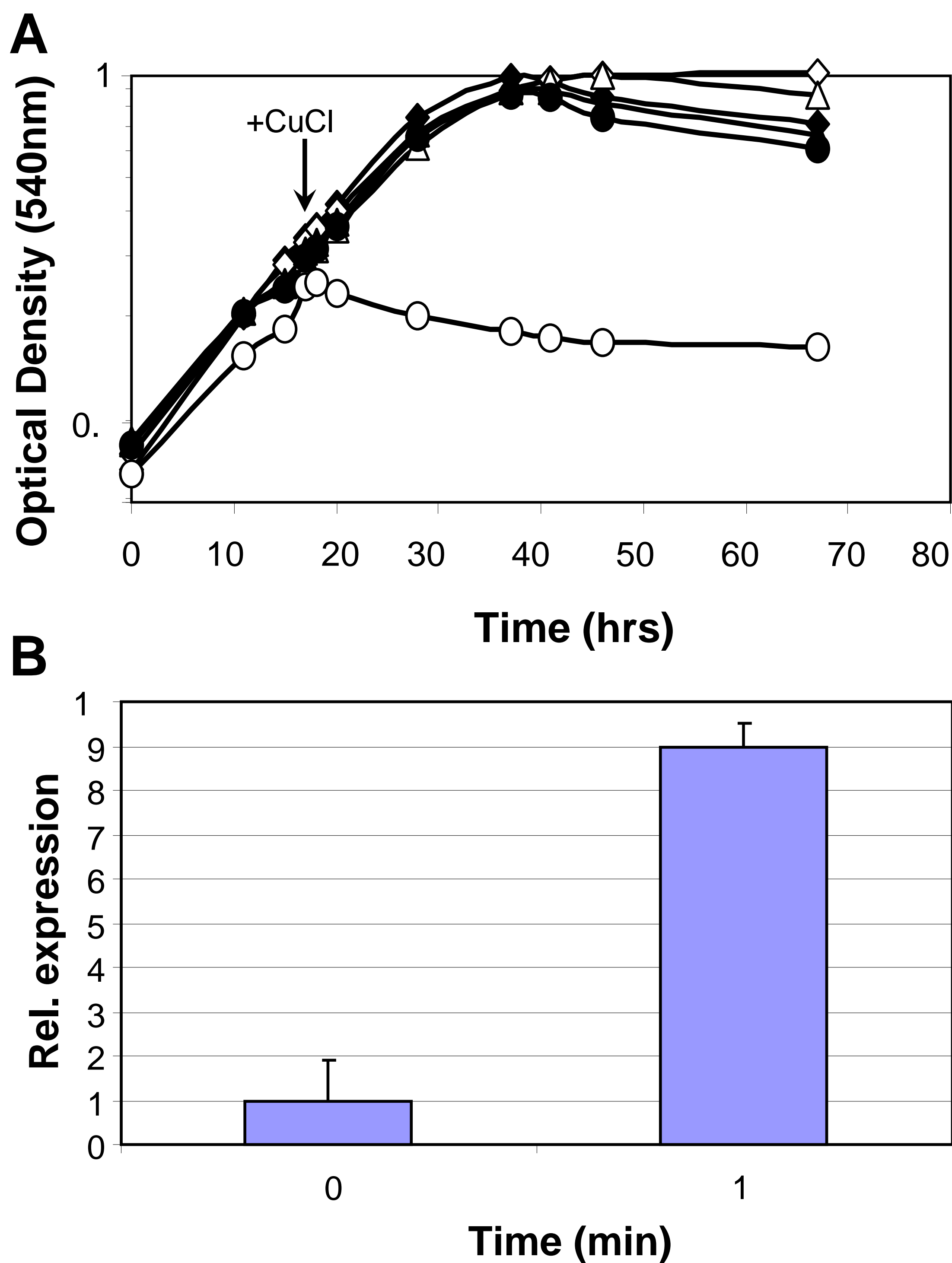


Fig. 3.7. Effect of $CuCl_2$ on growth of knockout and transformed strains and *copA* induction in PBL2070/pJmalA-copR. (A) Growth curves of: (●) PBL2070, (○) PBL2070 +Cu, (▲) PBL2070/pJmalA-copR in sucrose, (△)PBL2070/pJmalA-copR in sucrose +Cu, (♦)PBL2070/pJmalA-copR in maltose, (◊)PBL2070/pJmalA-copR in maltose +Cu. Copper was added to the indicated cultures after 17 hours. (B) Expression of *copA* in PBL2070/pJmalA-copR before and after treatment with 0.75 mM $CuCl_2$ for 10 minutes. Results are expressed as means ± standard errors.

also tested with maltose as the sole source of carbon and energy. This was not affected by copper stress either. To further prove that *copR* is responsible for upregulation of the *copA* transcript, qRT-PCR was performed using total RNA from PBL2070/pJmalA-copR cell cultures exposed to copper during log phase (Fig. 3.7B). *CopA* is constitutively expressed at basal levels in untreated cultures of PBL2070/pJmalA-copR. After treatment with copper for 10 min, the level of *copA* transcript increased nine-fold. This is consistent with the growth behavior of PBL2070/pJmalA-copR cells and the levels of expression are also consistent with those observed with wild type *S. solfataricus* 98/2 cells in response to copper exposure.

Chapter 4

Characterization of a new reporter gene for hyperthermophiles: A thermostable beta glucuronidase from *Sulfolobus solfataricus*

To be submitted to the Journal of Bacteriology

Introduction

The use of a reporter gene is of utmost importance in gene expression studies and promoter analysis because converts the information on the expression level of a protein into an easily detectable signal. So far, in archaea, efficient genetic systems with reporter genes have only been established for euryarchaeota, and in particular for halophilic archaea (Patenge *et al* 2000; Gregor and Pfeifer, 2001). Almost every genetic system used for thermophilic archaea depends solely on the *lacS* gene coding for a β-glycosidase, whose activity can be both qualitative and quantitative determined using specific substrates (Berkner *et al* 2007, Lubeska *et al* 2006). An attempt was made to create a reporter system based on the green fluorescent protein but unfortunately the constructs tested did not yield any fluorescent signal in *Sulfolobus* (Berkner and Lipps 2008). Therefore the discovery and characterization of new reporter genes for use in thermophilic organisms is of extreme interest for monitoring the expression of proteins or for systematic tests of promoter activities.

Sulfolobus solfataricus belongs to the crenarchaeal phylum of the archaea, and occupies niches that are biologically extreme in both temperature and pH. Its optimum temperature is 80 °C with a pH at about 3 (Brock *et al* 1972). It is on of the most widely studied organisms for molecular and biochemical investigations among hyperthermophilic archaea. Our laboratory has been using *S. solfataricus* strain 98/2

to study copper homeostasis. The genes that comprise the *cop* operon, responsible for copper transport, have been identified and characterized (Villafane *et al* 2009, and Villafane *et al*, in preparation), but the promoters of the transcribed genes have not been investigated in detail. To this end, we have identified in the *S. solfataricus* genome a sequence, Sso3036, encoding a protein annotated as GusB. The enzyme β-glucuronidase (GusB) belongs to the Glyco-hydro-2N and Glyco-hydro-2C super families (Schlaman *et al* 1994). GusB is to date the most frequently used reporter gene in plants (Basu *et al* 2004), and its activity is found in many bacterial species, all tissues of vertebrates and is also present in organisms of various invertebrate taxa. Its popularity comes as a result of its high stability in plant tissues and lack of toxicity even at high expression levels. Moreover, the glucuronidase (Gus) protein is considered safe for humans and the environment. The *Sulfolobus gusB* gene can be used as a reporter in strain PBL2025, which is a spontaneous double mutant Δ*lacS*, Δ*gusB* (Haseltine *et al* 1999). Using a standard GUS assay that we have modified for *Sulfolobus*, crude extracts of the wild-type strain 98/2 and of PBL2025 were assayed. Significant GusB activity was detected in wild-type, whereas it was negligible in PBL2025. Here we report the cloning, heterologous expression and initial characterization of the thermostable enzyme GusB. The gene is 1713 bp long and encodes a protein of 570 amino acids, with a mass of 66796 Daltons. Moreover, a promoter fusion was created with *gusB* to measure the transcriptional activity of the promoter of the copper transporting ATPase gene, *copA*, in response to excess copper.

Materials and methods

Growth conditions. *S. solfataricus* strains 98/2 and PBL2025 were cultured at 80°C in a defined standard medium (SM) as described elsewhere (Villafane *et al*, 2009), the medium was supplemented with 0.2% sucrose, or lactose as the carbon and energy source. Batch cultures were inoculated to obtain a density corresponding to an OD_{540} of about 0.025, with aliquots withdrawn from mid-log phase cultures. Growth was monitored at a wavelength of 540 nm on a Beckman DU-520 spectrophotometer (Beckman Coulter, USA).

Cloning and heterologous expression of the Sulfolobus GusB in E. coli. The *gusB* gene of *Sulfolobus*, ORF Sso3036, was amplified by PCR from the genomic DNA of *S. solfataricus* using the pair of primers GUSpET-F and GUSpET-R (Table 4.1). The construction of the plasmid, named pET21-*gusB*, was carried out in two steps. First, the 1203 bp NdeI-EcoRI fragment of the PCR amplified *gusB* of *Sulfolobus*, and including the start codon, was inserted at the corresponding sites of the expression vector pET21, to generate pET21-gus1. Next, the 513 bp EcoRI-XhoI fragment of the glucuronidase gene, that includes the stop codon, was inserted into EcoRI-XhoI of pET21-gus1 to generate pET21-*gusB* (Fig. 4.2A), carrying the entire *gusB* gene. The sequence of the recombinant *gusB* gene was verified through sequencing. The vector pET21-gusB, cloned in *E. coli* strain DH5α, was subsequently transformed into *E. coli* BL21 for expression. Genomic and plasmid DNA isolation and PCR were carried out using standard molecular techniques. For heterologous expression, *E. coli* cells were cultured in 50 ml of LB with 100 μg/ml ampicillin.

Partial purification of the recombinant β-glucuronidase. When cells reached approximately 0.6 OD_{600}, they were collected by centrifugation at 5000 rpm in an Eppendorf 5810R centrifuge (Brinkmann Instruments Inc., USA) for 10 min. Cell pellets were resuspended in 700 ml of assay buffer (see below) and lysed by sonication on ice (130 Watts, 20 Khz, 30 sec on 30 sec off for 3 min). The recombinant enzyme was partially purified by heating for 30 min at 80°C and the lysate was centrifuged at 14000 rpm for 1 min. This cleared lysate (400 µl per experiment) was used for all the biochemical characterization of the recombinant enzyme, as described in the results section.

GUS activity assays. A modified method for quantifying GUS activity (Jefferson *et al* 1987, Wickes and Edman 1995) was applied to measurements conducted on *Sulfolobus* and the recombinant protein expressed in *E. coli*. *S. solfataricus* cells from strains 98/2, PBL2025 and PBL2025/pJlacS-Pcop::gusB (a volume corresponding to 6 ODs) were harvested during mid-log phase and centrifuged for 10 minutes at 5000 rpm. Supernatant was removed and the cell pellet was resuspended in ddH_2O followed by centrifugation for 10 minutes at 5000 rpm. After removal of the supernatant, cell pellet was resuspended in 700µl of assay buffer (50 mM $NaPO_4$, pH 6.5; 1mM EDTA). Triton X-100 was added to a final concentration of 0.1% and left for 10 minutes at room temperature. Cells were sonicated for 3 minutes (30 seconds on, 30 seconds off) on ice. Cell lysate was centrifuged for 1 minute at maximum speed. 400 µl of supernatant were collected for GUS activity assays. Fifty µl of *Sulfolobus* crude extract, or *E. coli* cleared lysate, were mixed with β-mercaptoethanol and MUG (4-methylumbelliferyl-β-D-glucuronide) to final

concentrations of 10 mM and 1 mM, respectively. Reaction was incubated for 30 minutes at 65°C in the dark. After incubation, 950 µl of stop buffer (0.2 M Na_2CO_3, pH 9.5) were added. Fluorescence was then measured with excitation at 365 nm, emission at 510 nm on a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco). Results were normalized to total protein content, assessed using the BCA Protein Assay (Pierce, USA).

Construction of a shuttle plasmid carrying the copA*promoter fused to the gusB-reporter gene*. Overlap extension PCR was used to combine two PCR fragments to create the promoter-gene fusion construct (Horton *et al*. 1990; Taylor and Logan 1995). To prepare the two PCR products to be used in this procedure, a PCR was conducted with two different primer sets: Pcop2-F and fus2-R were used to amplify the promoter of *copTA* (fragment 1), while fus2-F and gus2-R were used to amplify the coding sequence of the β-glucuronidase gene (fragment 2). The primers fus2-R and fus2-F are complementary, and create an overlap between the two fragments to be joined; the primers Pcop2-F and gus2-R encode SphI restriction sites for insertion into the corresponding site of the *Sulfolobus*-*E. coli* shuttle vector pJlacS (Lipps 2008). The two fragments, 1 and 2, were purified using the QIAEX II Gel extraction kit (Qiagen, USA) and then used in equal amounts as template in an overlap extension PCR, with the pair of primers Pcop2-F and gus2-R, to obtain a DNA fragment of 1920 bp that consisted of the promoter region of *copTA* fused to the β-glucuronidase gene. Cloning in *E. coli*, selection, plasmid isolation and sequencing were performed according to standard procedures (Sambrook *et al* 1989).

Transformation of Sulfolobus *with pJlacS-Pcop::gusB*. Mid-logarithmic cultures (OD_{540} 0.3-0.5) of *S. solfataricus* were harvested and prepared for electroporation as previously described (see Materials and Methods, Chapter 3). Electroporation was performed with a Gene Pulser apparatus (Bio-Rad, USA). Milliseconds were recorded and competent cells were mixed with 1 μg of plasmid or sterile water (control), resuspended in 500 μl of preheated 1x Brock salts, and transferred into tubes with 8 ml of 1x Brock salts + 0.2% tryptone. Tubes were incubated at 80°C for 8 hours followed by centrifugation at 5000 rpm for 10 minutes. Cell pellets were resuspended with 1 ml of 1x Brock salts and transferred to 8 ml of 1x Brock salts + 0.2% lactose. Tubes were incubated at 80°C for eight days and growth was followed and recorded. Transformants were further purified by plating on selective solid media.

Phylogenetic analysis. The amino acid sequences of gusB from *S. solfataricus*, *Caldivirga maquilingensis* and from nine other bacterial species were aligned with

Table 4.1 Oligonucleotides used in chapter 4.

ID	**Sequence (5'-3')**	**Purpose**
GUSpET-F	GATATCATATGAGATCGTTTTATAGAC	Cloning in pET21
GUSpET-R	TATACTCTTGCTCTCGAGTTAAGAACGC	“
Pcop2-F	TTGCACGCATGCTAGTTTTAGAGTCATTTTGACTG	Overlap extension PCR, sequencing
Fus2-F	GGTCTATAAAACGATCTCATTTTTCCCTTATACATTGTCT	“
Fus2-R	AGACAATGTATAAGGGAAAAATGAGATCGTTTTATAGACC	“
gus2-R	AAGCATGCAACCATAATATACTCTTGCTATC	“

CLUSTAL_X v. 1.8 (Thompson *et al* 1997) and adjusted manually using SeaView (Galtier *et al* 1996). Phylogenetic distances were calculated using the Observed Divergence matrix and the neighbour-joining method was used to evaluate tree topologies. PHYLO_WIN was used to plot tree topologies (Galtier *et al* 1996) and their robustness was tested by bootstrap analysis with 100 resamplings.

Results and discussion

Phylogenetic analysis: Conservation and thermostability

The glucuronidase gene, *uidA*, was first isolated from *E. coli* (Jefferson *et al* 1986) and is still to date the most widely used reporter gene in plants. Its usefulness is due mainly to the absence of GUS activity in most plants, therefore providing a perfect scenario for developments of genetic systems. The GUS enzyme from *E. coli*, designated β-D-glucuronide glucuronoso-hydrolase (EC 3.2.1.31), catalyses the hydrolysis of β-D-glucuronides or β-D-glucuronosides into D-glucuronic acid and the aglycone (Oshima *et al* 1987). GUS activity is found in many other bacterial species including the enterobacterium *Shigella sonnei* which is closely related to *E. coli*, as well as in non-enterobacterial microorganisms such as *Bacteroides* and *Clostridium* (Hawkesworth *et al* 1971). GUS activity is commonly found in all tissues of vertebrates given its role in the degradation of glycosamino-glucuronides and in the release of active hormones (Kyle *et al* 1992). To examine the relationship between the β-glucuronidase from *S. solfataricus* and those from other prokaryotes, a phylogenetic analysis was performed with amino acid sequences from nine different bacteria and the archaeon *Caldivirga maquilingensis*, and a neighbor

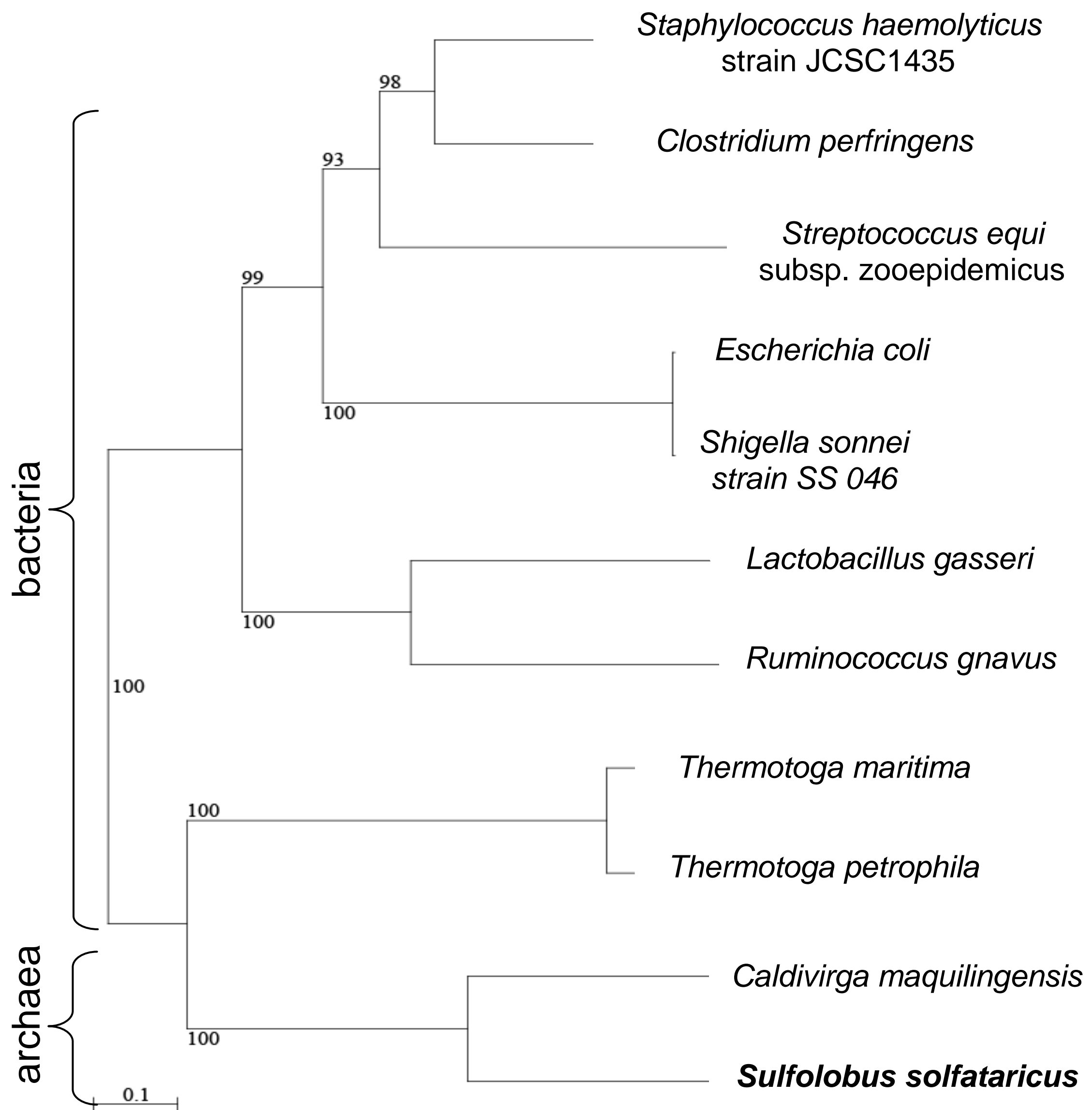


Fig. 4.1 A Neighbor-Joining tree was generated using an alignment of β-glucuronidase sequences from two archaeal and seven bacterial species. Boostrap analysis was done with 100 resamplings.

joining tree was generated (Fig. 4.1). As expected, the sequences from *E. coli* and *S. sonnei* paired up together and so did the sequences of *S. solfataricus* and *C. maquilingensis*. Interestingly, both sequences from the thermophilic bacteria species *Thermotoga* cluster with the archaeal sequences. This might be explained either by convergent evolution as a result of the environmental pressure exerted by the extreme high temperatures on the structures of these proteins, or it might be the result of gene lateral transfer. This question could be answered by including in the analysis glucuronidase homologs from mesophilic archaea. However, no such sequences were identified searching databases of protein sequences and sequenced genomes. Because β-glucuronidase are ubiquitously found in all three domains of life, its properties as a model reporter gene could not be exploited for use in most systems, with the exception of plants, that do encode this enzyme. Although *Sulfolobus* expresses a functional GusB, a mutant strain, PBL2025, is available that carry a deletion of *gusB* and can be used as recipient for reporter plasmids.

Cloning of S. solfataricus gusB and heterologous expression in E. coli

To characterize the *Sulfolobus* β-glucuronidase, the *gusB* gene was amplified by PCR from the chromosome of strain 98/2. The 1,713 bp insert, from start to stop, was ligated in an expression vector to obtain pET21-gusB (Fig. 4.2A) and transformed into *E. coli* BL21 for expression. To measure the activity of the recombinant GusB enzyme, a modified GUS assay (Wickes and Edman 1995) was performed on partially purified extracts from a culture of *E. coli* BL21 carrying the newly constructed pET21-*gusB* plasmid, while a culture of *E. coli* BL21 carrying the pET21 plasmid was used as negative control. Cells were harvested 1, 2 and 3 hours

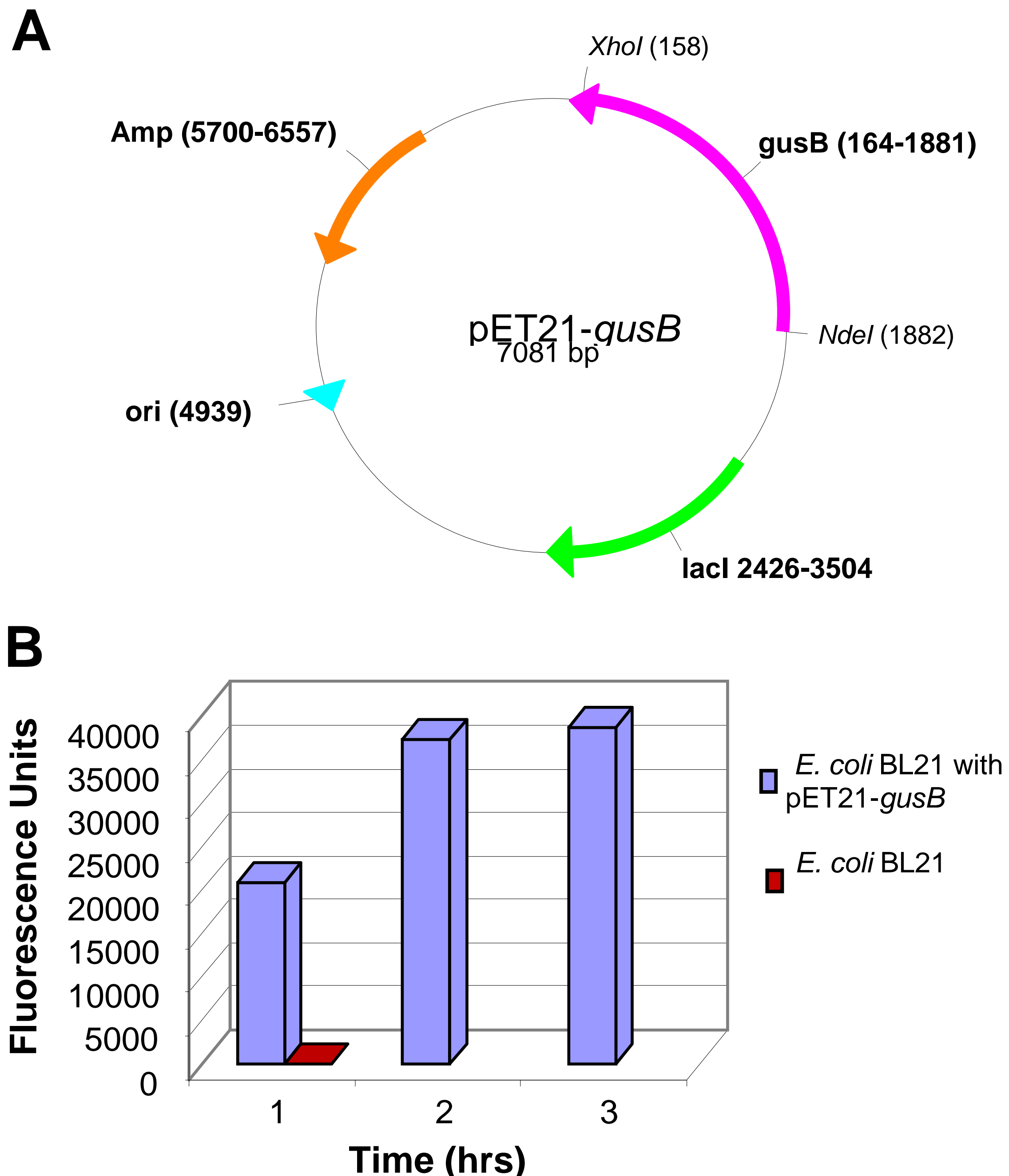


Fig. 4.2 Heterologous expression of the recombinant archaeal β-glucuronidase. (A) The *Sulfolobus gusB* gene was cloned into the expression vector pET21 and expressed in *E. coli* BL21. (B) GusB activity after IPTG induction of a control culture (red bar) carrying pET21 without insert and the experimental culture carrying the construct pET21-gusB (blue bars). One aliquot of the control culture was taken one hour after IPTG addition. Aliquots from the experimental culture where taken after addition of IPTG for three consecutive hours.

after IPTG induction. After extraction by sonication and heat denaturation, the GUS assay was performed at 80°C with the supernatant of the crude extract (Fig. 4.2B). Although *E. coli* possesses an intrinsic GUS activity, the treatment at high temperature of the crude extract causes the precipitation of most *E. coli* protein, resulting in an enrichment of the thermostable *Sulfolobus* GusB. Negligible results were obtained one hour after IPTG induction of the control culture: non-transformed *E. coli* BL21 yielded 932 fluorescence units (FU), while *E. coli* BL21 transformed with pET21-*gusB* yielded 20900 FU. GusB activity kept increasing for two hours after IPTG induction up to 37500 FU but it appeared to be leveling off after three hours (38800 FU). Without IPTG induction, the intensity of the fluorescence detected was comparable to the levels measured one hour after IPTG induction (data not shown). Thus, we opted for employing crude lysates from uninduced cells in the following experiments aimed to the biochemical characterization of the enzyme.

Optimization of GUS assay for thermophilic β-glucuronidase and determination of optimal temperature and pH

The standard GUS assay designed for mesophilic proteins is usually conducted at 37°C, and had to be optimized for the thermophilic glucuronidase. To determine the optimal temperature for the assay, the modified GUS assay previously applied to the recombinant GusB from *S. solfataricus,* was performed on crude extracts of *E. coli* BL21 transformed with pET21-*gusB* at temperatures ranging from 50°C to 100°C (Fig. 4.3A). The GusB activity obtained at 50°C was 25600 FU. The highest activity was recorded at both 60°C and 70°C with 55300 and 55000 FU, respectively. The activity shows a rapid drop of about 46000 FU between 70°C and 80°C, and

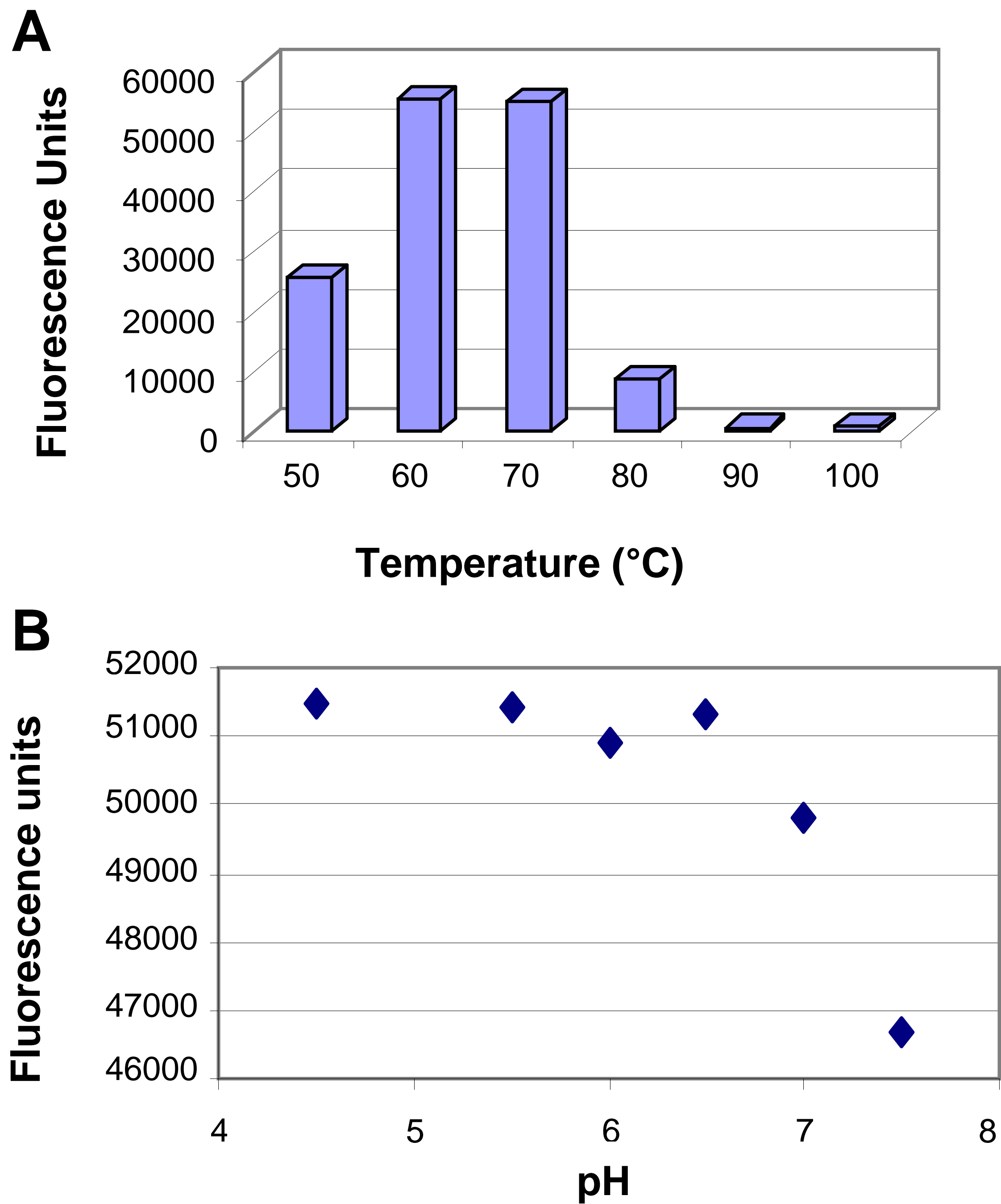


Fig. 4.3 GUS assay optimization. (A) Determination of optimal temperature for *S. solfataricus* GusB. Crude extracts of *E. coli* BL21 transformed with pET21-*gusB* were assayed for GUS activity at temperatures ranging from 50 to 100°C for 30 min. (B) Determination of optimal pH. Cleared lysates were prepared using buffers ranging from pH 4.5 to 7.5 and assayed at 65°C (optimal temperature for *S. solfataricus*'s GusB).

continues to decrease at 90°C and 100°C with the lowest activity measured at 673 FU. Therefore, the temperature of 65°C was selected to carry out all the experiments that follow. To start characterizing gusB from *S. solfataricus*, its active pH range as well as its optimal temperature was determined. Cleared lysate from transformed *E. coli* BL21 with pET21-*gusB* were prepared using buffers ranging from 4.5 to 7.5 and assayed at 65°C for 30 minutes (Fig. 4.3B). GusB remained highly active in the pH range of 4.5 to 6.5 and values of about 51000 FU were recorded across this range. Although a loss of activity is observed at pH greater than 6.5, its values are still relatively high (lowest activity recorded was 46666 FU at pH 7.5), demonstrating a broad active pH range of the recombinant thermophilic enzyme. For our experiments, we selected pH 6.5 since it is the recommended pH to use in the original GUS assay (Wickes and Edman 1995). The natural environment of *Sulfolobus* is characterized by low pH (2 to 3). However, its cytoplasmic pH is maintained at approximately 6.5 (Sørensen and Dandanell 2002), thus it was not surprising to find that the *Sulfolobus* *β*-glucuronidase has maximum activity at this pH.

Thermostability of GusB

To continue the characterization of gusB from *S. solfataricus* its active temperature range was investigated. Clarified lysate of *E. coli* BL21 transformed with pET21-*gusB* was pre-incubated for 30 minutes at 45, 55, 65, 75, 85 or 95°C, and the activity of the enzyme was then determined using the GUS assay at 65°C (Fig. 4.4A). GusB was very active in the temperature range from 45°C to 75°C, with a maximum activity of 68800 FU recorded at 65°C. There is, however, a drastic loss

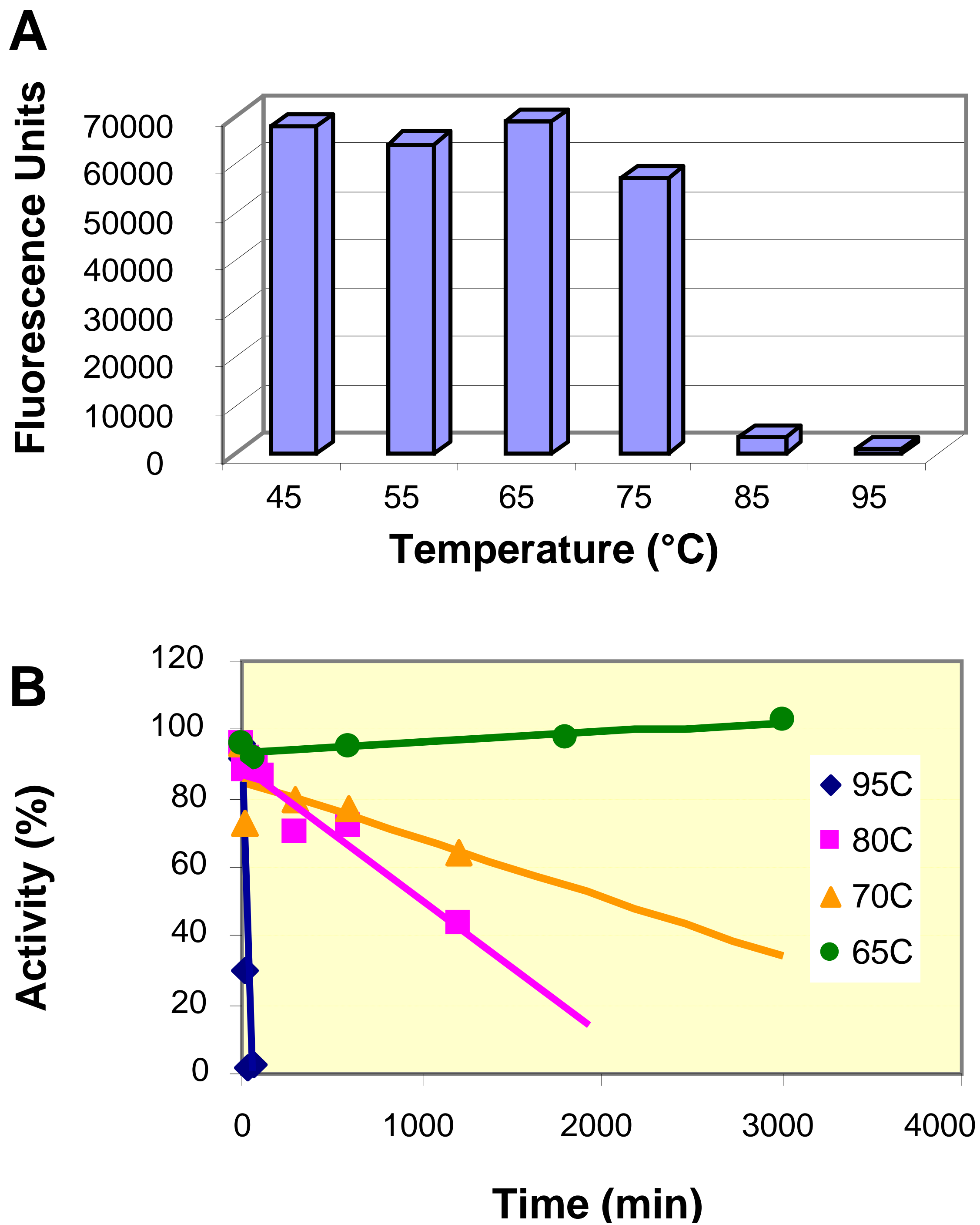


Fig. 4.4 Thermal stability of GusB. (A) Thermal stability was tested by pre-incubating the recombinant GusB for 30 min at different temperatures ranging from 45 to 95°C, and the activity of the enzyme was determined using the GUS assay at 65°C. (B) Thermostability of GusB was monitored over time. Activity of GusB at 65, 70, 80 and 95°C was measured as a percentage of its activity at optimal conditions (pH 6.5, 65°C for 30 minutes). Best fit line was calculated and drawn.

of activity at temperatures of 85°C and higher, suggesting that incubation times of 30 minutes or longer at these high temperatures result in inactivation of the enzyme. There is a marked difference between the optimal temperature of the mesophilic β-glucuronidase and the thermophilic GusB from *S. solfataricus*, being 37°C (Jefferson *et al* 1987), and approximately 65°C, respectively. To study the rate of decay of enzyme activity over time at different temperatures, stability measurements as a function of the incubation time were conducted at pH 6.5. Measurements of GUS activity were conducted on aliquots collected after 1, 10, 30 and 50 hours of incubation at 65°C, giving 54800, 56900, 58166 and 61533 FU, respectively, illustrating the decay of enzyme activity after extended period of times. Furthermore, the assay was performed on samples incubated for extended periods of time (more than 4 days and a half), without losing activity (98.9% activity, data not shown). The enzyme retained most of its activity after incubation times of 70°C and 80°C with the lowest activity recorded at 38166 FU. The samples incubated at 95°C showed a rapid loss of activity after 10 minutes of incubation and reached negligible levels after 30 minutes of incubation. This shows that the *Sulfolobus* GusB is stable after a prolonged exposure to the temperature of growth of *Sulfolobus*, and thus it can be a useful reporter for extremophilic microorganisms growing at temperature up to 80°C.

Use of the Sulfolobus GusB as a reporter

In previous studies we have demonstrated that in *S. solfataricus* 98/2, the *copTA* transcript is overexpressed with addition of copper to the culture, specifically after treatment with 0.75 mM $CuCl_2$. We have also showed that CopR is the regulator responsible for the induction of *copTA*, but we still have to define the exact location

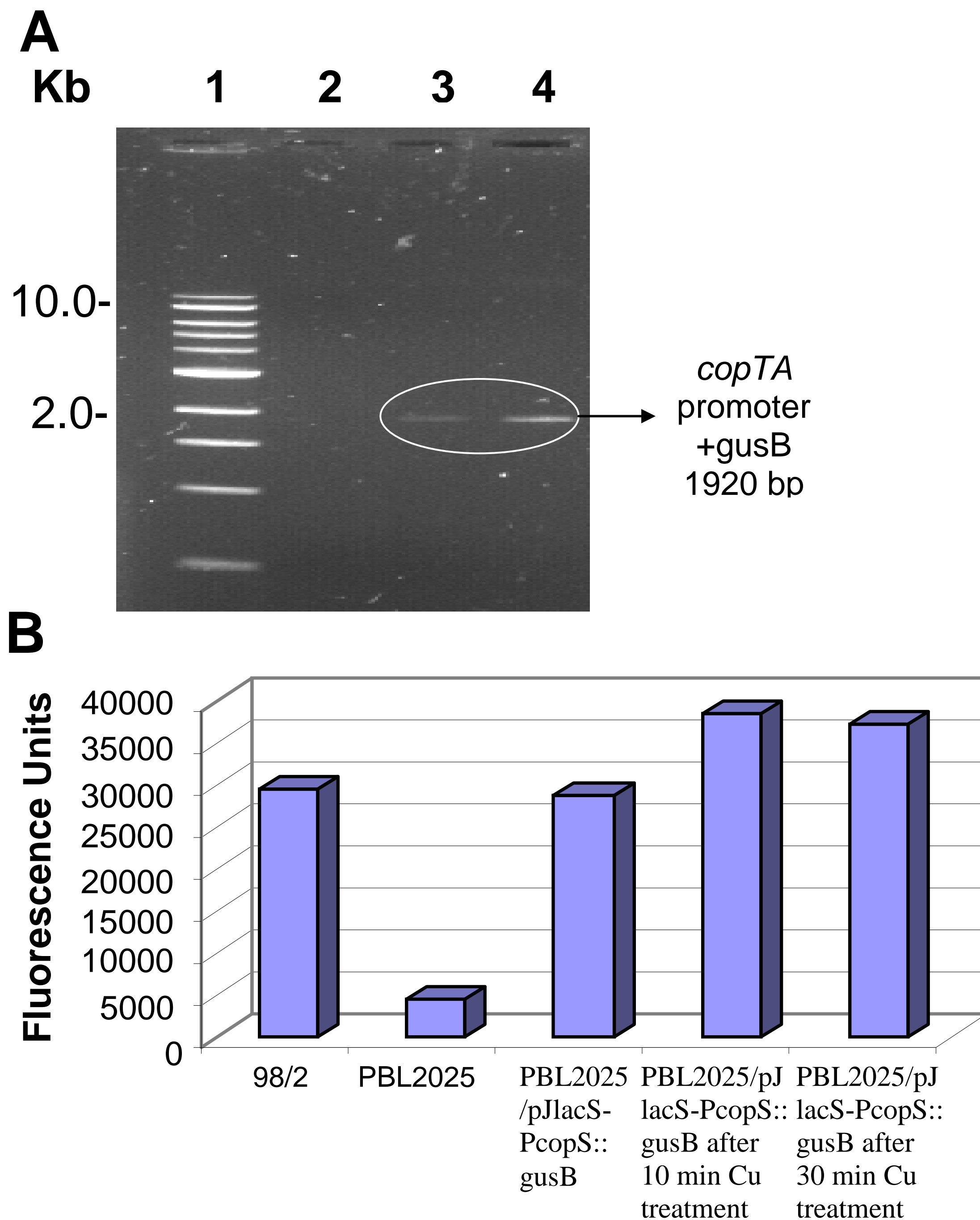


Fig. 4.5 Complementation of the Δ*lacS*, Δ*gusB* double mutant and promoter fusion experiment. (A) Agarose gel electrophoresis of the amplicons corresponding to the promoter region of *copTA* fused to the beta-glucoronidase gene. Lane 1 is a 1 kb standard. Lane 2 is amplification from PBL2025 genomic DNA. Lane 3 shows the amplicon from a genomic DNA extraction from PBL2025/pJlacS-PcopS::gusB. Lane 4 is amplification from of the extracted pJ*malA*,*copTA-gusB* plasmid from transformed DH5α cells. (B) Promoter fusion assay. A GUS assay was performed on crude extracts from *S. solfataricus* 98/2, PBL2025 and PBL2025/pJlacS-PcopS::gusB cells.

of the promoter of *copTA* and dissect the details of its regulation. To this end, and as a proof of concept for the establishment of the thermostable β-glucuronidase from *S. solfataricus* as a reporter gene, we used a reporter fusion plasmid where the *copTA* promoter was fused to the *gusB* gene from *S. solfataricus* by overlap extension PCR and used to transform the *S. solfataricus ΔgusB* mutant strain PBL2025. A plasmid was created through the insertion of the *PcopTA::gusB* in the unique *Sph*I site of pJ*lacS* (Shelert *et al* 2006) and was subsequently transformed in *E. coli* DH5α cells. The resulting plasmid, named pJ*lacS-PcopTA::gusB*, was then used to electroporate PBL2025 electrocompetent cells. After selection on lactose, to confirmthe presence of the fusion vector, the promoter gene fusion was verified through PCR using genomic DNA isolated from both electroporated and non-electroporated PBL2025 (control) cultures using the pair of primers Pcop2-F and gus2-R. A band of expected size was obtained (Lane 3, Fig. 4.5A) for transformed PBL2025 cultures with the plasmid containing the promoter gene fusion (1920 bp) while no PCR amplicon was obtained using the genomic DNA from non-transformed PBL2025 cells (Lane 2, Fig. 4.5A). As a further control, retransformation of *E. coli* DH5α cells with DNA extracted from PBL2025/pJlacS-PcopS::gusB cells was performed followed by plasmid isolation and PCR amplification of the promoter gene fusion. After gel electrophoresis a fragment of about 1920 bp was obtained (Lane 4, Fig. 4.5A) corresponding to the promoter gene fusion. All of this proves a successful transformation of PBL2025 cells. To determine whether the promoter region was susceptible to regulation by copper and could direct the transcription of the reporter, crude extracts from *S. solfataricus* 98/2, PBL2025, PBL2025/pJlacS-PcopS::gusB,

and PBL2025/pJlacS-PcopS::gusB cells treated for 10 and 30 minutes with 0.75 mM $CuCl_2$ were collected, and assayed for GUS activity (Fig. 4.5B). Crude extracts from wild type *S. solfataricus* 98/2 showed moderate activity with 29500 FU, encoding a functional GusB enzyme. As expected, PBL2025 displayed little residual activity with 4433 FU. PBL2025 complemented with pJlacS-PcopS::gusB had a GusB activity of 28733 FU, very similar to the measurements of wild type 98/2, indicating that even in the absence of copper the *copTA* promoter is transcribed, although at low level. Finally, induction of transcription of the promoter of *copTA* was demonstrated by copper treatment of PBL2025/pJlacS-PcopS::gusB cultures for 10 and 30 minutes and a corresponding increase in GusB activity of 38567 and 37233 FU, respectively.

Chapter 5

Conclusions

When *S. solfataricus* cells are challenged with copper, their rate of growth slows down, and this decrease is proportional to the concentration of copper tested. Although immediately after copper challenge a MIC of 1.5 mM copper has been determined, cells become resistant to environs containing up to 2.5 mM of $CuCl_2$ after prolonged exposure. An increased level of the transcripts of genes directly involved in copper detoxification, namely *copA* and *copT*, which are co-transcribed, is observed after treatment with 0.75 mM copper. One hour after copper challenge, the level of *copA* transcript declines until a steady-state level corresponding to an approximately 2-fold induction is reached. Similarly, after treatment with excess copper, the amount of the metal associated with the cells is maintained constant, after a slight accumulation followed by a decrease. These results are consistent with the existence of a feedback-like mechanism of action, where accumulation of the CopA transporter, derived from the burst of copA transcription, causes a drop in the levels of cytoplasmic copper ions. Sequestration of the metal by CopT contributes to limit the availability of free copper. The lowered copper level results in a reduced effect on CopR and consequently a decline of copA transcription. The different copper sensitivity of the two *Sulfolobus* strains P2 and 98/2, both having identical *copR* and *copT* genes and *copTA* promoter sequences, supports the hypothesis that additional factors probably have a role in the response of the cells to copper concentrations, and that these factors might be different in the two strains studied. Further research is required to elucidate the steps involved and to identify the additional genetic

determinants of the response of the cell to varying levels of copper in the surroundings.

Using a well established knockout system (Worthtington *et al* 2003) two *S. solfataricus* strains carrying a disruption of the *copR* gene were obtained and named PBL2050 and PBL2070. Although both strains have the same genetic background, PBL2070 has a deletion of the α- glucosidase gene necessary for growth on maltose.Growth of cultures of strains PBL2050 and PBL2070was inhibited by treatment 0.75 mM $CuCl_2$. Furthermore, the mutant strains were unable to grow even on copper concentrations as low as 0.1 mM (data not shown), consistent with the hypothesis that CopR has a direct role in copper homeostasis, as a regulator of transcription. When the levels of the *copA* transcript were investigated in PBL2050 cells after copper addition, they remained at their basal uninduced levels, consistent with their growth behavior and more importantly, clarifying the nature of *copR* as a positive regulator of transcription. We have thus herein identified one of the first positive archaeal regulators,along with the transcriptional regulator LysM from *S. solfataricus* and the euryarchaeal activator Ptr2 from *M. jannaschii* (Brinkman *et al* 2002; Ouhammouch *et al*, 2003) When PBL2070 cells were successfully transformed and complemented with a wild type copy of the *copR* gene, their physiological response to copper was tested, in order to see if the phenotype was rescued. After addition of sub-lethal concentrations of copper, PBL2070/pJcopR cells now became resistant to it and were able to maintain a growth rate similar to its parent strain. Ten minutes after copper challenge, the level of *copA* transcript increased to an approximate nine-fold induction. These results provide solid proof

that when CopR is present and when there are copper stress conditions, it activates transcription of the *copA* transcript by binding to the promoter region upstream of *copTA*. Additional research will be required to elucidate where and how strong is the promoter region of *copTA* where CopR binds and to solve the structure of this novel archaeal regulator. A model summarizing the data obtained thorugh this work is shown in Fig. 5.1.

The *S. solfataricus* gene Sso3036 encoding a functional β-glucuronidase protein (GusB) was first cloned in *E. coli* DH5α and then over expressed in *E. coli* BL21. A phylogenetic analysis carried out using archaeal and prokaryotic sequences, showed that the GusB protein from *S. solfataricus* P2 clustered together not only with the archaeal sequences but also with sequences from thermophilic prokaryotes, suggesting that different organisms living in the same environment often have similar if not identical protein sequences that provides them an evolutionary advantage over closely related organisms. GusB from *S. solfataricus* has an activity optimum at 65°C and remains highly active in the temperature range of 45 to 75°C, this is unusual and in contrast with other thermophilic enzymes that display optimal temperatures above the temperature of growth of the source organism, as is the case of a newly characterized L-threonine dehydrogenase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* that can be fully active up to 90°C (Bashir *et al* 2009). The GusB of *Sulfolobus* could have a broader temperature range extending to temperatures lower than 45°C, but further tests need to be done to assess its lower range of activity. GusB optimal pH is 6.5, but it also has a broad active range of pH of 4.5 to 7.5. More research needs to be done to determine if the pH range of GusB

from *S. solfataricus* could extend to more acidic pHs, given that the natural environment of *Sulfolobus* is characterized by low pH (2-3). Furthermore, the archaeal GusB is able to maintain its activity for extended period of times (greater than 3000 minutes at 65°C and up to 1200 minutes at 80°C), this thermostability makes it a convenient reporter for monitoring the activity of thermophilic promoters. When the gene encoding the thermostable GusB was used for promoter fusion experiments using the *copTA* promoter region, the GusB activity was easily detected. An increased level of fluorescence was observed in crude extracts from PBL2025/pJlacS-PcopS::gusB cells challenged with copper for 10 and 30 minutes when compared to crude extracts from PBL2025/pJlacS-PcopS::gusB cells without addition of copper, indicating that the fusion vector based on GusB is an effective tool for measuring the activity of regulated promoters. These features make it an ideal reporter gene for use in *Sulfolobus* and thermophilic microorganisms in general. Because its activity is easily separated from mesophilic activities by heat treatment, the enzyme has also potential applications as reporter in eukaryotes other than plants.

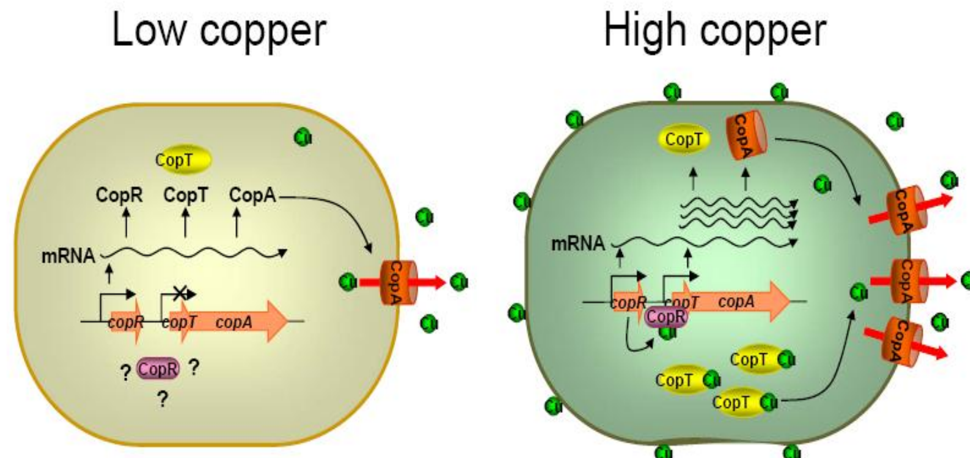


Fig. 5.1. A model for transcription of copper-responsive genes in *S. solfataricus*. The genes *copR, copT* and *copA* are constitutively cotranscribed from the *copR* promoter. In the presence of excess copper, the supplementary transcription of *copTA* is provided by the induction of the promoter of *copTA*. A high rate of *copA* transcription and CopA synthesis is necessary to reestablish homeostasis during early copper exposure. As a consequence of the decrease in intracellular concentration of copper, transcription of *copA* declines to reach a low basal induced level, which is maintained during long term exposures to copper. This might be explained with the accumulation of a stable CopA in the early stages of exposure, which would sustain the rate of copper efflux during prolonged exposures.

References

1. Adachi, N., M. Senda, R. Natsume, T. Senda, and M. Horikoshi, *Crystal structure of Methanococcus jannaschii TATA box-binding protein.* Genes Cells, 2008. **13**(11): p. 1127-40.

2. Albers, S.V. and A.J. Driessen, *Conditions for gene disruption by homologous recombination of exogenous DNA into the Sulfolobus solfataricus genome.* Archaea, 2008. **2**(3): p. 145-9.

3. Albers, S.V., M. Jonuscheit, S. Dinkelaker, T. Urich, A. Kletzin, R. Tampe, A.J. Driessen, and C. Schleper, *Production of recombinant and tagged proteins in the hyperthermophilic archaeon Sulfolobus solfataricus.* Appl Environ Microbiol, 2006. **72**(1): p. 102-11.

4. Alekshun, M.N., S.B. Levy, T.R. Mealy, B.A. Seaton, and J.F. Head, *The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution.* Nat Struct Biol, 2001. **8**(8): p. 710-4.

5. Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman, *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* Nucleic Acids Res, 1997. **25**(17): p. 3389-402.

6. Alvarez, S. and C.A. Jerez, *Copper ions stimulate polyphosphate degradation and phosphate efflux in Acidithiobacillus ferrooxidans.* Appl Environ Microbiol, 2004. **70**(9): p. 5177-82.

7. Anemuller, S. and G. Schafer, *Cytochrome aa3 from Sulfolobus acidocaldarius. A single-subunit, quinol-oxidizing archaebacterial terminal oxidase.* Eur J Biochem, 1990. **191**(2): p. 297-305.

8. Aravalli, R.N. and R.A. Garrett, *Shuttle vectors for hyperthermophilic archaea.* Extremophiles, 1997. **1**(4): p. 183-91.

9. Arguello, J.M., E. Eren, and M. Gonzalez-Guerrero, *The structure and function of heavy metal transport P(1B)-ATPases.* Biometals, 2007.

10. Arguello, J.M., E. Eren, and M. Gonzalez-Guerrero, *The structure and function of heavy metal transport P1B-ATPases.* Biometals, 2007. **20**(3-4): p. 233-48.

11. Arguello, J.M., A.K. Mandal, and S. Mana-Capelli, *Heavy metal transport CPx-ATPases from the thermophile Archaeoglobus fulgidus.* Ann N Y Acad Sci, 2003. **986**: p. 212-8.

12. Aucelli, T., P. Contursi, M. Girfoglio, M. Rossi, and R. Cannio, *A spreadable, non-integrative and high copy number shuttle vector for Sulfolobus solfataricus based on the genetic element pSSVx from Sulfolobus islandicus.* Nucleic Acids Res, 2006. **34**(17): p. e114.

13. Baker-Austin, C., M. Dopson, M. Wexler, R.G. Sawers, and P.L. Bond, *Molecular insight into extreme copper resistance in the extremophilic archaeon 'Ferroplasma acidarmanus' Fer1.* Microbiology, 2005. **151**(Pt 8): p. 2637-46.

14. Baker-Austin, C., M. Dopson, M. Wexler, R.G. Sawers, A. Stemmler, B.P. Rosen, and P.L. Bond, *Extreme arsenic resistance by the acidophilic archaeon 'Ferroplasma acidarmanus' Fer1.* Extremophiles, 2007. **11**(3): p. 425-34.

15. Baker-Austin, C., M.S. Wright, R. Stepanauskas, and J.V. McArthur, *Co-selection of antibiotic and metal resistance.* Trends Microbiol, 2006. **14**(4): p. 176-82.

16. Baliga, N.S. and S. DasSarma, *Saturation mutagenesis of the TATA box and upstream activator sequence in the haloarchaeal bop gene promoter.* J Bacteriol, 1999. **181**(8): p. 2513-8.

17. Baliga, N.S., S.P. Kennedy, W.V. Ng, L. Hood, and S. DasSarma, *Genomic and genetic dissection of an archaeal regulon.* Proc Natl Acad Sci U S A, 2001. **98**(5): p. 2521-5.

18. Banci, L., I. Bertini, S. Ciofi-Baffoni, M. D'Onofrio, L. Gonnelli, F.C. Marhuenda-Egea, and F.J. Ruiz-Duenas, *Solution structure of the N-terminal domain of a potential copper-translocating P-type ATPase from Bacillus subtilis in the apo and Cu(I) loaded states.* J Mol Biol, 2002. **317**(3): p. 415-29.

19. Bandeiras, T.M., C.A. Salgueiro, H. Huber, C.M. Gomes, and M. Teixeira, *The respiratory chain of the thermophilic archaeon Sulfolobus metallicus: studies on the type-II NADH dehydrogenase.* Biochim Biophys Acta, 2003. **1557**(1-3): p. 13-9.

20. Banuelos, G.S., Z.Q. Lin, I. Arroyo, and N. Terry, *Selenium volatilization in vegetated agricultural drainage sediment from the San Luis Drain, Central California.* Chemosphere, 2005. **60**(9): p. 1203-13.

21. Barkay, T., S.M. Miller, and A.O. Summers, *Bacterial mercury resistance from atoms to ecosystems.* FEMS Microbiol Rev, 2003. **27**(2-3): p. 355-84.

22. Bartlett, M.S., *Determinants of transcription initiation by archaeal RNA polymerase.* Curr Opin Microbiol, 2005. **8**(6): p. 677-84.

23. Bartlett, M.S., M. Thomm, and E.P. Geiduschek, *Topography of the euryarchaeal transcription initiation complex.* J Biol Chem, 2004. **279**(7): p. 5894-903.

24. Bashir, Q., N. Rashid, F. Jamil, T. Imanaka, and M. Akhtar, *Highly thermostable L-threonine dehydrogenase from the hyperthermophilic archaeon Thermococcus kodakaraensis.* J Biochem, 2009. **146**(1): p. 95-102.

25. Basu, C., A.P. Kausch, and J.M. Chandlee, *Use of beta-glucuronidase reporter gene for gene expression analysis in turfgrasses.* Biochem Biophys Res Commun, 2004. **320**(1): p. 7-10.

26. Bathe, S. and P.R. Norris, *Ferrous iron- and sulfur-induced genes in Sulfolobus metallicus.* Appl Environ Microbiol, 2007. **73**(8): p. 2491-7.

27. Bell, S.D., S.S. Cairns, R.L. Robson, and S.P. Jackson, *Transcriptional regulation of an archaeal operon in vivo and in vitro.* Mol Cell, 1999. **4**(6): p. 971-82.

28. Bell, S.D., P.L. Kosa, P.B. Sigler, and S.P. Jackson, *Orientation of the transcription preinitiation complex in archaea.* Proc Natl Acad Sci U S A, 1999. **96**(24): p. 13662-7.

29. Berkner, S., D. Grogan, S.V. Albers, and G. Lipps, *Small multicopy, non-integrative shuttle vectors based on the plasmid pRN1 for Sulfolobus acidocaldarius and Sulfolobus solfataricus, model organisms of the (cren-)archaea.* Nucleic Acids Res, 2007. **35**(12): p. e88.

30. Bernander, R. and A. Poplawski, *Cell cycle characteristics of thermophilic archaea.* J Bacteriol, 1997. **179**(16): p. 4963-9.

31. Bevers, L.E., E. Bol, P.L. Hagedoorn, and W.R. Hagen, *WOR5, a novel tungsten-containing aldehyde oxidoreductase from Pyrococcus furiosus with a broad substrate Specificity.* J Bacteriol, 2005. **187**(20): p. 7056-61.

32. Bevers, L.E., P.L. Hagedoorn, G.C. Krijger, and W.R. Hagen, *Tungsten transport protein A (WtpA) in Pyrococcus furiosus: the first member of a new class of tungstate and molybdate transporters.* J Bacteriol, 2006. **188**(18): p. 6498-505.

33. Bini, E., *Archaea-metal interactions: metabolism and strategies of resistance* in *Archaea: New models for prokaryotic biology*, P. Blum, Editor. 2008, Caister Academic Press. p. 27-43.

34. Bode, M.L., S.R. Buddoo, S.H. Minnaar, and C.A. du Plessis, *Extraction, isolation and NMR data of the tetraether lipid calditoglycerocaldarchaeol (GDNT) from Sulfolobus metallicus harvested from a bioleaching reactor.* Chem Phys Lipids, 2008. **154**(2): p. 94-104.

35. Borremans, B., J.L. Hobman, A. Provoost, N.L. Brown, and D. van Der Lelie, *Cloning and functional analysis of the pbr lead resistance determinant of Ralstonia metallidurans CH34.* J Bacteriol, 2001. **183**(19): p. 5651-8.

36. Bramkamp, M., M. Gassel, B. Herkenhoff-Hesselmann, J. Bertrand, and K. Altendorf, *The Methanocaldococcus jannaschii protein Mj0968 is not a P-type ATPase.* FEBS Lett, 2003. **543**(1-3): p. 31-6.

37. Brantl, S., *Bacterial gene regulation: metal ion sensing by proteins or RNA.* Trends Biotechnol, 2006. **24**(9): p. 383-6.

38. Brochier-Armanet, C., B. Boussau, S. Gribaldo, and P. Forterre, *Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota.* Nat Rev Microbiol, 2008. **6**(3): p. 245-52.

39. Brock, T.D., *Life at High Temperatures.* Science, 1985. **230**(4722): p. 132-138.

40. Brock, T.D., K.M. Brock, R.T. Belly, and R.L. Weiss, *Sulfolobus: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature.* Arch Mikrobiol, 1972. **84**(1): p. 54-68.

41. Brocklehurst, K.R., J.L. Hobman, B. Lawley, L. Blank, S.J. Marshall, N.L. Brown, and A.P. Morby, *ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of zntA in Escherichia coli.* Mol Microbiol, 1999. **31**(3): p. 893-902.

42. Brown, N.L., J.V. Stoyanov, S.P. Kidd, and J.L. Hobman, *The MerR family of transcriptional regulators.* FEMS Microbiol Rev, 2003. **27**(2-3): p. 145-63.

43. Brown, N.M., A.S. Torres, P.E. Doan, and T.V. O'Halloran, *Oxygen and the copper chaperone CCS regulate posttranslational activation of Cu,Zn superoxide dismutase.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5518-23.

44. Bushnell, D.A., K.D. Westover, R.E. Davis, and R.D. Kornberg, *Structural basis of transcription: an RNA polymerase II-TFIIB cocrystal at 4.5 Angstroms.* Science, 2004. **303**(5660): p. 983-8.

45. Butcher, B.G. and D.E. Rawlings, *The divergent chromosomal ars operon of Acidithiobacillus ferrooxidans is regulated by an atypical ArsR protein.* Microbiology, 2002. **148**(Pt 12): p. 3983-92.

46. Camakaris, J., I. Voskoboinik, and J.F. Mercer, *Molecular mechanisms of copper homeostasis.* Biochem Biophys Res Commun, 1999. **261**(2): p. 225-32.

47. Cannio, R., P. Contursi, M. Rossi, and S. Bartolucci, *An autonomously replicating transforming vector for Sulfolobus solfataricus.* J Bacteriol, 1998. **180**(12): p. 3237-40.

48. Cantini, F., L. Banci, and M. Solioz, *The copper-responsive repressor CopR of Lactococcus lactis is a 'winged helix' protein.* Biochem J, 2009. **417**(2): p. 493-9.

49. Cattoni, D.I., F.L. Flecha, and J.M. Arguello, *Thermal stability of CopA, a polytopic membrane protein from the hyperthermophile Archaeoglobus fulgidus.* Arch Biochem Biophys, 2008. **471**(2): p. 198-206.

50. Cervantes, C., G. Ji, J.L. Ramirez, and S. Silver, *Resistance to arsenic compounds in microorganisms.* FEMS Microbiol Rev, 1994. **15**(4): p. 355-67.

51. Chae, J.C., E. Kim, E. Bini, and G.J. Zylstra, *Comparative analysis of the catechol 2,3-dioxygenase gene locus in thermoacidophilic archaeon Sulfolobus solfataricus strain 98/2.* Biochem Biophys Res Commun, 2007. **357**(3): p. 815-9.

52. Changela, A., K. Chen, Y. Xue, J. Holschen, C.E. Outten, T.V. O'Halloran, and A. Mondragon, *Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR.* Science, 2003. **301**(5638): p. 1383-7.

53. Charlebois, R.L., Q. She, D.P. Sprott, C.W. Sensen, and R.A. Garrett, *Sulfolobus genome: from genomics to biology.* Curr Opin Microbiol, 1998. **1**(5): p. 584-8.

54. Chintalapati, S., R. Al Kurdi, A.C. van Scheltinga, and W. Kuhlbrandt, *Membrane structure of CtrA3, a copper-transporting P-type-ATPase from Aquifex aeolicus.* J Mol Biol, 2008. **378**(3): p. 581-95.

55. Chintalapati, S., R. Al Kurdi, A.C. van Scheltinga, and W. Kuhlbrandt, *Membrane structure of CtrA3, a copper-transporting P-type-ATPase from Aquifex aeolicus.* J Mol Biol, 2008. **378**(3): p. 581-95.

56. Chivers, P.T. and R.T. Sauer, *Regulation of high affinity nickel uptake in bacteria. Ni2+-Dependent interaction of NikR with wild-type and mutant operator sites.* J Biol Chem, 2000. **275**(26): p. 19735-41.

57. Chivers, P.T. and T.H. Tahirov, *Structure of Pyrococcus horikoshii NikR: nickel sensing and implications for the regulation of DNA recognition.* J Mol Biol, 2005. **348**(3): p. 597-607.

58. Ciaramella, M., F.M. Pisani, and M. Rossi, *Molecular biology of extremophiles: recent progress on the hyperthermophilic archaeon Sulfolobus.* Antonie Van Leeuwenhoek, 2002. **81**(1-4): p. 85-97.

59. Cobine, P.A., G.N. George, C.E. Jones, W.A. Wickramasinghe, M. Solioz, and C.T. Dameron, *Copper transfer from the Cu(I) chaperone, CopZ, to the repressor, Zn(II)CopY: metal coordination environments and protein interactions.* Biochemistry, 2002. **41**(18): p. 5822-9.

60. Cobine, P.A., C.E. Jones, and C.T. Dameron, *Role for zinc(II) in the copper(I) regulated protein CopY.* J Inorg Biochem, 2002. **88**(2): p. 192-6.

61. Cox, D.W. and S.D. Moore, *Copper transporting P-type ATPases and human disease.* J Bioenerg Biomembr, 2002. **34**(5): p. 333-8.

62. Croal, L.R., J.A. Gralnick, D. Malasarn, and D.K. Newman, *The genetics of geochemistry.* Annu Rev Genet, 2004. **38**: p. 175-202.

63. Cromie, M.J., Y. Shi, T. Latifi, and E.A. Groisman, *An RNA sensor for intracellular Mg(2+).* Cell, 2006. **125**(1): p. 71-84.

64. Culotta, V.C., M. Yang, and T.V. O'Halloran, *Activation of superoxide dismutases: putting the metal to the pedal.* Biochim Biophys Acta, 2006. **1763**(7): p. 747-58.

65. De Hertogh, B., A.C. Lantin, P.V. Baret, and A. Goffeau, *The archaeal P-type ATPases.* J Bioenerg Biomembr, 2004. **36**(1): p. 135-42.

66. de Kock, S.H., P. Barnard, and C.A. du Plessis, *Oxygen and carbon dioxide kinetic challenges for thermophilic mineral bioleaching processes.* Biochem Soc Trans, 2004. **32**(Pt 2): p. 273-5.

67. De Pina, K., V. Desjardin, M.A. Mandrand-Berthelot, G. Giordano, and L.F. Wu, *Isolation and characterization of the nikR gene encoding a nickel-responsive regulator in Escherichia coli.* J Bacteriol, 1999. **181**(2): p. 670-4.

68. Degtyarenko, K., *Bioinorganic motifs: towards functional classification of metalloproteins.* Bioinformatics, 2000. **16**(10): p. 851-64.

69. Deigweiher, K., T.L.t. Drell, A. Prutsch, A.J. Scheidig, and M. Lubben, *Expression, isolation, and crystallization of the catalytic domain of CopB, a putative copper transporting ATPase from the thermoacidophilic archaeon Sulfolobus solfataricus.* J Bioenerg Biomembr, 2004. **36**(1): p. 151-9.

70. Dello Russo, A., R. Rullo, G. Nitti, M. Masullo, and V. Bocchini, *Iron superoxide dismutase from the archaeon Sulfolobus solfataricus: average hydrophobicity and amino acid weight are involved in the adaptation of proteins to extreme environments.* Biochim Biophys Acta, 1997. **1343**(1): p. 23-30.

71. Deng, L., H. Zhu, Z. Chen, Y.X. Liang, and Q. She, *Unmarked gene deletion and host-vector system for the hyperthermophilic crenarchaeon Sulfolobus islandicus.* Extremophiles, 2009. **13**(4): p. 735-46.

72. Dey, S. and B.P. Rosen, *Dual mode of energy coupling by the oxyanion-translocating ArsB protein.* J Bacteriol, 1995. **177**(2): p. 385-9.

73. Dhawan, I.K., R. Roy, B.P. Koehler, S. Mukund, M.W. Adams, and M.K. Johnson, *Spectroscopic studies of the tungsten-containing formaldehyde ferredoxin oxidoreductase from the hyperthermophilic archaeon Thermococcus litoralis.* J Biol Inorg Chem, 2000. **5**(3): p. 313-27.

74. Diekert, G., U. Konheiser, K. Piechulla, and R.K. Thauer, *Nickel requirement and factor F430 content of methanogenic bacteria.* J Bacteriol, 1981. **148**(2): p. 459-64.

75. Diekert, G., U. Konheiser, K. Piechulla, and R.K. Thauer, *Nickel requirement and factor F430 content of methanogenic bacteria.* J Bacteriol, 1981. **148**(2): p. 459-64.

76. Dixit, V., E. Bini, M. Drozda, and P. Blum, *Mercury inactivates transcription and the generalized transcription factor TFB in the archaeon Sulfolobus solfataricus.* Antimicrob Agents Chemother, 2004. **48**(6): p. 1993-9.

77. Dopson, M., C. Baker-Austin, and P. Bond, *Towards determining details of anaerobic growth coupled to ferric iron reduction by the acidophilic archaeon 'Ferroplasma acidarmanus' Fer1.* Extremophiles, 2007. **11**(1): p. 159-68.

78. Dopson, M., C. Baker-Austin, A. Hind, J.P. Bowman, and P.L. Bond, *Characterization of Ferroplasma isolates and Ferroplasma acidarmanus sp. nov., extreme acidophiles from acid mine drainage and industrial bioleaching environments.* Appl Environ Microbiol, 2004. **70**(4): p. 2079-88.

79. Dopson, M., C. Baker-Austin, P.R. Koppineedi, and P.L. Bond, *Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms.* Microbiology, 2003. **149**(Pt 8): p. 1959-70.

80. Dungan, R.S., S.R. Yates, and W.T. Frankenberger, Jr., *Transformations of selenate and selenite by Stenotrophomonas maltophilia isolated from a seleniferous agricultural drainage pond sediment.* Environ Microbiol, 2003. **5**(4): p. 287-95.

81. Edmondson, S.P. and J.W. Shriver, *DNA binding proteins Sac7d and Sso7d from Sulfolobus.* Methods Enzymol, 2001. **334**: p. 129-45.

82. Eide, D.J., *The molecular biology of metal ion transport in Saccharomyces cerevisiae.* Annu Rev Nutr, 1998. **18**: p. 441-69.

83. Eitinger, T. and M.A. Mandrand-Berthelot, *Nickel transport systems in microorganisms.* Arch Microbiol, 2000. **173**(1): p. 1-9.

84. Ellefson, W.L., W.B. Whitman, and R.S. Wolfe, *Nickel-containing factor F430: chromophore of the methylreductase of Methanobacterium.* Proc Natl Acad Sci U S A, 1982. **79**(12): p. 3707-10.

85. Ellis, P.J., T. Conrads, R. Hille, and P. Kuhn, *Crystal structure of the 100 kDa arsenite oxidase from Alcaligenes faecalis in two crystal forms at 1.64 A and 2.03 A.* Structure, 2001. **9**(2): p. 125-32.

86. Ermakov, V.V., *Biogeochemical regioning problems and the biogeochemical selenium provinces in the former USSR.* Biol Trace Elem Res, 1992. **33**: p. 171-85.

87. Espariz, M., S.K. Checa, M.E. Audero, L.B. Pontel, and F.C. Soncini, *Dissecting the Salmonella response to copper.* Microbiology, 2007. **153**(Pt 9): p. 2989-97.

88. Ettema, T.J., A.B. Brinkman, P.P. Lamers, N.G. Kornet, W.M. de Vos, and J. van der Oost, *Molecular characterization of a conserved archaeal copper resistance (cop) gene cluster and its copper-responsive regulator in Sulfolobus solfataricus P2.* Microbiology, 2006. **152**(Pt 7): p. 1969-79.

89. Ettema, T.J., M.A. Huynen, W.M. de Vos, and J. van der Oost, *TRASH: a novel metal-binding domain predicted to be involved in heavy-metal sensing, trafficking and resistance.* Trends Biochem Sci, 2003. **28**(4): p. 170-3.

90. Fani, R., R. Gallo, and P. Lio, *Molecular evolution of nitrogen fixation: the evolutionary history of the nifD, nifK, nifE, and nifN genes.* J Mol Evol, 2000. **51**(1): p. 1-11.

91. Forget, D., M.F. Langelier, C. Therien, V. Trinh, and B. Coulombe, *Photo-cross-linking of a purified preinitiation complex reveals central roles for the RNA polymerase II mobile clamp and TFIIE in initiation mechanisms.* Mol Cell Biol, 2004. **24**(3): p. 1122-31.

92. Forterre, P., *A hot topic: the origin of hyperthermophiles.* Cell, 1996. **85**(6): p. 789-92.

93. Franke, S., G. Grass, C. Rensing, and D.H. Nies, *Molecular analysis of the copper-transporting efflux system CusCFBA of Escherichia coli.* J Bacteriol, 2003. **185**(13): p. 3804-12.

94. Fuhrman, J.A., K. McCallum, and A.A. Davis, *Novel major archaebacterial group from marine plankton.* Nature, 1992. **356**(6365): p. 148-9.

95. Gajiwala, K.S. and S.K. Burley, *Winged helix proteins.* Curr Opin Struct Biol, 2000. **10**(1): p. 110-6.

96. Gao, S., J. Ryu, K.K. Tanji, and M.J. Herbel, *Arsenic speciation and accumulation in evapoconcentrating waters of agricultural evaporation basins.* Chemosphere, 2007. **67**(5): p. 862-71.

97. Gao, Y.G., S.Y. Su, H. Robinson, S. Padmanabhan, L. Lim, B.S. McCrary, S.P. Edmondson, J.W. Shriver, and A.H. Wang, *The crystal structure of the hyperthermophile chromosomal protein Sso7d bound to DNA.* Nat Struct Biol, 1998. **5**(9): p. 782-6.

98. Gauss, G.H., P. Benas, B. Wiedenheft, M. Young, T. Douglas, and C.M. Lawrence, *Structure of the DPS-like protein from Sulfolobus solfataricus reveals a bacterioferritin-like dimetal binding site within a DPS-like dodecameric assembly.* Biochemistry, 2006. **45**(36): p. 10815-27.

99. Gihring, T.M., P.L. Bond, S.C. Peters, and J.F. Banfield, *Arsenic resistance in the archaeon "Ferroplasma acidarmanus": new insights into the structure and evolution of the ars genes.* Extremophiles, 2003. **7**(2): p. 123-30.

100. Gilbert, W. and B. Muller-Hill, *Isolation of the Lac Repressor.* Proc Natl Acad Sci U S A, 1966. **56**(6): p. 1891-1898.

101. Goede, B., S. Naji, O. von Kampen, K. Ilg, and M. Thomm, *Protein-protein interactions in the archaeal transcriptional machinery: binding studies of isolated RNA polymerase subunits and transcription factors.* J Biol Chem, 2006. **281**(41): p. 30581-92.

102. Golyshina, O.V., T.A. Pivovarova, G.I. Karavaiko, T.F. Kondrateva, E.R. Moore, W.R. Abraham, H. Lunsdorf, K.N. Timmis, M.M. Yakimov, and P.N. Golyshin, *Ferroplasma acidiphilum gen. nov., sp. nov., an acidophilic,*

autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the Archaea. Int J Syst Evol Microbiol, 2000. **50 Pt 3**: p. 997-1006.

103. Golyshina, O.V. and K.N. Timmis, *Ferroplasma and relatives, recently discovered cell wall-lacking archaea making a living in extremely acid, heavy metal-rich environments.* Environ Microbiol, 2005. **7**(9): p. 1277-88.

104. Gonzalez-Guerrero, M. and J.M. Arguello, *Mechanism of Cu+-transporting ATPases: soluble Cu+ chaperones directly transfer Cu+ to transmembrane transport sites.* Proc Natl Acad Sci U S A, 2008. **105**(16): p. 5992-7.

105. Grass, G. and C. Rensing, *Genes involved in copper homeostasis in Escherichia coli.* J Bacteriol, 2001. **183**(6): p. 2145-7.

106. Gregor, D. and F. Pfeifer, *Use of a halobacterial bgaH reporter gene to analyse the regulation of gene expression in halophilic archaea.* Microbiology, 2001. **147**(Pt 7): p. 1745-54.

107. Grogan, D.W., *Phenotypic characterization of the archaebacterial genus Sulfolobus: comparison of five wild-type strains.* J Bacteriol, 1989. **171**(12): p. 6710-9.

108. Grogan, D.W., *Cytosine methylation by the SuaI restriction-modification system: implications for genetic fidelity in a hyperthermophilic archaeon.* J Bacteriol, 2003. **185**(15): p. 4657-61.

109. Grogan, D.W. and R.P. Gunsalus, *Sulfolobus acidocaldarius synthesizes UMP via a standard de novo pathway: results of biochemical-genetic study.* J Bacteriol, 1993. **175**(5): p. 1500-7.

110. Grohmann, D., A. Hirtreiter, and F. Werner, *Molecular mechanisms of archaeal RNA polymerase.* Biochem Soc Trans, 2009. **37**(Pt 1): p. 12-7.

111. Hain, J., W.D. Reiter, U. Hudepohl, and W. Zillig, *Elements of an archaeal promoter defined by mutational analysis.* Nucleic Acids Res, 1992. **20**(20): p. 5423-8.

112. Hartzell, P.L. and R.S. Wolfe, *Requirement of the nickel tetrapyrrole F430 for in vitro methanogenesis: reconstitution of methylreductase component C from its dissociated subunits.* Proc Natl Acad Sci U S A, 1986. **83**(18): p. 6726-30.

113. Haseltine, C., R. Montalvo-Rodriguez, A. Carl, E. Bini, and P. Blum, *Extragenic pleiotropic mutations that repress glycosyl hydrolase expression*

in the hyperthermophilic archaeon Sulfolobus solfataricus. Genetics, 1999. **152**(4): p. 1353-61.

114. Hawksworth, G., B.S. Drasar, and M.J. Hill, *Intestinal bacteria and the hydrolysis of glycosidic bonds.* J Med Microbiol, 1971. **4**(4): p. 451-9.

115. Heider, J., K. Ma, and M.W. Adams, *Purification, characterization, and metabolic function of tungsten-containing aldehyde ferredoxin oxidoreductase from the hyperthermophilic and proteolytic archaeon Thermococcus strain ES-1.* J Bacteriol, 1995. **177**(16): p. 4757-64.

116. Helmann, J.D., B.T. Ballard, and C.T. Walsh, *The MerR metalloregulatory protein binds mercuric ion as a tricoordinate, metal-bridged dimer.* Science, 1990. **247**(4945): p. 946-8.

117. Herbel, M.J., T.M. Johnson, K.K. Tanji, S. Gao, and T.D. Bullen, *Selenium stable isotope ratios in California agricultural drainage water management systems.* J Environ Qual, 2002. **31**(4): p. 1146-56.

118. Hochstein, L.I. and H. Stan-Lotter, *Purification and properties of an ATPase from Sulfolobus solfataricus.* Arch Biochem Biophys, 1992. **295**(1): p. 153-60.

119. Hollenstein, K., D.C. Frei, and K.P. Locher, *Structure of an ABC transporter in complex with its binding protein.* Nature, 2007. **446**(7132): p. 213-6.

120. Huber, C. and G. Wachtershauser, *Peptides by activation of amino acids with CO on (Ni,Fe)S surfaces: implications for the origin of life.* Science, 1998. **281**(5377): p. 670-2.

121. Huber, C. and G. Wachtershauser, *alpha-Hydroxy and alpha-amino acids under possible Hadean, volcanic origin-of-life conditions.* Science, 2006. **314**(5799): p. 630-2.

122. Huber, R., M. Sacher, A. Vollmann, H. Huber, and D. Rose, *Respiration of arsenate and selenate by hyperthermophilic archaea.* Syst Appl Microbiol, 2000. **23**(3): p. 305-14.

123. Irimia, A., H. Zang, L.V. Loukachevitch, R.L. Eoff, F.P. Guengerich, and M. Egli, *Calcium is a cofactor of polymerization but inhibits pyrophosphorolysis by the Sulfolobus solfataricus DNA polymerase Dpo4.* Biochemistry, 2006. **45**(19): p. 5949-56.

124. Islam, S.M., K. Fukushi, and K. Yamamoto, *Development of an enumeration method for arsenic methylating bacteria from mixed culture samples.* Biotechnol Lett, 2005. **27**(23-24): p. 1885-90.

125. Jamers, A., K. Van der Ven, L. Moens, J. Robbens, G. Potters, Y. Guisez, R. Blust, and W. De Coen, *Effect of copper exposure on gene expression profiles in Chlamydomonas reinhardtii based on microarray analysis.* Aquat Toxicol, 2006. **80**(3): p. 249-60.

126. Jarrell, K.F. and G.D. Sprott, *Nickel transport in Methanobacterium bryantii.* J Bacteriol, 1982. **151**(3): p. 1195-203.

127. Jarrell, K.F. and G.D. Sprott, *Nickel transport in Methanobacterium bryantii.* J Bacteriol, 1982. **151**(3): p. 1195-203.

128. Jefferson, R.A., T.A. Kavanagh, and M.W. Bevan, *GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants.* Embo J, 1987. **6**(13): p. 3901-7.

129. Johnson, J.L., K.V. Rajagopalan, S. Mukund, and M.W. Adams, *Identification of molybdopterin as the organic component of the tungsten cofactor in four enzymes from hyperthermophilic Archaea.* J Biol Chem, 1993. **268**(7): p. 4848-52.

130. Jonuscheit, M., E. Martusewitsch, K.M. Stedman, and C. Schleper, *A reporter gene system for the hyperthermophilic archaeon Sulfolobus solfataricus based on a selectable and integrative shuttle vector.* Mol Microbiol, 2003. **48**(5): p. 1241-52.

131. Kadukova, J. and E. Vircikova, *Comparison of differences between copper bioaccumulation and biosorption.* Environ Int, 2005. **31**(2): p. 227-32.

132. Kappler, U., L.I. Sly, and A.G. McEwan, *Respiratory gene clusters of Metallosphaera sedula - differential expression and transcriptional organization.* Microbiology, 2005. **151**(Pt 1): p. 35-43.

133. Kashefi, K. and D.R. Lovley, *Reduction of Fe(III), Mn(IV), and toxic metals at 100 degrees C by Pyrobaculum islandicum.* Appl Environ Microbiol, 2000. **66**(3): p. 1050-6.

134. Keeling, P.J., H.P. Klenk, R.K. Singh, O. Feeley, C. Schleper, W. Zillig, W.F. Doolittle, and C.W. Sensen, *Complete nucleotide sequence of the Sulfolobus islandicus multicopy plasmid pRN1.* Plasmid, 1996. **35**(2): p. 141-4.

135. Kidd, S.P. and N.L. Brown, *ZccR--a MerR-like regulator from Bordetella pertussis which responds to zinc, cadmium, and cobalt.* Biochem Biophys Res Commun, 2003. **302**(4): p. 697-702.

136. Kim, B.K., T.D. Pihl, J.N. Reeve, and L. Daniels, *Purification of the copper response extracellular proteins secreted by the copper-resistant methanogen Methanobacterium bryantii BKYH and cloning, sequencing, and transcription of the gene encoding these proteins.* J Bacteriol, 1995. **177**(24): p. 7178-85.

137. Kletzin, A. and M.W. Adams, *Tungsten in biological systems.* FEMS Microbiol Rev, 1996. **18**(1): p. 5-63.

138. Kletzin, A., A. Lieke, T. Urich, R.L. Charlebois, and C.W. Sensen, *Molecular analysis of pDL10 from Acidianus ambivalens reveals a family of related plasmids from extremely thermophilic and acidophilic archaea.* Genetics, 1999. **152**(4): p. 1307-14.

139. Kletzin, A., T. Urich, F. Muller, T.M. Bandeiras, and C.M. Gomes, *Dissimilatory oxidation and reduction of elemental sulfur in thermophilic archaea.* J Bioenerg Biomembr, 2004. **36**(1): p. 77-91.

140. Komorowski, L., W. Verheyen, and G. Schafer, *The archaeal respiratory supercomplex SoxM from S. acidocaldarius combines features of quinole and cytochrome c oxidases.* Biol Chem, 2002. **383**(11): p. 1791-9.

141. Korencic, D., I. Ahel, J. Schelert, M. Sacher, B. Ruan, C. Stathopoulos, P. Blum, M. Ibba, and D. Soll, *A freestanding proofreading domain is required for protein synthesis quality control in Archaea.* Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10260-5.

142. Korkhin, Y., U.M. Unligil, O. Littlefield, P.J. Nelson, D.I. Stuart, P.B. Sigler, S.D. Bell, and N.G. Abrescia, *Evolution of Complex RNA Polymerases: The Complete Archaeal RNA Polymerase Structure.* PLoS Biol, 2009. **7**(5): p. e102.

143. Kurosawa, N. and D.W. Grogan, *Homologous recombination of exogenous DNA with the Sulfolobus acidocaldarius genome: properties and uses.* FEMS Microbiol Lett, 2005. **253**(1): p. 141-9.

144. Lebrun, E., M. Brugna, F. Baymann, D. Muller, D. Lievremont, M.C. Lett, and W. Nitschke, *Arsenite oxidase, an ancient bioenergetic enzyme.* Mol Biol Evol, 2003. **20**(5): p. 686-93.

145. Lee, H.H., J.Y. Yoon, H.S. Kim, J.Y. Kang, K.H. Kim, D.J. Kim, J.Y. Ha, B. Mikami, H.J. Yoon, and S.W. Suh, *Crystal structure of a metal ion-bound IS200 transposase.* J Biol Chem, 2006. **281**(7): p. 4261-6.

146. Lee, J.W. and J.D. Helmann, *The PerR transcription factor senses H2O2 by metal-catalysed histidine oxidation.* Nature, 2006. **440**(7082): p. 363-7.

147. Lee, S.M., G. Grass, C. Rensing, S.R. Barrett, C.J. Yates, J.V. Stoyanov, and N.L. Brown, *The Pco proteins are involved in periplasmic copper handling in Escherichia coli.* Biochem Biophys Res Commun, 2002. **295**(3): p. 616-20.

148. Lee, S.W., E. Glickmann, and D.A. Cooksey, *Chromosomal locus for cadmium resistance in Pseudomonas putida consisting of a cadmium-transporting ATPase and a MerR family response regulator.* Appl Environ Microbiol, 2001. **67**(4): p. 1437-44.

149. Leigh, J.A., *Nitrogen fixation in methanogens: the archaeal perspective.* Curr Issues Mol Biol, 2000. **2**(4): p. 125-31.

150. Li, E., C.I. Reich, and G.J. Olsen, *A whole-genome approach to identifying protein binding sites: promoters in Methanocaldococcus (Methanococcus) jannaschii.* Nucleic Acids Res, 2008. **36**(22): p. 6948-58.

151. Limauro, D., E. Pedone, L. Pirone, and S. Bartolucci, *Identification and characterization of 1-Cys peroxiredoxin from Sulfolobus solfataricus and its involvement in the response to oxidative stress.* Febs J, 2006. **273**(4): p. 721-31.

152. Lin, Y.F., A.R. Walmsley, and B.P. Rosen, *An arsenic metallochaperone for an arsenic detoxification pump.* Proc Natl Acad Sci U S A, 2006. **103**(42): p. 15617-22.

153. Linder, M.C., L. Wooten, P. Cerveza, S. Cotton, R. Shulze, and N. Lomeli, *Copper transport.* Am J Clin Nutr, 1998. **67**(5 Suppl): p. 965S-971S.

154. Liochev, S.I., L. Benov, D. Touati, and I. Fridovich, *Induction of the soxRS regulon of Escherichia coli by superoxide.* J Biol Chem, 1999. **274**(14): p. 9479-81.

155. Lipps, G., *Molecular biology of the pRN1 plasmid from Sulfolobus islandicus.* Biochem Soc Trans, 2009. **37**(Pt 1): p. 42-5.

156. Littlefield, O., Y. Korkhin, and P.B. Sigler, *The structural basis for the oriented assembly of a TBP/TFB/promoter complex.* Proc Natl Acad Sci U S A, 1999. **96**(24): p. 13668-73.

157. Livak, K.J. (1997) *ABI Prism 7700 Sequence Detection System*. User Bulletin no. 2

158. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

159. Lubelska, J.M., M. Jonuscheit, C. Schleper, S.V. Albers, and A.J. Driessen, *Regulation of expression of the arabinose and glucose transporter genes in the thermophilic archaeon Sulfolobus solfataricus.* Extremophiles, 2006. **10**(5): p. 383-91.

160. Macomber, L., C. Rensing, and J.A. Imlay, *Intracellular copper does not catalyze the formation of oxidative DNA damage in Escherichia coli.* J Bacteriol, 2006. **189**(5): p. 1616-26

161. Maden, B.E., *No soup for starters? Autotrophy and the origins of metabolism.* Trends Biochem Sci, 1995. **20**(9): p. 337-41.

162. Magnani, D., O. Barre, S.D. Gerber, and M. Solioz, *Characterization of the CopR regulon of Lactococcus lactis IL1403.* J Bacteriol, 2008. **190**(2): p. 536-45.

163. Magnani, D. and M. Solioz, *Copper chaperone cycling and degradation in the regulation of the cop operon of Enterococcus hirae.* Biometals, 2005. **18**(4): p. 407-12.

164. Maiers, D.T., P.L. Wichlacz, D.L. Thompson, and D.F. Bruhn, *Selenate reduction by bacteria from a selenium-rich environment.* Appl Environ Microbiol, 1988. **54**(10): p. 2591-3.

165. Mana-Capelli, S., A.K. Mandal, and J.M. Arguello, *Archaeoglobus fulgidus CopB is a thermophilic Cu2+-ATPase: functional role of its histidine-rich-N-terminal metal binding domain.* J Biol Chem, 2003. **278**(42): p. 40534-41.

166. Mandal, A.K. and J.M. Arguello, *Functional roles of metal binding domains of the Archaeoglobus fulgidus Cu(+)-ATPase CopA.* Biochemistry, 2003. **42**(37): p. 11040-7.

167. Mandal, A.K., W.D. Cheung, and J.M. Arguello, *Characterization of a thermophilic P-type Ag+/Cu+-ATPase from the extremophile Archaeoglobus fulgidus.* J Biol Chem, 2002. **277**(9): p. 7201-8.

168. Mandal, A.K., Y. Yang, T.M. Kertesz, and J.M. Arguello, *Identification of the transmembrane metal binding site in Cu+-transporting PIB-type ATPases.* J Biol Chem, 2004. **279**(52): p. 54802-7.

169. Martin, W. and M.J. Russell, *On the origins of cells: a hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells.* Philos Trans R Soc Lond B Biol Sci, 2003. **358**(1429): p. 59-83; discussion 83-5.

170. McNicholas, P.M. and R.P. Gunsalus, *The molybdate-responsive Escherichia coli ModE transcriptional regulator coordinates periplasmic nitrate reductase (napFDAGHBC) operon expression with nitrate and molybdate availability.* J Bacteriol, 2002. **184**(12): p. 3253-9.

171. Michalke, K., E.B. Wickenheiser, M. Mehring, A.V. Hirner, and R. Hensel, *Production of volatile derivatives of metal(loid)s by microflora involved in anaerobic digestion of sewage sludge.* Appl Environ Microbiol, 2000. **66**(7): p. 2791-6.

172. Miller, K.W., S.S. Risanico, and J.B. Risatti, *Differential tolerance of Sulfolobus strains to transition metals.* FEMS Microbiology Letters, 1992. **93**(1): p. 69-73.

173. Mills, S.D., C.K. Lim, and D.A. Cooksey, *Purification and characterization of CopR, a transcriptional activator protein that binds to a conserved domain (cop box) in copper-inducible promoters of Pseudomonas syringae.* Mol Gen Genet, 1994. **244**(4): p. 341-51.

174. Miura, Y., M. Kettoku, M. Kato, K. Kobayashi, and K. Kondo, *High level production of thermostable alpha-amylase from Sulfolobus solfataricus in high-cell density culture of the food yeast Candida utilis.* J Mol Microbiol Biotechnol, 1999. **1**(1): p. 129-34.

175. Monchy, S., M.A. Benotmane, R. Wattiez, S. van Aelst, V. Auquier, B. Borremans, M. Mergeay, S. Taghavi, D. van der Lelie, and T. Vallaeys, *Transcriptomic and proteomic analyses of the pMOL30-encoded copper resistance in Cupriavidus metallidurans strain CH34.* Microbiology, 2006. **152**(Pt 6): p. 1765-76.

176. Mukhopadhyay, R., B.P. Rosen, L.T. Phung, and S. Silver, *Microbial arsenic: from geocycles to genes and enzymes.* FEMS Microbiol Rev, 2002. **26**(3): p. 311-25.

177. Mukund, S. and M.W. Adams, *Molybdenum and vanadium do not replace tungsten in the catalytically active forms of the three tungstoenzymes in the hyperthermophilic archaeon Pyrococcus furiosus.* J Bacteriol, 1996. **178**(1): p. 163-7.

178. Muller, D., D. Lievremont, D.D. Simeonova, J.C. Hubert, and M.C. Lett, *Arsenite oxidase aox genes from a metal-resistant beta-proteobacterium.* J Bacteriol, 2003. **185**(1): p. 135-41.

179. Multhaup, G., D. Strausak, K.D. Bissig, and M. Solioz, *Interaction of the CopZ copper chaperone with the CopA copper ATPase of Enterococcus hirae*

assessed by surface plasmon resonance. Biochem Biophys Res Commun, 2001. **288**(1): p. 172-7.

180. Munson, G.P., D.L. Lam, F.W. Outten, and T.V. O'Halloran, *Identification of a copper-responsive two-component system on the chromosome of Escherichia coli K-12.* J Bacteriol, 2000. **182**(20): p. 5864-71.

181. Naji, S., S. Grunberg, and M. Thomm, *The RPB7 orthologue E' is required for transcriptional activity of a reconstituted archaeal core enzyme at low temperatures and stimulates open complex formation.* J Biol Chem, 2007. **282**(15): p. 11047-57.

182. Napoli, A., Y. Zivanovic, C. Bocs, C. Buhler, M. Rossi, P. Forterre, and M. Ciaramella, *DNA bending, compaction and negative supercoiling by the architectural protein Sso7d of Sulfolobus solfataricus.* Nucleic Acids Res, 2002. **30**(12): p. 2656-62.

183. Navarro, C., L.F. Wu, and M.A. Mandrand-Berthelot, *The nik operon of Escherichia coli encodes a periplasmic binding-protein-dependent transport system for nickel.* Mol Microbiol, 1993. **9**(6): p. 1181-91.

184. Nies, D.H., *Microbial heavy-metal resistance.* Appl Microbiol Biotechnol, 1999. **51**(6): p. 730-50.

185. Nies, D.H., *Efflux-mediated heavy metal resistance in prokaryotes.* FEMS Microbiol Rev, 2003. **27**(2-3): p. 313-39.

186. Odermatt, A. and M. Solioz, *Two trans-acting metalloregulatory proteins controlling expression of the copper-ATPases of Enterococcus hirae.* J Biol Chem, 1995. **270**(9): p. 4349-54.

187. Odermatt, A., H. Suter, R. Krapf, and M. Solioz, *Primary structure of two P-type ATPases involved in copper homeostasis in Enterococcus hirae.* J Biol Chem, 1993. **268**(17): p. 12775-9.

188. O'Halloran, T.V., *Transition metals in control of gene expression.* Science, 1993. **261**(5122): p. 715-25.

189. Oremland, R.S. and J.F. Stolz, *The ecology of arsenic.* Science, 2003. **300**(5621): p. 939-44.

190. Oshima, A., J.W. Kyle, R.D. Miller, J.W. Hoffmann, P.P. Powell, J.H. Grubb, W.S. Sly, M. Tropak, K.S. Guise, and R.A. Gravel, *Cloning, sequencing, and expression of cDNA for human beta-glucuronidase.* Proc Natl Acad Sci U S A, 1987. **84**(3): p. 685-9.

191. Ouhammouch, M., R.E. Dewhurst, W. Hausner, M. Thomm, and E.P. Geiduschek, *Activation of archaeal transcription by recruitment of the TATA-binding protein.* Proc Natl Acad Sci U S A, 2003. **100**(9): p. 5097-102.

192. Ouhammouch, M., G.E. Langham, W. Hausner, A.J. Simpson, N.M. El-Sayed, and E.P. Geiduschek, *Promoter architecture and response to a positive regulator of archaeal transcription.* Mol Microbiol, 2005. **56**(3): p. 625-37.

193. Outten, F.W., C.E. Outten, J. Hale, and T.V. O'Halloran, *Transcriptional activation of an Escherichia coli copper efflux regulon by the chromosomal MerR homologue, cueR.* J Biol Chem, 2000. **275**(40): p. 31024-9.

194. Outten, F.W., C.E. Outten, J. Hale, and T.V. O'Halloran, *Transcriptional activation of an Escherichia coli copper efflux regulon by the chromosomal MerR homologue, cueR.* J Biol Chem, 2000. **275**(40): p. 31024-9.

195. Patenge, N., A. Haase, H. Bolhuis, and D. Oesterhelt, *The gene for a halophilic beta-galactosidase (bgaH) of Haloferax alicantei as a reporter gene for promoter analyses in Halobacterium salinarum.* Mol Microbiol, 2000. **36**(1): p. 105-13.

196. Patteson, K.G., N. Trivedi, and T.C. Stadtman, *Methanococcus vannielii selenium-binding protein (SeBP): chemical reactivity of recombinant SeBP produced in Escherichia coli.* Proc Natl Acad Sci U S A, 2005. **102**(34): p. 12029-34.

197. Pecou, E., A. Maass, D. Remenik, J. Briche, and M. Gonzalez, *A mathematical model for copper homeostasis in Enterococcus hirae.* Math Biosci, 2006. **203**(2): p. 222-39.

198. Pena, M.M., J. Lee, and D.J. Thiele, *A delicate balance: homeostatic control of copper uptake and distribution.* J Nutr, 1999. **129**(7): p. 1251-60.

199. Peng, N., Q. Xia, Z. Chen, Y.X. Liang, and Q. She, *An upstream activation element exerting differential transcriptional activation on an archaeal promoter.* Mol Microbiol, 2009.

200. Pennella, M.A. and D.P. Giedroc, *Structural determinants of metal selectivity in prokaryotic metal-responsive transcriptional regulators.* Biometals, 2005. **18**(4): p. 413-28.

201. Permina, E.A., A.E. Kazakov, O.V. Kalinina, and M.S. Gelfand, *Comparative genomics of regulation of heavy metal resistance in Eubacteria.* BMC Microbiol, 2006. **6**: p. 49.

202. Petersen, C. and L.B. Moller, *Control of copper homeostasis in Escherichia coli by a P-type ATPase, CopA, and a MerR-like transcriptional activator, CopR.* Gene, 2000. **261**(2): p. 289-98.

203. Petris, M.J., I. Voskoboinik, M. Cater, K. Smith, B.E. Kim, R.M. Llanos, D. Strausak, J. Camakaris, and J.F. Mercer, *Copper-regulated trafficking of the Menkes disease copper ATPase is associated with formation of a phosphorylated catalytic intermediate.* J Biol Chem, 2002. **277**(48): p. 46736-42.

204. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.

205. Pomposiello, P.J., M.H. Bennik, and B. Demple, *Genome-wide transcriptional profiling of the Escherichia coli responses to superoxide stress and sodium salicylate.* J Bacteriol, 2001. **183**(13): p. 3890-902.

206. Portmann, R., D. Magnani, J.V. Stoyanov, A. Schmechel, G. Multhaup, and M. Solioz, *Interaction kinetics of the copper-responsive CopY repressor with the cop promoter of Enterococcus hirae.* J Biol Inorg Chem, 2004. **9**(4): p. 396-402.

207. Qin, J., B.P. Rosen, Y. Zhang, G. Wang, S. Franke, and C. Rensing, *Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite S-adenosylmethionine methyltransferase.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2075-80.

208. Qureshi, S.A. and S.P. Jackson, *Sequence-specific DNA binding by the S. shibatae TFIIB homolog, TFB, and its effect on promoter strength.* Mol Cell, 1998. **1**(3): p. 389-400.

209. Rae, T.D., P.J. Schmidt, R.A. Pufahl, V.C. Culotta, and T.V. O'Halloran, *Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase.* Science, 1999. **284**(5415): p. 805-8.

210. Rasmussen, B., *Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit.* Nature, 2000. **405**(6787): p. 676-9.

211. Remonsellez, F., A. Orell, and C.A. Jerez, *Copper tolerance of the thermoacidophilic archaeon Sulfolobus metallicus: possible role of polyphosphate metabolism.* Microbiology, 2006. **152**(Pt 1): p. 59-66.

212. Renfrow, M.B., N. Naryshkin, L.M. Lewis, H.T. Chen, R.H. Ebright, and R.A. Scott, *Transcription factor B contacts promoter DNA near the transcription start site of the archaeal transcription initiation complex.* J Biol Chem, 2004. **279**(4): p. 2825-31.

213. Rensing, C., B. Fan, R. Sharma, B. Mitra, and B.P. Rosen, *CopA: An Escherichia coli Cu(I)-translocating P-type ATPase.* Proc Natl Acad Sci U S A, 2000. **97**(2): p. 652-6.

214. Rensing, C., M. Ghosh, and B.P. Rosen, *Families of soft-metal-ion-transporting ATPases.* J Bacteriol, 1999. **181**(19): p. 5891-7.

215. Rensing, C. and G. Grass, *Escherichia coli mechanisms of copper homeostasis in a changing environment.* FEMS Microbiol Rev, 2003. **27**(2-3): p. 197-213.

216. Reyes, A., A. Leiva, V. Cambiazo, M.A. Mendez, and M. Gonzalez, *Cop-like operon: structure and organization in species of the Lactobacillale order.* Biol Res, 2006. **39**(1): p. 87-93.

217. Rice, W.J., A. Kovalishin, and D.L. Stokes, *Role of metal-binding domains of the copper pump from Archaeoglobus fulgidus.* Biochem Biophys Res Commun, 2006. **348**(1): p. 124-31.

218. Roberts, S.A., G.F. Wildner, G. Grass, A. Weichsel, A. Ambrus, C. Rensing, and W.R. Montfort, *A labile regulatory copper ion lies near the T1 copper site in the multicopper oxidase CueO.* J Biol Chem, 2003. **278**(34): p. 31958-63.

219. Rocha, E.R., G. Owens, Jr., and C.J. Smith, *The redox-sensitive transcriptional activator OxyR regulates the peroxide response regulon in the obligate anaerobe Bacteroides fragilis.* J Bacteriol, 2000. **182**(18): p. 5059-69.

220. Rocha, R., S.S. Leal, V.H. Teixeira, M. Regalla, H. Huber, A.M. Baptista, C.M. Soares, and C.M. Gomes, *Natural domain design: enhanced thermal stability of a zinc-lacking ferredoxin isoform shows that a hydrophobic core efficiently replaces the structural metal site.* Biochemistry, 2006. **45**(34): p. 10376-84.

221. Rodionov, D.A., P. Hebbeln, M.S. Gelfand, and T. Eitinger, *Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: evidence for a novel group of ATP-binding cassette transporters.* J Bacteriol, 2006. **188**(1): p. 317-27.

222. Rolfsmeier, M. and P. Blum, *Purification and characterization of a maltase from the extremely thermophilic crenarchaeote Sulfolobus solfataricus.* J Bacteriol, 1995. **177**(2): p. 482-5.

223. Rosenstein, R., A. Peschel, B. Wieland, and F. Gotz, *Expression and regulation of the antimonite, arsenite, and arsenate resistance operon of*

Staphylococcus xylosus plasmid pSX267. J Bacteriol, 1992. **174**(11): p. 3676-83.

224. Rother, M., A. Resch, W.L. Gardner, W.B. Whitman, and A. Bock, *Heterologous expression of archaeal selenoprotein genes directed by the SECIS element located in the 3' non-translated region.* Mol Microbiol, 2001. **40**(4): p. 900-8.

225. Rowlands, T., P. Baumann, and S.P. Jackson, *The TATA-binding protein: a general transcription factor in eukaryotes and archaebacteria.* Science, 1994. **264**(5163): p. 1326-9.

226. Roy, R. and M.W. Adams, *Characterization of a fourth tungsten-containing enzyme from the hyperthermophilic archaeon Pyrococcus furiosus.* J Bacteriol, 2002. **184**(24): p. 6952-6.

227. Roy, R. and M.W. Adams, *Tungsten-dependent aldehyde oxidoreductase: a new family of enzymes containing the pterin cofactor.* Met Ions Biol Syst, 2002. **39**: p. 673-97.

228. Rubio, L.M. and P.W. Ludden, *Maturation of nitrogenase: a biochemical puzzle.* J Bacteriol, 2005. **187**(2): p. 405-14.

229. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular cloning : a laboratory manual*. 2nd ed. 1989, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

230. Santini, J.M. and R.N. vanden Hoven, *Molybdenum-containing arsenite oxidase of the chemolithoautotrophic arsenite oxidizer NT-26.* J Bacteriol, 2004. **186**(6): p. 1614-9.

231. Sazinsky, M.H., S. Agarwal, J.M. Arguello, and A.C. Rosenzweig, *Structure of the actuator domain from the Archaeoglobus fulgidus Cu(+)-ATPase.* Biochemistry, 2006. **45**(33): p. 9949-55.

232. Sazinsky, M.H., A.K. Mandal, J.M. Arguello, and A.C. Rosenzweig, *Structure of the ATP binding domain from the Archaeoglobus fulgidus Cu+-ATPase.* J Biol Chem, 2006. **281**(16): p. 11161-6.

233. Schafer, G. and S. Kardinahl, *Iron superoxide dismutases: structure and function of an archaic enzyme.* Biochem Soc Trans, 2003. **31**(Pt 6): p. 1330-4.

234. Schelert, J., V. Dixit, V. Hoang, J. Simbahan, M. Drozda, and P. Blum, *Occurrence and characterization of mercury resistance in the*

hyperthermophilic archaeon Sulfolobus solfataricus by use of gene disruption. J Bacteriol, 2004. **186**(2): p. 427-37.

235. Schelert, J., M. Drozda, V. Dixit, A. Dillman, and P. Blum, *Regulation of mercury resistance in the crenarchaeote Sulfolobus solfataricus.* J Bacteriol, 2006. **188**(20): p. 7141-50.

236. Schlaman, H.R., E. Risseeuw, M.E. Franke-van Dijk, and P.J. Hooykaas, *Nucleotide sequence corrections of the uidA open reading frame encoding beta-glucuronidase.* Gene, 1994. **138**(1-2): p. 259-60.

237. Schleper, C., G. Jurgens, and M. Jonuscheit, *Genomic studies of uncultivated archaea.* Nat Rev Microbiol, 2005. **3**(6): p. 479-88.

238. Schleper, C., K. Kubo, and W. Zillig, *The particle SSV1 from the extremely thermophilic archaeon Sulfolobus is a virus: demonstration of infectivity and of transfection with viral DNA.* Proc Natl Acad Sci U S A, 1992. **89**(16): p. 7645-9.

239. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.

240. Schonheit, P., J. Moll, and R.K. Thauer, *Nickel, cobalt, and molybdenum requirement for growth of Methanobacterium thermoautotrophicum.* Arch Microbiol, 1979. **123**(1): p. 105-7.

241. Schumacher, M.A., K.Y. Choi, F. Lu, H. Zalkin, and R.G. Brennan, *Mechanism of corepressor-mediated specific DNA binding by the purine repressor.* Cell, 1995. **83**(1): p. 147-55.

242. Sehlin, H.M. and E.B. Lindstroem, *Oxidation and reduction of arsenic by Sulfolobus acidocaldarius strain BC.* FEMS Microbiology Letters, 1992. **93**(1): p. 87-92.

243. Self, W.T., A.M. Grunden, A. Hasona, and K.T. Shanmugam, *Molybdate transport.* Res Microbiol, 2001. **152**(3-4): p. 311-21.

244. Self, W.T., R. Pierce, and T.C. Stadtman, *Cloning and heterologous expression of a Methanococcus vannielii gene encoding a selenium-binding protein.* IUBMB Life, 2004. **56**(8): p. 501-7.

245. Semsey, S., A.M. Andersson, S. Krishna, M.H. Jensen, E. Masse, and K. Sneppen, *Genetic regulation of fluxes: iron homeostasis of Escherichia coli.* Nucleic Acids Res, 2006. **34**(17): p. 4960-7.

246. She, Q., R.K. Singh, F. Confalonieri, Y. Zivanovic, G. Allard, M.J. Awayez, C.C. Chan-Weiher, I.G. Clausen, B.A. Curtis, A. De Moors, G. Erauso, C. Fletcher, P.M. Gordon, I. Heikamp-de Jong, A.C. Jeffries, C.J. Kozera, N. Medina, X. Peng, H.P. Thi-Ngoc, P. Redder, M.E. Schenk, C. Theriault, N. Tolstrup, R.L. Charlebois, W.F. Doolittle, M. Duguet, T. Gaasterland, R.A. Garrett, M.A. Ragan, C.W. Sensen, and J. Van der Oost, *The complete genome of the crenarchaeon Sulfolobus solfataricus P2.* Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7835-40.

247. Siddique, T., B.C. Okeke, Y. Zhang, M. Arshad, S.K. Han, and W.T. Frankenberger, Jr., *Bacterial diversity in selenium reduction of agricultural drainage water amended with rice straw.* J Environ Qual, 2005. **34**(1): p. 217-26.

248. Siddique, T., Y. Zhang, B.C. Okeke, and W.T. Frankenberger, Jr., *Characterization of sediment bacteria involved in selenium reduction.* Bioresour Technol, 2006. **97**(8): p. 1041-9.

249. Sitthisak, S., L. Knutsson, J.W. Webb, and R.K. Jayaswal, *Molecular characterization of the copper transport system in Staphylococcus aureus.* Microbiology, 2007. **153**(Pt 12): p. 4274-83.

250. Smaldone, G.T. and J.D. Helmann, *CsoR regulates the copper efflux operon copZA in Bacillus subtilis.* Microbiology, 2007. **153**(Pt 12): p. 4123-8.

251. Smith, W.L. and G.M. Gadd, *Reduction and precipitation of chromate by mixed culture sulphate-reducing bacterial biofilms.* J Appl Microbiol, 2000. **88**(6): p. 983-91.

252. Solioz, M. and J.V. Stoyanov, *Copper homeostasis in Enterococcus hirae.* FEMS Microbiol Rev, 2003. **27**(2-3): p. 183-95.

253. Sorensen, G. and G. Dandanell, *A new type of dihydroorotate dehydrogenase, type 1S, from the thermoacidophilic archaeon Sulfolobus solfataricus.* Extremophiles, 2002. **6**(3): p. 245-51.

254. Spadoni, M., M. Voltaggio, M. Carcea, E. Coni, A. Raggi, and F. Cubadda, *Bioaccessible selenium in Italian agricultural soils: Comparison of the biogeochemical approach with a regression model based on geochemical and pedoclimatic variables.* Sci Total Environ, 2007.

255. Stedman, K.M., Q. She, H. Phan, H.P. Arnold, I. Holz, R.A. Garrett, and W. Zillig, *Relationships between fuselloviruses infecting the extremely thermophilic archaeon Sulfolobus: SSV1 and SSV2.* Res Microbiol, 2003. **154**(4): p. 295-302.

256. Stewart, E.J., F. Aslund, and J. Beckwith, *Disulfide bond formation in the Escherichia coli cytoplasm: an in vivo role reversal for the thioredoxins.* Embo J, 1998. **17**(19): p. 5543-50.

257. Stolz, J.F., P. Basu, and R.S. Oremland, *Microbial transformation of elements: the case of arsenic and selenium.* Int Microbiol, 2002. **5**(4): p. 201-7.

258. Stolz, J.F., P. Basu, J.M. Santini, and R.S. Oremland, *Arsenic and selenium in microbial metabolism.* Annu Rev Microbiol, 2006. **60**: p. 107-30.

259. Stoyanov, J.V. and N.L. Brown, *The Escherichia coli copper-responsive copA promoter is activated by gold.* J Biol Chem, 2003. **278**(3): p. 1407-10.

260. Stoyanov, J.V., J.L. Hobman, and N.L. Brown, *CueR (YbbI) of Escherichia coli is a MerR family regulator controlling expression of the copper exporter CopA.* Mol Microbiol, 2001. **39**(2): p. 502-11.

261. Stoyanov, J.V., D. Magnani, and M. Solioz, *Measurement of cytoplasmic copper, silver, and gold with a lux biosensor shows copper and silver, but not gold, efflux by the CopA ATPase of Escherichia coli.* FEBS Lett, 2003. **546**(2-3): p. 391-4.

262. Studholme, D.J. and R.N. Pau, *A DNA element recognised by the molybdenum-responsive transcription factor ModE is conserved in Proteobacteria, green sulphur bacteria and Archaea.* BMC Microbiol, 2003. **3**: p. 24.

263. Studholme, D.J. and R.N. Pau, *A DNA element recognised by the molybdenum-responsive transcription factor ModE is conserved in Proteobacteria, green sulphur bacteria and Archaea.* BMC Microbiol, 2003. **3**: p. 24.

264. Tanaka, Y., K. Tsumoto, T. Nakanishi, Y. Yasutake, N. Sakai, M. Yao, I. Tanaka, and I. Kumagai, *Structural implications for heavy metal-induced reversible assembly and aggregation of a protein: the case of Pyrococcus horikoshii CutA.* FEBS Lett, 2004. **556**(1-3): p. 167-74.

265. Tapiero, H., D.M. Townsend, and K.D. Tew, *Trace elements in human physiology and pathology. Copper.* Biomed Pharmacother, 2003. **57**(9): p. 386-98.

266. Thomm, M., C. Reich, S. Grunberg, and S. Naji, *Mutational studies of archaeal RNA polymerase and analysis of hybrid RNA polymerases.* Biochem Soc Trans, 2009. **37**(Pt 1): p. 18-22.

267. Traven, A., B. Jelicic, and M. Sopta, *Yeast Gal4: a transcriptional paradigm revisited.* EMBO Rep, 2006. **7**(5): p. 496-9.

268. Uhrigshardt, H., S. Zoske, and S. Anemuller, *Sulfolobus aconitase, a regulator of iron metabolism?* Biochem Soc Trans, 2002. **30**(4): p. 685-7.

269. Valko, M., H. Morris, and M.T. Cronin, *Metals, toxicity and oxidative stress.* Curr Med Chem, 2005. **12**(10): p. 1161-208.

270. Vargas, M., K. Kashefi, E.L. Blunt-Harris, and D.R. Lovley, *Microbiological evidence for Fe(III) reduction on early Earth.* Nature, 1998. **395**(6697): p. 65-7.

271. Villafane, A.A., Y. Voskoboynik, M. Cuebas, I. Ruhl, and E. Bini, *Response to excess copper in the hyperthermophile Sulfolobus solfataricus strain 98/2.* Biochem Biophys Res Commun, 2009. **385**(1): p. 67-71.

272. Voskoboinik, I., J. Camakaris, and J.F. Mercer, *Understanding the mechanism and function of copper P-type ATPases.* Adv Protein Chem, 2002. **60**: p. 123-50.

273. Voskoboinik, I., R. Fernando, N. Veldhuis, K.M. Hannan, N. Marmy-Conus, R.B. Pearson, and J. Camakaris, *Protein kinase-dependent phosphorylation of the Menkes copper P-type ATPase.* Biochem Biophys Res Commun, 2003. **303**(1): p. 337-42.

274. Wachtershauser, G., *From volcanic origins of chemoautotrophic life to Bacteria, Archaea and Eukarya.* Philos Trans R Soc Lond B Biol Sci, 2006. **361**(1474): p. 1787-806; discussion 1806-8.

275. Wang, G., S.P. Kennedy, S. Fasiludeen, C. Rensing, and S. DasSarma, *Arsenic resistance in Halobacterium sp. strain NRC-1 examined by using an improved gene knockout system.* J Bacteriol, 2004. **186**(10): p. 3187-94.

276. Wang, J., P.X. Jiang, H. Feng, Y. Feng, and Z.G. He, *Three eukaryote-like Orc1/Cdc6 proteins functionally interact and mutually regulate their activities of binding to the replication origin in the hyperthermophilic archaeon Sulfolobus solfataricus P2.* Biochem Biophys Res Commun, 2007. **363**(1): p. 63-70.

277. Wang, T. and Z. Guo, *Copper in medicine: homeostasis, chelation therapy and antitumor drug design.* Curr Med Chem, 2006. **13**(5): p. 525-37.

278. Werner, F. and R.O. Weinzierl, *Direct modulation of RNA polymerase core functions by basal transcription factors.* Mol Cell Biol, 2005. **25**(18): p. 8344-55.

279. Wernimont, A.K., D.L. Huffman, A.L. Lamb, T.V. O'Halloran, and A.C. Rosenzweig, *Structural basis for copper transfer by the metallochaperone for the Menkes/Wilson disease proteins.* Nat Struct Biol, 2000. **7**(9): p. 766-71.

280. White, M.F., *Archaeal DNA repair: paradigms and puzzles.* Biochem Soc Trans, 2003. **31**(Pt 3): p. 690-3.

281. White, M.F. and S.D. Bell, *Holding it together: chromatin in the Archaea.* Trends Genet, 2002. **18**(12): p. 621-6.

282. Wickes, B.L. and J.C. Edman, *The Cryptococcus neoformans GAL7 gene and its use as an inducible promoter.* Mol Microbiol, 1995. **16**(6): p. 1099-109.

283. Wilting, R., S. Schorling, B.C. Persson, and A. Bock, *Selenoprotein synthesis in archaea: identification of an mRNA element of Methanococcus jannaschii probably directing selenocysteine insertion.* J Mol Biol, 1997. **266**(4): p. 637-41.

284. Winge, D.R., *Copper-regulatory domain involved in gene expression.* Prog Nucleic Acid Res Mol Biol, 1998. **58**: p. 165-95.

285. Worthington, P., V. Hoang, F. Perez-Pomares, and P. Blum, *Targeted disruption of the alpha-amylase gene in the hyperthermophilic archaeon Sulfolobus solfataricus.* J Bacteriol, 2003. **185**(2): p. 482-8.

286. Wu, H., L. Sun, F. Blombach, S.J. Brouns, A.P. Snijders, K. Lorenzen, R.H. van den Heuvel, A.J. Heck, S. Fu, X. Li, X.C. Zhang, Z. Rao, and J. van der Oost, *Structure of the ribosome associating GTPase HflX.* Proteins, 2009.

287. Wu, J. and B.P. Rosen, *The ArsR protein is a trans-acting regulatory protein.* Mol Microbiol, 1991. **5**(6): p. 1331-6.

288. Wu, J. and B.P. Rosen, *The arsD gene encodes a second trans-acting regulatory protein of the plasmid-encoded arsenical resistance operon.* Mol Microbiol, 1993. **8**(3): p. 615-23.

289. Wu, L., *Review of 15 years of research on ecotoxicology and remediation of land contaminated by agricultural drainage sediment rich in selenium.* Ecotoxicol Environ Saf, 2004. **57**(3): p. 257-69.

290. Xu, C., T. Zhou, M. Kuroda, and B.P. Rosen, *Metalloid resistance mechanisms in prokaryotes.* J Biochem (Tokyo), 1998. **123**(1): p. 16-23.

291. Yamamoto, K. and A. Ishihama, *Transcriptional response of Escherichia coli to external copper.* Mol Microbiol, 2005. **56**(1): p. 215-27.

292. Yamamoto, K. and A. Ishihama, *Characterization of copper-inducible promoters regulated by CpxA/CpxR in Escherichia coli.* Biosci Biotechnol Biochem, 2006. **70**(7): p. 1688-95.

293. Yang, Y., A.K. Mandal, L.M. Bredeston, F. Luis Gonzalez-Flecha, and J.M. Arguello, *Activation of Archaeoglobus fulgidus Cu(+)-ATPase CopA by cysteine.* Biochim Biophys Acta, 2006.

294. Yokoyama, K., S.A. Ishijima, L. Clowney, H. Koike, H. Aramaki, C. Tanaka, K. Makino, and M. Suzuki, *Feast/famine regulatory proteins (FFRPs): Escherichia coli Lrp, AsnC and related archaeal transcription factors.* FEMS Microbiol Rev, 2006. **30**(1): p. 89-108.

295. Zehr, J.P., B.D. Jenkins, S.M. Short, and G.F. Steward, *Nitrogenase gene diversity and microbial community structure: a cross-system comparison.* Environ Microbiol, 2003. **5**(7): p. 539-54.

296. Zheng, M., F. Aslund, and G. Storz, *Activation of the OxyR transcription factor by reversible disulfide bond formation.* Science, 1998. **279**(5357): p. 1718-21.

297. Zheng, M. and G. Storz, *Redox sensing by prokaryotic transcription factors.* Biochem Pharmacol, 2000. **59**(1): p. 1-6.

298. Zheng, M., X. Wang, B. Doan, K.A. Lewis, T.D. Schneider, and G. Storz, *Computation-directed identification of OxyR DNA binding sites in Escherichia coli.* J Bacteriol, 2001. **183**(15): p. 4571-9.

299. Zheng, M., X. Wang, L.J. Templeton, D.R. Smulski, R.A. LaRossa, and G. Storz, *DNA microarray-mediated transcriptional profiling of the Escherichia coli response to hydrogen peroxide.* J Bacteriol, 2001. **183**(15): p. 4562-70.

300. Zhu, W., Q. Zeng, C.M. Colangelo, M. Lewis, M.F. Summers, and R.A. Scott, *The N-terminal domain of TFIIB from Pyrococcus furiosus forms a zinc ribbon.* Nat Struct Biol, 1996. **3**(2): p. 122-4.

301. Zivarts, M., Y. Liu, and R.R. Breaker, *Engineered allosteric ribozymes that respond to specific divalent metal ions.* Nucleic Acids Res, 2005. **33**(2): p. 622-31.

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Publications

1. Villafane A. A., Voskoboynik Y., Cuebas M., Ruhl I. and Bini E. Response to excess copper in the hyperthermophile *Sulfolobus solfataricus* strain 98/2. Biochem Biophys Res Commun. 2009. 385: 67-71.