The *Sulfolobus solfataricus* Lrp-like Protein LysM Regulates Lysine Biosynthesis in Response to Lysine Availability*

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Although the archaeal transcription apparatus resembles the eukaryal RNA polymerase II system, many bacterial-like regulators can be found in archaea. Particularly, all archaeal genomes sequenced to date contain genes encoding homologues of Lrp (leucine-responsive regulatory protein). Whereas Lrp-like proteins in bacteria are involved in regulation of amino acid metabolism, their physiological role in archaea is unknown. Although several archaeal Lrp-like proteins have been characterized recently, no target genes apart from their own coding genes have been discovered yet, and no ligands for these regulators have been identified so far. In this study, we show that the Lrp-like protein LysM from *Sulfolobus solfataricus* is involved in the regulation of lysine and possibly also arginine biosynthesis, encoded by the *lys* gene cluster. Exogenous lysine is the regulatory signal for *lys* gene expression and specifically serves as a ligand for LysM by altering its DNA binding affinity. LysM binds directly upstream of the TFB-responsive element of the intrinsically weak *lys* promoter, and DNA binding is favored in the absence of lysine, when *lysWXJK* transcription is maximal. The combined *in vivo* and *in vitro* data are most compatible with a model in which the bacterial-like LysM activates the eukaryal-like transcriptional machinery. As with transcriptional activity by *Escherichia coli* Lrp, activation by LysM is apparently dependent on a co-activator, which remains to be identified.

Since the discovery of archaea as a distinct domain of life, many studies have focused on archaeal transcription. It has become clear that although archaea resemble bacteria with respect to their cellular and genetic organization, their transcriptional apparatus is fundamentally different from that of bacteria. Their RNA polymerase (RNAP) is much more related to the eukaryal RNAPII system regarding subunit complexity and sequence homology (1). Thus, archaeal RNAP consists of at least 10 subunits in contrast to the five-subunit bacterial RNAP core enzyme. As in eukarya, archaeal transcription initiation is preceded by the binding of the TATA-binding protein (TBP) to a TATA-like sequence called the TATA-box and subsequent binding of transcription factor B (TFB). Archaeal TBP and TFB are highly homologous to the eukaryal TBP and TFIIIB, respectively. However, archaeal TBP is not complexed with TBP-associated factors as in eukarya (2), and there is no evidence that archaeal genomes encode TBP-associated factor homologues. The archaeal TATA-box is 8 bp in length and is located ~25 bp upstream of the start of transcription. Directly upstream of the TATA-box, a purine-rich sequence is present, called the TFB-responsive element (BRE). The BRE was shown to be an important determinant in directionality of transcription and promoter strength through interaction with a C-terminal helix-turn-helix domain of TFB (3, 4). The TFIIIB-BRE interaction is a conserved feature between archaea and eukarya. Once TBP and TFB are bound to the promoter, RNAP is recruited, involving an interaction between the RpoK subunit of RNAP and the N-terminal zinc ribbon domain of TFB (5).

Although no archaeal homologues of eukaryal TFIIA, TFIIIF, and TFIIH have been identified, a protein homologous to the N-terminal region of the α-subunit of eukaryal TFIIIE is present in archaea. This archaeal TFE stimulates transcription from promoters with suboptimal TATA-box sequences or in cases where TBP is limiting (6, 7). Whereas eukaryal TFIIIE is strictly necessary for transcription, archaeal TFE appears to be dispensable for basal transcription *in vitro*, although it may play a stimulatory role in transcription initiation at specific promoters.

Although the basal components of the archaeal and eukaryal transcription machineries are very similar, regulatory proteins do not appear to be conserved between the two domains. Instead, archaeal genomes contain many regulators previously identified only in bacteria, so-called bacterial-archaeal regulators (8). In particular, homologues of the Lrp/AsnC family of regulators appear to be widely distributed among both bacteria and archaea. Several bacterial as well as archaeal genomes contain up to 10 Lrp-like paralogues. *Escherichia coli* Lrp (leucine-responsive regulatory protein) is the paradigm that has been studied extensively (9, 10). It is a global regulator controlling the expression of up to 75 genes (11, 12). *E. coli* Lrp either represses or activates transcription, the effect of which is sometimes modulated by leucine. The target genes of *E. coli* Lrp encode enzymes that are directly or indirectly related to amino acid metabolism. This also appears to be the case for several specific (nonglobal) bacterial Lrp-like regulators from different bacteria. In archaea, the exact role of the numerous Lrp-like proteins has not been established. Several archaeal Lrp-like proteins have been characterized recently (13–16).
two of these proteins, Lrn14 from Sulfolobus solfataricus and LrpA from Pyrococcus furiosus, in an *in vitro* regulatory function could be assigned; both showed negative autoregulation independent of any amino acid ligand (14, 15). Moreover, the three-dimensional structure of *P. furiosus* LrpA was determined, providing the structural basis for understanding LrpA-DNA as well as LrpA-ligand interactions (17). However, neither the identity of this ligand nor the role of archaeal Lrp-like proteins in the expression of other genes has been determined.

To provide a suitable model system for analyzing the function of Lrp-like proteins in archaea, we have screened the genome of the hyperthermophilic archaeon *S. solfataricus* for the presence of Lrp-like proteins whose function, target, and identity of this ligand nor the role of archaeal Lrp-like proteins providing the structural basis for understanding LrpA-DNA as dimensional structure of *S. solfataricus* LysM is a regulator for lysine biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Growth of *S. solfataricus*—** *S. solfataricus* P2 was grown in defined medium containing 3.1 g/liter KH₂PO₄, 2.5 g/liter (NH₄)₂SO₄, 0.2 g/liter MgSO₄·7H₂O, 0.25 g/liter CaCl₂·2H₂O, 1.8 mg/ml MnCl₂·4H₂O, 4.5 mg/liter Na₂B₄O₇·10H₂O, 0.22 mg/liter ZnSO₄·7H₂O, 0.06 mg/liter CuCl₂·0.03 mg/liter Na₂MoO₄·2H₂O, 0.03 mg/liter VOSO₄·2H₂O, and 0.01 mg/liter CoCl₂, supplemented with 2-glutamine, 0.02 g/liter FeCl₃, and vitamins, adjusted to pH 3.0 with H₂SO₄. Amino acids, if present, were added to a final concentration of 1 mM.

**Analysis of *S. solfataricus* RNA—** Total RNA was isolated from *S. solfataricus* using the RNeasy method (Qiagen). 35 ml of midlog phase culture (A₆₀₀ nm of 0.4) was washed in 1 ml of medium and resuspended in 100 μl of Tris-SCC, 1.5 mM EDTA, pH 7.0. 10 μl of 4× Denaturing PAGE buffer (Amersham Biosciences) was added and then the sample was applied onto the gel. The gel was run at 90 V for 1 h, and the RNA was transferred to a positively charged nylon membrane (Ambion) as described by Sambrook et al. (18). Radiolabeled DNA probes were hybridized using UitraHyb™ solution (Ambion), according to the manufacturer’s prescriptions.

For primer extension analysis, 10 μg of total RNA and 2.5 ng of radiolabeled oligonucleotide BG815 or BG876 was resuspended in 2× AMV-RT buffer (Promega) in a final volume of 25 μl. Samples were heated to 70 °C for 10 min and slowly cooled to room temperature. MgCl₂, dNTPs, RNasin, and AMV-RT (Promega) were added to a final concentration of 5 mM, 0.4 mM, 0.8 units/μl, and radioactive [γ-³²P]ATP (Amersham Biosciences) was added to a final concentration of 50 μM. The reaction mixture was incubated at 42 °C for 10 min and slowly cooled to room temperature. The samples were incubated at 42 °C for 10 min and slowly cooled to room temperature. The samples were then placed on ice and RNase protection was performed using RPA III™ and MAXiscript™ kits (Ambion), according to the manufacturer’s prescriptions. 10 ng of total *S. solfataricus* RNA was used, isolated according the method described above. For the generation of a labeled lysW antisense RNA probe, a 267-bp PCR fragment, amplified using the oligonucleotides BG814 and BG876 (see Table I), was cloned into an Xcm1-digested pBluescriptII SK(+) derivative (19), yielding pLUW646. The orientation of the fragment was selected so that it was transcribed with HamHI-digested pLWU646 and T7 RNA polymerase yielded a 367-nucleotide lysW antisense RNA probe. For the generation of labeled RNA markers, an unrelated 76 bp was cloned as described above, yielding pLWU647, which was digested either with EcoRI, EcoRV, ClaI, XhoI, or ApaI and used in an *in vitro* transcription reaction with T7 RNA polymerase, yielding RNA marker fragments of 153, 161, 172, 186, and 199 nucleotides, respectively. RNase protection samples were analyzed on an 8% denaturing sequencing gel.

**Production and Purification of *S. solfataricus* LysM—** The gene encoding lysW was PCR-amplified using primers BG774 and BG775 (see Table I). Underlined sequences indicate the restriction sites NcoI and BamHI. The resulting PCR fragment was digested with NcoI and BamHI and cloned into the T7 expression vector pET24d (20) (Novagen, Inc.), resulting in the construct pLUW632. This construct was transformed into E. coli BL21(DE3) (Novagen, Inc.). A single colony was used to inoculate 5 ml of LB medium with 50 μg/ml kanamycin, and the culture was incubated in a rotary shaker at 37 °C until log phase growth was observed. Subsequently, the culture was used to inoculate 1 liter of identical medium, and incubation was continued until an A₆₀₀ of 0.5 was reached. Expression was induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and incubation was continued for 2.5 h. After expression, the cells were harvested and washed in 20 mM Tris-HCl buffer, pH 8.0. Cells from 250 ml of culture were resuspended in 15 ml of 20 mM Tris-HCl buffer, pH 8.0, and lysed by a single passage through a French pressure cell at 10,000 p.s.i. The lysate was centrifuged at 20,000 rpm for 20 min and loaded on a 25-ml Q-Sepharose column (Amersham Biosciences) that had been equilibrated with 20 mM Tris, pH 8.0. The flow-through containing LysM was collected and subjected to a heat incubation at 80 °C for 30 min and subsequently centrifuged for 20 min at 20,000 rpm. The supernatant contained the purified LysM. His₆-LysM was used for the production of a rabbit antiserum. For this purpose, we cloned and expressed His₆-LysM as described above, using the oligonucleotide BG938 instead of BG775 (see Table I). The underlined sequence indicates a BglII restriction site. His₆-LysM precipitated spontaneously from a cell extract after over-night storage at 4 °C, and we used this precipitated material to purify His₆-LysM under denaturing conditions using 8 M urea and Ni²⁺-nitrilotriacetic acid spin columns (Qiagen), according to the manufacturer’s prescriptions. The purified His₆-LysM was dialyzed stepwise against 50 mM Tris, pH 8.0. His₆-LysM was only used for immunization, since the protein lost its DNA binding activity, most likely as the result of unsuccessful renaturation.

**EMSAs and DNAase I Footprinting—** DNA probes used for gel mobility shift experiments were generated using PCR. The following primers were used: BG816 and BG815 for a 421-bp P₆⁻⁷ fragment; BG878 and BG877 for a 199-bp P₆⁻⁷ fragment; BG1087 and BG1088 for a 207-bp P₆⁻⁷ fragment (see Table I). PCR products were end-labeled using T4 kinase and radioactive [γ-³²P]ATP (Amersham Biosciences) and purified from a 6% acrylamide gel as described (18). Binding reactions were performed in a total volume of 10 μl, containing 50 mM Tris, pH 8.0, 1 mM diethiothreitol, 10% glycerol, and varying concentrations of purified LysM. Standard reactions contained 1–10 ng of [γ-³²P]ATP end-labeled DNA and 50 ng of poly(dC-dC)-poly(dC-dC) as nonspecific competitor DNA (Amersham Biosciences). L-Lysine, if present, was added to a final concentration of 0.5 mM. Reactions were incubated at room temperature or at 48 °C for at least 10 min and separated on a nondenaturing 6% acrylamide gel, buffered in 1× TBE buffer. Gels were dried, exposed to phosphor screens, and analyzed. Probes for DNAase I footprinting were generated using PCR with the oligonucleotides BG877 and BG878, where one of the two oligonucleotides was end-labeled using T4 kinase and radioactive [γ-³²P]ATP. Probes were purified from 6% acrylamide

**Table I**

| Oligonucleotides used in this study |
|-----------------------------------|
| BG774 | CGCCGCGACTTCCTTGGAACTGCTATTTGATAGAAG |
| BG775 | GCCCGCGACTTCCTTGGAACTGCTATTTGATAGAAG |
| BG876 | CTCTCGGAATCCCTTGGATAGGAAC |
| BG875 | GTATGCGGATATCTTCTTCGCG |
| BG906 | CTGAGAAATCCCTTGGATAGGAAC |
| BG905 | CTCTCGGAATCCCTTGGATAGGAAC |
| BG977 | CCATATGGAATCTGATAGGATCTACAA |
| BG938 | GGCGCCGATATCCGACCCCGACCCCGACCCCGACCCCGACCCCGG |
| BG387 | GTGAGAACATTTGCAGTGAAGT |
| BG1087 | TCTAAGAAGCTTCTGATGAAAGC |
| BG1108 | AGGTGCGCCGCCGACCTTCCC |
| BG1133 | GAATAGCGCGATAGCCCTTACAG |
| BG1134 | AAATTGCAGTATGCAATGATCTGQG |
| BG1198 | 5'CGTATGCGGATATCTTCTTCGCG 3' |
| BG1199 | TACTCTGGAATCCCTTGGATAGGAAC |
RESULTS

Identification of the Lysine Biosynthesis Gene Cluster—In the genome of *S. solfataricus* P2 (22), a gene encoding an Lrp-like protein (lysM, Sso0157; see Fig. 1A) is present. The *S. solfataricus* LysM protein is 29% and 33% identical to the archaeal Lrp-like proteins Ppr2 from *Methanococcus jannaschii* and LrpA from *F. purificus*, respectively, and 15 and 27% identical to the bacterial Lrp-like proteins Lrp and AemC from *Escherichia coli*, respectively (Fig. 1B). In *S. solfataricus*, the gene encoding LysM is part of a gene cluster. Four genes of this cluster are homologous to classical arginine biosynthesis genes (argBCDE); one is homologous to *Escherichia coli* rim*K*, encoding a ribosomal protein modification enzyme; and one of the genes is homologous to *Thermus thermophilus* orfF, encoding a small hypothetical protein. Similar gene clusters are also present in at least seven archaeal genomes and one bacterial genome (Fig. 1A). Whereas argD of *T. thermophilus* is not clustered with argBCE, it is present elsewhere on the genome (23) (see Fig. 1A). The role of the *T. thermophilus* cluster has been studied using gene disruption of the argB, argC, argD, orfF, or rimK gene, which resulted in lysine auxotrophy (23, 24). Because *T. thermophilus* does not synthesize lysine via the diaminopimelic acid pathway, believed to be common to all bacteria, but via α-aminoacidic acid (AAA) as an intermediate (25, 26), it was proposed that the *T. thermophilus* orfF-rimK-argCBD genes are involved in lysine biosynthesis through a modified AAA pathway, in which the conversion of AAA to lysine is similar to the conversion of glutamate to ornithine in the arginine biosynthesis pathway. The cluster was therefore renamed as the lys operon (24). Hence, we will refer to the respective *S. solfataricus* gene cluster as the lys gene cluster, and we have renamed the genes of the described *S. solfataricus* gene cluster accordingly (see Fig. 1A). The gene encoding the Lrp-like protein LysM is only present within the lys clusters of the three *Sulfolobus* species, and *A. pernix* *S. solfataricus* LysM has 74, 79, and 44% identity with the *Sulfolobus acidocaldarius*, *Sulfolobus tokodaii*, and *Aeropyrum pernix* orthologues, respectively. BLAST and genomic context analysis revealed that no additional genomes present in the current data base contain close homologues (>94% identity) of LysM, suggesting that LysM is restricted to crenarchaea. Since the lysM gene is clustered with putative lysine biosynthesis genes of *S. solfataricus*, we hypothesized that LysM could be involved in the regulation of these genes. In analogy, most bacterial Lrp-like proteins are involved in regulation of amino acid metabolism, and their regulatory effect is modulated by one or more amino acids.2

Expression of the Lys Genes in Vivo—To study in vivo expression of the lys gene cluster, Northern blotting experiments were performed using total RNA isolated from *S. solfataricus* cells grown to mid-logarithmic phase in defined medium either lacking or containing combinations of amino acids. Probes specific for lysT and lysM hybridized to identically sized mRNA species of about 2.3 kb expected for a polycistronic mRNA containing lysYZM, the level of which appeared to be almost identical under all tested growth conditions (Fig. 2A). Western blotting experiments with a polyclonal antiserum raised against *E. coli*–produced LysM confirmed these results, since the LysM concentration was constant under the growth conditions tested in Northern blotting (Fig. 2C). Using probes against lysW, lysX, and lysK, we detected two mRNA species of 2.3 and 3.2 kb, whereas an additional 0.2 kb transcript was detected only with a probe against lysW (Fig. 2A). This suggested that there are three different transcripts, one containing only lysW, one containing lysWXJ, and one containing lysWXJ/K. Theoretically, probes against lysW and lysX should also hybridize to the 3.2-kb lysWXJK mRNA; however, although this mRNA species was visible on the membrane, it was less abundant compared with the shorter 2.3-kb lysWXJ mRNA. Alternatively, the

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larger 3.2-kb transcript could be the result of initiation at some place within lysWXJK and transcription read-through downstream of lysK. To rule out this possibility, we used a probe against the cpds gene immediately downstream of lysK, which encodes a putative cis-polyprenyl diphosphate synthase. Only background levels were obtained using this probe, indicating that the cpds gene is not co-transcribed with the lys gene cluster, and that lysK is the 3′-terminal gene of the lys operon.

Fig. 1. A, the lys gene cluster of S. solfataricus, compared with those of the archaea S. tokodai (53), S. acidocaldarius (Q. She, personal communication), P. furiosus (http://www.genome.utah.edu), P. horikoshii (54), Pyrococcus abyssi (http://www.genoscope.cns.fr), A. pernix (27), Ferroplasma acidarmanus (http://www.jgi.doe.gov/JGI_microbial/html), and the thermophilic bacterium T. thermophilus (24). Patterns indicate homology between the encoded proteins. Promoters and length of produced transcripts in S. solfataricus are indicated by the arrows. B, multiple alignment of archaeal LysM proteins with other archaeal and bacterial Lrp-like proteins. The helix-turn-helix motif of Pyrococcus furiosus LrpA (17) is indicated. Ss_LysM, Sso0157; Sa_LysM, not annotated; St_LysM, Sto0193; Ap_LysM, not annotated; Mj_Ptr2, Q58133; Pf_LrpA, P42180; Ec_Lrp, P19494; Ec_AsC, P03809.
It is most likely that only a small fraction of mRNAs read past \textit{lysJ}, which explains the differential band intensity for 3.2- and 2.3-kb mRNAs when probed with \textit{lysW} or \textit{lysX}.

The \textit{lysWXJK} genes are all strongly regulated specifically by the presence of lysine in the medium. Arginine alone or a mixture of 18 amino acids (arginine and lysine omitted) did not affect the abundance of the \textit{lysWXJK} transcript. However, when lysine alone, both lysine and arginine, or all 20 amino acids were present, the amount of transcript decreased drastically. Thus, transcription of \textit{lysWXJK} is induced specifically in the absence of lysine. Quantification of the results obtained from Northern blotting and primer extension analysis revealed that transcript levels under induced conditions are over 8-fold higher compared with that of noninduced conditions. It is unclear why expression levels of this mRNA are below maximum in the absence of any of the amino acids. Taken together, we conclude that the \textit{lys} gene cluster is transcribed in two separate polycistronic mRNAs. The transcription of \textit{lysYZM} is almost constitutive, whereas the level of \textit{lysWXJK} mRNA falls drastically whenever lysine is present in the medium.

To determine the 5' end of the two transcripts, we performed primer extension analysis. We found that the tran-
scriptional start of lysY is located at an adenine preceding the predicted ATG start codon of lysY (Fig. 2, D and F). Putative BRE and TATA promoter sequences are present 25 bp upstream from the lysY transcriptional start. The transcriptional start of lysW is located 9 bp upstream from the predicted lysW ATG start codon. Noncanonical BRE and TATA sequences can be recognized 25 bp upstream from the lysW transcriptional start. No obvious Shine-Dalgarno sequences are present upstream of the lysY and lysW transcriptional starts. This is not uncommon in S. solfataricus; a complete genome analysis showed that that genes at the 5′ extremity of putative polycistronic mRNAs often lack ribosome binding sites upstream of their translational starts, whereas downstream genes within operons do contain Shine-Dalgarno sequences (28). A detailed sequence analysis of the lysW promoter revealed the presence of a perfect 15-bp inverted repeat directly upstream the identified lysW transcriptional start that could form a hairpin structure in the mRNA, causing stalling and eventually termination of reverse transcriptase (Fig. 2F). Since this may have obstructed the primer extension analysis, we performed RNase protection to confirm the lysW transcriptional start detected by primer extension. Assuming that the detected 5′ terminus is the true transcriptional start, a 177-nucleotide labeled antisense RNA fragment is expected in RNase protection. As shown in Fig. 2E, an RNA fragment with this size is present, confirming that the 5′ terminus detected by primer extension is the true transcriptional start. As expected, the intensity of this band decreased when we used RNA isolated from a culture containing lysine.

To verify the variation in transcription levels observed by Northern blotting, we performed quantitative primer extension with the same RNA samples as used for Northern blotting (Fig. 2B). We found the same modulation in transcript levels as shown by Northern blotting experiments. We therefore conclude that transcription of lysYZM is driven by the lysY promoter, whereas transcription and regulation of lysWXJK occurs from the lysW promoter.

LysM Binds to the lysWPromoter—To study the role of LysM in the regulation of the lysY gene cluster, we overproduced LysM in E. coli to facilitate its purification. Whereas significant LysM overproduction was reached, its purification was severely hampered by the tendency of LysM to precipitate irreversibly from the cell extract at a pH lower than 8.0 or in the presence of several salts like MgCl₂, NaCl, KCl, or (NH₄)₂SO₄. However, using both anion exchange chromatography and heat incubation, we were able to purify the recombinant LysM to homogeneity, as judged from SDS-PAGE analysis (Fig. 3A). The purified LysM protein was used in electrophoretic mobility shift assays (EMSAs), to determine whether it binds to the mapped promoters. A 421-bp DNA fragment containing the lysY promoter (P₂lysY) overlapping the BRE and TATA sequences was used. However, no binding of LysM to this fragment was observed (Fig. 3B). To assay LysM binding to the lysW promoter (P₂lysW), a 199-bp DNA fragment containing the P₂lysW BRE and TATA sequences was used in the EMSA. The assay revealed that LysM binds to this DNA fragment, forming four protein-DNA complexes of distinct electrophoretic mobility (Fig. 3B, I–IV). Binding of LysM to P₂lysW appeared to be specific, since pLUW641, a plasmid containing P₂lysW, competed for binding, whereas no competition occurred with the control plasmid pBluescript II SK⁺ (Stratagene) or pT6, containing the Sulfolobus islandicus virus SSV1 T6 promoter (2) (Fig. 3C). Using an antisera raised against purified recombinant LysM, we verified whether the bands appearing upon the addition of LysM represented LysM-DNA complexes. Upon the addition of the antisera, the LysM-DNA complexes either disappeared or supershifted, due to the binding of antibodies to the LysM protein (Fig. 3D). This effect was not observed using the LysM preimmune serum or an antisera against P. furiosus LrpA (15). To screen for additional LysM binding sites outside the two tested fragments, 1-kb DNA fragments overlapping the lysY and lysW promoters were digested with several restriction enzymes to obtain fragments between 50 and 500 bp. These fragments were subsequently used in an EMSA with LysM, revealing that only a restriction fragment overlapping the 199-bp P₂lysW fragment shifted (data not shown). We therefore conclude that LysM binds to a single site at P₂lysW but not to P₂lysY. Binding experiments were performed at room temperature, 48 °C, or 65 °C, but no difference in affinity was observed (not shown).

Lysine Specifically Affects LysM-DNA Binding—Since transcription from P₂lysW varies strongly in response to the presence of lysine in the growth medium (Fig. 2), we hypothesized that lysine acts as a ligand for LysM, which in turn regulates transcription from P₂lysW. Using EMSAs, we studied the effect of lysine and several other amino acids on LysM-DNA binding. The addition of lysine in the binding reaction decreased the affinity of LysM for P₂lysW, but did not completely eliminate binding (Fig. 4A). We tested higher lysine concentrations for complete inhibition of DNA binding, but we found that throughout the tested concentration range (0.2–30 mM) the inhibition is constant (Fig. 4B). Binding inhibition was specific for lysine, since the addition of other amino acids had no effect on LysM-DNA binding (Fig. 4A). To rule out the possibility that lysine is an aspecific DNA-binding inhibitor for Lrp-like proteins, we tested whether it also affected binding of the previously characterized P. furiosus LrpA protein to its promoter (15). However, lysine had no effect on LrpA-DNA binding (Fig. 4C). To analyze the effect of lysine in more detail, we performed EMSAs with gradually increasing concentrations of LysM, in the presence or absence of lysine (Fig. 4, D and E). Two major effects of lysine were apparent. First, lysine decreased the overall LysM-DNA binding affinity. Quantification of the results obtained in Fig. 4D revealed that the dissociation constant (Kd), defined as the LysM concentration at which 50% of the DNA is in complex with LysM, is about 10 nM in the absence of lysine and 330 nM in the presence of lysine, reflecting a 33-fold decrease in DNA binding affinity. Second, lysine changed the relative abundance of individual complexes. For example, complex IV is almost absent without lysine, whereas it is more abundant when lysine is present. This possibly reflects a structural change in one of the LysM-DNA complexes (e.g., increased compaction) or the formation of a novel complex, dependent on the presence of lysine. For E. coli Lrp, it has been shown recently that its ligand, leucine, induces dissociation of the Lrp hexadecameric form to the octameric form (29), which may alter the affinity for sites within its different target operons. Using chemical cross-linking of LysM we analyzed its oligomeric state (not shown). LysM was incubated with or without lysine and cross-linked using dimethyl suberimidate (30). LysM cross-linked as a protein with a maximum molecular mass of about 66 kDa, which is indicative of a LysM tetramer. The addition of lysine had no apparent effect on cross-linking, suggesting that LysM multimerization is unaffected by lysine. However, this interpretation should be taken with care, since dimethyl suberimidate specifically cross-links the amino groups of lysine residues, and the addition of free lysine could therefore quench or interfere with the cross-linking reaction.

LysM Binds Directly Upstream of the BRE of P₂lysW—Using DNase I footprinting, we mapped the LysM binding site at the lysW promoter. We found that LysM protects a region of at least 15 bp directly upstream of the BRE of P₂lysW (Fig. 5A).
Furthermore, bands representing sites hypersensitive to DNase I cleavage appear outside of the LysM footprint. These sites are indicative of secondary structure changes of DNA, induced by LysM. The addition of lysine had no obvious effect on the footprint pattern, although some hypersensitive sites appeared to be slightly less abundant when lysine was present.

To confirm that the LysM binding site is indeed the sequence protected from DNase I cleavage directly upstream of the PlysW BRE, an EMSA was performed with LysM and a 24-bp synthetic DNA fragment containing this LysM binding site (Fig. 5C). LysM bound to this fragment, and as with the larger fragment used for Fig. 4A and B, lysine decreased the affinity of LysM for this fragment (Fig. 5B). In contrast to LysM binding to a larger fragment, which gives rise to the formation of four distinct LysM-DNA complexes, only two LysM-DNA complexes were formed (I and II). In addition, the overall affinity of LysM for this DNA is lower than for a larger fragment (compare Figs. 4 and 5B). Most likely, LysM specifically recognizes and binds the 24-bp sequence directly upstream of PlysW, whereas interactions between LysM and the DNA flanking this sequence may contribute to stronger binding and the formation of additional LysM-DNA complexes.

In Vitro Transcription from PlysY and PlysW—To study the effect of LysM on transcription of the lys cluster, we performed in vitro transcription experiments with Sulfolobus TBP, TFB, and RNAP (2). Transcription from the previously characterized T6 control promoter (2) was efficient, and a transcript of the expected size was obtained (Fig. 6). Using the PlysY promoter as a template also resulted in a transcript of the expected length, but transcription was less efficient compared with T6. Presumably, PlysY is an intrinsically weak promoter, and we previously showed that this promoter (referred to as the argC promoter) could be stimulated by the transcription factor TFE, which is dispensable for basal transcription in vitro but stimulates transcription from suboptimal promoters (6). In accordance with data obtained from EMSA experiments, the addition of LysM had no effect on transcription from PlysY, either in the presence or absence of lysine (not shown). Using PlysW in vitro transcription experiments, we could not detect any transcription, and the addition of TFE or LysM, either with or without lysine, had no effect (not shown). Transcription reactions were therefore performed with cell extracts from S. solfataricus grown in the absence of lysine (where transcription from PlysW in vivo is maximal, see Fig. 2A), either in the presence (not shown) or absence (Fig. 6) of purified TBP, TFB, and RNAP. However, no transcription from PlysW could be detected.

PlysY and PlysW are Bona Fide Promoters in Vivo—Because PlysW had no detectable activity under the tested conditions,
it had to be verified whether P_{lysW} is a true promoter in vivo. In an alternative model for regulation of the lysWXJK transcript, transcription could be initiated from the (constitutive) lysY upstream promoter, and read-through into lysWXJK would be allowed only when lysine is absent. In this hypothetical model, LysM could act as a transcriptional roadblock, which, in response to lysine, permits RNA polymerase to read through or terminate transcription at or near the LysM binding site. In the absence of lysine, this would give rise to a transcript of about 5.5 kb containing lysYZMWXJK. Since no mRNA of this size was detected by Northern blotting (Fig. 2A), a subsequent RNase processing event would be responsible for cleavage of lysYZMWXJK into lysYZM and lysWXJK, whose 5’ terminus we have mapped in primer extension and RNase protection experiments (Fig. 2, D and E). To investigate the validity of this hypothetical model, it was crucial to determine whether the 5’ terminus of the lysWXJK is initiated or processed. Therefore, we performed ligation-mediated RT-PCR as described by Bensing et al. (21). Briefly, total RNA is either treated or untreated with tobacco acid phosphatase (TAP), which converts 5’-triphosphate termini, indicative of transcription initiation, into 5’-monophosphate termini.
When a 5'-monophosphate is present, an RNA adapter can subsequently be ligated to these termini. In an RT-PCR with adapter-specific and gene-specific primers, only mRNAs that had 5'-monophosphates can thus be amplified, and in this way it is possible to discriminate between initiated and processed 5' mRNA termini. With this technique, we analyzed 5' termini of lysY and lysW as well as the 16S gene. The latter has been shown to be processed in *S. solfataricus* (31, 32). For all three tested 5' ends, we obtained PCR products of the expected length, based on our primer extension and RNase protection data. As expected, 16S control RNA could be PCR-amplified both from TAP-untreated and TAP-treated samples (Fig. 7), which indicated that its 5' end is the result of RNA processing. lysY and lysW RNA could only be PCR-amplified from TAP-treated samples, which demonstrated that both lysY and lysW promoters have initiated 5' ends (Fig. 7). We concluded therefore that *P*<sub>lysY</sub> and *P*<sub>lysW</sub> are *bona fide* promoters in *S. solfataricus*; however, under the used *in vitro* transcription reaction conditions, transcription does not take place.

**Fig. 5.** A, DNase I footprinting of LysM at *P*<sub>lysW</sub> DNA. Reactions contained 0, 30, or 60 ng/µl purified LysM. 0.5 mM L-lysine was present as indicated. The LysM footprint, TATA element, and BRE are indicated by *open* and *gray boxes*, respectively, and hypersensitive DNase I sites are indicated by *arrows*. The position relative to the lysW transcriptional start site is indicated at the left. B, a 24-bp fragment overlapping the 15-bp LysM footprint was used in an EMSA with LysM. 0, 10, 40, 150, and 600 ng of LysM was added to the binding reaction, and 0.5 mM L-lysine was present as indicated. Two distinct LysM-DNA complexes are present (and **II**). C, summary of A and B. The location of the LysM footprint, BRE, and TATA-box are depicted as *gray boxes* in the lysW promoter. *Boldface characters* display the lysM stop codon and the lysW start codon. The sequence position relative to the transcriptional start site is indicated at the *top*, and the 24-bp DNA fragment used in B is shown at the *bottom*. 
arginine biosynthesis, as was proposed for the lys gene cluster of Pyrococcus horikoshii (24). Moreover, dual activity has been measured for LysJ and LysK of T. thermophilus, homologues of S. solfataricus LysJ and LysK, respectively, suggesting that the lys gene cluster could indeed be involved in arginine as well as lysine biosynthesis (23, 33).

Why is the lys gene cluster of S. solfataricus organized in a constitutive (lysYZM) and a regulated part (lysWXJK)? Possibly, down-regulation of the lysYZM is not permitted because this would abolish regulation of the lysWXJK genes through LysM, the gene of which is co-transcribed with lysY. Our data do not exclude the possibility that LysM has additional targets in the genome of S. solfataricus, but if this were the case, down-regulation of lysY could result in an even more serious loss of regulatory capacity in S. solfataricus. Alternatively, one of the enzyme activities encoded by lysYZM could be dispensable for growth under the conditions tested. The genomic organization of lysM in the lys cluster is identical in other Sulfolobus species and functionally comparable with that of A. pernix, where the lysYZM cluster is inverted (Fig. 1A), most likely allowing a similar mode of regulation.

Although the role of lysW and lysX has not been demonstrated experimentally, we speculate that they are specific for the prokaryotic AAA lysine biosynthesis route proposed by Nishida et al. (24), since they are clustered within the lys clusters of T. thermophilus, S. acidocaldarius, S. tokodai, A. pernix, Pyrococcus species, and Ferroplasma acidarmanus and because the classical arginine or lysine pathways do not involve such genes. LysX is 24% identical to RimK of E. coli, which was shown to be a post-translational modification enzyme, catalyzing the coupling of four glutamate residues to the C terminus of the S6 ribosomal protein (34). However, we found that in several prokaryotic genomes rimK-like genes are clustered with amino acid biosynthesis genes (not shown), suggesting that the catalytic activity of the rimK-encoded protein here is not utilized for a post-translational modification but rather in amino acid biosynthesis. Interestingly, Galperin et al. (35) demonstrated that RimK belongs to a superfAMILY of enzymes with a so-called “ATP grasp” fold. This family includes enzymes like β-alanine-β-alanine ligase, glutathione synthetase, biotin carboxylase, and carbamoyl phosphate synthetase. All of these enzymes possess ATP-dependent carboxylate-amino ligase activity (i.e., the capacity to form a peptide bond). In the proposed AAA-dependent lysine biosynthesis route described by Nishida et al. (24), LysX was predicted to catalyze a similar reaction, namely connecting the amino group of AAA to the carboxyl group of a yet unidentified molecule. By doing so, LysX catalyzes a reaction functionally analogous to that of ArgA (N-acetylglutamate synthase) in the classical arginine biosynthesis pathway, the gene of which is absent in all lys clusters shown in Fig. 1A.

The small protein encoded by lysW has no homologues in the data base, apart from lysW genes found in the lys clusters depicted in Fig. 1A. A PSI-BLAST analysis (36) of LysW showed that this small protein is homologous to the N-terminal domain of archaeal TFB transcription factors (not shown). After four iterations, an expect value of ≥10−11 was obtained with TFB N-terminal domains from several archaeal. This domain consists of a zinc ribbon (37), and the two CPXCG “zinc knuckle” motifs that bind the zinc atom are well conserved in LysW. The zinc ribbon domain of Sulfolobus TFB has been shown to be involved in the recruitment of RNA polymerase (RNAP) (38), through interaction with the RpoK subunit of RNAP (5). Generally, zinc ribbon domains mediate protein-protein or protein-DNA interactions and can be found for instance in several eukaryal transcription factors. LysW

**DISCUSSION**

Genomic sequence data have revealed that Lrp-like proteins are ubiquitously present in bacteria and archaea. Although Lrp from E. coli is the archetype Lrp-like protein, more than a dozen bacterial Lrp-like proteins have been studied either in vitro or using genetic approaches, and it is clear that these proteins are all involved in the regulation of amino acid metabolism and that amino acids serve as ligands for Lrp-like proteins. Efficient genetic tools to study gene regulation (like recombination or gene disruption) are not yet available for hyperthermophilic archaea, and we have used a bioinformatics approach in combination with in vivo and in vitro analyses to identify and study the archaeal Lrp-like protein LysM from S. solfataricus, the gene of which is present in the lys gene cluster. This allowed us for the first time to study the role and function of an archaeal Lrp-like protein in relation to its physiological target genes.

Regulation of lys transcription in S. solfataricus is responsive to lysine only, which strongly suggests that the lys genes are involved in lysine biosynthesis, most likely via a pathway similar to that of T. thermophilus, where gene disruption of the homologous lys cluster resulted in lysine auxotrophy (24). The pathway utilized here involves AAA rather than diaminopimelic acid, the typical bacterial precursor (25, 26). Although arginine has no effect on lys transcription, we cannot rule out the possibility that lys-encoded enzymes are also functional in...
may therefore interact with one of the encoded enzymes of the lys gene cluster, perhaps acting as a regulatory subunit for the respective enzymatic activity, or it could play a regulatory role in lys transcription. However, LysW is also encoded by the lys gene cluster of *T. thermophilus*, which possesses a bacterial basal transcription machinery, and it is questionable whether involvement in transcription here is possible. It should be noted, however, that the RpoK subunit, with which the TFB zinc ribbon interacts, is a conserved subunit in RNAPs of eukarya (RPB6) and bacteria (ω). Unfortunately, our attempts to produce the LysW protein in *E. coli* were unsuccessful.

The data presented in this study strongly suggest that LysM acts as a transcriptional activator for P$_{lysY}$ and P$_{lysW}$. We found that transcription from P$_{lysY}$ is maximal when lysine is absent, and under these conditions the affinity of LysM for binding this promoter is the highest. Conversely, in the presence of lysine, transcription is lowest, and the binding affinity of LysM is decreased. It is most likely that LysM occupies P$_{lysW}$ preferably when lysine is absent, thereby somehow activating this promoter. Although lysine reduces rather than eliminates LysM-DNA binding, this reduction is expected to be physiologically important. As shown in Fig. 4, D and E, the effect of lysine is maximal at a low LysM concentration, presumably a relevant cellular condition (using Western blotting analysis, we roughly estimated the abundance of LysM to be about 0.01% of total soluble protein, not shown). This reduction rather than elimination of binding has also been observed for other (bacterial) Lrp-like proteins (39–43) and may therefore be a general feature of Lrp-like proteins. Using *in vivo* formaldehyde cross-linking followed by immunoprecipitation of LysM-DNA complexes, we have attempted to relate results from *in vitro* binding studies with *in vivo* LysM promoter occupation, but unfortunately the results of these experiments were irreproducible. Nevertheless, to a certain extent, our study is comparable with the regulation of *E. coli* iloIH by Lrp. For example, Lrp activates iloIH transcription, and this activation is decreased when leucine is present in the medium (44). In accordance, the Lrp-iloIH affinity *in vitro* is reduced but not eliminated by leucine (45). For iloIH, this reduction of binding *in vitro* could be related to an *in vivo* decrease in promoter occupancy *using in vivo* footprinting experiments (46), and we anticipate that this *in vitro-*in *vivo* relationship can also be made for LysM.

Our DNase I footprint data support the possibility that LysM is an activator, since it showed that LysM protects the bases −46 to −59 relative to the transcriptional start site, whereas the TBP-TFB-RNAP preinitiation complex has previously been shown to protect the bases −43 to +8 at *Sulfolobus* viral T6 promoter (38). Hence, LysM binding is not expected to interfere with or occupy the target sites for TBP, TFB, or RNAP but rather binds upstream of these proteins, as is usually the case for activators. In many cases, these activators recruit components or stabilize binding of the preinitiation complex (PIC) through direct contacts. However, such direct contacts have not yet been shown for Lrp-like proteins. Alternatively, activation could involve promoter remodeling in which the secondary structure of the promoter DNA is changed, for instance by bending the DNA in a certain angle. This altered DNA structure could subsequently be recognized more efficiently by one of the components of the PIC. In this case, activation of transcription would be independent of direct contacts between the activator and the PIC components. As shown in Fig. 5A, binding of LysM induces several DNase I-hypersensitive sites, indicative of DNA secondary structure changes. Interestingly, one of the hypersensitive sites is located between the BRE and TATA-box, representing a structural alteration that is potentially able to alter the interaction properties of TBP/TFB with the DNA. In addition, the intensity of some of the LysM-induced hypersensitive sites is somewhat changed in the presence of lysine. However, the magnitude of this effect is less obvious than the effect of lysine observed in EMSAs (Fig. 4). We have tested the ability of LysM to bend its target DNA by using the pBEND2 system described by Kim *et al.* (47). For this purpose, we cloned the 24-bp fragment used for Fig. 5, B and C, into pBEND2 and used it as described (47), but no LysM-induced bending was observed, either in the presence or absence of lysine. The possibility cannot be ruled out, however, that low affinity binding to sequences outside the chosen sequence fragment contributes to the LysM-DNA interaction and bending.

Our *in vitro* transcription experiments showed that compared with the T6 control promoter (2), transcription from P$_{lysY}$ and P$_{lysW}$ is very weak. We showed previously that P$_{lysY}$ could be stimulated by the addition of TFE (6) (referred to as P$_{ TE}$, but this was not possible for P$_{lysW}$. Apparently, both promoters are intrinsically weak promoters, which is in agreement with the low homology to the *Sulfolobus* consensus promoter sequence. It is therefore possible that binding of TBP and/or TFB might be impaired at P$_{lysW}$. In agreement with this, in an EMSA we could not observe any interaction between P$_{lysW}$ DNA and (combinations of) these transcription factors, and the addition of LysM or lysine had no effect (not shown). Altogether, our results thus suggest that the intrinsic activity of P$_{lysW}$ promoters is very low. In contrast, *lysWXJK* mRNA could easily be detected in Northern blotting and primer extension experiments, suggesting efficient transcription *in vivo*. Since we have proven that both P$_{lysY}$ and P$_{lysW}$ are true promoters in *vivo*, we suggest that at least for P$_{lysW}$, additional factors like co-regulators may be required for efficient transcription. Thus, under our experimental conditions, LysM is not able to activate transcription, but in the presence of an unidentified factor, transcription may take place. In comparison, some *E. coli* promoters that belong to the Lrp regulon require an additional DNA-binding protein (e.g. integration host factor (IHF) (48), histone-like protein H-NS (49), or catabolite activator protein (CAP) (50, 51)). To identify such proteins in *S. solfataricus*, we have taken several approaches. First, in our *in vitro* transcription experiments, we have added cell extracts of *S. solfataricus*
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grown in the absence of lysine, but no stimulation of transcription in vitro was observed. Second, we have performed pull-down experiments in which glutathione S-transferase (GST) was fused to LysM and immobilized on glutathione-agarose beads. An S. solfataricus cell extract was subsequently screened for proteins interacting with GST-LysM. We found that a single protein interacted with LysM, but this protein was identified as the LysM protein itself, most likely being the result of multimerization of GST-LysM and wild-type LysM during the experiment (data not shown).

The observation that LysM is conserved in the lys clusters of three Sulfolobus species and in A. pernix suggests that it plays a similar role in these organisms. We have therefore compared the sequence of their putative lysW promoters to identify a possible consensus LysM binding site. Indeed, a conserved GGTTC inverted repeat element is present, as shown in Fig. 8. For the putative lysW promoters of Sulfolobus species, the position of the presumptive LysM binding site is very similar (overlapping the lysM stop codon), whereas in A. pernix, where the lys gene cluster is organized in a somewhat different way (see Fig. 1A), the putative LysM site is centered between the lysY and lysW genes. It is remarkable that this LysM binding site is conserved and highly palindromic, since this is usually not the case for naturally occurring binding sites of Lrp-like proteins (9, 15, 52). We have derived a consensus LysM site from the alignment given in Fig. 8, and we used this sequence to screen the S. solfataricus and A. pernix genomes for LysM sites, but no additional LysM binding sites could be identified.

Several archaean Lrp-like proteins have been characterized recently (13–16), but LysM is the first for which a clear physiological role has been demonstrated. Unlike previously studied archaean Lrp-like proteins, LysM is expressed constitutively and not negatively autoregulated. Moreover, LysM strongly resembles bacterial Lrp-like proteins and appears to be a specific rather than a global regulator, since it is clustered with its target genes. However, we cannot exclude the possibility that LysM has additional targets in the S. solfataricus genome, and experiments are necessary to confirm this. Furthermore, all bacterial Lrp-like proteins characterized to date act as transcriptional repressors or activators involved in the regulation of amino acid metabolism, and all ligands identified so far were found to be amino acids. In analogy, the previously characterized archaean Lrps are repressors, whereas our data is most compatible with LysM being an activator. Thus, Lrp-like proteins appear to be functionally equivalent in the bacterial and archaean domains, despite the fundamental differences in transcriptional machineries.

In conclusion, we have studied the role of the S. solfataricus lysW-like protein LysM in the regulation of the S. solfataricus lysY/ZMWXXJK gene cluster, which is involved in lysine and possibly arginine biosynthesis. Transcription of this cluster arises from two promoters, PlysY and PlysW, and the addition of lysine (but not arginine) represses the internal PlysW activity 8-fold without affecting PlysY. LysM binds to a site directly upstream from the BRE of PlysY, and since the affinity of LysM for this binding site in vitro is highest in the absence of lysine, it is most likely that LysM acts as an activator for lysW/XJK transcription. The fact that we could not confirm this in an in vivo transcription system does suggest that activation by LysM requires one or more (yet unidentified) additional factors, as is the case for transcriptional activation of some promoters by the homologous E. coli Lrp. Future research will be focused on the identification of these cofactors using the yeast two-hybrid system. Furthermore, transcription and regulation of the PlysW promoter in vitro could be further analyzed in conjunction with the recently characterized chromatin-associated protein Alba, which has been shown to block transcriptional regulatory potential.

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