Characterization of an Archaeal Two-Component System That Regulates Methanogenesis in Methanosaeta harundinacea

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Abstract

Two-component signal transduction systems (TCSs) are a major mechanism used by bacteria in response to environmental changes. Although many sequenced archaeal genomes encode TCSs, they remain poorly understood. Previously, we reported that a methanogenic archaeon, Methanosaeta harundinacea, encodes FilI, which synthesizes carboxyl-acyl homoserine lactones, to regulate transitions of cellular morphology and carbon metabolic fluxes. Here, we report that filI, the cotranscribed filI-filR1, and the adjacent filI-filR2 constitute an archaeal TCS. FilI possesses a cytoplasmic kinase domain (histidine kinase A and histidine kinase-like ATPase) and its cognate response regulator. FilR1 carries a receiver (REC) domain coupled with an ArsR-related domain with potential DNA-binding ability, while FilR2 carries only a REC domain. In a phosphorelay assay, FilI was autophosphorylated and specifically transferred the phosphoryl group to FilR1 and FilR2, confirming that the three formed a cognate TCS. Through chromatin immunoprecipitation–quantitative polymerase chain reaction (ChIP-qPCR) using an anti-FilR1 antibody, FilR1 was shown to form in vivo associations with its own promoter and the promoter of the filI-filR2 operon, demonstrating a regulatory pattern common among TCSs. ChIP-qPCR also detected FilR1 associations with key genes involved in acetoclastic methanogenesis, acs4 and acs1. Electrophoretic mobility shift assays confirmed the in vitro tight binding of FilR1 to its own promoter and those of filI-filR2, acs4, and mtrABC. This also proves the DNA-binding ability of the ArsR-related domain, which is found primarily in Archaea. The archaeal promoters of acs4, fill, acs1, and mtrABC also initiated FilR1-modulated expression in an Escherichia coli lux reporter system, suggesting that FilR1 can up-regulate both archaeal and bacterial transcription. In conclusion, this work identifies an archaeal FilI/FilR1 TCS that regulates the methanogenesis of M. harundinacea.

Introduction

Methanogenesis is a major contributor to global warming, and methanogens are the only organisms known to perform this metabolism [1]. Acetate-derived methane contributes about 70% of the global methane production and is produced by acetoclastic methanogens such as Methanosarcina and Methanosaeta. Although these archaea possess prokaryotic cells, their genetic machinery for replication, transcription, and translation more closely resembles that of Eukarya than Bacteria [2]. Methanogens are archaea that are distributed in diverse anoxic environments and can develop complex regulation mechanisms in response to environmental changes [3,4].

Two-component signal transduction systems (TCSs) are one of the principal means by which bacteria respond to environmental changes [5–7]. Gao and Stock summarized the diverse bacterial TCSs, indicating that the typical TCS comprises a membrane-bound sensor histidine kinase (HK) and a cognate response regulator (RR) and catalyzes a phosphotransfer between the two [8]. Typically, upon sensing environmental stimuli, the HK is autophosphorylated at a conserved histidine residue. The phosphoryl group is then transferred to its cognate RR at a conserved aspartate residue. In prototypical HKs, the cytoplasmic kinase core consists of a well-conserved C-terminal HK-like ATPase (HATPase) and a less-conserved histidine kinase A (HisKA) domain [9], which can be coupled to an overwhelmingly diverse array of sensory domains, including PAS, GAF, and HAMP domains, enabling HKs to sense a wide variety of stimuli. Characteristic RR superfamily proteins contain a receiver (REC) domain, which is linked to variable effector domains that mediate nucleic acid binding or enzymatic and protein/ligand-binding activity [8]. Moreover, approximately 17% of RR exist as standalone REC domains that lack an effector domain [8,10,11]; these RRUs usually implement a more intricate regulation of a TCS [12,13].
TCS genes are found in many sequenced archaeal genomes; however, they are poorly studied [7,14,15]. *Methanosaeta harundinacea* 6Ac is an obligate acetoclastic methanogenic archaeon that was isolated from an upflow anaerobic sludge blanket reactor [16]. It contributes to granule formation and the efficacy of waste removal in the reactor. Previously, we determined that Fill, a bacterial LuxI homolog from *M. harundinacea*, synthesized the quorum sensing (QS) signal molecules carboxyl-acyl homoserine lactones (AHLs), which regulate the cell morphological transition from short cells to filaments and carbon metabolic flux from biomass formation to methane production [17].

In this work, based on in silico analysis, Fill, the cotranscribed gene fill2, and the adjacent gene that we tentatively named fill1 were predicted to encode the only TCS in this archaeon. Then, through a combination of in vivo and in vitro approaches, Fill and the two FillRs were shown to possess many of the properties of bacterial TCSs. Moreover, they were found to be involved in the regulation of methanogenesis in *M. harundinacea*.

**Results**

**In silico analysis predicted a putative TCS in *M. harundinacea***

To gain a general view of the TCSs in *M. harundinacea* 6Ac, bacterial prototypical kinase core domains of HKs and REC core domains of RRss were used as probes to query the genome (CP003117) [17]. The search found only three putative HKs (Mhar_0446, Mhar_0936, and Mhar_1766) with characteristic cytoplasmic HisKA and HATPase domains, and it identified five RRss (Mhar_0169, Mhar_0445, Mhar_0447, Mhar_1520, and Mhar_2042) with REC core domains. These results were consistent with those of the P2CS and MIST2 databases [18,19]. Further, transmembrane regions of the three putative HKs were analyzed using three programs: TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) [20], Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html), and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) [21]. All three programs revealed that only Mhar_0446 was likely to possess the two transmembrane regions that are common in prototypical membrane-bound HKs (Figure 1A, 1B). Next, functional domains of the five RRss were analyzed using the Pfam database (http://pfam.sanger.ac.uk/) [9]. Mhar_0447 and Mhar_1520 were stand-alone RRss that possessed only the REC domain (Figure 1C), while Mhar_0169 and Mhar_2042 had REC domains that were fused with PAS sensory domains, which is an unusual RR organization in which the REC domain is fused to a ligand-binding domain [10]. Only Mhar_0445 appeared to encode a typical TCS RR with a REC core domain coupled with PB005323, a predicted ArsR-related domain with potential DNA-binding ability (Figure 1C). Moreover, Mhar_0446 and Mhar_0447 could constitute an operon because they are only separated by a short intergenic distance of 37 bp and are adjacent to Mhar_0445. Therefore, the three proteins might constitute the only typical TCS in *M. harundinacea*.

Mhar_0446 encodes a protein of 886 amino acid residues that exhibits 39.6% identity to AhlI, a LuxI family autoinducer synthase of *Escherichia coli* [17]. The recombinant His6-tagged protein had carboxyl-AHL synthase activity; therefore, Mhar_0446 was named Fill [17]. Using the Pfam database, six functional domains were predicted in Fill, including a CHASE4 domain, a HAMP (potential signal transmission) domain, a PAS intracellular stimuli sensory domain, a GAF intracellular stimuli sensory domain, a C-terminal HisKA domain, and an HATPase domain (Figure 1A, 1B). Moreover, the transmembrane region prediction programs TMHMM, Tmpred, and SOSUI indicated that the CHASE4 domain and the HAMP domain are extracytoplasmic or transmembrane, while the other four domains are all cytoplasmic (Figure 1B). In addition, the AHL synthase activity of Fill suggested a potential connection between the predicted Fill/FillRss TCS and quorum sensing (QS) in *M. harundinacea*.

An alignment of the two RRss in this putative TCS (Figure 1C), named FilR1 and FilR2, indicated a sequence similarity of 48% between the C-terminal REC domains of FilR1 and FilR2 (Figure 1D). Moreover, according to the domain compositions, FilR1, with a potential DNA-binding domain, was predicted to be the direct effector of this putative TCS. FilR2, as a stand-alone RR, was predicted to be involved in yet-unknown regulation mechanisms.

**fill and fill2 constituted an operon and were cotranscribed**

The in silico analysis predicted that fill2 and fill1 constitute an operon from which fil1 is divergently transcribed (Figure 2A). To test this prediction, a reverse transcription-polymerase chain reaction (RT-PCR) assay was performed using the total RNA of *M. harundinacea* as a template. As shown in Figure 2B, a PCR product was amplified from the cDNA of the fil1/fill2 intergenic region, indicating the cotranscription of fill1 and fill2 (Figure 2B). As a control, no amplification was produced from the intergenic region between fill2 and the downstream ferredoxin gene (Figure 2C).

**Autophosphorylation of Fill and phosphotransfer among Fill and the FillRs**

A functional TCS relies on the autophosphorylation of the HK and a phosphotransfer from the HK to the cognate RR [8]. To determine if these activities are present in the predicted Fill/FillRss TCS system, recombinant Fill, FilR1, and FilR2 were purified from *Escherichia coli* (Figure 3A). Fill was readily autophosphorylated within 45 min of incubation with [γ-32P]ATP (Figure 3B). Phosphorylated Fill was capable of robust phosphoryl group transfers to both FilR1 and FilR2 within a short time (Figure 3B). Autophosphorylation of the truncated Fill proteins, containing only the C-terminal kinase core domain, was also detected. This domain was able to phosphorylate FilR2, but its efficiency was lower than that of the entire Fill protein (Figure 3C). In contrast, the truncated Fill only weakly phosphorylated the other two potential RR transcriptional regulators Mhar_0169 and Mhar_1520 after 30 min (Figure 3D). These results demonstrated the specificity of the phosphorelay from Fill to FilR2 and to FilR1, and demonstrated the activity of the Fill HK domain. Further, the Fill-synthesized carboxyl-AHls (N-carboxyl-C10-HSL, m/z 318, or N-carboxyl-C12-HSL, m/z 346) were added directly to the autophosphorylation reaction of Fill and phosphotransfer reactions to FillRs. However, very weak enhancing effects were observed (Figure S1). Therefore, the Fill-synthesized carboxyl-AHls did not have an evident direct effect on the autophosphorylation of Fill or phosphotransfer to FillRs in the in vitro experiment.

**Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assay of the in vivo associations of FillR1 with the promoters of the TCS system and the key genes of methanogenesis**

Because of the lack of a genetic system for *M. harundinacea* 6Ac, the in vivo TCS action of Fill and FillR1 was determined using ChIP assays. *M. harundinacea* 6Ac possesses a thick cell envelope;
therefore, a novel method to gently lyse the cells with dithiothreitol (DTT) treatment at alkaline pH was developed from a procedure for *Methanospirillum* [22]. As shown in Figure 4A, the promoter regions of *filR1* and the *filI-filR2* operon were enriched in the anti-FilR1 antibody-immunoprecipitated DNA (lane AbFilR1) but not in the mock-IP control DNA (lane CK). Moreover, compared with the mock-IP DNA (CK), qPCR indicated 11.7- and 24.8-fold enrichments for the promoters of *filR1* and *filI-filR2* operon in the anti-FilR1 antibody-immunoprecipitated DNA (Figure 4B), respectively. In the same experiment, there was no enrichment for a fragment of similar length from the 16S rRNA gene (Figure 4). Therefore, ChIP-PCR and ChIP-qPCR assays verified the *in vivo* association of FilR1 with its promoter and the promoter of the *filI-filR2* operon, a common characteristic of TCSs.

Previously, FilI-synthesized AHLs were found to regulate a transition of the cellular morphology from short to long cells and a carbon metabolic flux leading to the formation of more methane and less cellular biomass from acetate in *M. harundinacea* [17]. To test whether the FilI cognate RR FilR1 was involved in the regulation of methanogenesis, associations of FilR1 with the key genes in acetoclastic methanogenesis were assayed using the same protocol. As shown in Figure 5, the promoter regions of the *acs1* operon, *acs4* gene, *mtr* operon, *fwdCABD* operon, and *omp* gene (Table S1) were all amplified by PCR assays from the anti-FilR1 antibody-immunoprecipitated DNA samples (Figure 5A). Also, qPCR indicated 11- and 13-fold enrichments of the promoter regions of the *acs1* operon and the *acs4* gene, respectively, and modest enrichment of the *mtr* and *fwd* operons and the *omp* gene (6.34- to 7.88-fold enrichment) (Figure 5B). Therefore, FilR1 interacted with the promoters of a number of genes that are essential for methanogenesis in *M. harundinacea* 6Ac as summarized in Table S1.

Figure 1. Schematic representation of the domain structures of Fill and FilR proteins. (A) Domain structure of Fill analyzed using programs of Pfam and NCBI blast. (B) Location analysis of domains in Fill through the programs of TMHMM, TMpred and SOSUI. (C) Domain structures of FilR1 and FilR2 were analyzed by programs of Pfam and NCBI blast. In addition, the amino acid identity (%) for the aligned fragments of FilR1 and FilR2 is shown on the right. (D) Protein sequence alignment of the REC domains of FilR1 (C-terminal 276–446aa) and FilR2 (the whole length) by software GeneDoc. Identical amino acids are shown with a black background, while similar amino acids are shown with a gray background.

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FiIR1 bound in vitro to the promoters of the TCS system and key genes of methanogenesis

Next, electrophoretic mobility shift assays (EMSAs) were performed to confirm the in vivo binding of FiIR1 to the gene promoters studied above. Using a biotin-labeled DNA amplified from the tested gene promoters as probes, DNA-protein complexes with the recombinant FiIR1 were detected by native polyacrylamide gel electrophoresis (PAGE). As shown in Figure 6, FiIR1 bound efficiently to its promoter and that of the filI-filR2 operon in a dose-dependent manner, demonstrating its auto-regulation and regulation of the filI-filR2 operon.

Similarly, EMSA also detected the in vitro binding of FiIR1 to promoters of the acs1 operon, acs4 gene, mtr operon, fwdCABD operon, and omp gene (Figure 7). However, no binding was detected for the promoter of Mhar_0449 and an internal fragment of filI, which served as negative controls (Figure 7 and data not shown). These data further confirmed the direct regulation acetyl-CoM methanogenesis and the methyl oxidative shunt by FiIR1; these findings were consistent with the in vivo binding results determined by the ChIP assays described above. In addition, the phosphorylated FiIR1 via phosphotransfer from FilI exhibited an obviously enhanced DNA-binding affinity to the promoters of the acs1 and fwdCABD operons (Figure S2), implying the possible positive regulation of methanogenesis by the FiIR1/FiIRs TCS in Methanosaeta.

However, EMSA did not detect the binding of FiIR1 to the promoters of some other differentially expressed genes in the QS-mediated transition. These genes included the cdhCD and cdhBE4 operons, which encode the key enzyme complex for the acetyl-CoM reaction; the mcr operon, which encodes the methyl-CoM reductase; and the fpo operon, which encodes the only membrane-associated protein complex for electron transfer in Methanosaeta (data not shown). Therefore, the expression of these genes is probably not directly regulated by FiIR1.

The ArsR-related domain is found primarily in Archaea, and it has not yet been shown to bind DNA. This work also provides the first experimental evidence of the DNA-binding ability of the ArsR-related domain.

An ex vivo E. coli promoter-reporter system detected FiIR1 regulation of the genes involved in methanogenesis

An E. coli-based promoter-reporter ex vivo system [23,24] was used to further examine the regulation of FiIR1. First, the filI promoter-reporter system detected a protein band of 54 kDa, the predicted size of FiIR1, in extracts of transformed E. coli (data not shown). Meanwhile, the examined promoters were fused upstream of a promoterless lux operon inserted into pCS26-Pac to construct a variety of methanogenic promoter-lux reporter plasmids denoted as pOs-lux (x: the tested genes) (Table 1). pFiIR1 and each of the pOs-lux promoter-lux reporter plasmids were cotransformed into E. coli, and they were recognized by E. coli RNA polymerase and promoted the transcription of the downstream lux gene, yielding luminescence comparable to that of bacterial promoters. Coexpression with FiIR1 caused a remarkable stimulation of the acs4 (46-fold) and filI-filR2 (33-fold) promoters. FiIR1 also increased the bioluminescence from the mcr (13-fold), filI (9-fold), acs1 (6-fold), and mtr (7-fold) promoters (Table 1), suggesting that FiIR1 regulated these genes as well. In contrast, the FiIR1-stimulated expression by the promoter of the fwdCABD operon was increased less than 2-fold in this promoter-reporter system (Table 1).
which are genes whose expression was not directly regulated by FilR.

To determine if archaeal promoters share the characteristics of bacterial promoters and, therefore, can be recognized by *E. coli* RNA polymerase, we first predicted the promoters in the above-studied genes using the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html) [25] (Table S2). In addition to a typical archaeal promoter, a bacteria-like promoter was found upstream of the *mtr* coding region (Figure S3). Coincidently, the highest bioluminescence was detected for the *mtr* and *acs4* promoters in the *E. coli* promoter-reporter system (Table 1). Furthermore, a GC substitution was made for a six base-AT tract in the predicted promoters of *mtr*, *acs4*, *filR1*, and *filI-filR*. The bioluminescence from the mutated *filI-filR2* and *mtr* promoters was reduced 6.4- and 31.5-fold, respectively, and FilR1-induced bioluminescence was also reduced (Table S3). The bioluminescence was not affected by mutations in the *acs4* and *filR1* promoters; this observation can probably be attributed to an incorrect promoter prediction. This indicates that *E. coli* RNA polymerase can recognize some archaeal promoters.

**Discussion**

TCSs are distributed widely in bacteria and are well characterized [7,14,19]. They are abundant in free-living species like *E. coli* and *Bacillus subtilis*, which contain 30 and 36 TCSs [19], respectively, but they are absent in many parasites, such as *Mycoplasma genitalium* and *Mycoplasma pneumonia* [14,15]. Because TCSs enable organisms to adapt to environments, an abundance of TCSs is considered a measure of the bacterial IQ [26]. Authentic genes of archaeal TCSs are only found in some Euryarchaeota and Thaumarchaeota genomes, and they are found in neither Crenarchaeota nor Nanoarchaeota genomes [14,15]. In these phyla, one-component systems of a single protein containing both sensor and regulator domains are widely distributed [27]. Compared with bacteria, fewer TCSs are annotated in the sequenced archaeal genomes, and TCS HKs and RRs are not always present proportionally. Within Archaea, the most TCSs are found in *Methanobacterium thermoautotrophicum* (16 HKs and 10 RRs) and *Archaeoglobus fulgidus* (14 HKs and 11 RRs) [7]. In contrast, *Pyrococcus horikoshii* contains a single HK...
and two RRs (chemotaxis proteins CheA, CheY, and CheB), and none are found in *Methanococcus jannaschii*, *Aeropyrum pernix*, and *Thermoplasma acidophilum* [7].

Hitherto, archaeal TCSs have not been well studied although this group of organisms is assumed to be highly adapted to extreme environments. In this work, an archaeal FilI/FilRs TCS, which is also the only *in silico* predicted TCS in the methanogenic archaon *M. haruniageta* 6Ac, was identified experimentally, and the genes regulated directly by this TCS were determined.

TCSs are predicted to be of bacterial origin, and they were acquired by Archaea and some eukaryotes like plants, fungi, and protozoa by horizontal gene transfer [7,15]. Though distinct from...
Bacteria, *Methanosaeta 6Ac* employs a TCS with properties that are similar to the bacterial system, i.e., catalyzing the phosphotransfer between FilI and FilRs, the HK and RRs of the TCS. Through ChIP-based *in vivo* assays and an EMSA-dependent *in vitro* approach, FilR1 was shown to bind the promoters of *filI* and its own gene, indicating that auto-regulation by the direct effector RR occurs in this archaean TCS, a behavior that is typical of bacterial TCSs. FilR1 was also determined to bind to the promoters of genes involved in methanogenesis: the *acs1* operon and *acs4* gene encode acetyl-CoA synthetase for acetate activation at the initial and rate-limiting steps of acetoclastic methanogenesis; the *mtr* operon encodes a protein complex for methyl transfer from methyltetrahydrofolate to coenzyme M; and the *fwdCAB* operon encodes tungsten formylmethanofuran dehydrogenase subunits CABD in the methyl oxidative shunt. In addition, FilR1 was also determined to bind to the promoter of the *omp* gene, a cell envelope protein gene. These results indicate the direct regulation of acetoclastic methanogenesis and the methyl oxidative shunt by FilR1, which is inconsistent with the genes that are differentially expressed in QS-mediated filaments and short rod cells. Remarkably, a second RR protein, FilR2, is included in this archaean TCS. This RR only has a REC domain and no output domain. The *filR2* gene constitutes an operon with the gene encoding FilI, and the two genes are cotranscribed; therefore, an equal level of the two proteins might be maintained in cells. In addition, phosphoryl group transfer occurred from FilI not only to FilR1 but also to FilR2, implying the involvement of FilR2 in this phosphorelay. However, details of its function remain to be

**Figure 6.** EMSAs showed FilR1 binding to the promoters of its own and the operon *filI-filR2*. Purified recombinant FilR1 protein was incubated with 0.5 nM of biotin-labeled DNA in the standard binding reaction mixture at 25°C for 20 min, and then run on a native PAGE. Concentration of purified FilR1 protein was shown at the top of each lane. Unlabeled *filI-filR2* promoter (NP_{filI-filR2}) was used as a competitor substrate of FilR1, which was added at the final concentrations of 5, 25, 125 and 250 nM in lane 6 and 15, 7 and 16, 8 and 17, and 9 and 18, respectively. (A) P_{filI-filR2}, promoter of the *filI-filR2* operon; (B) P_{filR1}, promoter of *filR1*, and (C) U_{filI} an internal DNA fragment of gene *filI*. doi:10.1371/journal.pone.0095502.g006

**Figure 7.** EMSAs showed FilR1 binding to the promoters of genes key to methanogenesis in *M. harundinacea*. Purified recombinant FilR1 protein was incubated with 0.5 nM of biotin-labeled DNA in the standard binding reaction mixture for 20 min at 25°C and then run on a native PAGE. Concentration of purified FilR1 protein used was shown at the top of each lane. (A) P_{acs1}, promoter of the *acs1* operon; (B) P_{acs4}, promoter of *acs4*; (C) P_{omp}, promoter of the *mtr* operon; (D) P_{fwdCABD}, promoter of the *fwdCABD* operon; (E) P_{omp}, promoter of *omp*; and (F) P_{Mhar_0449}, promoter of *Mhar_0449*. doi:10.1371/journal.pone.0095502.g007
understood. Therefore, this work demonstrates that FilI, FilR1, and FilR2 constitute a methanogen TCS that is involved in the regulation of methanogenesis.

QS regulation has been extensively studied in Bacteria [28–31]. Recently, a new signal transduction pathway in *Vibrio harveyi* was identified in which the QS signals, AHLs, are transduced via a TCS circuit [8,31–33]. However, QS regulation seems to be overlooked in Archaea. In our previous work, FilI, presumably the related RR in this archaeal TCS, presumably through cooperation with FilR1 or other regulators.

This work determined that the Methanosaeta *mtr*, *fwd*, *cdhCD*, and *acs* promoters induced the transcription of a bacterial *lux* gene in *E. coli*. Because the expression was activated by the coexpression of FilR1, it mimics the natural expression in *Methanosaeta* and is unlikely to have resulted from fortuitous expression of cloned DNA near to but not including the promoter. Thus, some archaeal promoters appear to be recognized by bacterial transcription machinery, which is partially verified by mutation of the predicted promoters. This can also be anticipated on the basis of conserved promoter modularity and general basal transcription machinery among various lineages, as reviewed by Decker et al. [34]. Recent biochemical and structural studies have revealed significant similarities of the basal transcription machinery among Bacteria, Archaea and Eukarya that include the following: 1) structural and functional similarity of the multi-subunit polymerases; 2) conserved promoter modularity from bacteria to humans; 3) functional equivalence between bacterial *σ* factors and the basal transcription factors, TFB and TBP, in Archaea and Eukarya; and 4) similar strategies for transcription factors (TFs, activators, or repressors) to alter the core promoter specificity within Bacteria, Archaea, and Eukarya. While the detailed mechanisms for the action of the methanogen FilR1 with the *E. coli* transcription system need to be clarified through further studies, this work provides experimental evidence for the similarities in transcriptional machinery and regulation mechanisms between Bacteria and Archaea.

In conclusion, an archaeal FilI/FilRs TCS present in a methanogenic archaeon, *M. harundinacea*, was identified, and its regulation of methanogenesis was determined in this work. Moreover, this work indicated a possible connection between the FilI/FilRs TCS and QS regulation in this methanogenic archaeon;

### Table 1. Expression of the archaean promoters in the *E. coli* ex vivo reporter system.

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<th>Reporter plasmids&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bioluminescence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold stimulation by FilR1&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup>Values are shown as relative light units and the average of at least three independent readings.

<sup>b</sup>Genes and operons shown in each reporter plasmid are listed in Table S1.

<sup>c</sup>Fold difference of the luciferase activity are calculated from those determined for *E. coli* strain carrying plasmid pairs of FilR1-vacant p184 plus po<sup>OR2</sup>-lux over that of the strain carrying pOx-lux alone (+p184/ -- ), strain with FilR1 plus po<sup>OR2</sup>-lux over that with po<sup>OR2</sup>-lux alone (+pFilR1/ -- ), and strain with FilR1 plus po<sup>OR2</sup>-lux over that with FilR1-vacant p184 plasmid (+pFilR1+p184).

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Materials and Methods

Strains, plasmids, and primers

*M. harundinacea* 6Ac was preserved in our laboratory and routinely cultured in a pre-reduced basal medium containing 100 mM sodium acetate in anaerobic bottles sealed with butyl rubber stoppers as described previously [16,17]. *E. coli* DH5α served as the host for cloning experiments, while *E. coli* BL21 (DE3) was used for overproduction of recombinant proteins. *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) medium with shaking. Kanamycin (50 µg/mL) and chloramphenicol (34 µg/mL) were supplied when necessary.

The expression vector pET28a was obtained from Novagen (Madison, WI, USA). Plasmids pCS26-pac and pACYC184 for the bioluminescence assay were kindly provided by Professor Keqian Yang (Institute of Microbiology, CAS, Beijing, China). The primers used for DNA amplification in this study (Table S4, S5, S6) were synthesized by Sangon (Beijing, China).

Construction of expression plasmids

The ORFs of *filR1* and *filR2* were amplified from genomic DNA of *M. harundinacea* with the primer pairs P1/P2 and P3/P4 (Table S4), respectively. The PCR products were digested with NcoI and Ncol/HindIII, and then they were cloned into the same sites of the vector pET28a, resulting in the expression plasmids p28FilR1 and p28FilR2, respectively. The p28Fil plasmid was used to express FilI as previously described [17]. The DNA fragment encoding the C-terminal HK domain of FilI (amino acids 642–886) was amplified from genomic DNA with the primer pair P5/P6 (Table S4) and inserted into the Nhel/HindIII sites of pET28a, resulting in the expression plasmid p28Fil-C. Two ORFs (Mhar_0169 and Mhar_1520) encoding proteins with predicted REC domains served as the negative control for the phosphorylation assay and were amplified with the primer pairs P7/P8 and P9/P10 (Table S4) and cloned into plasmid pET28a to construct expression plasmids p28-0169 and p28-1520, respectively. PCR-amplified sequences were verified by DNA sequencing of all constructs.

Protein expression and purification

To overexpress and purify FilI, FilR1, FilR2, Fil-C, Mhar_0169, and Mhar_1520, *E. coli* BL21 (DE3) harboring each of the expression plasmids was cultivated in LB medium containing kanamycin to an optical density at 600 nm of 0.4 to 0.6 when isopropyl-D-thiogalactopyranoside was added at a final concentration of 0.1–0.5 mM. The induced cultures were allowed to grow for an additional 3 h. Cells were harvested by centrifugation, resuspended in lysis buffer containing 0.3 M NaCl, 20 mM imidazole, and 50 mM sodium phosphate buffer (NaH2PO4/Na2HPO4, pH 8.0), and lysed by ultrasonication. The cell lysates were centrifuged at 13,400 g for 30 min, and the His6-tagged recombinant proteins were purified from the supernatant by Ni2þ-nitrilotriacetic acid-agarose column (Novagen), followed by ion change chromatography through a Resource Q column (GE Healthcare, Pittsburgh, PA, USA) or by gel filtration chromatography through a Superdex 200 10/300 GL column (GE Healthcare) when further purification was needed. Purified proteins were examined by sodium dodecyl sulfate (SDS)-PAGE, and protein concentrations were determined using a bicinchoninic acid (BCA) protein concentration assay kit (Pierce, Rockford, IL, USA) combined with a Bradford protein concentration assay kit (Pierce).

Phosphorylation assay

To test for autophosphorylation, 2 µg of recombinant FilI was incubated with 10 µCi [γ-32P] ATP (3000 Ci/mmol) in 10 µL of phosphorylation buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 50 mM KCl, 10 mM MgCl2, and 10% glycerol (v/v)) at 37°C for 45 min. For the phosphotransfer assay, 10 µL of phosphorylation buffer containing 4 µg of the recombinant FilI1 or FilI2 was subsequently added. After 2 min or 5 min, reactions were stopped by addition of 5× SDS sample buffer (250 mM Tris-HCl, pH 6.8, 5% SDS, 50% glycerol, 5% β-mercaptoethanol, 0.1% bromphenol blue) supplemented with 2.5 µL 0.5 M EDTA (pH 8.0). In control reactions, 4 µg of the recombinant Mhar_0169 and Mhar_1520 was used and incubated for 30 min or longer. The phosphorylated products were resolved by 15% SDS-PAGE, and isotope-labeled proteins were visualized by autoradiography with X-ray film.

ChIP assay

*M. harundinacea* 6Ac was grown to the late exponential phase, corresponding to the high cell density mode of QS [17], and the cells were immediately fixed with 1% (v/v) formaldehyde for 10 min. Fixation was terminated by the addition of glycine to a final concentration of 125 mM. Culture (450 mL; ~5×10¹⁰ cells) was harvested and pelleted at 10,000 g. Cell pellets were washed twice with Na2CO3/NaHCO3 buffer containing 100 mM DTT, resuspended in an equal volume of Na2CO3/NaHCO3 buffer containing 100 mM DTT, and incubated for 2 h in anaerobic bottles. Cells were collected at 5000 g and stored frozen at −80°C. Upon thawing, cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 0.05% (v/v) SDS, 1 mM phenylmethyl-sulfonyl fluoride (PMSF) and incubated in an ice-bath for 30 min. The protein concentration in the supernatant was determined by the BCA protein concentration assay kit (Pierce). The protein in the supernatant was diluted to 3 mg/mL with immunoprecipitation (IP) buffer (10 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 0.05% (v/v) SDS, 0.05% sodium deoxycholate, 200 mM NaCl, 1 mM PMSF), and sonicated using a Bioruptor UC300 (Diagenode, Denville, NJ, USA) until the chromatin DNA was sheared to an average size of 200–500 bp (2.75 min/cycle, 30 s on/30 s off, high-power setting) according to the manufacturer’s instructions. Cell debris was removed by centrifugation, and the supernatant was retained. A 500-µL aliquot of the sample was used for each IP experiment, and at least 10 µL of each sample was reserved as an input control. Each 500-µL sample was first incubated with 20 µL Dynabeads Protein A/G beads (Life Technology, Carlsbad, CA, USA) for 1 h at 4°C and mixed on a gently rotating wheel. The beads were separated from the supernatant by binding DynaMag-2 (Life Technology) and discarded. Next, the supernatant was incubated with 50 µL anti-FilR1 rabbit polyclonal antibody, which was purified by antigen affinity chromatography, overnight at 4°C with gentle mixing. A parallel experiment without antibody was conducted as a negative control (mock-IP). The supernatant was then incubated with an additional 20 µL Dynabeads Protein A/G beads that were pre-incubated with 5 mg/mL bovine serum albumin for another 1 h at 4°C with gentle mixing. Then, immunoprecipitated complex-bound beads were separated as above and washed twice with IP buffer, twice with IP buffer containing 500 mM NaCl, and once with Tris-EDTA buffer (pH 7.5).
Immunoprecipitated complexes were eluted in 53 μL Stock Reverse Crosslinking Buffer (Life Technology), combined with 1 μL proteinase K and incubated at 55°C for 60 min to de-crosslink. Control samples (10 μL) were treated with the same procedure. Samples were separated by DynaMag-2, and the beads were discarded. Supernatants and input control were then incubated at 65°C for 30 min to inactivate the proteinase K. Finally, the non-crosslinked DNA was purified using DNA Purification Magnetic Beads and the provided buffers (Life Technology) or by the QIAquick PCR Purification Kit (Qiagen, Dusseldorf, Germany). DNA concentrations were determined by the Quant-iT DNA Assay Kit (Life Technology), and DNA fragment sizes were checked by an Agilent 2100 Bioanalyzer. All ChIP assays were performed at least three times to ensure the reproducibility.

PCR and real-time qPCR assay

PCR and real-time qPCR were performed to determine the enrichment of FilR1-bound targets in the immunoprecipitated DNA samples. For PCR amplification, 1 μL input, IP, or mock-IP (CK) DNA samples were used in a 25-μL reaction mix containing 0.4 μM of each oligonucleotide primer. PCR amplification used rTaq DNA polymerase (Takara, Dalian, China) for 25 to 30 cycles. Then, 5–10 μL reaction mixture was analyzed by electrophoresis on a 1.5% agarose gel. For qPCR analysis, a 25-μL reaction mixture was prepared that contained 1× SYBR Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan), 0.4 μM each of forward and reverse primers (Table S4), and 1 μL input, IP, or mock-IP (CK) DNA samples as templates. The qPCR analysis was performed on a Mastercycler ep realplex real-time PCR machine (Eppendorf, Hamburg, Germany). Fold differences between samples were calculated as described previously [35]. Briefly, the ΔCt value (normalized to the input samples) for each sample was calculated according to the equation: ΔCt [Ct (sample) − Ct (input)]. Next, the ΔΔCt was calculated by ΔΔCt = ΔCt (IP sample) − ΔCt (mock-IP control). Finally, the fold difference between the IP sample and mock-IP control was calculated as 2−ΔΔCt.

EMSA

All the probes that were used were amplified with a biotin-labeled forward primer. Double-stranded DNA (dsDNA) probes targeting the intergenic regions upstream of the studied genes (Table S1) including their DNA promoter regions were amplified using the genomic DNA of M. harundinacea as the template (Table S5), while the ORF of filI and the predicted DNA promoter region of Mhar_0449 were amplified as control probes. The DNA probes generated via PCR amplification were purified by an agarose gel DNA Purification Kit (Qiagen). A standard EMSA was performed using a Light Shift Chemiluminescent EMSA Kit (Pierce) as recommended by the manufacturer with some modifications as follows. A standard binding reaction mixture (20 μL) contained 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM MgCl2, 1 mM DTT, 2.5% glycerol, 1% Nonidet P 40, 0.5 mM EDTA, 1 μg/μL poly dI-dC, 10 fmol labeled DNA probe, and the indicated amount of purified His6-FilR1 used in each reaction. After incubating at 25°C for 20 min, samples were immediately loaded on a non-denaturing 5% polyacrylamide gel (with an acrylamide to bisacrylamide weight ratio of 80:1) in 0.5× Tris-borate-EDTA buffer and run at 130 V for 2.5 h. Then, the DNA-protein complex was transferred onto a nylon membrane and cross-linked using a GS Gene Linker UV Chamber (Bio-Rad, Hercules, CA, USA). The biotin-labeled DNA was detected by chemiluminescence.

To determine the effect of phosphorylation on FilR DNA-binding, Fil protein (5 pmol) was autophosphorylated by incubation with 50 pmol ATP for 45 min at 37°C. Then, autophosphorylated Fil was mixed with 0, 0.1, 0.5, 1, and 2.5 pmol FilR1 for 10 min. EMSA of the phosphorylated FilR1 protein was performed as above.

Construction of promoter-reporter strains for luciferase assays in E. coli

E. coli-based promoter-reporter strains were constructed to detect the possible regulation of genes of interest by FilR1. Intergenic regions upstream of the tested genes including their promoters were amplified by PCR with primer pairs listed in Table S6 and genomic DNA of M. harundinacea as a template. The amplified DNA fragments were then ligated to BamHI/Xhol-digested pCS26-Pac to yield the reporter plasmids pO²-lux (x refers to the tested gene). To express the FilR1 protein in E. coli, the filR1 gene was amplified with the primer pair P37/P38 (Table S6) using the plasmid p28FilR as a template and introducing E. coli Shine-Dalgarno sequences and recognition sites for BamHI [23]. After digestion with BamHI, the fragment was ligated to BamHI/EcoRV-digested pACYC184 to yield the plasmid pFilR1. Attract mutants were constructed by PCR with primer pairs listed in Table S6 and pO²-lux reporter plasmids as templates; PCR products were digested with DpnI to yield the pO²-mut-lux reporter plasmids. The bioluminescence of the E. coli reporter cultures was measured using a TD 20/20a single tube luminometer (Turner Biosystems, Sunnyvale, CA, USA) as described previously [23]. All measurements were performed with duplicate samples, and all experiments were repeated at least three times.

Supporting Information

Figure S1 Effect of FilI synthetic carboxyl-AHLs on the autophosphorylation of FilI and phosphotransfer from FilI to FilRs visualized on SDS-PAGE. (A) autophosphorylation of the recombinant FilI protein (2 μg) incubated with [γ-32P]ATP for 45 min at 37°C in the presence (+) or absence (−) of a carboxyl-AHL (N-carboxyl-C10-HSL, m/z 318, or N-carboxyl-C12-HSL, m/z 346) at final concentration 2 ng. (B) Phosphotransfer of the autophosphorylated FilI to His-tagged RRs (4 μg) for 5 min in the presence (+) or absence (−) of carboxyl-AHLs (N-carboxyl-C10-HSL, m/z 318, or N-carboxyl-C12-HSL, m/z 346) at final concentration 2 ng. Autophosphorylation and phosphotransfer reactions without FilI included as negative controls. Solid arrows indicate the phosphorylated proteins: pi-FilI, pi-FilR1 and pi-FilR2. Dotted arrows indicate nonspecific bands. (TIF)

Figure S2 EMSAs showed phosphorylation enhancing the DNA binding affinity of FilR1. Fil protein (5 pmol) was first autophosphorylated by incubation with ATP (50 pmol) for 45 min at 37°C, and then mixed with 0, 0.1, 0.5, 1, and 2.5 pmol FilR1 for 10 min, respectively. The mixtures including phosphorylated FilR1 protein were incubated with 0.5 nM of biotin-labeled DNA in the standard binding reaction mixture at 25°C for 20 min before electrophoresis on native PAGE. The final concentrations of the FilR1 proteins for EMSA were shown at the top of each lane. (A) P_acs1, promoter of the acs1 operon; (B) P_FilI, promoter of the filI operon. FilR1, purified recombination FilR1 protein; Pi-FilR1, FilR1 protein incubated with autophosphorylated Fil protein before EMSA. (TIF)
Figure S3 Schematic architecture of the predicted promoter of mtr. Two transcription start sites (TSS), TSS1 (at −20 nt) and TSS2 (defined as +1), were predicted upstream the mtr coding region. Predicted TATA box, BRE and bacteria promoter character −55 region are shadowed, and the distances (nt) from TSS2 are indicated. TATA box (AATTAA) was mutated by a substitution of GGACCC in the experiment of E. coli RNA polymerase recognizing archaeal promoters.

(TIF)

Table S1 Genes of M. harundinacea 6ac studied.
(PDF)

Table S2 Promoter searching for some genes of M. harundinacea 6ac using Neural Network Promoter Prediction Program.
(PDF)

Table S3 Effect of mutation of predicted TATA box in archaean promoters on their expression in the E. coli ex vivo reporter system.
(PDF)

References