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Genomics update

Control of methionine metabolism by the SahR transcriptional regulator in Proteobacteria

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Summary

Sulphur is an essential element in the metabolism. The sulphur-containing amino acid methionine is a metabolic precursor for S-adenosylmethionine (SAM), which serves as a coenzyme for ubiquitous methyltrtansferases. Recycling of organic sulphur compounds, e.g. via the SAM cycle, is an important metabolic process that needs to be tightly regulated. Knowledge about transcriptional regulation of these processes is still limited for many free-living bacteria. We identified a novel transcription factor SahR from the ArsR family that controls the SAM cycle genes in diverse microorganisms from soil and aquatic ecosystems. By using comparative genomics, we predicted SahR-binding DNA motifs and reconstructed SahR regulons in the genomes of 62 Proteobacteria. The conserved core of SahR regulons includes all enzymes required for the SAM cycle: the SAH hydrolase AhcY, the methionine biosynthesis enzymes MetE/MetH and MetF, and the SAM synthetase MetK. By using a combination of experimental techniques, we validated the SahR regulon in the sulphate-reducing *Deltaproteobacterium Desulfovibrio alaskensis*. SahR functions as a negative regulator that responds to the S-adenosylhomocysteine (SAH). The elevated SAH level in the cell dissociates SahR from its DNA operators and induces the expression of SAM cycle genes. The effector-sensing domain in SahR is related to SAM-dependent methylases that are able to tightly bind SAH. SahR represents a novel type of transcriptional regulators for the control of sulphur amino acid metabolism.

Introduction

Sulphur is an ubiquitous element both in bacterial and eukaryotic cells. The sulphur-containing amino acids methionine and cysteine are essential for protein synthesis, but also play other important roles in microbial metabolism. There are several alternative pathways for *de novo* methionine biosynthesis in bacteria that use homoserine and either cysteine or inorganic sulphur to synthesize homocysteine (Sekowska *et al*., 2000; Rodionov *et al*., 2004a) (Fig. 1). Methionine is a metabolic precursor for S-adenosylmethionine (SAM), which serves as an essential cofactor for a variety of SAM-dependent methyltransferase reactions involved in DNA and RNA metabolism, protein post-translational modifications, as well as in formation of variety of small metabolites. The main product of these ubiquitous reactions, S-adenosylhomocysteine (SAH), is a strong inhibitor of SAM-dependent methytransferases (Clarke and Banfield, 2001), which needs to be rapidly eliminated by one of two recycling pathways in bacteria. First, SAH can be directly degraded to adenosine and homocysteine using the AhcY hydrolase. Alternatively, the SAH nucleosidase Mtn can produce S-ribosylhomocysteine, which is then converted to homocysteine and 4,5-dihydroxypentan-2,3-dione using a LuxS-like lyase. The latter product serves as a precursor of N-acylhomoserine lactones, which are part of the quorum-sensing mechanism in Proteobacteria. To maintain a proper cellular balance of SAM, SAH and methionine, the SAM recycling and methionine biosynthesis pathways are tightly regulated on the transcriptional level in bacteria.

Although the methionine metabolism is mostly conserved among Bacteria, its transcriptional regulation is rather labile and involves multiple RNA and DNA regulatory systems (Rodionov, 2007). In particular, in

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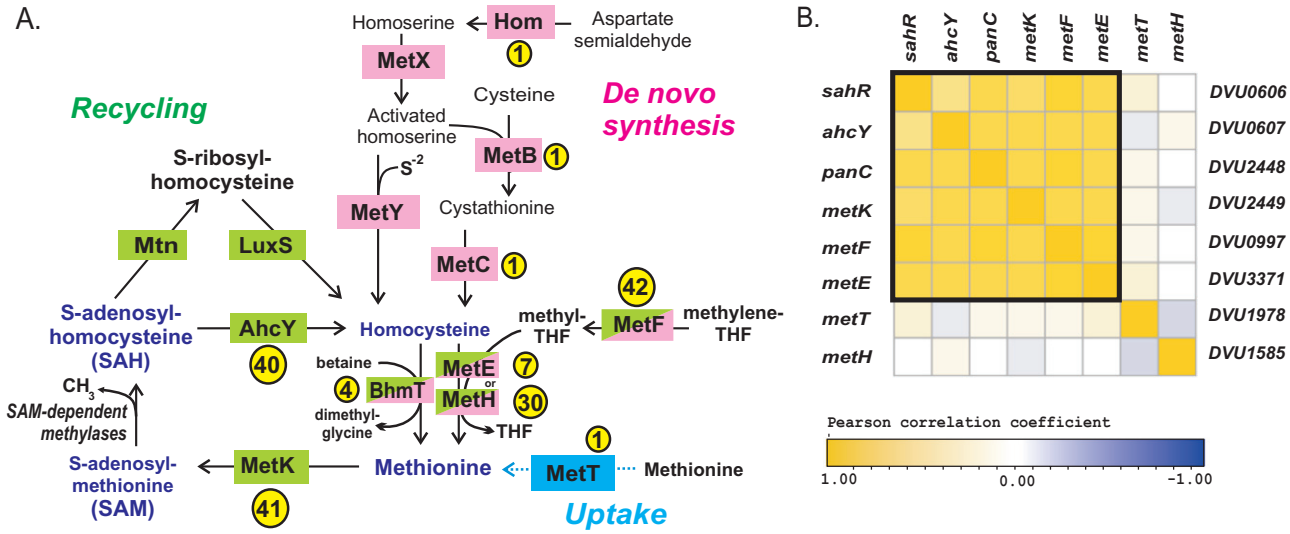

Fig. 1. Metabolic content of SahR regulons in bacterial genomes.
A. Methionine biosynthesis and SAM cycle pathways in 62 studied genomes of Proteobacteria. Genes controlled by SahR regulators are indicated by yellow circles with a number showing the number of genomes where the target gene is preceded by a candidate SahR-binding site.
B. SahR regulon in the sulphate-reducing bacterium DvH. Heat map of gene expression correlations for the methionine metabolic genes in DvH. Pairwise gene correlations were calculated in the MicrobesOnline web resource based on the expression of each gene in 383 microarray experiments. The shading of each box corresponds to the Pearson correlation coefficient between the gene pair according to the scale shown below the heat map. Genes from the SahR regulon are boxed.

most Firmicutes, it is controlled by the SAM-sensing riboswitch (SAM-I or S-box) and the methionine T-box mechanisms (Rodionov *et al.*, 2004a), whereas in the Streptococci group the methionine regulon is operated by the homocysteine-responsive regulator MtaR (LysR family) (Ravcheev *et al.*, 2013). In *Escherichia coli* and related γ-*Proteobacteria*, two transcription factors, MetJ and MetR, are implicated in the control of the methionine metabolism (LaMonte and Hughes, 2006; Rodionov *et al.*, 2011). The SAM-dependent repressor MetJ controls all methionine biosynthesis genes, the methionine uptake transporters *metNPQ* and *metT*, and the SAM synthetase gene *metK* (Augustus and Spicer, 2011). In addition, the homocysteine-responsive activator MetR activates the methionine synthase genes *metE* and *metH* in Enterobacteria and Vibrionales (Bogard *et al.*, 2012). In the actinobacterium *Corynebacterium glutamicum*, the SAH-responsive repressor McbR (TetR family) co-regulates the methionine biosynthesis, sulphur assimilation and cysteine biosynthesis genes (Rey *et al.*, 2005). At least six classes of riboswitches that sense SAM, or its metabolic derivative SAH, are known (Wang and Breaker, 2008). Similarly to SAM-I in Firmicutes, SAM-II riboswitches control the methionine biosynthesis genes and *metK* in some lineages of Proteobacteria and Bacteroidetes. SAM-III was found only in Lactobacillales controlling a single *metK* gene, whereas SAM-IV regulates the *de novo* homocysteine synthesis pathway in Actinobacteria. A distinct class of riboswitch that sense SAH, and strongly discriminate against SAM, was found to control some SAM cycle genes in several γ- and β-*Proteobacteria* (Wang *et al.*, 2008).

Transcriptional regulation of the SAM/methionine metabolism is poorly understood in other divisions of the Proteobacteria phylum, including numerous free-living microorganisms from the α and δ divisions. In our previous analysis of transcriptional regulatory elements in the genomes of δ-*Proteobacteria*, we described the SAM-I regulon controlling the *de novo* methionine biosynthesis genes *metIC* and *metX* in *Geobacter* spp. (Rodionov *et al.*, 2004b); however, regulatory mechanisms for other methionine metabolism genes in these and many other proteobacterial genomes remained unknown. In the current study, we identify a novel ArsR-family transcription factor (termed SahR) that controls the methionine biosynthesis and SAM cycle in most lineages of α- and δ-*Proteobacteria*, as well as in some γ-*Proteobacteria*. We report the bioinformatic identification of DNA-binding motifs for SahR regulators and provide a detailed description of corresponding regulons in the genomes of 62 bacteria from nine proteobacterial lineages. By using a combination of experimental techniques, we validated the predicted SahR-dependent regulatory network in the sulphate-reducing *Desulfovibrio* spp. and confirmed that SahR functions as a SAH-responsive transcriptional repressor of the SAM cycle genes.

Results

Genomic reconstruction of SahR regulons in Proteobacteria

Orthologs of the *sahR* gene are broadly distributed in the genomes of δ-, α-, and γ-*Proteobacteria*, where they are often co-localized with the *ahcY*, *metF*, *metH* and *metK* genes implicated in the SAM cycle (Fig. 2). Among γ-*Proteobacteria*, SahR was identified in only 3 out of 10 families (all *Pseudomonadales* species, and some *Alteromonadales* and *Oceanospirillales* species). Among α-*Proteobacteria*, SahR is present in most lineages except *Rhodobacteraceae* and *Bradyrizobiaceae*, whereas in δ-*Proteobacteria* its presence is limited to *Desulfovibrionales* and *Desulfuromonadales*. Interestingly, additional *sahR* orthologs were found in *Leptospira* spp. (the *Spirochaetes* phylum), where they co-transcribe with the *ahcY* and *metH* genes. The phylogenetic analysis of SahR proteins suggests that the *sahR* genes in *Leptospira* were likely obtained via horizontal gene transfer from δ-Proteobacteria.

To perform the comparative genomics reconstruction of SahR regulons, we selected the reference set of 62 genomes from nine taxonomic groups of Proteobacteria that are available in the RegPredict Web server (Table S2). By applying the DNA recognition procedure to a training set of upstream regions of *sahR*-containing operons from three subdivisions of Proteobacteria, we have identified its candidate DNA-binding motifs in each of these groups (Fig. 2). The predicted SahR operators in Proteobacteria share a similar 20 bp palindromic structure, which is common for other characterized regulators from the ArsR family. The candidate SahR binding motifs in δ- and γ-*Proteobacteria* have a common consensus (ATATC AAnnnnnnTTTGATAT), whereas in α-*Proteobacteria*, the modified SahR binding motif has consensus AYATAAAG nnnnCTTTATRT (where Y and R denote C or T and A or G respectively).

To find new members of the SahR regulon, the derived lineage-specific profiles were used to scan the *sahR*-containing genomes. The content of reconstructed SahR regulon is quite uniform in the representatives of all three Proteobacteria groups, although individual genomes may have between one and five target sites controlling up to five target operons (Table S2). Among the predicted target genes the most conserved regulatory interaction is an autoregulation of the SahR (*sahR* is preceded by a SahR binding site in 60 out of 62 studied genomes). Other highly conserved targets of SahR include the *ahcY* (40 cases), *metK* (41 cases), *metF* (42 cases) and *metH* genes (30 cases) that are all involved in SAM cycle pathway (Fig. 1).

Among other less conserved candidate members of SahR regulons, there are methionine synthases of two other types, *metE* in seven genomes and *bhmT* in four genomes. In addition, two lineage-specific expansions of the SahR regulon include the *de novo* methionine biosynthesis operon *hom-metBC* in *Oceanicaulis alexandrii* and the methionine transporter *metT* in *Desulfovibrio alaskensis*. Interestingly, in five out of nine *Desulfovibrionales* genomes, the SahR-regulated SAM synthetase *metK* gene is co-transcribed with the pantothenate synthetase gene *panC*, whereas in the remaining four genomes only the *metK* but not *panC* gene is regulated by SahR.

Structural and functional analysis of SahR proteins

The SahR proteins belong to the ArsR family of transcription factors that have a characteristic DNA-binding domain on their N terminus (Pfam accession number PF01022). In agreement with this fact, the identified SahR-binding DNA motifs share a common consensus (nnATnAAnnnnnnTTnATnn) with DNA motifs of ArsR repressors from Actinobacteria, Cyanobacteria and Bacilli (see the ArsR family in the RegPrecise database). However, the C-terminal domains in SahR proteins are quite unusual for ArsR-family regulators, as they are homologous to SAM-dependent enzymes catalysing the methyl transfer reactions (Pfam accession number PF01209). We propose that the methylase-like domains in SahR regulators are involved in the SAH effector sensing. SAH, as a product of the methylation reactions, binds more tightly to methylases than does SAM. We compared effector-binding domains in SahR proteins with a homologous methylase, TT1324 from *Thermus thermophilus*, whose tertiary structure was solved in complex with SAH (PDB accession number 2GS9). Protein sequence comparison revealed conservation in SahR proteins of eight residues involved in SAH binding in TT1324 (Glu43, Gly45, Gly47, Glu64, Ser66, Met69, Gly87 and Glu88), suggesting that SahR regulators can potentially bind SAH as an effector (Fig. S1).

SahR regulators in Proteobacteria represent another example of metabolite-sensing transcription factor emerging via a fusion between DNA-binding and enzymatic domains. Among other regulators in bacteria 'designed' using similar principles are the biotin repressor BirA, which represents a fusion of functional biotin ligase and a DNA-binding domain (Rodionov *et al.*, 2002); NrtR-family regulators that contain an effector-sensing domain, which is homologous to adenosine diphosphate (ADP)-ribose pyrophosphatases (Rodionov *et al.*, 2008); and sugar-responsive repressors from the ROK family that contain a sugar kinase-like sensor domain (Kazanov *et al.*, 2013).

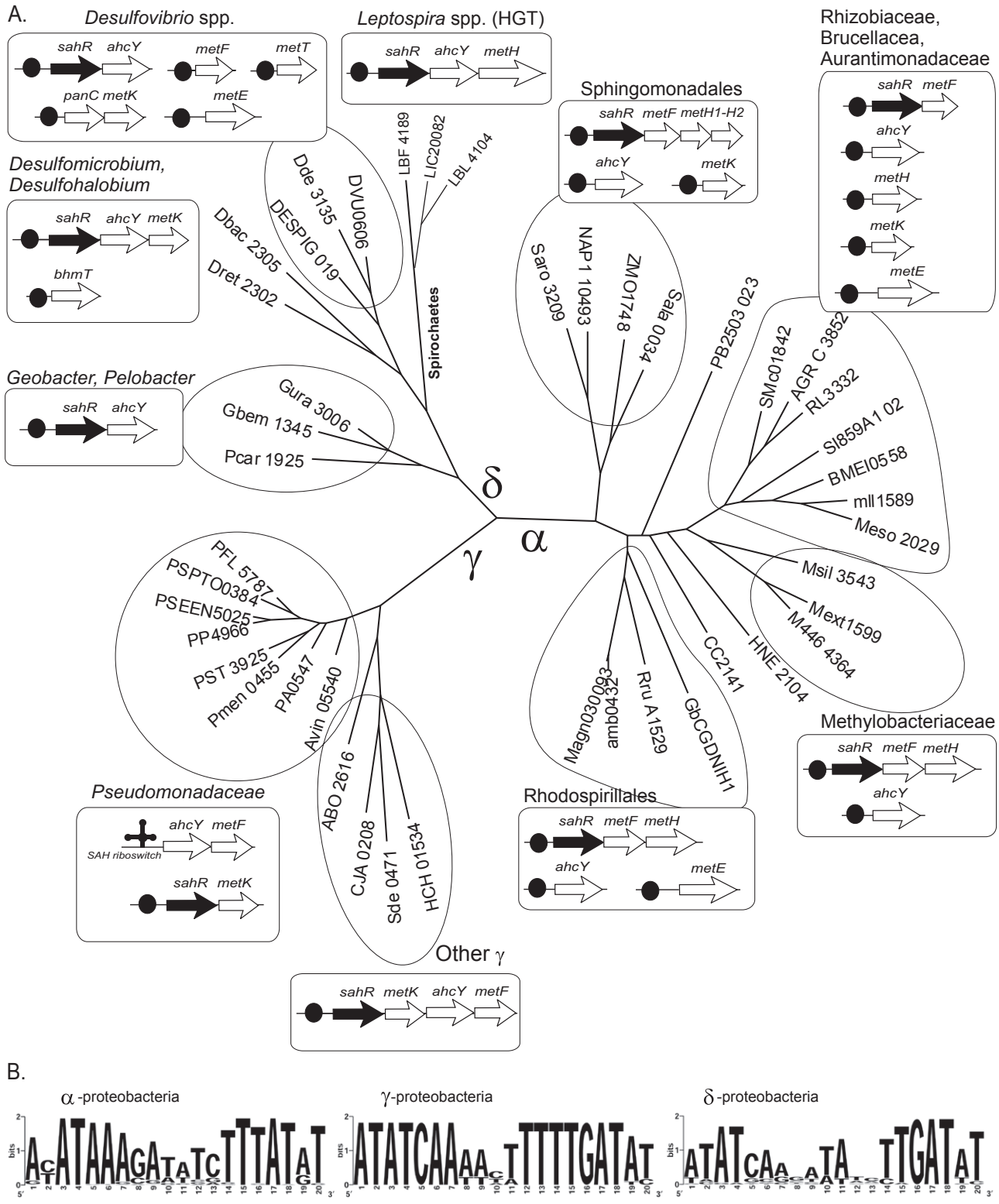

Fig. 2. Comparative genomics analysis of SahR proteins and regulons in bacterial genomes.
A. Maximum likelihood phylogenetic tree for SahR proteins in the selected studied genomes of δ-, α-, and γ-*Proteobacteria*, and in *Leptospira* spp. Genomic context of *sahR* regulators and the content of reconstructed SahR regulons are shown for each SahR branch. Candidate SahR-regulated genes and SahR-binding sites are shown by arrows and black circles respectively.
B. Sequence logos for the predicted binding DNA-motifs of SahR in three taxonomic divisions of Proteobacteria.

Systems biology validation of SahR regulon in Desulfovibrio *spp.*

To assess the reconstructed SahR regulon in the sulphate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough (DvH), we used the publicly available microarray expression profiles in the MicrobesOnline database (Dehal *et al*., 2010), containing the results of at least 383 experiments for DvH grown under different conditions. We calculated pairwise Pearson correlations for the predicted SahR target genes and two other methionine-related genes, the methionine synthase *metH* and methionine transporter *metT*, which do not belong to the predicted SahR regulon. The obtained expression correlation matrix demonstrates good correlation between all predicted members of the SahR regulon (Fig. 1B). As expected, the expression of *metH* and *metT* is not correlated with the SahR regulon genes. Thus, the predicted SahR regulon in DvH is consistent with a large-scale expression data set.

Because in most cases SahR-binding sites are located close to the translational start sites of the proximal regulated genes, we propose that SahR acts as a transcriptional repressor by sterically interfering with RNA polymerase binding to a promoter. We analysed the genome-wide sets of transcripts experimentally determined using high-resolution tiling microarrays and 5′ RNA sequencing in DvH (Price *et al*., 2011) and *D. alaskensis* G20 (J.V. Kuehl, unpubl. data). For all target genes, the predicted SahR operators either overlap with or are located downstream of the transcription start sites and their respective −35 and −10 promoter regions (Table S1). Also, the structure of predicted SahR-regulated operons, including *panC-metK* and *sahR-ahcY*, is corroborated by the results of high-throughput transcript determination in the two *Desulfovibrio* species. These results suggest that SahR is a negative regulator of its target operons.

Experimental validation of SahR regulon in D. alaskensis *G20*

The predicted specificities towards DNA sites and potential effectors were further assessed *in vitro* using fluorescence polarization assay (FPA) and electrophoretic mobility shift assay (EMSA) binding assays with the recombinant SahR (Dde_3135) protein from *D. alaskensis* G20 and the synthetic-labelled DNA fragments containing predicted SahR operator sites (Table S1). By using FPA, we assessed the specific binding of five predicted SahR-binding sites from *D. alaskensis* G20 with increasing concentrations of SahR protein (Fig. S2). All tested DNA fragments except *Dde_3135* demonstrated the SahR concentration-dependent increase of fluorescence polarization, confirming specific interactions between SahR and DNA. The apparent K_d values for SahR protein interacting with the tested DNA fragments were in the range of 28–68 nM (Table S1). Additional EMSA experiments confirmed the specific binding between the recombinant SahR protein and its cognate operators (Fig. S2). The SahR concentration-dependent shift of DNA bands was observed with four tested DNA fragments (2 nM) containing SahR-binding sites upstream of the *Dde_2476*, *Dde_1498*, *Dde_2525* and *Dde_3135* genes, whereas the negative control DNA fragment was not shifted.

To test the influence of potential effectors on the interaction between the SahR protein and its DNA sites, we performed another EMSA experiment. Binding of the purified SahR with the *Dde_2328* DNA fragment was assessed in the presence of intermediary metabolites associated with the SahR-regulated metabolic pathway. SAH, homocysteine and methionine demonstrated suppression of the SahR-DNA binding-mediated shift, whereas the effect of SAM was not detected (Fig. S2). The most pronounced negative effect on SahR-DNA complex formation was measured for SAH, which can affect the binding even at minimal concentration of 0.5 mM, whereas ~fourfold higher concentration of homocysteine or methionine was required to have a comparable effect on the complex formation. These results suggest that SahR is a SAH-responsive regulator that binds specifically to the predicted operator sites in *D. alaskensis* G20.

To validate the predicted negative effect of SahR on gene expression *in vivo*, we examined two mutant strains of *D. alaskensis* G20 that have a transposon inserted within the *sahR* gene. We compared gene expression in these mutants with gene expression in G20 with intact *sahR* (Fig. S3). The predicted targets of SahR tended to be up-regulated in the *sahR* mutant (between 1.9- and 4.6-fold), as expected if SahR functions as a repressor. The expression of many other genes was also changed, and the overall correlation of $\log_2$ expression levels between the *sahR*$^-$ and G20 strains was just 0.66. Because the differences tended to be consistent within operons (correlation of 0.79), they do not appear to be experimental noise. We found that several other mutants, in functionally unrelated genes, had expression patterns that were similar to the *sahR*$^-$ strains ($r > 0.9$). We suspect that there are non-specific effects on expression, perhaps because the *sahR*$^-$ and other mutant strains grow slightly more slowly than the G20 strain. We used linear regression on the $\log_2$ expression from the other strains to fit the $\log_2$ expression for the *sahR*$^-$ strains. This should separate the generic effects on growth from the specific effects of the mutation in SahR. Indeed, the regression fit very well: the correlation was very high ($r = 0.99$), and there were few outliers aside from predicted targets of SahR. Most of the predicted targets of SahR were expressed

more highly than expected in both *sahR*⁻ strains. The only exception was *ahcY*, which is downstream of *sahR* and is co-transcribed with it: the expression of *ahcY* in the *sahR*⁻ mutant may be affected by polar effects. Overall, the expression analysis confirmed that SahR is a repressor and that the regulon inferred by comparative genomics is accurate.

Conclusions

Transcriptional control of the methionine metabolism in bacteria is mediated by a variety of regulatory systems, including both DNA-dependent regulators (e.g. transcription factors) and RNA regulatory elements (e.g. riboswitches). The comparative genomic approach was applied to identify and characterize a novel SahR transcription factor for control of the SAM cycle pathway in 62 diverse Proteobacteria. The cognate DNA motifs of SahR orthologs have a common consensus in δ- and γ-*Proteobacteria*, whereas α-*Proteobacteria* possess a slightly different consensus (Fig. 2). The *in silico* predicted SahR regulon was tested by a combination of *in vitro* and *in vivo* techniques in two sulphate-reducing *Desulfovibrio* species. The results of these experiments confirmed that SahR is a negative regulator that binds its DNA operators in the absence of effector, whereas the addition of SAH disrupts the SahR-DNA complex and induces the target gene expression (Fig. 3). SahR is analogous to another SAH-responsive regulatory system, SAH riboswitch, which also controls the SAM cycle genes including *ahcY* (Sun *et al.*, 2013). Interestingly, these two regulatory systems not only function by different mechanisms (DNA and RNA-dependent), but also utilize two opposite modes for SAH-dependent control. Thus, SAH riboswitch activates gene expression in the presence of SAH (Wang *et al.*, 2008), while SahR functions as a transcriptional repressor in the absence of SAH.

The SAH-responsive regulator SahR plays an important role in the transcriptional control of the SAM cycle in bacteria. SAH is the product of ubiquitous SAM-dependent methyl transfer reactions in the cell. The cellular level of SAH directly reflects the consumption of SAM. Most SAM-dependent methylases are strongly inhibited by a low concentration of SAH. The SAH hydrolase AhcY is one of the major targets of SahR regulons in Proteobacteria (Fig. 1). These facts suggest that the physiological function of SahR is to sense and dispose of the excess of SAH in the cell. Our *in vitro* experiments suggest that SahR discriminates between SAH and SAM, and its DNA-binding activity is affected by the first metabolite but not by its methylated precursor. Thus, the SahR-dependent metabolic regulation should maintain a high SAM/SAH ratio in the cell. Homocysteine, the product of SAH hydrolysis, is reutilized for the biosynthesis of methionine and SAM by using several downstream enzymes that are also transcriptionally regulated by SahR (Fig. 1). Interestingly, EMSA experiments demonstrated that homocysteine and methionine are weakly active as inducers for SahR as they are able to disrupt the SahR-DNA complex at higher concentrations compared with SAH. Further experiments are required to test the *in vivo* effect of SahR regulator on the level of SAH cycle intermediates.

Experimental procedures

Full version of experimental procedures and any associated references are available in the Supporting Information online.

Bioinformatics techniques

For SahR regulons reconstruction, we used the established comparative genomics approach based on identification of candidate regulator-binding sites in closely related bacterial genomes implemented in RegPredict Web server tool (Novichkov *et al.*, 2010b). The details of reconstructed SahR regulons are captured in RegPrecise database (Novichkov *et al.*, 2010a).

Cloning and expression of sahR

The *sahR* gene from *D. alaskensis* G20 was amplified by polymerase chain reaction and inserted into the pSMT3 vector. The recombinant SahR protein containing an N-terminal six-histidine and Smt3 polypeptide fusion tag (to enhance protein solubility) was overexpressed in *E. coli* and purified using Ni^{2+}-chelating chromatography followed by gel filtration, and the tag was then cleaved from the SahR protein.

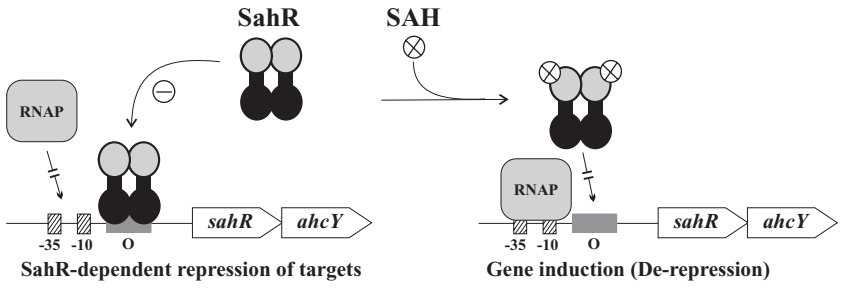

Fig. 3. Model of SahR-dependent regulation in *Desulfovibrio* spp. In the unliganded form, SahR binds to the operator (*O*) regions in the target operons and inhibit their transcription. When the product of SAM-dependent methyl transfer reactions, SAH, is accumulated in the cell, it causes SahR to dissociate from the DNA, which induces the transcription of its target genes.

DNA binding assays with SahR

The interaction of the purified recombinant SahR protein with its cognate DNA-binding sites in *D. alaskensis* G20 was assessed using two techniques: EMSA and FPA, with 40 bp synthetic fluorescein-labelled DNA fragments labelled with 6-carboxyfluorescein (Table S1). The effect of methionine, SAM, SAH and homocysteine on the protein–DNA interaction was tested by EMSA.

Whole-genome transcriptional analysis

Microarray gene expression measurements were conducted for *D. alaskensis* G20 and the respective mutant strains containing transposon insertions within the *sahR* gene. In addition, nine other mutant strains containing transposon insertions within other genes were used to perform the statistical analysis of expression patterns of the SahR regulon genes.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Multiple alignment of effector-binding domains in SahR proteins and the SAM-dependent methylase TT1324.
Fig. S2. *In vitro* assesment of interactions of SahR with cognate DNA operators in *D. alaskensis* G20.
Fig. S3. *In vivo* assesment of SahR regulon in *D. alaskensis* G20.
Table S1. SahR-binding sites in *Desulfovibrio* spp. and their *in vitro* validation in *D. alaskensis* G20.
Table S2. Distribution of SahR orthologs and reconstructed regulons in Proteobacteria.
Methods S1. Bioinformatics and experimental methods for identification and characterization of SahR regulons.