Phosphorylation of the acyl-CoA binding pocket of the FadR transcription regulator in Sulfolobus acidocaldarius

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Highlights

• FadR is phosphorylated within its acyl-CoA-binding pocket
• The eukaryotic-type kinase ArnC phosphorylates FadR in vitro at threonine 134
• The phosphomimetic mutant of FadR is less sensitive for acyl-CoA
• FadR phosphorylation may control metabolism and chromatin conformation

Abstract

The archaeal model organism Sulfolobus acidocaldarius possesses a TetR-like transcription factor that represses a 30-kb gene cluster encoding fatty acid metabolism enzymes. Interaction of this regulator, FadR, with acyl-CoA molecules causes a DNA dissociation, which may lead to a derepression of the gene cluster. Previously, a phosphoproteome analysis revealed the phosphorylation of three consecutive amino acids in the acyl-CoA ligand binding pocket. Here, we study this phosphorylation event and show that ArnC, a Hanks-type protein kinase, targets a threonine within the phosphoacceptor motif in vitro. Electrophoretic mobility shift assays using a phosphomimetic mutant of FadR demonstrate that the presence of negatively charged groups on the phosphoacceptor motif causes an inhibition of the ligand binding that desensitizes the responsiveness of the regulator to acyl-CoA molecules. Based on these observations, we propose a model in which phosphorylation of FadR in its ligand-binding pocket acts as an additional regulatory layer silencing acyl-CoA responsive derepression of fatty acid and lipid degradation. Moreover, given the recently discovered interplay between FadR and the chromosome structuring protein Coalescin, FadR phosphorylation could also influence local chromosome conformation under specific cellular conditions.

Keywords

protein phosphorylation – transcription factor – FadR – Sulfolobus – archaea – acyl-CoA
1. Introduction

Protein phosphorylation is an abundant posttranslational modification in the thermoacidophilic crenarchaeon Sulfolobus acidocaldarius and the related species Saccharolobus solfataricus, as evidenced by phosphoproteomics [1,2]. About ten kinases have been identified in S. acidocaldarius, belonging to the families of the typical eukaryotic-like Hanks-type protein kinases (ePK) or of the atypical protein kinases (aPK) [3]. Collectively, these kinases are responsible for the phosphorylation of a large number of target proteins: at least 801 unique phosphoproteins, belonging to various functional classes, including 18 transcription regulators [2,3]. This suggests that direct phosphorylation of single-component transcription regulators constitutes an important signal transduction mechanism in S. acidocaldarius, especially given the complete absence of two-component systems in Crenarchaeota [3], which are regarded as the bacterial paradigm of phosphorylation-mediated transcription regulation. With the exception of phosphorylation of regulators acting on the expression of the archaellum [4], the archaeal motility structure, and on biofilm formation in S. acidocaldarius [5], very little is currently known about the functional role of phosphorylation of single-component regulators in this organism.

Archaea harbor typical bacterial-like transcription regulators [6], exemplified by the TetR-family regulator FadRsa in S. acidocaldarius [7]. FadRsa is a transcriptional repressor of genes involved in lipid and fatty acid catabolism. In response to interacting with long-chain acyl-CoA molecules, the regulator dissociates from DNA resulting in a derepression. FadRsa is capable of exerting long-range repressive effects on a 30-kb gene cluster comprising 23 genes by binding to only four genomic binding sites, in contrast to bacterial TetR-family FadR regulators, for which such a regulatory mechanism has never been observed [7]. Although the exact underlying mechanism is unknown, a correlation between binding of FadRsa and of the chromosome structuring factor coalescin has been observed [8], which may explain such a global mode of repression. In the phosphoproteomics reported in [2], FadRsa was found to be phosphorylated. Here, we report on the impact of phosphorylation on FadRsa function and discuss how this process may impact the regulation of fatty acid and lipid metabolism in S. acidocaldarius and possibly even chromosome organization.

2. Materials and methods

2.1 Cloning, site-directed mutagenesis and protein purification

Kinase genes Saci_0965, Saci_1193 (arnC) and Saci_1694 (arnD) were PCR-amplified from genomic DNA of S. acidocaldarius and cloned into pET28b plasmid vector using the restriction sites Ndel/BamHI (Saci_0965), NheI/HindIII (arnC) and Ndel/EcoRI (arnD), respectively, enabling expression of N-terminally His-tagged proteins. Kinase gene Saci_1041 was cloned into the vector pET-DueT-1, omitting the transmembrane domains by PCR to improve protein
solubility. To generate a triple phosphomimetic mutant \((Y^{133}\text{D}-T^{134}\text{E}-T^{135}\text{E})\) of FadR\textsubscript{Sa},
\[
\text{FadR}_{\text{Sa}}^{\text{PM}},
\]
site-directed mutagenesis of the \(fadR_{\text{Sa}}\) coding region was performed with the
overlap PCR mutagenesis approach \cite{9} using pET24ax\textit{fadR}_{\text{Sa}}Ndenu\textit{ll} \cite{7} as a template.
Similarly as for the wild-type (WT) FadR\textsubscript{Sa} protein, the resulting construct enables expression of
a C-terminally His-tagged recombinant protein. All oligonucleotides, plasmids and strains used
in this work are provided in \textbf{Supplementary Tables S1, S2 and S3}, respectively.
All recombinant proteins were heterologously expressed in \textit{Escherichia coli} employing the \(E.\ 
\text{coli}\) strains Rosetta (DE3) (FadR\textsubscript{Sa}, FadR\textsubscript{Sa}^{\text{PM}}, Saci0965 and ArnD) or BL21 CodonPlus (ArnC).
This was followed by purification with an affinity chromatography approach as described \cite{7}
with the following exceptions for the kinases Saci0965, ArnC and ArnD: induction of
heterologous proteins was performed by adding 1 mM isopropyl \(\beta\)-\textit{D}-\textit{1-thiogalactopyranoside}
to the cultures at an optical density (OD\textsubscript{600}) of 0.7, followed by overnight incubation at 37°C.
The resulting cell extracts were not subjected to heat treatment as was done for FadR\textsubscript{Sa} and
its mutant variant. Saci1041 was, after dialysis against 20 mM Tris-\textit{HCl} [pH 7.0], further
subjected to anion exchange chromatography (UNO Q-17) using a linear salt gradient to 1 M
NaCl. Subsequently, Saci1041, as well as ArnC and ArnD, were further purified using a size
exclusion chromatography step with a HiLoad\textsuperscript{®} Superdex 200 16/60 column and an ÄKTA
Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare Life Sciences). All protein
preparations with the exception of Saci1041 were ultimately dialyzed using storage buffer (20
mM Na\textsubscript{2}HPO\textsubscript{4}/NaH\textsubscript{2}PO\textsubscript{4} [pH 7.4], 0.5 M NaCl). The Saci1041 preparation was stored in 50 mM
Tris-\textit{HCl} [pH 7.0], 0.3 M NaCl.

\textbf{2.2 In vitro phosphorylation assay}
Between 0.2 and 4 μg of purified proteins were incubated in a total volume of 30 μl reaction
buffer (25 mM Tris-\textit{HCl} [pH 7.4], 100 mM NaCl, 10 mM MgCl\textsubscript{2} and 1 mM 1,4-dithiothreitol
(DTT)) containing 100 μM non-labeled adenosine triphosphate (ATP) and 33 nM (3 μCi) \(^{32}\text{P}\).
\(\gamma\)-ATP (3000 Ci mmol\textsuperscript{-1}, PerkinElmer). Reactions were incubated for 30 minutes at 65°C and
stopped by adding 5 μl 4x NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) and by
heating at 95°C for 5 minutes. Subsequently, proteins were separated by sodium dodecyl
sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie-stained.
Autoradiograph imaging was performed by drying the gel and employing a Personal Molecular
Imager system (Bio-Rad Laboratories).

\textbf{2.3 Mass spectrometry analysis}
Experimental procedures for mass spectrometry analyses are provided in \textbf{Supplementary
Materials} (\textbf{Supplementary Methods}).
2.4 Electrophoretic mobility shift assay

In vitro protein-DNA binding assays were performed using \(^{32}\)P-radiolabeled DNA fragments harboring the \(Saci\_1106\) promoter region [7]. One primer was first labeled with \(^{32}\)P-\(\gamma\)-ATP (Perkin Elmer) with the help of T4 polynucleotide kinase (Thermo Scientific). A PCR was then performed with the labeled and the non-labeled primers (Supplementary Table S1) after which amplified double stranded (ds) DNA fragments were purified by acrylamide gel electrophoresis. Binding assays were done using 4 μg FadR\(_{Sa}\) or FadR\(_{Sa}\)\(^{PM}\) proteins (at a final concentration of 6.8 μM), 1 μl radiolabeled DNA (15000 cpm/μl), and 0.5 μg sonicated salmon sperm DNA as competitor DNA in binding buffer (20 mM Tris-HCl [pH 8.0], 0.1 mM DTT, 1 mM MgCl\(_2\), 12.5% glycerol, 50 mM NaCl, 0.4 mM EDTA). Stearoyl-CoA was added to the reactions in an increasing concentration gradient (between 0.1 and 100 μM), using dimethylsulfoxide as a solvent to dissolve acyl-CoA. Reactions were incubated during 20 minutes at 37°C prior to an electrophoresis on a 6% native polyacrylamide gel. Visualization was achieved by autoradiography and scanning using a Bio-500 Microtek scanner. Integrated densities of free DNA bands were measured using ImageJ (NIH) and the values were normalized taking into account the background and then converted into DNA-bound fractions. The inhibition coefficient (IC\(_{50}\)) was calculated using Prism7 (GraphPad) by a non-linear fitting using an inhibitor vs. normalized response equation.

3. Results and Discussion

3.1 FadR\(_{Sa}\) is phosphorylated in its ligand-binding pocket \(in\) \(vivo\) and \(in\) \(vitro\)

FadR\(_{Sa}\) was previously found to be phosphorylated on three consecutive residues, Y\(^{133}\), T\(^{134}\) and T\(^{135}\), in a phosphoproteomic survey [2] (Figure 1A). These amino acids are located in the α7 helix within the ligand-binding pocket, as shown in the native FadR\(_{Sa}\) crystal structure (PDB 5MWR) [7]. This dimeric protein structure is characterized by an asymmetric ligand binding, with lauroyl-CoA present in the ligand-binding pocket of subunit B, but not subunit A [7]. A closer view at the lauroyl-CoA-FadR\(_{Sa}\) interactions in subunit B unveils an important role for Y\(^{133}\) in ligand binding at the initial part of the binding cavity, by establishing hydrophobic interactions with the pantothenic acid moiety of lauroyl-CoA and by forming a hydrogen bond with its \(\beta\)-mercapto-ethylamine group. The electron density map [7] indicated that Y\(^{133}\) in subunit A is phosphorylated (Figure 1B). This was an unexpected finding, since the protein was heterologously produced in \(E.\) \(coli\) [7]. Upon comparing the Y\(^{133}\) conformation in both subunits in the molecular model, it can be suggested that the presence of a phosphate group on Y\(^{133}\) could prevent Y\(^{133}\)-acyl-CoA interactions and that it might even sterically hinder the presence of acyl-CoA in the binding pocket.
That hypothesis prompted us to investigate which kinases might be responsible for FadR<sub>sa</sub> phosphorylation. A set of four <i>S. acidocaldarius</i> kinases (ArnC, ArnD, Saci1041 and Saci0965) were analyzed in an <i>in vitro</i> phosphorylation assay, and while all four kinases displayed autophosphorylation signals (Figure 1C), only the addition of the ePKs ArnC and Saci1041 generated additional phosphorylation signals at the level of FadR<sub>sa</sub>. In the autoradiograph, two different bands representing phosphorylated FadR<sub>sa</sub> were observed (Figure 1C). The lower-MW band (FadR<sub>sa</sub>′) was not observed in the corresponding Coomassie-stained SDS-PAGE (Figure 1C), however, it was observed upon loading higher FadR<sub>sa</sub> amounts for electrophoresis (Supplementary Figure S1). Mass spectrometry analysis indicated that both bands consisted of full-length FadR<sub>sa</sub> protein, suggesting that the lower-MW band represents a conformationally distinct FadR<sub>sa</sub> population. Despite that this FadR<sub>sa</sub> population represents a minor fraction, the Saci1041 kinase preferentially phosphorylated this conformational state of the protein in contrast to ArnC (Figure 1C). Upon evaluating the corresponding Coomassie-stained SDS-PAGE, there were no indications that phosphorylation causes a shift in the relative abundance of these two populations.

To identify the phosphorylated FadR<sub>sa</sub> residues, the proteins present in the reaction mixtures were subjected to mass spectrometry analysis (Figure 1A). After incubating FadR<sub>sa</sub> with either ArnC or Saci1041, residues T<sup>78</sup>, T<sup>80</sup> and T<sup>114</sup> were found to be phosphorylated. The first residue T<sup>78</sup> was also found to be phosphorylated in the non-treated FadR<sub>sa</sub> preparation, albeit with low localization probability, demonstrating that this phosphorylation was already present after heterologous expression in <i>E. coli</i>. In addition, ArnC targeted T<sup>134</sup> which is among the <i>in vivo</i> phosphorylated tripartite target site. Phosphorylation of Y<sup>133</sup>, which was reported in the previous phosphoproteome study [2], was not detected in this assay leaving the kinase responsible for the phosphorylation of that tyrosine in FadR<sub>sa</sub> unknown.

3.2 Phosphomimetic mutation of FadR<sub>sa</sub> sites targeted for phosphorylation inhibits ligand response

A triple phosphomimetic mutant of FadR<sub>sa</sub>, FadR<sub>sa</sub><sup>PM</sup> (Y<sup>133</sup>D-T<sup>134</sup>E-T<sup>135</sup>E), was prepared thereby introducing residues with a negative electrostatic potential to mimic the phosphorylation observed <i>in vivo</i> [2]. In electrophoretic mobility shift assays (EMSAs), it was observed that FadR<sub>sa</sub><sup>PM</sup> displays a similar DNA-binding behavior as the WT protein, albeit with a somewhat lower affinity (Supplementary Figure S2). Binding of acyl-CoA disrupts FadR<sub>sa</sub>−DNA complex formation by altering protein conformation [7]. In testing the hypothesis that phosphorylation inhibits acyl-CoA binding, EMSAs were performed with FadR<sub>sa</sub><sup>WT</sup> and FadR<sub>sa</sub><sup>PM</sup> (Figure 2). Whereas the addition of the long-chain acyl-CoA stearoyl-CoA abrogated DNA binding of FadR<sub>sa</sub><sup>WT</sup> at low concentrations (starting at 0.3 µM), FadR<sub>sa</sub><sup>PM</sup> was clearly less responsive with an inhibition coefficient (IC<sub>50</sub>) being about 56 times higher (Figure 2).
FadR<sub>Sa</sub><sup>PM</sup>-DNA complexes were therefore more resistant to disruption even at high acyl-CoA concentrations, suggesting that phosphorylation in the ligand-binding pocket renders FadR<sub>Sa</sub> insensitive to acyl-CoA binding.

4. Conclusions

FadR<sub>Sa</sub> is phosphorylated <i>in vitro</i> by two kinases, ArnC and Saci1041, with ArnC targeting a threonine in a three-residue stretch previously found to be phosphorylated <i>in vivo</i> [2] and located at the initial entry section of the acyl-CoA binding cavity. ArnC is considered to be a globally acting kinase in <i>S. acidocaldarius</i>, a master regulatory kinase in the archaellum regulatory network [10] and capable of phosphorylating almost all other kinases as shown in the related <i>Sulfolobus islandicus</i> [11]. It is unknown which kinase(s) target(s) Tyr<sup>133</sup>, which is ideally positioned to inhibit ligand binding upon phosphorylation as shown in the FadR<sub>Sa</sub> crystal structure [7]. Of note, a tyrosine kinase has not yet been identified in archaea [3]. Nevertheless, the observation that a phosphomimetic mutant of FadR<sub>Sa</sub> is less responsive to acyl-CoA <i>in vitro</i> strongly suggests that phosphorylation of this site <i>in vivo</i> acts as a signal to switch off ligand response of the regulator, resulting in a continuous repression of genes encoding lipid and fatty acid degradation. Given the interplay between FadR<sub>Sa</sub> and the chromosome-structuring factor coalescin [8], phosphorylation of the FadR<sub>Sa</sub> ligand-binding pocket could thus lead to maintaining a large 30-kb genomic region in a condensed and transcriptionally repressed state, regardless of intracellular acyl-CoA concentrations. Phosphorylation-mediated inhibition of ligand binding emerges as a unique mechanism not observed before for prokaryotic TetR-family regulators. Instead, bacterial single-component regulators are more commonly found to be phosphorylated in the helix-turn-helix domain thereby inhibiting DNA binding [12].

**Figure captions**

Figure 1. FadR<sub>Sa</sub> is phosphorylated in its acyl-CoA binding pocket. A. Secondary structure map of FadR<sub>Sa</sub> showing domain organization. Phosphorylated residues, observed <i>in vivo</i> [2] or <i>in vitro</i> by mass spectrometry analysis are indicated in a table, with their locations also annotated on the secondary structure map. The asterisk indicates that the phosphorylation was detected with low localization probability and that it only occurred on one of these possible residues. B. Cartoon representation of the FadR<sub>Sa</sub> crystal structure (PDB 5MWR) [7] with indication of the phosphorylated tyrosine (pY<sup>133</sup>) modeled in subunit A in a colored ball-and-stick representation, as well as a heptanoyl-CoA ligand modeled in subunit B. Zooms are presented for the ligand-binding pocket of each subunit. C. In <i>vitro</i> phosphorylation assay testing phosphorylation of FadR<sub>Sa</sub> by various S. <i>acidocaldarius</i> kinases. Following protein concentrations were used in the assay: 2.30 µM (FadR<sub>Sa</sub>), 0.51 µM (ArnC), 2.55 µM...
(Saci0965), 0.70 µM (ArnD) and 0.08 µM (Saci1041). The Coomassie-stained SDS-PAGE and autoradiographs are aligned to each other, with the two last lanes being displayed using the same autoradiograph but at a longer exposure time. Two electrophoretically different FadRsa species are annotated as FadRsa and FadRsa', respectively, with the second species not being visible in the SDS-PAGE (see also Supplementary Figure S1). The assay was performed three times with similar results. Only one of the assays is shown as a representative result.

**Figure 2.** The FadRsa$^{PM}$ mutant protein is less sensitive to acyl-CoA binding *in vitro* than the WT protein. Representative autoradiographs of electrophoretic mobility shift assays (EMSAs) of the binding of FadRsa$^{WT}$ (A) and FadRsa$^{PM}$ (B) to a 154-bp DNA fragment representing the operator/promoter region of the *Saci_1106* target gene in the presence of increasing stearoyl-CoA concentration. Calculated inhibition coefficient (IC$_{50}$) values, based on three replicate EMSAs, are displayed next to the autoradiographs.

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**References**


L. Hoffmann, A. Schummer, J. Reimann, M.F. Haurat, A.J. Wilson, M. Beeby, et al., 
Expanding the archaellum regulatory network - the eukaryotic protein kinases ArnC and 
ArnD influence motility of *Sulfolobus acidocaldarius*, MicrobiologyOpen, 6 (2017).

phosphorylation is a major functional determinant of the Lrs14-type biofilm and motility 
regulator AbfR1 in *Sulfolobus acidocaldarius*, Molecular Microbiology. 105 (2017) 777–
793.

L. Lemmens, H.R. Maklad, I. Bervoets, E. Peeters Transcription regulators in archaea: 
homologies and differences with bacterial regulators. J. Mol. Biol. 431 (2019) 4132-
4146.

transcription factor regulates fatty acid metabolism in the archaeal model organism 

N. Takemata, R.Y. Samson, S.D. Bell, Physical and functional compartmentalization of 

R. Higuchi, B. Krummel, R.K. Saiki, A general method of *in vitro* preparation and specific 
mutagenesis of DNA fragments: study of protein and DNA interactions, Nucleic Acids 

L. Hofmann, A. Schummer, J. Reimann, M.F. Haurat, A.J. Wilson, M. Beeby, W. 
Warscheid, S.-V. Albers, Expanding the archaellum regulatory network – the eukaryotic 
protein kinases ArnC and ArnD influence motility of *Sulfolobus acidocaldarius*, 
MicrobiologyOpen 6 (2017) e00414.

Q. Huang, Q. Zhong, J.B.A. Mayaka, J. Ni, Y. Shen, Autophosphorylation and cross-
phosphorylation of protein kinases from the crenarchaeon *Sulfolobus islandicus*. Front. 
Microbiol. 8 (2017) 2173.

A. Derouiche, V. Bidnenko, R. Grenha, N. Pigonneau, M. Ventroux, M. Franz-Wachtel 
et al, Interaction of bacterial fatty-acid-displaced regulators with DNA is interrupted by 
tyrosine phosphorylation in the helix-turn-helix domain, Nucleic Acids Res. 41 (2013) 
9371-9381.
Figure 1

A

DNA binding

acyl-CoA binding

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B

subunit A

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subunit A

subunit B

acetyl-CoA

pY133

pY133

C

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Coomassie-stained

autoradiograph

Figure 1
Figure 2

A

C18:0 (μM) - - 0.1 0.3 0.5 1.0 2.5 5.0 10 25 50
FadR_{Sa}^{WT} - + + + + + + + + +

FadR_{Sa}-DNA complex
free DNA

IC_{50} = 0.3±0.2 μM

B

C18:0 (μM) - - -
FadR_{Sa}^{PM} - + + + + + + +

FadR_{Sa}-DNA complex
free DNA

IC_{50} = 17±0.3 μM
Title of Paper: Phosphorylation of the acyl-CoA binding pocket of the FadR transcription regulator in Sulfolobus acidocaldarius

Author(s): Hassan R. Maklad, Gustavo J. Gutierrez, Dominik Esser, Bettina Siebers, Eveline Peeters

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Supplementary Materials

Phosphorylation of the acyl-CoA binding pocket of the FadR transcription regulator in *Sulfolobus acidocaldarius*

Hassan R. Maklad, Gustavo J. Gutierrez, Dominik Esser, Bettina Siebers and Eveline Peeters
**Supplementary Tables**

### Supplementary Table S1. Overview of oligonucleotides used in this work.

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<tr>
<td>EP333</td>
<td>5’-GAAGCTTTATCAGATCAGAATAGCTC-3’</td>
<td>Amplification <em>Saci</em>_1106 promoter fragment for EMSA</td>
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<td>EP411</td>
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<td>Cloning <em>fadR</em>&lt;sub&gt;PM&lt;/sub&gt; (NdeI restriction site)</td>
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<td>EP412</td>
<td>5’-CTTCGACTCCCCATATGGGATCT-3’</td>
<td>Cloning <em>fadR</em>&lt;sub&gt;PM&lt;/sub&gt; (Saci1106 promoter fragment for EMSA)</td>
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<tr>
<td>HM012</td>
<td>5’-GGAATTCCATATGGGATCT-3’</td>
<td>Mutagenic primer <em>fadR</em>&lt;sub&gt;PM&lt;/sub&gt;</td>
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<tr>
<td>HM013</td>
<td>5’-GGAATTCCATATGGGATCT-3’</td>
<td>Mutagenic primer <em>fadR</em>&lt;sub&gt;PM&lt;/sub&gt;</td>
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### Supplementary Table 2. Overview of plasmids used in this work.

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<td>pET24a</td>
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<td>Novagen</td>
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<tr>
<td>pET28b</td>
<td>Protein overexpression vector</td>
<td>Novagen</td>
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<tr>
<td>pET-DueT-1</td>
<td>Protein overexpression vector</td>
<td>Novagen</td>
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<tr>
<td>pET24afadR&lt;sub&gt;SA&lt;/sub&gt;Ndeull</td>
<td>FadR&lt;sub&gt;SA&lt;/sub&gt; expression vector</td>
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<td>FadR&lt;sub&gt;SA&lt;/sub&gt;PM expression vector</td>
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### Supplementary Table 3. Overview of strains used in this work.

<table>
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<th>Reference or source</th>
</tr>
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<td><em>E. coli</em> BL21 (DE3)</td>
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<td>Novagen</td>
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<td><em>E. coli</em> BL21-CodonPlus (DE3)</td>
<td>Protein overexpression strain</td>
<td>Agilent</td>
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Supplementary Figures

**Supplementary Figure S1.** Full-length FadR<sub>sa</sub> is separated as two electrophoretically distinct populations in SDS-PAGE. Coomassie-stained SDS-PAGE of FadR<sub>sa</sub> purification displaying crude extract (CE), flowthrough (FT) and first and second elution fractions (E1 and E2, respectively). MWL = molecular weight ladder with indication of molecular weights in kDa. The two distinct FadR<sub>sa</sub> species that have been subjected to LC-MS/MS analysis after excision from the SDS-PAGE gel are boxed. Both species were shown to contain full-length FadR<sub>sa</sub> proteins phosphorylated on T78 or Y79 with low localization probability (see Figure 1A).
Supplementary Figure S2. FadRsa<sup>WT</sup> and FadRsa<sup>PM</sup> have similar DNA-binding behaviours. Autoradiograph of an electrophoretic mobility shift assay (EMSA) of the binding of FadRsa<sup>WT</sup> and FadRsa<sup>PM</sup> to a 154-bp DNA fragment representing the operator/promoter region of the Saci_1106 target gene. The calculated dissociation equilibrium constant, calculated based on densitometry of the scanned autoradiograph and fitting to a Hill equation [2], is displayed for both proteins.
Supplementary Methods

Mass spectrometry analysis

For mass spectrometry analyses, non-radioactive in vitro phosphorylation assays were performed using reaction mixtures containing 20 μg FadRsa and either 2 μg ArnC or 0.2 μg SacI1041 in 30 μl as described above but in a different reaction buffer (25 mM MES [pH 6.5], 150 mM KCl, 1 mM MnCl₂ and 0.1 mM ATP) and incubated at 55°C. Proteins were then diluted by adding 70 μl 9 M urea in 20 mM HEPES pH 8.0, reduced by adding 4.5 mM DTT and incubating the mixtures during 30 minutes at 55°C. Alkylation of the proteins was performed by adding 10 mM iodoacetamide and incubating the mixtures during 15 minutes at room temperature in the dark. Samples were subsequently diluted with 100 μl 20 mM HEPES pH 8.0 and proteins were digested with 0.4 μg lysyl endopeptidase (Wako, 1/50, w/w) for 4 hours at room temperature. Proteins were further diluted with 200 μl 20 mM HEPES pH 8.0 and digested with 0.1 μg trypsin (Promega, 1/200, w/w) using an overnight incubation at 37°C. Finally, peptides were purified on OMIN C18 pipette tips (Agilent), dried completely by vacuum drying and re-dissolved in loading solvent A (0.1% trifluoroacetic acid (TFA) in water/acetonitrile (98:2, v/v)) for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The details of the LC-MS/MS procedure can be found below.

In addition, a sample of FadRsa was run on SDS-PAGE, leading to the separation of conformationally distinct protein species (Supplementary Figure S1), which were excised from the gel after staining, transferred to a tube and immediately cooled using dry ice. Gel bands were then washed with 500 μl H₂O, incubated in 500 μl H₂O/acetonitrile during 15 minutes and in 500 μl acetonitrile during another 15 minutes before being dried completely in a vacuum concentrator. Next, 0.15 μg sequencing-grade trypsin (Promega) in 50 mM ammonium bicarbonate in water/acetonitrile (9:1, v/v) was added to the dried gel bands and proteins were digested overnight at 37°C. Peptides eluted from every gel band were dried completely in a vacuum concentrator and re-dissolved in loading solvent A, which were then subjected to LC-MS/MS analysis. The LC-MS/MS procedure is described below.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed at the VIB Proteomics Expertise Center. Peptides were injected on an Ultimate 3000 RSLCnano system in-line connected to a Q Exactive mass spectrometer (Thermo). Trapping was performed at 10 μl/min for 4 minutes in loading solvent A on a 20 mm trapping column (made in-house, 100 μm internal diameter (I.D.), 5 μm beads, C18 Reprosil-HD, Dr. Maisch, Germany) and the sample was loaded on a 150 mm analytical column (made in-house, 75 μm I.D., 3 μm beads C18 Reprosil-HD, Dr. Maisch). Peptides were eluted by a linear increase from 2 to 55% MS solvent B (0.1% FA in water/acetonitrile (2:8, v/v)) over 30 minutes for the gel band samples and over 120 minutes for the in-solution samples, all at a constant flow rate of 300 nl/min, followed by a 5-minutes wash reaching 99% MS solvent B and re-equilibration with MS solvent A (0.1% FA
in water/acetonitrile (2:8, v/v)). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the most abundant ion peaks per MS spectrum. Full-scan MS spectra (400-2000 m/z) were acquired at a resolution of 70,000 in the orbitrap analyser after accumulation to a target value of 3,000,000. Depending on the instrument settings, the 5 or 10 most intense ions above a threshold value of 13,000 or 17,000 were isolated (window of 2.0 Th) for fragmentation at a normalized collision energy of 25% after filling the trap at a target value of 50,000 for maximum 80 or 60 ms. MS/MS spectra (200-2000 m/z) were acquired at a resolution of 17,500 in the orbitrap analyser.

Data analysis was performed with MaxQuant (version 1.5.3.8) using the Andromeda search engine with default search settings including a false discovery rate set at 1% on both the peptide and protein level. Spectra were searched against the *Escherichia coli* strain K12 proteins in the SwissProt database (database release version of May 2016 containing 4,306 protein sequences downloaded from [www.uniprot.org](http://www.uniprot.org)) supplemented with FadRsa, ArnC and Saci1041 sequences (Q4J9S1, Q4J9J0 and Q4J9X9, respectively). The mass tolerance for precursor and fragment ions was set to 4.5 and 20 ppm, respectively, during the main search. Enzyme specificity was set as C-terminal to arginine and lysine (trypsin), also allowing cleavage at arginine/lysine-proline bonds with a maximum of two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification for the in-solution samples only and variable modifications were set to oxidation of methionine residues (to sulfoxides), acetylation of protein N-termini and phosphorylation of serine, threonine and tyrosine residues.
Supplementary References
