Wing phosphorylation is a major functional determinant of the Lrs14-type biofilm and motility regulator AbfR1 in *Sulfolobus acidocaldarius*

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Summary

In response to a variety of environmental cues, prokaryotes can switch between a motile and a sessile, biofilm-forming mode of growth. The regulatory mechanisms and signaling pathways underlying this switch are largely unknown in archaea but involve small winged helix-turn-helix DNA-binding proteins of the archaea-specific Lrs14 family. Here, we study the Lrs14 member AbfR1 of *Sulfolobus acidocaldarius*. Small-angle X-ray scattering data are presented, which are consistent with a model of dimeric AbfR1 in which dimerization occurs via an antiparallel coiled coil as suggested by homology modeling. Furthermore, solution structure data of AbfR1-DNA complexes suggest that upon binding DNA, AbfR1 induces deformations in the DNA. The wing residues tyrosine 84 and serine 87, which are phosphorylated in vivo, are crucial to establish stable protein-DNA contacts and their substitution with a negatively charged glutamate or aspartate residue inhibits formation of a nucleoprotein complex. Furthermore, mutation abrogates the cellular abundance and transcription regulatory function of AbfR1 and thus affects the resulting biofilm and motility phenotype of *S. acidocaldarius*. This work establishes a novel wHTH DNA-binding mode for Lrs14-like proteins and hints at an important role for protein phosphorylation as a signal transduction mechanism for the control of biofilm formation and motility in archaea.

Introduction

Archaea possess a large variety of small DNA-binding proteins, which are generally classified as either transcription factors or chromatin-organizing proteins, although it is not always easy to make the distinction between both protein functions (Peeters et al., 2015). The predominant domain class in archaeal DNA-binding proteins is the winged helix-turn-helix (wHTH) motif (Aravind and Koonin, 1999; Pérez-Rueda and Janga, 2010), a structural variant in which a β hairpin ‘wing’ flanks the typical three-helical structure of the helix-turn-helix (HTH) motif. A well-studied example of a wHTH type family in archaea is the Lrs14 family. This is an archaeal-specific family of DNA-binding proteins that is particularly abundant and widespread in Crenarchaeota (Orell et al., 2013). For example, the hyperthermoadicophilic crenarchaeal model species *Sulfolobus*...
acidoocaldarius is predicted to harbor at least six Lrs14-like proteins of which five are conserved in the related Sulfolobus solfataricus.

Lrs14-type proteins are characterized by a basic isoelectric point (pl) and by a molecular mass between 12 and 14 kDa. Crystal structure determination of Sto12a, a Sulfolobus tokodaii Lrs14 member, revealed an antiparallel homodimeric structure with each subunit being characterized by a central wHTH fold flanked by two additional α-helices on either side (Shinkai et al., 2007). Dimerization is accommodated by the C-terminal α-helices of both subunits, which form an interchain, antiparallel coiled-coil structure. The Sto12a dimer is further stabilized by multiple disulfide bridges; while an interchain bridge links two corresponding cysteine residues (C15 and C16) in the N-terminal α-helices, another cysteine residue (C100) of the N-terminal α-helix is connected to a cysteine residue of the C-terminal α-helix by an intrachain bridge within each subunit. The Sto12a structure resembles that of the S. solfataricus nucleoid-associated protein Sso10a (Chen et al., 2004), although it harbors unique inter- and intrachain disulfide bridges (Shinkai et al., 2007) indicating a site for redox-dependent regulation.

Lrs14 members in Sulfolobales have been studied in the context of either DNA structuring and/or gene regulation (Napoli et al., 1999; Bell and Jackson, 2000; Napoli et al., 2001; Fiorentino et al., 2003; Orell et al., 2013). Different S. solfataricus Lrs14-like proteins have been retrieved in a number of independently performed pull-down assays with Sulfolobus crude cell extracts using entirely different double-stranded (ds) DNA substrates: while Smj12 was retrieved in a pull-down assay with a synthetic four-way DNA junction substrate (Napoli et al., 2001), the prototypical Lrs14 was pulled down together with chromatin proteins Sso7d and Alba using an alcohol dehydrogenase promoter as bait (Fiorentino et al., 2003). Ssa1 using a probe comprising the radA promoter (Abella et al., 2007) and its orthologue from the related Sulfolobus islandicus using rudiviral SIRV1 promoter probes (Kessler et al., 2006). Most of these Lrs14 proteins interact with DNA in a non-sequence specific manner, forming multiple nucleoprotein complexes with different electrophoretic mobilities (Napoli et al., 1999; Napoli et al., 2001; Orell et al., 2013), and/or interacting with a relatively long stretch of DNA (Napoli et al., 1999; Abella et al., 2007) in contrast to canonical prokaryotic transcription factors that interact with a short inverted repeat. For AbfR1 (archaeal biofilm regulator 1), the orthologue of Smj12 in S. acidocaldarius, the binding affinity is higher for promoter regions than for intragenic sequences although the DNA recognition mode is yet to be discerned (Orell et al., 2013). Smj12 has also been shown to introduce positive supercoiling and to be capable of stabilizing dsDNA against thermodenaturation (Napoli et al., 2001).

Lrs14-type proteins are capable of affecting gene expression in a positive and/or negative manner. For example, Sta1 has been shown to activate the radA promoter (Abella et al., 2007) and SIRV1 viral promoters (Kessler et al., 2006). On the other hand, Lrs14 directly represses transcription initiation of its own gene by interacting with the TATA box and factor B recognition element (BRE) thereby sterically inhibiting binding of the basal transcription factors (Bell and Jackson, 2000). AbfR1 is found to be a dual regulator that is capable of either repressing or activating gene expression depending on the target gene (Orell et al., 2013), although it is not yet known whether this is a direct or indirect regulation.

The exact physiological roles of Lrs14-type proteins in Sulfolobales remained largely enigmatic until transcriptomic studies revealed significant differential expression of lrs14-like genes in a variety of physiological states involving large genotypic and phenotypic changes. Interestingly, five lrs14-like genes were expressed at higher levels in biofilm-associated cells of S. acidocaldarius when compared to cells growing in a planktonic lifestyle (Koerdt et al., 2011; Orell et al., 2013) and genetic mutant analyses demonstrated a link between Lrs14-like proteins on the one hand and biofilm formation and cellular motility on the other hand (Orell et al., 2013).

Three Lrs14 mutant strains including the AbfR1-deleted strain displayed notable morphological differences in the formed biofilm. AbfR1 negatively regulates biofilm formation by decreasing the production of extracellular polymeric substance (EPS), increasing cell motility by activating the expression of flaB, encoding the structural component of the archaellum filament and decreasing the production of adhesive pili by repressing aapA expression (Orell et al., 2013). Also in other physiological processes Lrs14-type proteins appear to be involved. For example, heat shock treatment of S. solfataricus cells resulted in the upregulation of four lrs14-like genes (including Sta1, Smj12 and the Sto12a ortholog) (Tachdjian and Kelly, 2006) and during stationary growth of S. acidocaldarius five lrs14-like genes (including AbfR1 and the Lrs14, Sta1 and Sto12a orthologs) are upregulated when compared to exponential growth (Orell et al., 2013). Previously, it was already observed that the lrs14 transcript accumulates in the late growth phase of S. solfataricus (Napoli et al., 1999).

Many Sulfolobus chromatin proteins and transcription factors are post-translationally modified (Bell et al., 2002; Botting et al., 2010; Esser et al., 2012; Reimann et al., 2012, 2013; Niu et al., 2013). A phosphoproteomic study of S. acidocaldarius revealed a high number of phosphoproteins, including also AbfR1 (Reimann et al., 2007).
et al., 2013). Previously it was shown that transcription factors ArnA and ArnB, two components of the archael-

lum regulatory network, are both phosphorylated by the protein kinase ArnC (encoded by Saci_1193), whereas

ArnB but not ArnA is also phosphorylated by the kinase

ArnD (encoded by Saci_1694) (Reimann et al., 2012).

Despite Lrs14 being an important family of DNA-

binding proteins in Sulfolobales and AbfR1 being a crucial
determinant for the biofilm and motility phenotype of

S. acidocaldarius, the exact mode of DNA binding of this

wHTH protein is as yet uncharacterized. Furthermore, it is unknown how the expression and function of

AbfR1 is regulated for mediating the observed gene reg-

ulation in biofilm versus planktonic growth conditions. In

this work, we aimed to unravel how AbfR1 interacts with

DNA and we investigate if and how protein phosphoryla-
tion influences AbfR1 function. Our results suggest that

the phosphorylation status of AbfR1 plays a crucial role in

mediating the regulation of biofilm formation and cell motility in S. acidocaldarius.

Results

AbfR1 forms dimers in solution

AbfR1 is conserved in other Sulfolobales with sequence

identities between 62% and 67% (Fig. 1A). To learn

more about the structural properties of AbfR1, which

were still unknown, we built a homology model of the

protein structure based on the other Lrs14-like protein

Sto12a, which shows 32.6% sequence identity to AbfR1
(Fig. 1B). This model predicts the AbfR1 monomer to

comprise five α-helices (H0, H1, H2, H3 and H4) and

two β-sheets (S2, S3) in a topology in which an N-
terminal (H0) and an extended C-terminal α-helix (H4)

flank a central wHTH domain (Fig. 1A and B). Size

exclusion chromatography (SEC) was performed to ana-
lize the oligomeric structure of AbfR1 in solution (Fig.

1C and Table 1). In this experiment, AbfR1 eluted at a

terminal (H0) and an extended C-terminal

volume corresponding to a dimer.

Previously, a phosphoproteomic study demonstrated that AbfR1 is phosphorylated in vivo at a tyrosine and

serine residue, Y84 and S87 (Reimann et al., 2013). These

residues are both located on the wing region of the

wHTH domain, more specifically on β-strand S3 (Fig.

1A and B). Since the study of the role of these res-

aidues and their phosphorylation is a main objective of

this work, we prepared single and double protein

mutants in which phosphoacceptor sites were replaced

by either alanines or negatively charged amino acids.

More specifically, Y84 was substituted by a glutamate

(Y84E) and/or S87 by an aspartate (S87D) thereby

mimicking phosphorylation to some extent. Importantly,

SEC and SAXS analyses demonstrated that the double

mutant protein retains a wild-type (WT) conformation

with a dimeric state and negligible structural rearrange-

ments (Fig. 1C and D).

To obtain further low-resolution information about the

AbfR1 structure, we performed small angle X-ray scatter-
ing (SAXS) experiments of the protein, which further sup-
port the existence of AbfR1 as a homogenous population of
dimers (Fig. 1D and Table 1). In the pair distribution

function, \( P(r) \), the maximum particle diameter (D_{max}) in

AbfR1 is found to be 97.5 Å, which is in agreement with

the diameter of its modelled dimer (Fig. 1D). The profile

suggests that the two monomers are connected via a

coiled coil interaction of the H4 helices. Crystal structures

of similar archael winged helix domain proteins display a

dimeric conformation with an antiparallel coiled-coil

interaction of the H4 helices (Chen et al., 2004; Shinkai

et al., 2007). However, upon comparing both structures a

high degree of flexibility of the wHTH motifs is noticed:

while Sto12a builds a compact dimer, the dimer of a

wHTH protein from M. mazei attains a more relaxed and

stretched conformation (Fig. 1B). The ab initio envelope

of the AbfR1 solution structure can easily be superim-

posed on the structural model that was built with the

Sto12a crystal structure template, pointing to the forma-
tion of a compact dimer similar to Sto12a (Fig. 1D).

Nevertheless, a residual \( \chi^2 \) of 3.5 suggests that in

solution the domains of AbfR1 are not as rigidly arranged as

in the Sto12a crystal structure but that they might still

exert some conformational flexibility.

Interestingly, while the Sto12a structure is stabilized by

both inter- and intrachain disulfide bridges (Shinkai et al.,

2007), only one of three cysteine residues present in

Sto12a, the cysteine in H4, is conserved in AbfR1 (Fig.

1A). This suggests that the typical Sto12a disulfide

bridges are absent in AbfR1, a finding that was verified

by analytical SEC in the presence of 20 mM DTT or

1 mM β-mercaptoethanol. Clearly, reducing agents do

not affect the dimerization behavior of AbfR1. Altogether,

these results indicate that despite the absence of inter-

and intrachain disulfide bridges the AbfR1 dimer is tightly

stabilized by a coiled-coil interaction of the H4 helices.

Solution structure of the AbfR1-DNA complex

AbfR1 binds DNA in a non-sequence specific manner

with a higher binding affinity for promoter sequences

than for coding sequences (Orell et al., 2013). To

assess the minimal binding unit for AbfR1 binding, elec-
trophoretic mobility shift assays (EMSA) were per-
formed with probes of varying lengths all harboring at

least part of the abfR1 promoter region (Fig. 2A and B).

The abfR1 promoter region is bound by AbfR1 with a
high binding affinity (Orell et al., 2013). On a relatively long DNA fragment, AbfR1 binds multiple non-specific sites in a highly cooperative manner (Fig. 2A). Whereas a 20-bp probe did not display any interaction with AbfR1, incubation of the protein with 40-bp and 60-bp probes resulted in the formation of a distinct complex.

**Fig. 1.** Three-dimensional structure and oligomeric state of AbfR1.

A. Sequence alignment of AbfR1 and homologs with indication of identical (black) and similar (grey) residues with respect to S. acidocaldarius AbfR1. Homologs from S. acidocaldarius, S. tokodaii and S. solfataricus are indicated with Sa, St and Ss respectively. Position numbering is with respect to SaAbfR1. Predicted secondary structure elements based on homology modeling are indicated on top of the alignment. Residues that have been shown to be phosphorylated in a study of the S. acidocaldarius phosphoproteome (Reimann et al., 2013) are indicated in blue, cysteines in red.

B. Homology model of AbfR1 structure based on Sto12a (PDB: 2D1H), consisting of five helices (H0-H4) in cyan and three sheets (S2 and S3) in yellow. Polar charged residues are depicted in sticks. Phosphorylation sites (Y84 and S87) are indicated. Structures of winged helix members that served as a template for homology modeling are shown to demonstrate flexibility of the wing region: Sto12a from S. tokodaii (indicated by 2D1H) (Shinkai et al., 2007) and a putative transcriptional regulator from M. mazei (indicated by 3R0A).

C. Size exclusion chromatography analysis of AbfR1 WT and mutant proteins. SDS-PAGE of the purified proteins is shown in the inset, with indication of monomeric (M) and dimeric (D) populations.

D. $R(r)$ distribution profiles of AbfR1 (blue dashed line) and AbfR1$^{Y84E,S87D}$ (purple dashed line) as determined by SAXS analysis. On the right-hand side, the dimeric model of AbfR1 structure is shown with both domains highlighted. The SAXS-derived molecular envelope of AbfR1 solution structures is shown in the bottom right with superimposed dimeric AbfR1 in surface representation.
protein-DNA interaction. Lrs14-like proteins (Fig. 1A), are involved in cognate wing regions of AbfR1, which are highly conserved in results indicate that both the recognition helix and the bending sites within the dsDNA (Fig. 3E). Overall, these regions of AbfR1 might have direct contacts with the ab initio
tion,
B) are important for this major groove interaction. In addi-
tion, arginine and lysine residues in the H3 helix (Fig. 1A and recognition helix. We postulate that the positively charged envelope furthermore suggests that the AbfR1 dimer fold symmetry axis (Fig. 3E). The low resolution SAXS distorted element, in which binding of an AbfR1 dimer to the 40 bp dsDNA. Twenty different ab initio envelope models were generated using Dammin (Svergun, 1999) and imposing either P1 or P2 symmetry. Most of these ab initio models reflect the presence of a distorted element, in which binding of an AbfR1 dimer to DNA could cause considerable bending of the straight duplex DNA (Fig. 3C–E). In the AbfR1 dimer-dsDNA model the ends of the duplex DNA face opposite sides to the central dsDNA element that is perpendicular to the imposed two-fold symmetry axis (Fig. 3E). The low resolution SAXS envelope furthermore suggests that the AbfR1 dimer binds to duplex DNA in such a way that each monomer interacts with the major groove of DNA, probably via the recognition helix. We postulate that the positively charged arginine and lysine residues in the H3 helix (Fig. 1A and B) are important for this major groove interaction. In addition, ab initio models suggest that the flexible wing regions of AbfR1 might have direct contacts with the binding sites within the dsDNA (Fig. 3E). Overall, these results indicate that both the recognition helix and the wing regions of AbfR1, which are highly conserved in Lrs14-like proteins (Fig. 1A), are involved in cognate protein-DNA interaction.

| Table 1. SAXS parameters and estimated molecular masses for apo- and DNA-bound AbfR1 variants |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Rg (Å) | l0 (cm⁻¹) | Dmax (Å) | Volume (nm³) | Qp | MMcalc (kDa) | MMSEC (kDa) | MMSEX (kDa) |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| dsDNA | 35.6 ± 2.4 | 83.1 ± 0.14 | 120 | 27.48 | 1.25 | 24.0 | N.A. | 13.46 |
| AbfR1 | 26.4 ± 0.6 | 26.7 ± 0.05 | 97.5 | 46.80 | 2.95 | 28.6 | 30.3 ± 2.5 | 23.94 |
| AbfR1-DNA | 30.0 ± 4.9 | 51.3 ± 0.07 | 116 | 94.22 | 4.39 | 52.6 | N.A. | 46.97 |
| AbfR1Y84E | N.A. | N.A. | N.A. | N.A. | N.A. | 28.6 | 31.7 ± 2.5 | N.A. |
| AbfR1S87D | N.A. | N.A. | N.A. | N.A. | N.A. | 28.6 | 30.7 ± 2.5 | N.A. |
| AbfR1Y84ES87D | 26.6 ± 0.5 | 32.2 ± 0.05 | 97.5 | 50.9 | 2.96 | 28.6 | 31.8 ± 2.5 | 24.03 |

Rg = radius of gyration, Dmax = maximum diameter of the particle, Qp = particle mass determination coefficient, l0 = forward scattering, N.A. = not applicable. Calculated masses (MMcalc) for AbfR1 and its mutants refer to dimeric states.

Role of the wing for DNA binding and effect of its phosphorylation

To assess the importance of the S87 and Y84 wing residues for DNA binding we performed EMSAs with AbfR1 mutant proteins in which one or both residues are substituted with an alanine residue (Fig. 2C). Mutation of S87 and/or Y84 not only lowered the binding affinity (2.5 to more than 10-fold), but also inhibited the stable formation of lower-stoichiometric complexes. These effects demonstrate a crucial role for these wing residues for the AbfR1-DNA interaction. Hence it can be assumed that Y84 and S87 either establish direct interactions with the DNA or disrupt the cooperative multimerization on the DNA. Taking into account their position with respect to the AbfR1-DNA interface as found in the SAXS data we hypothesize that Y84 and S87 establish DNA-protein interactions at the bending interface.

Given the importance of both phosphoacceptor wing residues for DNA binding, it can be anticipated that their phosphorylation as observed in vivo (Reimann et al., 2013) might also affect the interaction with DNA. In order to evaluate this, EMSAs were performed with the AbfR1Y84E, AbfR1S87D and AbfR1Y84ES87D mutant proteins (Fig. 2D), which demonstrate that Y84E and S87D mutations inhibit DNA binding to an even larger degree than the corresponding alanine mutants (Fig. 2D). Therefore, mutation of Y84 and S87 by a glutamate and aspartate respectively, does not affect the protein conformation per se (Fig. 1C) but instead inhibits the interaction with DNA, presumably by creating negative charges on the wing domain. Thus, it can be concluded that for AbfR1-type wHTH proteins the wing-DNA interaction and bending of the DNA is necessary in order to form a stable complex.

AbfR1 interacts with abfR1 and flaB promoters in vivo

AbfR1 influences biofilm formation and affects motility in response to starvation by transcriptionally regulating the expression of, among other genes, the structural archaellum gene flaB (Orell et al., 2013). In order to verify if

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this regulation is due to a direct or indirect role of AbfR1 and whether or not AbfR1 binds the studied promoter regions in vivo, we performed a chromatin immunoprecipitation (ChIP) assay using a *S. acidocaldarius* strain with a genomically encoded HA-tagged *abfR1* gene and anti-HA antibodies (Fig. 4). This assay demonstrated that in starvation conditions, which are relevant conditions for *abfR1* regulation (see below), both the *flaB* and *abfR1* promoter region (the latter representing the probe used in the above-presented in vitro DNA-binding experiments) are significantly enriched as compared to a mock immunoprecipitation assay. In addition, AbfR1
association was also demonstrated for the secY open reading frame (ORF) (Fig. 4), which is not considered to be a regulatory target. This genomic association underlines the lack of DNA-binding specificity of AbfR1, as was demonstrated before in in vitro analyses (Orell et al., 2013).

To investigate if decreased interactions between the AbfR1 Y84 and S87 mutant proteins and promoter DNA as observed in vitro (Fig. 2C and D) are also relevant in vivo, we prepared S. acidocaldarius strains with each strain harboring one of the HA-tagged mutant abfR1 genes (abfR1_{Y84A}, abfR1_{S87A}, abfR1_{Y84AS87A} and abfR1_{Y84ES87D}). Western blotting of these strains demonstrated that under normal growth conditions the AbfR1 expression level itself is negatively affected by mutation of these residues (Supporting Information Fig. S1A), with the abfR1_{Y84ES87D} mutant displaying the largest negative effect, as it is characterized by an undetectable presence of AbfR1 protein similar as in the ΔabfR1 strain. Unexpectly, qRT-PCR analysis showed that the
abfR1 transcription level is not significantly affected in the abfR1Y84ES87D strain and that Y84- and S87-mediated regulation of AbfR1 expression is occurring at another level than the transcriptional level (Supporting Information Fig. S1B).

ChIP-qPCR analysis confirmed that AbfR1 is not associated with the abfR1 and flaB promoter regions in the abfR1Y84ES87D mutant strain (Fig. 4). This observation can be largely, if not at all, explained by the low abundance of the regulator in the abfR1Y84ES87D strain. However, it might also partially result from the abrogation of the DNA-binding function of the protein upon mutating Y84 and S87, similarly as observed in vitro. We can thus conclude that AbfR1 binds the promoter of flaB and of its own gene in vivo, that this binding most likely causes flaB regulation, and that the wing residues Y84 and S87 are crucial for AbfR1 expression and for the DNA-binding function of the protein.

The transcriptional regulatory function of AbfR1 is dependent on Y84 and S87

To further understand the role of residues Y84 and S87 for the transcription regulatory function of AbfR1, we subjected the S. acidocaldarius abfR1 mutant strains to an expression analysis of AbfR1 target genes. AbfR1 exerts an activation effect on different transcriptional units involved in archaellum synthesis (Orell et al., 2013): besides the flaB gene, encoding the structural component of the archaellum, the flaXGFHIJ operon is regulated as well. The mutant strains were thus assayed by qRT-PCR for relative expression levels of target genes flaB and flaX with respect to the WT strain in the two relevant conditions for AbfR1 regulation: biofilm growth and starvation (Fig. 5B–E). It should be noted that it has been shown that AbfR1 is not phosphorylated in a WT genetic background (Esser et al., 2012). As expected, in conditions of biofilm growth, all AbfR1 point mutations resulted in lower expression levels with respect to the isogenic WT strain pointing to a diminished activation (Fig. 5B) in a similar manner as when AbfR1 is deleted entirely, which can be correlated to the lower expression levels of AbfR1 itself (Supporting Information Fig. S1A). The level of downregulation was more pronounced for the charged residue mutations as compared to the corresponding alanine mutations. Furthermore, western-blot analysis of the same biofilm samples using anti-FlaB antibodies supported the flaB qRT-PCR results at the protein level (Fig. 5C) with the exception of the abfR1S87A mutant, where FlaB protein is hardly detectable.

The same experiment was performed under starvation conditions (Fig. 5D and E), which also cause an AbfR1-mediated activation of flaB expression (Lassak et al., 2012; Orell et al., 2013). Similar observations could be made as during biofilm growth although the observed downregulation was not as large when comparing mutant strains to the WT strain (Fig. 5D). Western blotting confirmed that upon starvation FlaB protein levels were nearly as low in the phosphoacceptor mutant strains as in the ΔabfR1 strain with again the abfR1S87A mutant as exception (Fig. 5E). Based on these observations, we can conclude that the substitution of Y84 and S87 by a charged residue, and to a large extent also their alanine substitution, abrogates the transcriptional activation of the archaellum genes.

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Phenotypic effects are determined by Y84 and S87 and their phosphorylation status

To verify if the inhibition of FlaB regulation resulting from the mutations of AbfR1 residues Y84 and/or S87 is also detected at the phenotypic level, we analyzed swimming motility of the abfR1 mutant strains on soft Gelrite plates in a medium lacking a sugar carbon source (Fig. 6A). As expected, all mutations resulted in a reduced motility. With the exception of the abfR1Y84ES87D mutant, which still retained 80% motility, all other point mutant strains displayed at least a 40% reduction in motility, similar to the ΔabfR1 strain.

Finally, we analyzed biofilm production with confocal laser scanning microscopy (CLSM) for all mutant strains (Fig. 6B). While the cells in the biofilms were stained with the DNA stain 4',6-diamidino-2-phenylindole (DAPI), produced EPS was visualized using the lectins ConA (green channel: α-D-mannopyranosyl and α-D-glucopyranosyl) and IB4 (yellow channel: α-galactosyl). Based on a qualitative assessment, we can conclude that most mutations resulted in markedly higher EPS levels as compared to the WT strain, but lower levels than those of the ΔabfR1 strain. Although for the abfR1Y84ES87D strain the effect on biofilm formation is not as strong as for the ΔabfR1 strain, the DAPI stain indicates higher cell densities and the height of the biofilm is higher in the mutant as compared to the MW001 strain (Fig. 6B). AbfR1 inhibits biofilm formation of *S. acidocaldarius* cells not only by upregulating motility, as

![Fig. 5. Expression analysis of AbfR1 and target genes.](image)
Fig. 6. Role of AbfR1 phosphoacceptor residues for motility and biofilm phenotypes of *S. acidocaldarius*.

A. Analysis of motility of the different mutant strains by soft Gelrite assays. Differences in the swimming radius are shown, as well as the mean values of the calculated swimming radius (diameter of outer swimming border was subtracted from the diameter of the colony border). Motility plates were inoculated with three biological replicates from which six technical replicates were grown for each.

B. CLSM analysis of three-day-old biofilms grown with strains harboring different AbfR1 phosphoacceptor residue mutant strains. The blue channel represents 4',6-diamidino-2-phenylindole (DAPI), which stains the DNA, the green channel represents the fluorescently labeled lectin ConA that binds to glucose and mannose residues. The lectin IB4 that binds to α-galactosyl residues is shown in yellow. Both lectins visualize the extracellular produced EPS during biofilm formation. Overlay images of all three channels are shown. Scale bar = 20 μm. Three biological replicates were imaged and a representative image is shown.

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shown above, but also by downregulating EPS production levels (Orell et al., 2013). Altogether, these results indicate that the effects of mutating Y84 and/or S87 observed at the level of gene expression are propagated to the very complex phenotypic level of biofilm formation.

Discussion

This work demonstrates that AbfR1 forms a homodimer, which is suggested to be formed by an antiparallel coiled-coil dimer interface similar to other non-specific DNA-binding proteins in Sulfolobales such as Sto12a and Sso10a/Sac10a (Chen et al., 2004; Kahsai et al., 2005; Shinkai et al., 2007). The wHTH motif is commonly found in DNA-binding proteins across all three domains of life (Gajiwala and Burley, 2000) and is even the predominant class of DNA-binding domains in archaea (Aravind and Koonin, 1999). While the overall structural topology of the motif has been conserved throughout the evolution, there is a large diversity in the nucleic acid interaction profiles of wHTH proteins (Gajiwala and Burley, 2000; Harami et al., 2013). In the ‘canonical’ DNA interaction mode (HNF3-γ-like), which is used by most wHTH-containing transcription factors, the recognition helix H3 interacts in a sequence-specific manner with the major groove of B-type DNA (Harami et al., 2013). Another distinct class of wHTH-containing DNA-binding proteins interacts with DNA through sequence-specific interactions of the wing domain with the major groove and minor-groove interaction of the recognition helix (RFX1-like) (Harami et al., 2013). Here, we provide low-resolution structural data that suggest that AbfR1 utilizes a novel non-sequence specific wHTH DNA-interaction mode, in which the DNA recognition helix might dock into the major groove and the wing region additionally contacts the dsDNA thereby inducing deformations in the straight B-type dsDNA.

We further provide in vivo data that suggest that AbfR1 activates its own expression and that of the flaB and flaX target operons through direct promoter interactions (Figs 4 and 5). Lrs14-like proteins are capable of activating and/or repressing transcription by binding the DNA in a non-sequence specific manner, but the molecular regulation mechanisms are as yet unclear, with the exception of the autoregulation mechanism by Lrs14 in S. solfataricus. For the latter, steric hindrance of TATA binding protein and transcription factor B binding has been proposed as the repression mechanism (Bell and Jackson, 2000). Given that AbfR1 is associated with the abfR1 and flaB promoters in vivo, activation is possibly exerted through direct interactions with the basal transcription machinery. Since kinking of dsDNA is a well-known mechanism for the control of transcription (Werner et al., 1996), e.g., by loop formation, we suggest that AbfR1-like members of the Lrs14 family like Smj12 might perform transcriptional regulation not only by directly interacting with the basal transcription machinery, but also by affecting the local DNA topology such as kinking and introducing supercoils, as shown for the S. solfataricus AbfR1 ortholog (Napoli et al., 2001). This is corroborated by the non-specific binding mode of AbfR1, as demonstrated by its in vivo association within the non-relevant secY coding region (Fig. 4), and the observation of similar regulatory effects for different targets (flaB and flaX) (Fig. 5), which are possibly only a subset of the genes regulated by AbfR1. Our observations suggest that AbfR1 is a global acting DNA-binding protein combining pleiotropic transcription regulation with a chromatin-structuring role (Peeters et al., 2015). This is reminiscent of H-NS in bacteria, a DNA-binding protein that also combines gene regulation with nucleoid organization and likewise plays a role in the regulation of motility and biofilm formation, among a wide variety of other physiological processes (Attung and Ingmer, 1997; Kim and Blair, 2015).

In contrast to HNF3-like wHTH proteins, in which wing interactions only have an auxiliary stabilizing function (Harami et al., 2013), the wings appears to be crucial for functioning of AbfR1. Alanine substitution of wing residues Y84 and S87 inhibited the formation of stable AbfR1-DNA complexes. It is an intriguing observation that the mutation (or corresponding phosphorylation) of only one of these residues has such large effects on AbfR1 function, almost comparable or even exceeding the effect of deleting the abfR1 gene entirely. Mutation of Y84 and/or S87 did not alter the oligomeric state or conformation of AbfR1 in vitro, as analyzed by SEC and SAXS. Therefore, it can be assumed that this is also the case in vivo and that the observed changes in gene regulation and phenotype are not due to a loss of function because of structural instability. Instead, the observed loss of function can be largely, if not entirely, explained by decreased intracellular AbfR1 protein levels. Although a hypothesis could be postulated that lowered AbfR1 protein levels are caused by diminished DNA binding upon mutation of Y84 and/or S87 and thus resulting in a decreased transcriptional auto-activation, this hypothesis is refuted by the observation of similar abfR1 transcription levels in the abfR1Y84ES87D mutant strain and the isogenic WT strain (Supporting Information Fig. S1B). As such, it can be concluded that Y84 and S87 residues are not only crucial for DNA binding of the protein, but also affect protein levels post-translationally through an unknown mechanism. This points to AbfR1-mediated regulation of biofilm formation and motility being an inherently complex process in which the effects of wing
phosphorylation on the DNA-binding characteristics of the protein appear to be an important but not the sole element.

Unlike the Lrs14 homologue Sto12a, AbfR1 forms a redox-independent dimer that is held together without the formation of disulfide bridges. Indeed, while some Lrs14-like proteins have been suggested to have only a single intrachain but no interchain disulfide bridge (Shinkai et al., 2007), AbfR1 and its orthologs comprise only a single cysteine residue that is conserved in the H0 helix and is not involved in any disulfide bridge formation. Here, we show that the function of AbfR1 is modulated by post-translational modification of wing residues instead of a redox signal as was proposed for Sto12a (Shinkai et al., 2007). Given the observed in vivo phosphorylation of these residues (Reimann et al., 2013) together with the results presented here, post-translational phosphorylation appears to be a major signaling mechanism for AbfR1 function, acting through the inhibition of DNA binding. This phosphorylation of residues in the wing region may either induce conformational changes or merely confer electrostatic repulsion to the negatively charged backbone of the cognate DNA target. Our data favor the latter given the lack of major structural changes observable for AbfR1 mutants in vitro. In this context, it is interesting to note the conservation of these residues in Sto12, although the corresponding residues were not phosphorylated in the *S. acidocaldarius* Sto12 orthologue (Fig. 1A; Reimann et al., 2013). Therefore, we assume that wing phosphorylation, which is a unique mechanism of regulation for archaeal transcription regulators and for single-component regulatory systems in general, is an AbfR1-specific phenomenon.

Phosphorylation is a well-described signaling mechanism in regulatory cascades underlying biofilm growth in bacteria. Our data suggest that phosphorylation could similarly contribute to the transition from a motile, planktonic to a sessile growth mode in *S. acidocaldarius* and its close relatives. Our data support the conclusions of Reimann et al. (2013) and conclude that the control of motility and biofilm is determined by a complex regulatory system in Crenarchaeota, involving multiple regulators and kinases. In *Sulfolobus* spp., the phosphorylation of the wing of AbfR1 is one of many strategies in the repertoire of regulatory strategies to ensure an appropriate growth mode in response to environmental conditions.

### Experimental procedures

#### Strains and growth conditions

*S. acidocaldarius* MW001 (Wagner et al., 2012), MW251 (Orell et al., 2013) and all derived mutant strains were grown aerobically at 75°C in Brock medium (Brock et al., 1972) at pH 3.0, supplemented with 0.1% (w/v) NZ-amine and 0.2% (w/v) dextrin and, unless otherwise stated, 10 μg/ml of uracil. Growth progression was monitored by measurement of the optical density at 600 nm (OD600). Growth on solid media was performed as described (Wagner et al., 2012) unless stated otherwise. Biofilm samples were grown as described (Koerd et al., 2010).

*Escherichia coli* strains DH5α and Top10 were used for the construction of recombinant plasmids. *E. coli* BL21 (DE3)-RIL was used as a host for recombinant protein overexpression. *E. coli* ER1821 was used for plasmid methylation prior to electrotransformation into *S. acidocaldarius*. All *E. coli* strains were grown at 37°C in standard Lysogeny Broth (LB) medium or on LB agar plates supplemented with 50 μg/ml ampicillin, 25 μg/ml kanamycin or 30 μg/ml chloramphenicol, if required.

#### Recombinant protein overexpression and purification

Site-directed mutagenesis of *abfR1* was performed by overlap PCR mutagenesis (Higuchi et al., 1988) with the WT AbfR1 expression plasmid pSVA2009 as a template (Orell et al., 2013) and by using primers listed in Table S2. The overlap extension PCR products were subsequently cloned into the EcoRI and NotI restriction sites of pETDuet-1 (Novagen), yielding plasmids pSVA3007–3009 and pSVA3016–3018 (Supporting Information Table S1). All constructed plasmid constructs were verified by sequencing.

Heterologous overexpression of recombinant WT and site-directed mutant AbfR1 proteins was performed as reported previously (Orell et al., 2013). For protein purification, pelleted cells were resuspended in 20 ml lysis buffer (50 mM KH₂PO₄, 300 mM KCl, 5 mM imidazole (pH 8)) containing Complete EDTA-free protease inhibitor cocktail (Roche) and were sonicated or passed through a microfluidizer (Microfluidics) three times at 1000 bar. The resulting crude extracts were centrifuged at 50,000 × g for 45 min at 4°C, and the supernatants were subsequently subjected to a heat precipitation for 10 min at 70°C followed by centrifugation at 5000 × g for 20 min at 4°C. Supernatants were applied to a Ni²⁺-affinity column (Native IMAC) using the Profinia™ protein purification system (Bio-Rad Laboratories) and bound protein was eluted with elution buffer.
were performed at 4 ATSAS software package (Petoukhov et al., 2012) and analyzed in PRIMUS (Konarev et al., 2003). The radius of gyration (Rg) of the complexes was determined by subtracting the buffer-only spectrum and then merged Scattering (Rambo, 2009). One-dimensional datasets were automatically resulting in scattering vectors q ranging from 0.0025 Å−1 to 0.50 Å−1. The scattering vector is defined as q = 4π sin(θ)/λ, with 2θ being the scattering angle. All experiments were performed at 4°C and data were processed using the ATSAS software package (Petoukhov et al., 2012) and ScÅtter (Rambo, 2009). One-dimensional datasets were subtracted from the buffer-only spectrum and then merged and analyzed in PRIMUS (Konarev et al., 2003). The radius of gyration (Rg) was calculated by AutoRg (Petoukhov et al., 2007). The molecular mass was calculated using the Qθ approach (Rambo and Tainer, 2013). The Guinier and Kraty plots can be found in SASBDB (Valentini et al., 2015) with accession codes SASDBF2 (40bp long dsDNA), SASDBG2 (AfrR1WT), SASDBH2 (AfrR1Y84ES87D) and SASDBJ2 (AfrR1WT in complex with 40 bp long dsDNA). The R(θ) functions were calculated by the program Gnom (Svergun, 1992) with the distance r at which the R(θ) function approaches zero intensity identifying the maximal dimension (Dmax) of the macromolecule. The ab initio envelope models were generated using Dammin (Svergun, 1999) using either P1 or P2 symmetry and were analyzed in CRYOSOL (Svergun et al., 1995). Theoretical atomic models of the dsDNA in straight B-DNA or in bent conformations were generated from the modelling server Haddock (van Dijk et al., 2006). The bending angles of the DNA as visualized in the envelope were calculated in PyMol (http://www.pymol.org). The overall shape of the protein in its bound conformation was manually fitted to the envelope. The fitting quality was further analyzed by CRYOSOL (Svergun et al., 1995).

**Homology modeling**

The three-dimensional structure of the AbfR1 at its potential dimeric conformation was modeled using the SWISS-MODEL workspace (Guex and Peitsch, 1997) with the Lrs14-like Sto12a from S. tokodaiii as a template (PDB: 2D1H) having a sequence identity of 32.6%. For this model, the QMEAN-score (Benkert et al., 2009) was 0.65.

**SAXS data collection and analysis**

SAXS datasets were collected at the synchrotron Bio-SAXS beamline BM29 (ESRF) Grenoble (France) (Pernot et al., 2013) using a 2D photon counting Pilatus1M-W pixel detector. A serial dilution of protein concentrations ranging from 1.25 mg/ml to 5 mg/ml was analyzed. Forty-bp long complementary DNA oligonucleotides representing the afr1 promoter region (Supporting Information Table S2) were annealed in 10 mM Tris/HCl pH 8, 50 mM NaCl, 1 mM EDTA containing buffer. A DNA-protein mixture was prepared in a 1:2 molar ratio (200 μM oligonucleotide duplex: 400 μM AbfR1), assuming that each duplex fragment is bound by a single AbfR1 dimer, followed by dialysis in 0.5X TBE buffer pH 8.5 (50 mM Tris, 50 mM boric acid, 1 mM EDTA).

SAXS experiments were performed with 20 mM HEPES pH 7.5 for apo-protein samples and with 0.5X TBE for protein-DNA complexes. The wavelength λ and sample-to-detector distance were set to 1.0 Å and 2.43 m respectively, resulting in scattering vectors q ranging from 0.0025 Å−1 to 0.50 Å−1. The scattering vector is defined as q = 4π sin(θ)/λ, with 2θ being the scattering angle. All experiments were performed at 4°C and data were processed using the ATSAS software package (Petoukhov et al., 2012) and ScÅtter (Rambo, 2009). One-dimensional datasets were subtracted from the buffer-only spectrum and then merged and analyzed in PRIMUS (Konarev et al., 2003). The radius of gyration (Rg) was calculated by AutoRg (Petoukhov et al., 2007). The molecular mass was calculated using the Qθ approach (Rambo and Tainer, 2013). The Guinier and Kraty plots can be found in SASBDB (Valentini et al., 2015) with accession codes SASDBF2 (40bp long dsDNA), SASDBG2 (AfrR1WT), SASDBH2 (AfrR1Y84ES87D) and SASDBJ2 (AfrR1WT in complex with 40 bp long dsDNA). The R(θ) functions were calculated by the program Gnom (Svergun, 1992) with the distance r at which the R(θ) function approaches zero intensity identifying the maximal dimension (Dmax) of the macromolecule. The ab initio envelope models were generated using Dammin (Svergun, 1999) using either P1 or P2 symmetry and were analyzed in CRYOSOL (Svergun et al., 1995). Theoretical atomic models of the dsDNA in straight B-DNA or in bent conformations were generated from the modelling server Haddock (van Dijk et al., 2006). The bending angles of the DNA as visualized in the envelope were calculated in PyMol (http://www.pymol.org). The overall shape of the protein in its bound conformation was manually fitted to the envelope. The fitting quality was further analyzed by CRYOSOL (Svergun et al., 1995).

**Electrophoretic mobility shift assays**

For protein-DNA interaction analysis, 32P-radiolabeled probes were prepared to harbor the afr1 promoter region. Primer labeling was performed with γ32P-ATP (Perkin Elmer) and T4 polynucleotide kinase (Thermo Scientific). A 175-bp fragment was prepared by performing a PCR with a labeled and non-labeled primer. Annealing of reverse complementary oligonucleotides, of which one labeled, yielded 20-bp, 40-bp and 60-bp probes for analysis of binding stoichiometry. All primer sequences are provided in Supporting Information Table S2. All dsDNA probes were gel-purified prior to use in EMSAs, which were performed as described previously (Enoru-Eta et al., 2000) using approximately 0.1 mM radiolabeled DNA and 25 μg/ml sonicated salmon sperm DNA as non-specific competitor DNA. AfrR1-DNA binding reactions were incubated in binding buffer (20 mM Tris-HCl (pH 8.0), 1 mM MgCl2, 0.1 mM dithiothreitol (DTT), 12.5% glycerol, 50 mM NaCl, 0.4 mM EDTA) (Peeters et al., 2006) during 20 min at 37°C prior to gel electrophoresis. EMSA autoradiographs were scanned followed by measurement of integrated densities of individual bands. After subtracting background densities, values were converted to fraction bound DNA. These data were plotted and fitted non-linearly using the Hill equation using Prism 6 software (GraphPad), yielding the apparent equilibrium dissociation constant (Kd).

**Construction of S. acidocaldarius mutant strains**

For the construction of the markerless site-directed mutant strains, plasmid constructs were prepared with mutant versions of afr1 with the overlap PCR mutagenesis method (Higuchi et al., 1988) similarly as for the expression plasmids, but by also adding the up- and downstream flanking regions to the open reading frame (see Supporting Information Table S2 for primer sequences). Overlap extension products were restricted with NcoI and BamHI and subsequently ligated into pSVA406, yielding markerless insertion plasmids.

For the construction of a chromosomally human influenza hemagglutinin (HA) epitope tagged strain, overlap extension PCR was performed to prepare a fragment with a sequence encoding a 683-bp region upstream of the afr1 stop codon, an HA-epitope coding sequence, a new stop codon and a sequence 439 bp downstream of the afr1 genomic stop codon (see Supporting Information Table S2 for primer
sequences). This product was cloned into the Apal and BamHI restriction sites of pSVA406 to generate the markerless insertion plasmid pSVA2028. For the generation of HA epitope tagged abfR1 mutant strains, similar markerless insertion plasmids were made but by using the respective abfR1 mutant expression plasmids as a template instead of a WT template.

Methylated markerless insertion plasmids were transformed by electroporation as described (Wagner et al., 2012) into the ΔabfR1 strain MW251 in case of the site-directed mutant constructions and MW001 in case of the epitope tag mutant construction. Integrants were selected on uracil-lacking selective medium and subsequently grown on solid medium containing uracil and supplemented with 100 μg/ml 5-fluoroorotic acid (5-FOA). All strains were confirmed to harbor the desired mutations by sequencing the abfR1 genomic region.

Chromatin immunoprecipitation assay

ChIP was mainly performed as described by (Nguyen Duc et al., 2012) with the following modifications: two biological replicates were analyzed of starvation-induced cultures of *S. acidocaldarius* strains encoding HA-tagged AbfR1 or AbfR1Y84E/S87D. Starvation induction was performed as described previously (Lassak et al., 2012). Cells were immediately fixed by adding 1% (v/v) formaldehyde and the reaction was quenched by adding glycine to a final concentration of 125 mM after 5 minutes. Cells were pelleted by centrifuging at 58,563 × g during 10 min, washed twice with PBS buffer and resuspended in 3.0 ml lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, pH 7.5), containing protease inhibitors (Roche). Resuspended pellets were sonicated (20% amplitude) until the DNA fragment size reached an average of 300 bp. Cell lysates were combined with 5 μg anti-HA antibody (Thermo Fisher Scientific) previously coupled with 1 mg Dynabeads® M-280 sheep anti-rabbit IgG (Life Technologies). Each cell lysate sample was also treated with 1 mg of non-anti-HA coupled Dynabeads as a mock control. Immunoprecipitation reactions were incubated for 1 h at 4°C and washing steps were performed as indicated by manufacturers using a magnetic stand. Subsequently, all ChIP and input DNA samples were purified with the iPure DNA extraction kit and analyzed for enrichment relative to input DNA by quantitative PCR (qPCR) with primers specific for abfR1 and flaB promoter regions and the secY (SacI, 0574) coding region (Supporting Information Table S2). qPCR reactions were performed as triplicates using a GoTaq® qPCR Master Mix (Promega). Reactions were run on an iCycler System (Bio-Rad) using a two-step protocol. Data were normalized using the Percent Input Method by dividing ChIP signals by input DNA signals after adjusting all signals according to their dilution factor.

RNA isolation and qRT-PCR analysis

Total RNA samples were isolated from 3-day mature biofilms and from shaking cultures after 4 hr of starvation or from exponential growth phase (OD<sub>600</sub> of about 0.4–0.5). To obtain mature biofilm, cells were incubated at 75°C for 3 days, the biofilm was harvested, immediately frozen in liquid nitrogen and stored at −80°C. Starvation samples were isolated as described (Lassak et al., 2012). TRIzol reagent (Invitrogen) was used for total RNA isolation following manufacturer's instructions. DNA digestion prior to cDNA synthesis was analyzed using secY-specific primers (Supporting Information Table S2). Preparation of cDNA was performed as described (Lassak et al., 2012). Quantitative reverse transcriptase (qRT-PCR) analysis of flaB and flaX was performed as described (Haurat et al., 2016), whereas qRT-PCR analysis of abfR1 was performed using Magnetic Induction Cycler (Bio Molecular Systems). qPCRBio Blue Mix Lo-ROX (PCR Biosystems) was used according to the manufacturer's protocol with qRT-Primers and Real-Time PCR Probes with BMN-Quencher and 5′-Fluorophores (Biomers) respectively (Table S2). As a control, qPCR reactions were also performed with RNA samples as a template. At least three biological replicates and two technical replicates were assayed. Fold-ratios of relative gene expression were calculated using the 2<sup>−ΔΔCt</sup> method (Livak & Schmittgen, 2001) and subjected to a statistical analysis (student's t-test) to validate differential expression.

Western blotting

For analysis of FlaB, FlaX and AbfR1-HA levels, the respective strains were harvested at an OD<sub>600</sub> between 0.4 and 0.5 by centrifuging at 4000 × g during 10 min, and resuspended to a theoretical OD<sub>600</sub> of 3. Proteins were separated by performing a 15% SDS-PAGE (Laemmli, 1970) and transferred to a PVDF membrane (Roche) by applying a semidry method. A primary anti-FlaB antibody (Lassak et al., 2012) (Eurogentec) or an anti-HA primary antibody (Sigma) in combination with an anti-rabbit IgG-HRP (Invitrogen) or alkaline-phosphatase coupled antibody (Sigma) were used for detection. Chemiluminescent signal was detected using ECL chemocam imager (Intas) and WesternSure ECL Substrate (Li-Cor).

Swimming motility on semi-solid Gelrite plates

Swimming motility on plates was analyzed on semi-solid Gelrite plates consisting of 0.15% Gelrite in Brock medium supplemented with 0.001% (w/v) NZamine. Cells grown in standard Brock medium were harvested during exponential growth and used to inoculate plates at a cell density of 10<sup>7</sup> cells per ml. Plates were incubated for 7 days in a humid chamber at 75°C. Swimming behavior of the different *S. acidocaldarius* strains was analyzed by measuring the swimming radius.

Biofilm growth and analysis by confocal laser scanning microscopy (CLSM)

Static biofilm cultures of *S. acidocaldarius* strains were grown in small Petri dishes (m-dishes, 35 mm, Ibidi, Martinsried) in Brock medium supplemented with 0.1% (w/v) NZ-amine and 10 μg/ml uracil. All strains were inoculated
with a starting OD_{600} 0.01 and statically grown for 3 days. Small-sized Petri dishes were placed in large Petri plate dishes (150 × 20 mm), which were subsequently put into a specially designed metal box (25 cm L × 20 cm W × 20 cm D) with about 500 ml of water in the bottom to minimize evaporation of the media, as described before (Koerdt et al., 2010). CLSM (Leica TCS SP8 STED 3X) and fluorescent probes were employed to visualize the biofilm. In brief, 15 μg/ml DAPI was used to stain the cells of the biofilm and images were taken at an excitation wavelength of 405 nm and emission wavelength of 415–477 nm. Fluorescently labeled lectins were employed to visualize the EPS of the biofilm. Before adding lectins to the biofilm, the growth medium was replaced with medium adjusted to pH 5.0 to ensure that binding of lectins was not inhibited by low pH. fluorescein conjugated concanavalin A (ConA) (37.5 μg/ml) (Invitrogen, Karlsruhe, Germany) binds to α-D-mannopyranosyl and α-D-glucopyranosyl residues and its signal was recorded at an excitation wavelength of 494 nm and emission wavelength of 497–550 nm. Alexa Fluor 594 conjugated IB4 (4 μg/ml) recognizes specifically α-D-galactosyl residues (isoelectin GS-IB4 from Griffonia simplicifolia; Invitrogen, Karlsruhe, Germany) and images were taken at an excitation wavelength of 597 nm and emission wavelength of 602–676 nm. The lectin-biofilm mixtures were incubated at room temperature for 20–30 min in the absence of light. After incubation, the biofilm was washed with Brock media (pH 5.0) to remove excess label and images were taken by CSLM. Image data obtained were deconvoluted by using the Huygens software package (SVI) and subsequently processed by using the IMARIS 7.6 software package (Bitplane).

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