Transcriptional regulation of the $N^\epsilon$-fructoselysine metabolism in *Escherichia coli* by global and substrate-specific cues

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Abstract
Thermally processed food is an important part of the human diet. Heat-treatment, however, promotes the formation of so-called Amadori rearrangement products, such as fructoselysine. The gut microbiota including *Escherichia coli* can utilize these compounds as a nutrient source. While the degradation route for fructoselysine is well described, regulation of the corresponding pathway genes *frlABCD* remained poorly understood. Here, we used bioinformatics combined with molecular and biochemical analyses and show that fructoselysine metabolism in *E. coli* is tightly controlled at the transcriptional level. The global regulator CRP (CAP) as well as the alternative sigma factor $\sigma^{32}$ (RpoH) contribute to promoter activation at high cAMP-levels and inside warm-blooded hosts, respectively. In addition, we identified and characterized a transcriptional regulator FrlR, encoded adjacent to *frlABCD*, as fructoselysine-6-phosphate specific repressor. Our study provides profound evidence that the interplay of global and substrate-specific regulation is a perfect adaptation strategy to efficiently utilize unusual substrates within the human gut environment.

Keywords
$1-(\epsilon-N\text{-lysyl})\text{-1-deoxy-D-fructose}$, Amadori rearrangement product, fructosyllysine, glycation, GntR transcriptional regulator
1 | INTRODUCTION

Glycation is a non-enzymatic form of glycosylation and means a spontaneous reaction of amino compounds with reducing sugars such as glucose (Lassak et al., 2019; Ulrich and Cerami, 2001). The phenomenon was first described by Louis-Camille Maillard in 1912 being predominantly responsible for the taste, aroma, and appearance of thermally processed food (Maillard, 1912a; 1912b). Simple condensation products of primary amino groups at the N-termini of polypeptides or the ε-amino group of lysine and reducing sugars such as glucose are the most prevalent Maillard reaction products in food (Henle, 2003). These “sugar-amino acids” are also called “Amadori rearrangement products” (ARPs) (Amadori, 1925; Hodge, 1955). Protein-bound Maillard reaction products can be the result of a condensation reaction, taking place between an aldose or ketose and a primary amine either in form of an α-amino group at the N-terminus or an ε-amino group of lysine residues within the polypeptide chain. Bacteria, including Escherichia coli, Bacillus subtilis, and Salmonella enterica have evolved efficient strategies to use these ARPs as sole carbon source (Ali et al., 2014; Miller et al., 2015; Wiame et al., 2002; 2004; 2005). Notably, the wide distribution of the ARP catabolism among the gut microbiota (Sabag-Daigle et al., 2018) further suggests that this carbon source plays an important role in colonizing the intestinal environment (Barroso-Batista et al., 2020).

While there are numerous uptake mechanisms and diverse specificities toward glycation products in distinct microorganisms (Miller et al., 2015; Sabag-Daigle et al., 2018; Wiame et al., 2004), degradation follows a conserved route and can be illustrated by the E. coli N₁-fructoselysine (ε-FrK) metabolism. Upon uptake—presumably by the putative permease FrlA—a kinase FrlD phosphorylates the sugar moiety at the C6-position (Figure 1) (Wiame et al., 2002). In a second step, the deglycase FrlB hydrolyses fructoselysine-6-phosphate (FrK-6P) into glucose-6-phosphate and lysine to be further processed via glycolysis and amino acid metabolism, respectively. Together with an additional N₁-psicoselysine/ε-FrK epimerase FrlC (Wiame and Van Schaftingen, 2004), the pathway is encoded in one single operon frlABCD of thus far unknown regulation. In the present study we show that the E. coli ε-FrK catabolism is tightly controlled by positive and negative regulation. On the one hand, the global transcription factor CRP (CAP) as well as the sigma factor σ₃₂ (RpoH) contribute to promoter activation. On the other hand, we identified the previously elusive regulator FrlREco, encoded adjacent to frlABCD, as an ε-FrK specific roadblock repressor. However, ε-FrK is not recognized directly but only upon phosphorylation. FrlREco itself is a member of the GntR/HutC family of transcriptional regulators recognizing the consensus sequence 5′-(N)yGT(N)xAC(N)y-3′. Binding of ε-FrK-phosphate presumably leads to structural rearrangements in the FrlREco transcriptionally active dimer, which in turn weakens DNA binding, and subsequently permits transcription of the frlABCD operon. Thus, we conclude that the interplay of global and substrate-specific regulation combined with a σ₃₂ mediated transcription activation as response of colonization of a warm-blooded host is a perfect adaptation to utilize thermally processed food within the human gut environment.

2 | RESULTS

2.1 | FrlREco is a putative GntR like transcriptional regulator

Wiame and coworkers noticed the presence of a putative regulator, termed FrlREco, in the genomic vicinity of the ε-FrK degradation
pathway (Wiame et al., 2002). However, its role in controlling the
friABCD operon remained enigmatic. To elucidate the function of
FrlR_Eco, we started with a bioinformatic comparison and performed
a multiple sequence alignment (Figure 2). This revealed sequence
similarities to the B. subtilis orthologous regulator FrlR of the
fri-BONMD operon (Deppe et al., 2011), encoding the genes to me-
tabolize various α-glycated amino acids (Wiame et al., 2004). We
also identified a distinct ortholog in S. enterica, termed FraR, being
encoded immediately upstream of the fraBDAE operon, a gene
cluster that is needed for degradation of fructosaeaparagine (Ali
et al., 2014). Taken together, E. coli FrlR is likely to be involved in
substrate-specific regulation of ARP metabolism. The outcome of
our blast search also suggests a common regulatory theme that ap-
plies to all ARP metabolizing organisms despite their distinct sub-
strate spectra.

We next used Phyre2 (Kelley et al., 2015) as well as the iTASSER
suite (Yang et al., 2015) and performed a homology modelling to
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To see whether FrlR Eco functions as a repressor similarly to NagR Bsu, we first performed a series of growth experiments with FrK. Initially, we reinvestigated the capability of E. coli to utilize this ARP as sole carbon source. To this end, we monitored bacterial growth in M9 minimal medium (Miller, 1972) supplemented with either 1 mM ε-FrK or 1 mM glucose, the latter serving as positive control (Figure 4a left). As demonstrated earlier (Griffiths and Pridham, 1980; Wiame et al., 2002), E. coli can grow on both carbon sources yielding similar biomass as can be concluded from their same maximal optical density at 600 nm. The major difference between the two curves is a prolonged lag phase and an increased doubling time of 210 min for ε-FrK supplemented cells, being around 20% longer compared to glucose (180 min). As further control, we tested a strain ΔfrlD lacking the FrK kinase, which catalyzes the first step in the degradation pathway. Expectedly, this mutant was no longer able to grow on ε-FrK (Figure 4a right). *Escherichia coli* FrK kinase FrID shows only little enzymatic activity toward α-glycated amino acids in vitro (Wiame et al., 2004) and hence we were curious whether α-FrK can substitute for the ε-glycated lysine in vivo. In line with the previously determined substrate specificity of FrID, we observed neither significant growth with the α-glycated ARP nor any degradation (Figure 4a left, 4b). Having confirmed these previous findings, we went on to investigate whether FrlR Eco is involved in the regulation of ε-FrK utilization (Figure 4b). We compared growth of an E. coli wild type with a ΔfrlR strain and found the phenotype with respect to total biomass yield indistinguishable from each other. At the same time, we noticed a shortened lag-phase, giving the first hint that FrlR Eco acts as a repressor. In line with this assumption, the overproduction of FrlR Eco (frlR++) prevents efficient ε-FrK utilization (Figure 4a right, 4b, Figure S1). PRESumably, the increased protein copy number decouples DNA binding from substrate recognition, and thus reveals FrlR Eco to be a transcriptional repressor. In parallel, we quantified metabolization of both derivatives of FrK by the wild type during 24 hr of incubation and using direct amino acid analysis. Whereas the concentration of α-FrK remained unchanged during the experiment, ε-FrK was almost completely degraded while at the same time lysine was formed in an equimolar amount.
for ε-FrK were similar in the ΔfrlR mutant. Less ε-FrK was degraded by frlR** cells, while no degradation and only little lysine formation was observed in the ΔfrlRD strain lacking the kinase. We conclude that growth of *E. coli* is retarded when metabolism of ε-FrK is inhibited.

### 2.3 frlABCD transcription is positively controlled by σ32 and CRP as well as negatively regulated by FrlR<sub>Eco</sub>

Having shown that ε-FrK utilization is subject to FrlR<sub>Eco</sub> regulation, we rigorously analyzed the 5′-UTR (untranslated region) of *frlABCD* employing P<sub>frlABCD</sub> promoter fusions to uncover further control elements. It is predicted that *frlABCD* transcription starts 75 nucleotides 5′ of the frlA open reading frame (+1) and depends on the housekeeping sigma factor σ<sub>70</sub> (RpoD) (Figure 5a) (Huerta and Collado-Vides, 2003). We further noticed a sequence matching the CRP/CAP (cAMP Response Protein/Catabolite Activator Protein) binding site centered around position −41.5 and thus being in perfect distance to constitute a class II promoter (Lawson et al., 2004). To test our hypotheses on transcriptional regulation of the P<sub>frlABCD</sub> promoter we initially fused 294 bp (−219/+75) 5′ of *frlABCD* with the lux-operon luxCDABE of *Photorhabdus luminescens* (Volkwein et al., 2017) and measured the light output over a time course of 24 hr in *E. coli* BW25113 wild-type cells grown in Lysogeny broth (LB/Miller) (Bertani, 2004). We reached a maximal luminescence per OD<sub>600</sub> of about 1 × 10<sup>5</sup> RLU, showing that this region comprises a fully functional promoter (Figure 5b–d). We note that a further sequence extension to 397 bp (−322/+75) did not change this emission significantly (Figure 5b), demonstrating that the −219/+75 lux fusion encompasses all required elements for transcriptional control. A truncation to 140 bp (−65/+75) reduced the light output by a factor of five to 2 × 10<sup>3</sup> RLU showing that this construct lacks an important part of the promoter. Using RegulonDB (Santos-Zavaleta et al., 2019) we became aware of putative −10 and −35 regions of the heat shock sigma factor σ<sub>32</sub> (RpoH). To investigate their involvement in P<sub>frlABCD</sub> activation, we ectopically expressed rpoH and measured the effect on the promoter lux fusions. A similar strategy was described earlier and is favored as the sigma factor is essential at growth temperatures higher than 20°C (Zhao et al., 2005). With the −219/+75 construct σ<sub>32</sub> overproduction led to a significant increase in luminescence compared to the wild-type situation (Figure 5c). Interestingly, −65/+75 also responded positively to σ<sub>32</sub> expression.

![Diagram](image.png)

**Figure 5** In vivo analysis of the *frlABCD* promoter region. (a) Illustration of the *frlABCD* promoter region with +1 as the putative transcriptional start site. DNA-recognition sites for RNA-polymerase are boxed in green (dependent on RpoH/σ<sub>32</sub>) and light green (dependent on RpoD/σ<sub>70</sub>). The binding motif of the cAMP-activated global transcriptional regulator CRP is boxed in dark green. The DNA-binding site of FrlR<sub>Eco</sub> is boxed in red. Consensus sequences for the transcription factors are highlighted with bold letters. (b–d) In vivo analyses of P<sub>frlABCD</sub> promoter lux fusions in *E. coli* cells containing the respective reporter plasmid. The maximal light emission from a 24 hr time course experiment of cells grown in LB (Miller) is given in RLU. The mean values and standard deviations of at least three biological replicates are shown. Naming of the P<sub>frlABCD</sub>-lux promoter truncations depicted on the abscissa gives information about the sequence length and numbering and relates to the illustration in (a). *E. coli* BW25113 wild-type cells (black bars) in comparison to the isogenic mutant strain JW5698 (ΔfrlR; grey bars) (Baba et al., 2006) and cells where frlR<sub>Eco</sub> overexpression was induced by the addition of 0.2% (w/v) arabinose (frlR**; white bars) from a pBAD33 backbone were analyzed in (b). To highlight the differences with Δ-48/+28 a second ordinate was introduced. (c) *E. coli* BW25113 wild-type cells (black bars) in comparison to cells where rpoH overexpression was induced by the addition of 0.2% (w/v) arabinose (rpoH**; grey bars) from a pBAD33 backbone. To highlight the differences with Δ-48/+28 a second ordinate was introduced. (d) *E. coli* BW25113 wild-type cells (black bars) in comparison to an isogenic mutant strain, which lacks the adenylyl cyclase CyaA. JW3778 (ΔcyaA) and cells ectopically overexpressing cyaA (cyaA**; dashed lined bars). A wild-type cell culture supplemented with 20 mM glucose (glc; white bars) was also analyzed.
(two-fold increase in light output), implying that the sigma factor also binds within the presumed $\sigma^{32}$ promoter region. Therefore, we included another reporter construct which lacks base pairs $-28$ to $-48$ ($\Delta-48/-28$). Cells harboring $\Delta-48/-28$ turned almost dark unless $\sigma^{32}$ was overproduced which increased light production fivefold. Thus, we can conclude on the presence of two promoters both of which are needed to fully induce $P_{frlABCD}$. One in close proximity to the transcription start site and presumably the major driver of $frlABCD$ expression, the other further upstream and important to reach high level activation. While both promoter regions respond to increased levels of $\sigma^{32}$, only the downstream one encompasses the DNA-binding elements for the house keeping sigma factor $\sigma^{70}$ as well as CRP.

Accordingly, we next assessed the regulation by catabolite repression. In an inverse correlation to the extracellular glucose concentration, CRP activates metabolic processes that facilitate the conversion of alternative carbon sources. Notably, the glucose content is not measured directly but is derived from the intracellular cAMP concentration which in turn relies on the activity of a sole cAMP-synthetizing adenylate cyclase CyaA (Lawson et al., 2004). Accordingly, luminescence was analyzed in the $-65/+75$ promoter fusion which should comprise the full CRP DNA-binding motif. Here, LB/Miller growth conditions, with amino acids as sole carbon source, were compared on the one hand to a culture supplemented with 10 mM glucose and on the other hand to a $\Delta$cyAA strain (Baba et al., 2006). For the latter two conditions, the light output was strongly diminished (Figure 5d) corroborating our assumption about carbon catabolite repression. This is in line with an earlier global study that suggested CRP-dependent regulation of the $frlABCD$ operon (Shimada et al., 2011). We also note that glucose and cAMP dependency is lost with the truncated promoter fusion $-32/+75$ (Figure 5d). However, with this construct the overall luminescence is strongly decreased. As $-32/+75$ no longer encompasses the full CRP-binding site, RNA-polymerase recruitment is impaired (Figure 5a). Similarly, $\Delta-48/-28$ does no longer respond to cAMP ($\Delta$cyAA), demonstrating that there is no second CRP-binding site. Next, we investigated whether inducer exclusion (Deutscher et al., 2014; Osumi and Saier, 1982; Sondej et al., 2002) might also play a role in $P_{frlABCD}$ regulation. Hence, we analyzed luminescence development with $-219/+75$ and in a mutant lacking component EIIA/Crr ($\Delta$crr) of the phosphotransferase system (Figure S2) (Deutscher et al., 2014). Compared to the wild type, the light output in the mutant was diminished (Figure S2a). This was expected as in $\Delta$crr cells CyaA adenylate cyclase activity can no longer be stimulated by phosphorylated EIIA. At the same time, an external supplement with 1 mM $\varepsilon$-FrK led to elevated light production showing that regulation by $\varepsilon$-FrK was retained. The negative regulation by glucose (Figure 5d), however, was mitigated in the mutant (Figure S2b). This in turn gives a first hint that EIIA might prevent $\varepsilon$-FrK internalization by inhibiting the assumed transport activity of FrI A, similar to those what was reported for other sugar transporters (Deutscher et al., 2014). Additional experiments are needed, to further corroborate this hypothesis.

Lastly, we employed the lux reporter to investigate transcriptional regulation by $\varepsilon$-FrK. Our initial growth experiments (Figure 4a) indicated the role of the protein as negative regulator repressing $P_{fiabc}$ under non-inducing conditions. However, the strong light emission for wild-type cells harboring $P_{frlABCD}$ lux fusions in LB/Miller seems to contradict this assumption. We hypothesized that the plasmid-borne nature of the reporter might imbalance the ratio of $\varepsilon$-FrK promoter and plasmid abundance in favor of the latter. Accordingly, increasing the $\varepsilon$-FrK copy number—by introducing an arabinose inducible ectopic copy of $\varepsilon$-FrK ($pBDA33$–$\varepsilon$-FrK (Eco))—should be enough to silence the promoter and this was in fact what we observed (Figure 5b). We also note that cells lacking $\varepsilon$-FrK produced 1.5-fold more the light of wild-type cells, further supporting that $\varepsilon$-FrK is a transcriptional repressor. We ultimately analyzed whether this repression is $\varepsilon$-FrK dependent. Consequently, bioluminescence development was now measured in $\Delta\varepsilon$-FrK cells in the concomitant presence of a $P_{BAD}$ controlled, plasmid-encoded copy of $\varepsilon$-FrK ($pBDA33$–$\varepsilon$-FrK (Eco)) and the $-219/+75$ $P_{frlABCD}$-lux reporter construct. In this scenario, light emission was strongly reduced (even without arabinose induction) but becomes elevated again when adding 1 mM $\varepsilon$-FrK (Figure 6). In contrast, promoter repression was maintained with 1 mM $\alpha$-FrK. These data confirm that specificity for the $\varepsilon$-glycated form is not limited to the metabolism, but also extends, directly or indirectly, to regulation. To define the sensitivity of the system we performed a titration series with $\varepsilon$-FrK (10 $\mu$M-10 mM), and thus determined that the system responds to concentrations as low as 10 $\mu$M (Figure 6, Figure S5). The strength of repression is gradual with a maximum in the low mM range. Considering that the $\varepsilon$-FrK uptake with our diet can reach such concentrations (Henle, 2003; 2005), $E. coli$ is perfectly adapted to its natural human gut habitat.

**FIGURE 6** $\varepsilon$-FrK sensitivity analysis toward $\varepsilon$-fructoselysine.

In vivo analyses of the $P_{fiabc}$ promoter lux fusion $-219/+75$ in *Escherichia coli* JW5698 cells ($\Delta\varepsilon$FrI) that concomitantly express $frlREco$. The maximal light emission from a 24 hr time course experiment of cells grown in LB (Miller) (without the addition of the $P_{BAD}$ inducing agent L-arabinose) is given in RLU. The mean values and standard deviations of at least three biological replicates are shown.
Knowing that FrlR<sub>Eco</sub> is a transcriptional repressor, we went on to identify its cognate DNA-binding motif. Therefore, we analyzed various P<sub>frlABCD</sub>-lux fusions on FrlR<sub>Eco</sub> dependent repression (Figure 5B). These were truncated 5′ (−322/+75, −219/+75, −65/+75, −33/+75 and Δ−48/−28) or 3′ (−219/+60 and −219/+25) relative to the transcriptional start site (+1). To our surprise we saw a strong drop in light output with −219/+60 and −219/+25. As the ribosome binding site driving LuxC production was kept the same in all fusion constructs, the reduction might be attributed to structural alterations of the mRNA that potentially interfere with proper binding to the ribosome.

Upon ectopic frlR<sub>Eco</sub> expression luminescence decreased dramatically irrespective of the promoter truncation tested. This in turn narrows down the putative binding site to a region of about 50 nucleotides. The proposed structural similarities of FrlR<sub>Eco</sub> to NagR<sub>Bsu</sub> (Figure 3) implied that both transcription factors belong to the same family of regulators, namely the GntR/HutC related ones (Hoskisson and Rigali, 2009; Rigali et al., 2002). Members of this group bind to a motif 5′-GT(N)<sub>x</sub>AC(N)<sub>y</sub>-3′ and so do NagR<sub>Bsu</sub> and FrlR<sub>Bsu</sub> (Deppe et al., 2011; Fillenberg et al., 2015). We recognized a putative operator 5′-GT CATGTT AC(N)<sub>y</sub>-3′ (frlR<sub>Eco</sub>) immediately downstream of the transcription start site indicating that RNA polymerase is occluded from binding to the σ<sub>70</sub> dependent promoter when frlR<sub>Eco</sub> is occupied by FrlR<sub>Eco</sub>. To prove this hypothesis, the putative FrlR<sub>Eco</sub> binding motif was placed between the T7-polymerase promoter (P<sub>T7</sub>) and a synthetic ribosome-binding site, all of which precede the ORF of the superfolder green fluorescent protein (sfGFP) (Figure 7a). Fluorescence intensity was measured as means of the transcriptional activity in the absence and presence of a plasmid-borne copy of frlR<sub>Eco</sub> while sfgfp expression was high without FrlR<sub>Eco</sub> cells turned dark with the repressor. Thus, the artificially introduced motif comprises frlR<sub>Eco</sub>.

When substituting the conserved GT/AC by CC/GG, sfgfp expression was no longer controlled by FrlR<sub>Eco</sub>. Similarly, the extension of the 6 bp long spacer by one additional “A” heavily interfered with DNA-binding as can be concluded from the heterogeneous fluorescence signal. As FrlR<sub>Eco</sub> is expected to work as a dimer (Fillenberg et al., 2015), a spacer length of six is indicative for a binding of each monomer at opposite sides of the operator region.

We were also curious what happens, when we mirror the core motif. Here, FrlR<sub>Eco</sub> was still able to diminish sfgfp expression, however not as efficient as with the native binding site. This led us to conclude that, beside the bracketing GT/AC and spacer length, their upstream and downstream residues—which were kept in the original order—play also a crucial role in frlR<sub>Eco</sub> recognition.

Our in vivo analysis was complemented by testing the operator sequence on FrlR<sub>Eco</sub> binding in vitro. We employed thermal shift assays (Huynh and Parth, 2015) to assess heat stability of FrlR<sub>Eco</sub> in dependence of frlR<sub>Eco</sub> (frlR<sup>+</sup>) (Figure S3). As controls we measured melting temperatures in the presence of a random DNA-fragment of similar size (frlR<sup>−</sup>) as well as in the absence of any DNA. In theory, DNA-binding to FrlR<sub>Eco</sub> should stabilize the protein and in turn, the melting temperature increases. In fact, we observed a FrlR<sub>Eco</sub> melting temperature of 62°C in combination with frlR<sub>Eco</sub> whereas it was 3°C lower in the mixture with the random DNA-fragment.

As these data are only qualitative, we next utilized Surface Plasmon Resonance spectroscopy (SPR) in order to determine the binding kinetics (association rate k<sub>a</sub> and dissociation rate k<sub>d</sub>) as well as the affinity (K<sub>d</sub>) of FrlR<sub>Eco</sub> and its cognate recognition motif (Figure 7b, Figure S3). To this end, different concentrations of FrlR<sub>Eco</sub> were combined with two immobilized DNA fragments (see the experimental procedures for details). One fragment includes the FrlR<sub>Eco</sub> binding site, the other was of random sequence composition and served as negative control. Before that, the absolute and “active” fraction of FrlR<sub>Eco</sub> was determined using calibration free concentration analysis (CFCA) to approximately 50% of the total protein concentration. A clear and stable binding could be observed for FrlR<sub>Eco</sub> to frlR<sub>Eco</sub> with a high association (k<sub>a</sub> = 7.0 × 10<sup>4</sup> M<sup>−1</sup> s<sup>−1</sup>) and low dissociation (k<sub>d</sub> = 3.4 × 10<sup>−2</sup> s<sup>−1</sup>) rate, whereas only a weak binding of FrlR was seen with the control DNA. Calculations were based on the association and dissociation rates and with this we derived an affinity of FrlR<sub>Eco</sub> for frlR<sub>Eco</sub> of 4.9 nM. In comparison, the GntR/HutC transcription regulator NagR<sub>Bsu</sub> has a 250 times lower dissociation constant of around 20 pM for the native nagAB operator (Fillenberg et al., 2015) which might reflect an adaption strategy to respond appropriately to specific stimuli.

We were also curious whether the E. coli FrlR binding motif is recognized by the orthologous regulators FrlR of B. subtilis and FraR of S. enterica. In this regard, the corresponding genes were placed under control of the arabinose inducible promoter P<sub>BAD</sub> analogous to frlR of E. coli. NagR of B. subtilis (recognition motif: 5′-GTGTCTAGACCAC-3′) was also included in the study and served as negative control. Binding to P<sub>frlABCD</sub> was tested once more employing the −219/+75 lux reporter. Despite significant differences in sequence composition of their cognate DNA-binding motifs (Figure 7c), the two FrlR<sub>Eco</sub> homologs FrlR<sub>Bsu</sub> and FraR but not NagR<sub>Bsu</sub> negatively regulated P<sub>frlABCD</sub> dependent luxCDAE expression under inducing conditions (Figure 7d). By contrast, leaky expression (no arabinose for promoter activation added) was only sufficient for efficient silencing by FrlR<sub>Eco</sub>. While FrlR<sub>Bsu</sub> retained a mild repressing phenotype, FraR completely lost its regulatory capability. Reportedly, such copy number dependent differences hint to alterations in DNA-binding affinity (Schlundt et al., 2017). The strength of repression matches the phylogenetic distance to FrlR<sub>Eco</sub> and decreases the more distantly related the transcription factor is (Figure 2).

It is notable, that the sequence length between the bracketing GT/AC differs among the three FrlR homologs. Whereas, in frlR<sub>Eco</sub> and frlR<sub>Bsu</sub> the spacer encompasses six bp, in the putative fraO it is seven bp long. This distinguishing feature might provide one rationale explaining the differences in repression efficiency.
To assess DNA binding by FrlR<sub>Eco</sub>, we first verified DNA binding by the putative wHTH-DNA binding domain (Figure 3). DNA-binding to the recognition sequence can be enforced by overproduction of the wHTH-domain solely (Schlundt et al., 2017). Accordingly, we truncated FrlR<sub>Eco</sub> and compared the fragment encompassing amino acids 1-77 with the full-length protein (244 aa). In E. coli cells, that simultaneously harbor the P<sub>PABCD-lux</sub> −219/+75 reporter plasmid, luminescence can be suppressed only when transcription of the FrlR<sub>Eco</sub> 1-77 aa fragment was arabinose induced while with the full-length FrlR<sub>Eco</sub> even low protein levels diminished the light output (Figure 7d). These data strongly imply that indeed the first 77 residues fold into a wHTH-domain that is sufficient to recognize the
**FIGURE 7** In vivo and in vitro analysis of protein/DNA interactions. (a) frlO<sub>Eco</sub>-dependent sfGFP production: sfGfp expression is driven by the T<sub>7</sub> promoter. A sequence was inserted either comprising the putative FrlR<sub>Eco</sub> recognition motif (frlO) or a mutated version (frlO*). The sequence of the motif inserted downstream to the T<sub>7</sub> promoter is shown with bold letters indicating the Escherichia coli frlO core motif. Mutations are colored in red and the transcription start site is underlined. The box plot graph shows the fluorescence intensity (given in relative fluorescence units RFU) of 300 individual cells in the presence or absence of FrlR<sub>Eco</sub>. Pictures were analyzed using ImageJ (Schneider et al., 2012). sfGFP production was visualized by Western blot analysis using anti-GFP specific antibodies (α-GFP). (b) In vitro DNA binding of FrlR<sub>Eco</sub> to frlO<sub>Eco</sub> analyzed by surface plasmon resonance spectroscopy (SPR) with the biotin-labeled DNA fragment frlO<sub>Eco</sub> (left), and the control fragment without the frlO<sub>Eco</sub> (right) using different concentrations of purified FrlR<sub>Eco</sub>. (c) Sequence comparison of the operator of frlO<sub>Eco</sub>, frlO<sub>BSu</sub>, and nagO<sub>BSu</sub> as well as the putative DNA-binding motif of FraR of Salmonella enterica. (d,e) Depicted is the relative P<sub>frlABCD-lux</sub> activity in % which was compared to the light output of JW5698 cells (ΔfrlR) harboring the lux fusion −219/+75. (e) Analyzed are the binding capabilities to frlO<sub>Eco</sub> by the FrlR orthologs depicted in (d) (left) and the E. coli FrlR winged helix-turn-helix DNA-binding domain (wHTH) (right). The corresponding genes were ectopically expressed utilizing P<sub>BAD</sub> with (black bars) and without (grey bars) the addition of 0.2% L-arabinose. The mean values and standard deviations of at least three biological replicates are shown. Protein production was confirmed by Western blot analysis using anti-His<sub>6</sub> specific antibodies (α-His<sub>6</sub>). (e) wHTH mutant analysis. Assessed is the capability of FrlR<sub>Eco</sub> mutant variants to repress the frlABCD promoter. Neg.: ΔfrlR Pos.: ΔfrlR+FrlR<sup>wt</sup>. Protein production was confirmed by Western blot analysis using anti-His<sub>6</sub> specific antibodies (α-His<sub>6</sub>).

**FIGURE 8** FrlR<sub>Eco</sub> domains in vivo/ in vitro interaction analyses. (a) Qualitative in vivo self-interaction analysis of a T18/T25-FrlR UTRA domain fusion as well as a T18/T25-wHTH-domain fusion. (b) Quantitative in vivo self-interaction analysis of a T18/T25-FrlR UTRA domain fusion with and without (−) supplement of α-FrK or ε-FrK. The maximal light emission from a 40 hr time course experiment is given in RLU. Ninety-five percent confidence intervals of at least six replicates are shown. Asterisks indicate significant (p < .05) differences in the maximal light emission determined by a T-test for paired samples between cells without supplement and those being exposed to ε-FrK. (c) Size exclusion chromatography of purified FrlR<sub>Eco</sub> UTRA domain. The elution profile is depicted as solid red line. The black line represents a calibration curve of three proteins with varying size (158 kDa, 44 kDa, and 13.7 kDa). Based on this the FrlR<sub>Eco</sub> UTRA domain migration behavior corresponds to a size of 38.7 kDa. (d) 1D-jump-and-return NMR experiments including 0.004, 2.0, and 2.5 ms relaxation delays from which the transverse relaxation time T<sub>2</sub> is estimated to be 9 ms, allowing to infer a rotational correlation time τ<sub>c</sub> of 21 ns assuming isotropic tumbling.
promoter. However, full-length FrIR\textsubscript{Eco} is needed for high affinity binding.

Next, we generated FrIR\textsubscript{Eco} mutants based on the sequence alignment with NagR of \textit{B. subtilis} (Figure 2). In NagR\textsubscript{Bbu}, the wing motif reaches into the minor groove and contacts the flanking guanosine via the carbonyl oxygen of Gly69 (Fillenberg et al., 2015). In addition, the two conserved arginines Arg38 and Arg48 specifically recognize further guanosines of the operator by forming bidental contacts with their corresponding guanine base. Taken together, these three amino acids are assumed to be the hallmark feature of the specific interaction of NagR\textsubscript{Bbu} with DNA (Fillenberg et al., 2015). Two of the contacts—Gly69 and Arg48—are also conserved throughout FrIR/ FraR homologs (Figure 2). Accordingly, we mutated the corresponding residues—Arg49 and Gly70—into alanine resulting in the FrIR\textsubscript{Eco} variants R49A and G70A. We additionally mutated the putative nucleic acid interacting residues N39, I50, R67, and K71 into alanine, being in the equivalent positions to NagR’s Arg38, Met49, and Arg70, respectively. The functionality of the FrIR\textsubscript{Eco} variants was tested in vivo on their capability to abolish luminescence in ΔfrIR cells encoding the ε−219/+75 \textit{P}\textsubscript{frlABCD}−lux reporter (Figure 7e). Three out of the six mutants—R49A, G70A, and K71A—clearly lost their repressing capability, indicating a role in DNA binding. This led us to conclude that FrIR\textsubscript{Eco} binds to its recognition motif in a way similar as described for NagR\textsubscript{Bbu}.

\subsection*{2.5 FrIR\textsubscript{Eco} dimerizes via its C-terminal UTRA domain}

The predicted structural similarities to NagR\textsubscript{Bbu} combined with our data on FrIR\textsubscript{Eco} DNA-binding implies that substrate binding might also occur analogously. Canonically, members of the GntR/HutC family of transcriptional regulators form antiparallel dimers via their UTRA domain to accommodate the two half sites of their palindromic GT/AC flanked DNA-binding motif (Fillenberg et al., 2015; Rigali et al., 2002; Suvorova et al., 2015). Whether this mode of action also applies for FrIR\textsubscript{Eco} we examined the dimerization tendency in vitro and in vivo. For the in vivo analysis, the bacterial two-hybrid system (BTH) described by Karimova \textit{et al.} was employed (Karimova \textit{et al.}, 1998) with the exception that all experiments were carried out with our recently published reporter strain, that has a luminescence reporter readout in addition to the original LacZ based colorimetric one (Volkwein \textit{et al.}, 2019). Based on the FrIR\textsubscript{Eco} homology modeling (Figure 3), we split the protein into two parts, one comprising the N-terminal DNA-binding domain (aa 1-77), the other one encompassing the C-terminal UTRA domain (aa 78-244) and fused them to the T25 and T18 fragments of \textit{Bordetella pertussis} adenylate cyclase. Bioluminescence was recorded qualitatively on agar plates together with the GCN4 leucine zipper as positive (bright phenotype) and T25/T18 solely as negative control (dark phenotype) (Figure 8a). When assessing the self-interaction of the UTRA domain we saw the bright phenotype, whereas clones with the wHTH-domain remained dark, confirming our initial assumption.

We also tested whether FrK influences interaction strength or might even interfere with dimer formation. Consequently, we measured bioluminescence development quantitatively by recording the light output in a 40 hr time course experiment. The maximal RLU are plotted as a bar diagram (Figure 8b). Neither the addition of the ε nor the ε−glycated FrK abolished light emission. However, we observed a slight but significant change in interaction strength with the latter. Reportedly, NagR\textsubscript{Bbu} derepression is not achieved by monomerization upon signal perception (Resch \textit{et al.}, 2010) as, for example, in the case of other one-component systems such as CadC (Buchner \textit{et al.}, 2015; Lindner and White, 2014). Instead, substrate binding to the UTRA domain is transduced into a structural rearrangement of the two wHTH-domains, which hinders proper DNA binding. Similarly, such movement might also reposition the two fragments T18 and T25 and could explain the reduction in bioluminescence with ε−FrK. This is plausible as the loop, which is transitioned into a helix upon GlcNacP/GlcNP binding to NagR\textsubscript{Bbu} (Fillenberg \textit{et al.}, 2015; Resch \textit{et al.}, 2010), was kept in our fusion construct.

To recapitulate our in vivo observation in vitro, we investigated the oligomerization behavior of the UTRA domain (FrIR-UTRA) by determining its molecular weight by size exclusion chromatography (Figure 8c). In its monomeric form, this would correspond to 19.3 kDa. As expected, and in line with the BTH data, FrIR-UTRA runs at the size of dimers. The addition of ε-FrK did not change the migration behavior of FrIR-UTRA (data not shown) further supporting that the regulation does not alter the oligomeric state of the protein. In addition to this, nuclear magnetic resonance (NMR) spectroscopy also confirmed the dimeric state of FrIR-UTRA. The line width of \textsuperscript{1}H-\textsuperscript{15}N resonances in \textsuperscript{1}H-\textsuperscript{15}N-NHSQC spectra is larger than what would be expected for a monomeric FrIR-UTRA (Figure S4a). Receptor-based titration with ε-FrK did not change the resonance line width in \textsuperscript{1}H-\textsuperscript{15}N-NHSQC spectra and we could only observe small chemical shift perturbations (Figure S4a) even with six-fold excess of ε-FrK, indicating that interaction with ε-FrK is weak and FrIR-UTRA does not become monomeric upon titration with ε-FrK. This qualitative assessment of FrIR-UTRA dimerization was followed up with 1D \textit{T}\textsubscript{2} experiments, in which a relaxation delay has been included in a 1D-\textit{T}\textsubscript{2} experiment to estimate the transverse relaxation time \textit{T}\textsubscript{2}. From three different relaxation delays (0.004, 2.0 and 2.5 ms) we could estimate \textit{T}\textsubscript{2} to be 9 ms (Figure 8d). Assuming isotropic tumbling, we estimate a rotational correlation time of 21 ns which would correspond to a molecular weight of 42 kDa. This indicates that FrIR\textsubscript{Eco} tumbles as a dimer in solution. The weak interaction with ε-FrK is confirmed by ligand-based titration using saturation transfer difference (STD)-NMR, where no magnetization transfer could be observed from protein to ligand suggesting an affinity weaker than \textsuperscript{10} M−1 for the FrIR−ε-FrK interaction (Figure S4b,c).

\subsection*{2.6 Fructoselysine-6-phosphate is the cognate effector substrate of FrIR}

We have conclusively shown, that ε-FrK efficiently induces \textit{E. coli} FrR dependent derepression in vivo. However, in vitro FrIR\textsubscript{Eco}
interaction analysis with the ligand showed only a weak response (Figure S3a) or even failed (Figure S3b,c). Thus, we hypothesized that not ε-FrK directly but instead one of its metabolic derivatives is the cognate substrate. This is a plausible explanation as the structural homolog of FrlR<sub>Eco</sub>—NagR<sub>Bsu</sub>—recognizes the phosphorylated form of GlcNAc—GlcNacP (Resch et al., 2010; Rigali et al., 2002). Analogous to this, the only rational candidate is FrK-6P, which is generated by the kinase FrlD as first step of the degradation pathway (Figure 1). If the assumption is true, loss of FrlD will prevent from ε-FrK mediated derepression of P<sub>frlABCD</sub>. We, therefore, utilized an E. coli ΔfrlD strain containing the −219/+75 P<sub>frlABCD</sub>-lux fusion and measured the light output in the presence and the absence of the ARP. Indeed, the frlD<sup>−</sup>-strain lost its ability to respond to ε-FrK, as can be inferred from the maximum light emission, which—unlike frlD<sup>+</sup>-cells—did not increase with external supplementation of the ARP (Figure 9).

The sequence comparison of FrlR<sub>Eco</sub> and NagR<sub>Bsu</sub> revealed high conservation of the phosphate-binding pocket (Figure 2). Therefore, we decided to investigate whether FrK-6P might be accommodated analogously as GlcNacP in NagR<sub>Bsu</sub> (Figure 3). Accordingly, we generated the substitution variants located in the UTRA domain, namely T91A, R133A, S166A, and Y168A, and investigated them on their capability to regulate promoter activity in an ε-FrK dependent manner once more utilizing the −219/+75 P<sub>frlABCD</sub>-lux fusion. With the wild-type protein and in the absence of ε-FrK we observed a strong repression, exhibited by an about 95% diminished light output compared to cells lacking frlR<sub>Eco</sub> (Figure 9). The luminescence increased by more than five-fold when the culture was supplemented with the ARP. Deviating from this behavior S166A and Y168A did not respond to the presence of ε-FrK with P<sub>frlABCD</sub> remaining repressed. Similarly, R133A became blind to the inductor but at the same time also lost its DNA-binding capability. We hypothesize that this variant is locked in its inactive state that precludes efficient operator recognition. In summary, these data strongly indicate that FrK-6P is the cognate substrate of FrlR<sub>Eco</sub> and is recognized similar to GlcNacP by NagR<sub>Bsu</sub>.

3 | DISCUSSION

In the present study, the transcriptional regulation of FrK metabolism in E. coli was investigated. Our data show that the expression of the frlABCD operon is controlled by global and specific stimuli. We identified two promoter regions, both stimulated by the alternative sigma factor RpoH (σ<sub>32</sub>). The induction of the other σ<sub>70</sub>-dependent promoter is subject to CAMP/CRP triggered catabolite repression. A repressor FrlR<sub>Eco</sub> further prevents transcription in the absence of FrK. However, this substrate has to be processed to FrK-6P by the kinase FrlD to be recognized as the cognate stimulus. Accordingly, a basal frlABCD gene expression is necessary even under repressive conditions. Several scenarios are conceivable to achieve such a goal. For instance, the copy number of the FrlR<sub>Eco</sub> protein could be limited to such an extent that not every cell has a sufficient amount for effective repression. In this case, an unequal distribution of the permease FrlA and kinase FrlD occurs within the population, which enables an individual response to the carbon source. The resulting heterogeneity might be particularly pronounced because of the complex regulation and the necessity to transport and modify ε-FrK to induce the cell response. The so-called “bet hedging” strategy (van Vliet and Ackermann, 2015) would be particularly advantageous here: Limiting the FrK metabolism to a subset of cells allows the simultaneous utilization of different carbon sources by the entire population. In the intestine, E. coli is confronted with exactly such a situation and is, thus, able to compete with other bacteria of the gut microbiota. In the same context, the specialization of the metabolism toward certain ARPs is also beneficial. While E. coli exclusively utilizes ε-fructose/psicoselysine, B. subtilis favors various α-glycated amino acids (Wiame et al., 2004, Wiame and Van Schaftingen, 2004), thus creating distinct niches and minimizing competition. However, such selectivity also demands for species specific regulation patterns. Global transcriptional control seems to be adjusted to the respective organism with B. subtilis being dependent on CodY, a transcriptional regulator that helps to adapt to changes in nutrient availability (Deppe et al., 2011) and E. coli where ARP metabolism is activated by CRP/cAMP and σ<sub>12</sub> instead. By contrast, the substrate-specific FrlR proteins from both organisms are 63% similar and even recognize the same operator sequence. It is therefore possible, that both FrlRs also respond to the same stimulus. However, in our assays.
we saw a highly specific response of *E. coli* FrIR toward ε-FrK. As only its phosphorylated form is the cognate signal for FrIR, an elegant way to achieve regulatory specificity could come through distinct substrate specificities of certain enzymes in the degradation pathways. Notably, the FrID kinases from *E. coli* and *B. subtilis* differ in their enzymatic properties (Wiame et al., 2004). The Km of ε-FrK for instance is 20 µM with FrIDeco the corresponding one for FrIDbiu (YurL) is three orders of magnitude higher (14 mM). Conversely, α-fructosealine is processed efficiently by the *B. subtilis* enzyme at a concentration of about 100 µM, whereas the *E. coli* counterpart has a Km above 20 mM. The selectivity of the metabolism can also explain how FrIRbiu is able to respond to chemically diverse α-glycated substrates. Specifically, substrate recognition could simply be achieved by the combination of sugar 6-phosphate and C2-N linkage, two structure elements shared by all phosphorylated ARPs derived from glucose.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, plasmids, and growth conditions

Bacterial strains, plasmids, and primers are listed in Tables S1–S3. *E. coli* was routinely cultivated in LB according to the Miller modification (Bertani, 1951; Miller, 1992) or M9 minimal medium (Miller, 1972) unless indicated otherwise. For solidification 1.5% (w/v) agar was added to the medium. If needed, carbon sources and other media supplements were added as indicated. Antibiotics were used at the following final concentrations: 100 µg/ml ampicillin sodium salt, 50 µg/ml kanamycin sulfate, 30 µg/ml chloramphenicol, or 20 µg/ml gentamicin sulfate. Growth was recorded by measuring the optical density at a wavelength of 600 nm. Plasmids carrying the pBAD (Guzman et al., 1995) or Tp promoter were induced with L-arabinose at a final concentration of 0.2% (w/v) or Isopropyl-β-D-thiogalactopyranosid (IPTG) at a final concentration of 1 mM.

All kits and enzymes employed for plasmid construction were used according to manufacturer's instructions: Plasmid DNA was isolated using the HiYield® Plasmid Mini Kit from SÜD-Laborbedarf GmbH. DNA fragments were purified from agarose gels using the HiYield® Gel/PCR DNA fragment extraction kit from SÜD-Laborbedarf GmbH. All restriction enzymes, DNA modifying enzymes, and the Q5® high fidelity DNA polymerase for PCR amplification were purchased from New England BioLabs GmbH. A detailed description for plasmid construction is given in Table S2.

4.2 | Synthesis and analysis of ARPs

*Nε- and Nα-FrK were synthesized and isolated according to previous publications (Hellwig et al., 2011; Krause et al., 2003) and met the spectroscopic properties given in those works. Analysis of both ARPs as well as lysine was performed by amino acid analysis with the analyzer S 433 (Sykam) on a cation-exchange column (LCA K07/Li; 150 mm × 4.6 mm, 7 µm). Before analysis, 10 µl of solutions from microorganism culture was diluted with 190 µl of loading buffer (0.12 M lithium citrate, pH 2.12), centrifuged (10.000× g, 10 min), and 40 µl of the diluted sample was injected. Separation was accomplished with custom lithium citrate buffers of increasing pH and ionic strength. Amino acids were detected by online post-column derivatization with ninhydrin and UV-detection (570 nm). Calibration was performed by an external standard of proteinogenic amino acids, and both FrK derivatives were quantified as lysine (Hellwig et al., 2011).

4.3 | SDS–PAGE and western blotting

For protein analyses cells were subjected to 12.5% (w/v) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (Laemmli, 1970). To visualize proteins by UV light 2,2,2-trichloroethanol was added to the polyacrylamide gels (Ladner et al., 2004). Subsequently, the proteins were transferred onto nitrocellulose membranes, which were then subjected to immunoblotting. In a first step the membranes were incubated either with 0.1 µg/ml anti-6×His® antibody (Abcam) or with 0.1 µg/ml anti-GFP (Sigma-Aldrich) antibody. These primary antibodies (rabbit) were targeted with 0.2 µg/ml anti-rabbit alkaline phosphatase-conjugated secondary antibody (Rockland) or 0.1 µg/ml anti-rabbit IgG (IRDye® 680RD) (donkey) antibodies (Abcam). Anti-rabbit IgG was detected by adding development solution [50 mM sodium carbonate buffer, pH 9.5, 0.01% (w/v) p-nitro blue tetrazolium chloride (NBT), and 0.045% (w/v) 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)]. Anti-rabbit IgG were visualized via Odyssey® CLx Imaging System (LI-COR, Inc).

4.4 | Bacterial two-hybrid assay

Protein-protein interactions were detected using the bacterial adenylate cyclase two-hybrid system kit (Euromedex) according to product manuals (Karimova et al., 2000). This system is based on functional reconstitution of split Bordetella pertussis adenylate cyclase CyaA, which catalyzes the formation of cyclic AMP from ATP. In *E. coli* KV1, the cAMP dependent lac promoter Plac precedes a translational fusion of the lux-operon and lacZ, allowing the indirect measurement of protein-protein interactions by light emission and colorimetric detection (Volkwein et al., 2019).

For measuring interaction strength, chemically competent (Inoue et al., 1990) *E. coli* KV1 cells were transformed with pKT25-frIR FL, pKT25-frIR UTRA domain, or pKT25-frIR HTH-domain and pUT18C-frIR FL, pUT18C-frIR UTRA domain, or pUT18C-frIR HTH-domain. Transformants containing pUT18-3C/pKT25-3C and pUT18C/pKT25 vector backbones were used as positive and negative controls, respectively. Single colonies were inoculated in LB (with 50 µg/ml kanamycin sulfate and 100 µg/ml ampicillin sodium salt, 5 mM α-/ε-FrK and 0.5 mM IPTG (w/v)) and grown aerobically at 37°C o/n. The next day, a microtiter plate with fresh LB (with
the appropriate antibiotics, 10 mM substrate and 0.5 mM IPTG (w/v) was inoculated with the cells at an OD_{600} of 0.01. The cells were grown aerobically in the Tecan Infinite F500 system (TECAN) at 30°C. OD_{600} and luminescence were recorded in 10 min intervals over the course of 16 hr. Each measurement was performed at least in triplicate.

For qualitative analysis, the bacterial two-hybrid KV1 strains were plated on LB agar (containing 50 µg/ml kanamycin sulfate, 100 µg/ml ampicillin sodium salt, and 0.5 mM IPTG) and grown overnight at 37°C. Pictures of the plates were taken in a Fusion-SL 3500 WL (PEQLAB) using 10 s of exposure time.

4.5 Luminescence activity assay

*E. coli* cells harboring a lux fusion plasmid (Table S2—reporter assays) were inoculated in LB (with appropriate antibiotics and 0.2% arabinose (w/v)) and grown aerobically at 37°C. The next day, a microwell plate with fresh LB (with the appropriate antibiotics and 0.2% arabinose (w/v)) was inoculated with the cells at an OD_{600} of 0.01. The cells were grown aerobically in the Tecan Infinite F500 system (TECAN) at 37°C. OD_{600} and luminescence were recorded in 10 min intervals over the course of 16 hr. Light units were normalized to OD_{600} and are thus expressed in relative light units (RLU). Each measurement was performed in triplicate.

To examine the regulatory role of rpoH, the growth temperature was adjusted to 20°C and 25°C during the measurement. The binding site for CAP was examined in LB with or without 20 mM glucose, thereby repressing or activating CyaA, respectively.

4.6 Surface plasmon resonance spectroscopy

Surface Plasmon Resonance Spectroscopy (SPR) spectroscopy and calibration-free concentration (CFCA) assays were performed using a Biacore T200 device (GE Healthcare) and streptavidin-precoated Xantec SAD500-L carboxymethyl dextran sensor chips (XanTec Bioanalytics GmbH, Düsseldorf, Germany). All experiments were conducted at 25°C with HBS-EP buffer [10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) detergent P20].

Before immobilizing the DNA fragments, the chips were equilibrated by three injections using 1 M NaCl/50 mM NaOH at a flow rate of 10 µl min⁻¹. Then, 10 nM of the respective double-stranded biotinylated DNA fragment was injected using a contact time of 420 s and a flow rate of 10 µl min⁻¹. As a final wash step, 1 M NaCl/50 mM NaOH/50% (v/v) isopropanol was injected. Approximately 250-350 RU of each respective DNA fragment were captured onto the respective flow cell. All interaction kinetics of FlrR with the respective DNA fragment were performed in HBS-EP buffer at 25°C at a flow rate of 30 µl min⁻¹ in the presence and absence of 1 mM ε-FrK. The proteins were diluted in HBS-EP buffer and passed over all flow cells in different concentrations (0.5 nM-125 nM) using a contact time of 180 s followed by a 300 s dissociation time before the next cycle started. After each cycle the surface was regenerated by injection of 2.5 M NaCl for 30 s at 60 µl min⁻¹ flow rate followed by a second regeneration step by injection of 0.5% (w/v) SDS for 30 s at 60 µl min⁻¹. All experiments were performed at 25°C. Sensorgrams were recorded using the Biacore T200 Control software 2.0 and analyzed with the Biacore T200 Evaluation software 2.0. The surface of flow cell 1 was not immobilized with DNA and used to obtain blank sensorgrams for subtraction of bulk refractive index background. The referenced sensorgrams were normalized to a baseline of 0. Peaks in the sensorgrams at the beginning and the end of the injection emerged from the run-time difference between the flow cells of each chip.

Calibration-free concentration analysis (CFCA) was performed using a 1 µM solution of purified FlrR_{Eco}, which was stepwise diluted 1:2, 1:5, 1:10, and 1:20. Each protein dilution was injected two-times, one at 5 µl min⁻¹ as well as 100 µl min⁻¹ flow rate. On the active flow cell DNA fragment including the P_{lux}-DNA was used for FlrR binding. CFCA basically relies on mass transport, which is a diffusion phenomenon that describes the movement of molecules between the solution and the surface. The CFCA therefore relies on the measurement of the observed binding rate during sample injection under partially or complete mass transport limited conditions. Overall, the initial binding rate (dR/dt) is measured at two different flow rates dependent on the diffusion constant of the protein. The diffusion coefficient of BceR-P was calculated using the Biacore diffusion constant calculator and converter webtool (https://www.biacore.com/lifesciences/Application_Support/online_support/Diffusion_Coefficient_Calculator/index.html), whereby a globular shape of the protein was assumed. The diffusion coefficient of FlrR was determined as D = 1.02 × 10⁻¹⁰ m²/s. The initial rates of those dilutions that differed in a factor of at least 1.5 were considered for the calculation of the “active” concentration, which was determined as 5 × 10⁻⁷ M (approximately 50% of the total protein concentration) for FlrR. The “active” protein concentration was then used for calculation of the binding kinetic constants and steady-state affinity.

4.7 Protein purification

His_{6}-SUMO-tagged FraR from *S. enterica* (pET-SUMO-fraR (Sen)), FlrR from *E. coli* (pET-SUMO-flrR (Eco)) and *B. subtilis* (pET-SUMO-flrR (Bsu)) (Table S2—Protein overexpression) were overproduced in *E.coli* BL21 (DE3) by addition of 1 mM IPTG to exponentially growing cells and subsequent cultivation at 18°C o/n. Cells were lysed by sonication in the respective buffer (Table S4). The proteins were purified using Ni-nitrotriacetic acid (Ni-NTA; Qiagen) according to the manufacturer’s instructions, using 20 mM imidazole for washing and 250 mM imidazole for elution. Subsequently, imidazole was removed by dialysis o/n at 4°C in buffer 1. The His_{6}-SUMO tag was cleaved by incubation with His_{6}-Ulp1 (Starosta et al., 2014) overnight.
Subsequently, tag-free FrlR\textit{Eco} was collected from the flow through after metal chelate affinity chromatography. Size exclusion chromatography was performed in the respective buffer (Table S4) using a Superdex 200 Increase 10/300-Gl column with a flow rate of 0.3 ml/min on an Äkta purifier (GE Healthcare). Four milligrams of protein were loaded in a volume of 0.4 ml (8.7 mg/ml). Eluting protein was detected at 280 nm. Fractions of 0.5 ml were collected.

4.8 NMR experiments

All NMR spectra were acquired using a Bruker Avance III NMR spectrometer with a magnetic field strength corresponding to a proton Larmor frequency of 700 MHz equipped with a room temperature triple resonance gradient probe head. NMR experiments were performed in a buffer containing 100 mM potassium phosphate, 300 mM NaCl, pH 6.5 at 298 K. Two dimensional $^1$H-$^1$N-HSQC titrations were performed with 200 $\mu$M FrlR-UTRA domain in the absence and presence of 1.2 mM excess of $\varepsilon$-FrK. For measuring $T_2$ relaxation, one dimensional experiments were performed with 200 $\mu$M FrlR-UTRA in the presence of 1.2 mM $\varepsilon$-FrK. 1D $T_2$ experiments were performed using a 1-1 echo pulse sequence with a relaxation delay varying between 0.004, 2.0 and 2.5 ms (Sklenář and Bax, 1987). $T_2$ was estimated using the equation $1/(5T_2) = 1$/(2$T_2$) where $T_2$ is expressed in seconds (Barbato et al., 1992; Kay et al., 1989). Saturation transfer difference NMR experiments (Mayer and Meyer, 1999) were performed on 10 $\mu$M of FrlR-UTRA + 1mM $\varepsilon$-FrK with irradiation at either 0.65 ppm (protein methyl region) or 8.5 ppm (protein amide region), far from $\varepsilon$-FrK signals to only saturate protein, with a relaxation delay of 5 s, which includes an effective saturation time of 4 s and an interscan delay of 1 s. For control experiments only 1 mM $\varepsilon$-FrK was used.

4.9 Fluorescence microscopy

The DNA-binding site of FrlR\textit{Eco} was examined by fluorescence microscopy of the \textit{E. coli} strain BL21 (DE3) transformed with pUC19 based fluorescent reporter plasmids (Table S2—Reporters assays). The cells were cultivated overnight at 30°C in LB medium supplemented with ampicillin. Expression of FrlR\textit{Eco} was induced or repressed by addition of 20 mM arabinose or 20 mM glucose, respectively. The overnight cultures were used to inoculate (OD\textit{600} of 0.1) fresh LB medium supplemented with ampicillin and arabinose or glucose. Cells were aerobically cultivated at 37°C and harvested by gentle centrifugation after 2 hr. The pellet was washed twice and resuspended in PBS (OD\textit{600} 0.5). 2 $\mu$l of the culture was spotted on 2% (w/v) agarose pads, placed onto microscopic slides and covered with a coverslip. Subsequently, images were taken on a Leica DMi8 inverted microscope equipped with a Leica DFC365 FX camera (Wetzlar). An excitation wavelength of 484 nm and a 535 nm emission filter with a 75-nm bandwidth was used for sfGFP fluorescence for 1 s, gain 5, and 75% intensity. Fluorescence intensity of at least 300 cells was measured and plotted using ImageJ.

4.10 Thermal shift assay

About 5 $\mu$M of the Protein (FrlR\textit{Eco}) was pipetted into 30 $\mu$l (2.5 $\mu$M) of DNA (cleaned up PCR product) on ice. About 0.3 $\mu$l of a 1:10 dilution of SYPRO\textsuperscript{TM} orange protein gel stain (Thermo Fisher Scientific) was added to the mix. The mixture was transferred into a 96-well semi-skirted PCR plate. To ensure that the liquid was at the bottom the plate was centrifuged for 30 s at 3,000 rpm. Subsequently, the plate was inserted into iQ\textsuperscript{TM}5 Real-Time PCR Detection Systems (Bio-Rad). The 96-well plate was incubated at 8°C for 10 min. Every 30 s the temperature was increased by 0.5°C until 90°C were reached. Fluorescence was recorded at a wavelength of 520 nm.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Biochemical and genetic analyses on FrlR/FraR/NagR were conducted by N.G., F.K., and B.G.V.A. NMR studies were performed by P.K.A.J and J.H. The corresponding proteins were produced and purified by B.G.V.A., N.G. and F.K. M.H. and T.H. synthesized ARPs and analyzed $\varepsilon$/a-FrK turnover. J.L. designed the study. The manuscript was written by B.G.V.A., N.G. and F.K., P.K.A.J., J.H., M.H. and J.L.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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