

Towards the control of biofilm formation in *Anabaena* (*Nostoc*) sp. PCC7120: novel insights into the genes involved and their regulation

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Summary

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- Cyanobacteria are major components of biofilms in light-exposed environments, contributing to nutrient cycling, nitrogen fixation and global biogeochemical processes. Although nitrogen-fixing cyanobacteria have been successfully used in biofertilization, the regulatory mechanisms underlying biofilm formation remain poorly understood.
- In this work, we have identified 183 novel genes in *Anabaena* sp. PCC7120 potentially associated with exopolysaccharide (EPS) biosynthesis and biofilm formation, unveiling conserved and novel regulatory connections shared with phylogenetically distant bacteria.
- *Anabaena* possesses homologues of two-component systems such as XssRS and ColRS from *Xanthomonas* spp., and AnCrpAB from *Methylobacillus*, suggesting that these homologues play essential or advantageous roles in biofilm formation across diverse bacterial lineages. Additionally, *Anabaena* features homologues of several proteins exhibiting the GG-secretion motif typical of small proteins required for biofilm formation in unicellular cyanobacteria. A wide array of biofilm-related genes in *Anabaena*, including major gene clusters participating in the synthesis and translocation of EPS and key regulatory proteins involved in the control of biofilms in other bacteria are modulated by ferric uptake regulator proteins.
- These findings link the control of biofilm formation in *Anabaena* to environmental cues such as metal availability, desiccation and nitrogen levels, providing new insights to improve the use of nitrogen-fixing cyanobacterial biofilms in sustainable agriculture and environmental management.

Introduction

Biofilms are complex communities of microorganisms that adhere to surfaces and are embedded within a self-produced matrix of extracellular polymeric substances mainly composed of exopolysaccharides (EPSs) and water. Biofilms are ubiquitous in nature and contribute to processes such as nutrient cycling, pollutant degradation and symbiotic interactions (Perera *et al.*, 2022; Vandana & Das, 2023). In light-exposed environments, photosynthetic microorganisms such as cyanobacteria, algae and photosynthetic bacteria are important biofilm components. These microorganisms significantly influence the health and stability of their habitats, such as freshwater bodies, oceans and soil surfaces.

Cyanobacteria play a crucial role in both the atmosphere and the oceans, significantly impacting global biogeochemical cycles and supporting marine ecosystems (Falkowski & Raven, 2007). Their low nutritional requirements and ability to adapt to adverse environmental conditions drive their ecological success. Cyanobacterial biofilms have been successfully used in the stabilization

of agricultural soils (Peng & Bruns, 2019) and in biofertilization approaches (Bao *et al.*, 2021), since some cyanobacterial strains have the ability to fix atmospheric nitrogen, making it accessible to plants and other organisms (Esteves-Ferreira *et al.*, 2017). In filamentous diazotrophic cyanobacteria, such as *Nostoc* spp., nitrogen fixation occurs in specialized cells called heterocysts, in which nitrogenase is protected against oxygen by a thickened cell envelope consisting of polysaccharide (HEP) and glycolipid (HGL) layers, which limit oxygen diffusion (Shvarev *et al.*, 2019). Cyanobacteria also release EPS, forming protective barriers that help them attach to surfaces, retain moisture and capture nutrients. The EPS exported by cyanobacteria exhibit unique features in comparison with those generated by other bacteria. Cyanobacterial EPS include a wider variety of monosaccharides, with the predominance of uronic acids and the presence of sulphate groups. Therefore, their anionic nature makes them very suitable to use in the bioremediation of heavy metals (Pereira *et al.*, 2011; Di Pippo *et al.*, 2013; Mota *et al.*, 2016). As many other organisms do, cyanobacteria produce two main types of polysaccharides: capsular polysaccharides (CPS) and released

polysaccharides (RPS). CPS are tightly bound to the cell surface and can form various structures such as sheaths, capsules or slimes. These structures offer protection to the cyanobacterial cells by forming a barrier against environmental stresses, playing a critical role in maintaining cell integrity and facilitating interactions with the environment (Kehr & Dittmann, 2015; Cruz *et al.*, 2020). Conversely, RPS are excreted into the surrounding medium, facilitating biofilm formation and providing a microenvironment that can trap nutrients and protect against toxins (Kehr & Dittmann, 2015). Therefore, both CPS and RPS have significant ecological and biotechnological importance and contribute to the survival and adaptability of cyanobacteria in adverse environments.

In silico analysis of cyanobacterial genomes revealed that many genes potentially involved in the biosynthesis and transport of extracellular glycans are clustered into operons and often occur in multiple copies, resulting in redundancy (Yoshimura *et al.*, 2007; Pereira *et al.*, 2009; Flores *et al.*, 2019). However, although most strains contain genes encoding proteins related to the three main export pathways (Wzy [the wzy gene encodes the O-antigen polymerase]-dependent, ATP-binding cassette (ABC) transporter-dependent and synthase-dependent), they often lack the complete set of genes required for any single pathway (Kehr & Dittmann, 2015; Pereira *et al.*, 2015). While the mechanisms involved in the synthesis, polymerization, chain length control and export of EPSs in cyanobacteria are relatively conserved, the sugar activation/modification enzymes and the glycosyltransferases (GTs) are strain-dependent (Pereira *et al.*, 2009, 2011). Interestingly, most cyanobacteria harbour a larger number of genes coding for GTs than those present in heterotrophic bacteria (Pereira *et al.*, 2009; Potnis *et al.*, 2021; Drula *et al.*, 2022). Among them is the nitrogen-fixing cyanobacterium *Anabaena* (*Nostoc*) sp. PCC 7120, which contains at least 81 genes coding for GTs or probable GTs with the corresponding motif (Potnis *et al.*, 2021), evidencing the intricate complexity of its glycan biosynthesis pathways. Furthermore, EPS composition and biofilm formation are affected by light conditions, as well as diverse environmental stresses and nutritional factors, such as metal availability or the C : N ratio (Kehr & Dittmann, 2015; Mota *et al.*, 2021; Kobayashi *et al.*, 2023; Madsen *et al.*, 2023; Moia *et al.*, 2023; Reignier *et al.*, 2023), suggesting the participation of numerous transcriptional regulators in the control of these processes.

Beyond their potential use in bioremediation, biofilms of nitrogen-fixing cyanobacteria have a profound impact on soil fertility. They enhance nutrient availability, reduce the need for chemical fertilizers and promote sustainable agricultural practices (Chamizo *et al.*, 2018). However, most studies focussed on identifying the genes involved in the synthesis and export of EPSs and the formation of biofilms have been performed on unicellular, nondiazotrophic cyanobacteria (Ozturk & Aslim, 2010; Jittawuttipoka *et al.*, 2013; Pereira *et al.*, 2019; Santos *et al.*, 2021; Kobayashi *et al.*, 2023), and the regulatory pathways related to those processes are poorly characterized in nitrogen-fixing models, such as *Anabaena* sp. PCC7120 (hereafter *Anabaena*). Therefore, identifying novel genes associated with EPS biosynthesis

and biofilm formation in *Anabaena*, along with studying their regulation, is crucial for optimizing diazotrophic cyanobacterial biofilms for biofertilization and enhancing their bioremediation capabilities, offering significant benefits for both agricultural and environmental applications.

Ferric uptake regulator (FUR) proteins are global regulators in cyanobacteria involved in the control of metal homeostasis, nitrogen metabolism and the oxidative stress response (Sevilla *et al.*, 2021). Therefore, considering the factors affecting EPS biosynthesis and biofilm formation, along with the role of the FUR family in managing stress responses, it is reasonable to suggest that these proteins might be involved in modulating key genes and operons responsible for the synthesis of EPSs and the formation of biofilms. In *Anabaena*, the FUR family consists of three paralogs, named Fur or iron uptake regulator (FurA), FurB or zinc uptake regulator (Zur) and FurC or peroxide response regulator (PerR). Besides their relevant function in the modulation of the stress responses in *Anabaena*, our previous work unveiled that the expression level of Zur was related to the ability of this cyanobacterium to establish biofilms (Olivan-Muro *et al.*, 2023). Therefore, with the aim of increasing our knowledge about which genes are involved in the formation of biofilms by *Anabaena* and their potential modulation by FUR proteins, a comprehensive search of biofilm-related genes in other bacterial strains has been performed, and the potential orthologues in *Anabaena* have been identified. The evaluation of the presence of FUR-binding boxes in their promoter regions, together with electrophoretic mobility shift assays (EMSA) and real-time polymerase chain reaction (real time RT-PCR) assays using different FUR-deregulated strains of *Anabaena*, evidenced that this family of regulators is either directly or indirectly involved in the modulation of pivotal genes related to the synthesis and export of EPS, and, in turn, to biofilm formation. By understanding the genetic and regulatory mechanisms behind these processes in *Anabaena*, including the role of FUR proteins in EPS biosynthesis, we can potentially engineer more efficient cyanobacterial biofilms. These biofilms could enhance nitrogen fixation, minimize the use of manufactured fertilizers and improve soil health. Additionally, the ability of cyanobacterial biofilms to aid in bioremediation offers further potential to address soil contamination and support plant growth, promoting environmentally friendly farming practices.

Materials and Methods

Bioinformatic identification of homologues

Protein sequences were retrieved from UniProt and used as query to run a BLASTp (Bateman *et al.*, 2023) against the *Nostoc* sp. (strain PCC 7120/SAG 25.82/UTEX 2576) proteome applying an e-threshold of E-03. Based on additional factors such as identity percentage, sequence coverage and cellular localization (both based on bioinformatic predictions and published proteomics data), up to four best homologues were selected for each query, with seven being selected in 10 exceptions in which the next best homologues showed a similar fit as the fourth.

Amyloidogenic hotspot prediction

The protein sequences of a selection of potential *Synechococcus elongatus* PCC7942 EbfG1–4 homologues were surveyed for amyloidogenic sequences applying five different methods: APPNN, Waltz, Aggrescan, ArchCandy and TANGO (Fernandez-Escamilla *et al.*, 2004; Conchillo-Solé *et al.*, 2007; Maurer-Stroh *et al.*, 2010; Ahmed *et al.*, 2015; Família *et al.*, 2015). Standard conditions were applied in all cases except for the latter, in which the threshold was increased to 0.5. Potential amyloidogenic hotspots were defined when a consensus was observed between the results of three or more different methods.

Bioinformatic prediction of putative FUR-binding sites

Position weight matrices for FurA, Zur and PerR were generated by MEME (<http://meme-suite.org/tools/meme>) (Bailey *et al.*, 2015) using binding sequences previously identified as direct targets of each FUR paralog, with the default parameters (Napolitano *et al.*, 2012; Gonzalez *et al.*, 2014; Sarasa-Buisan *et al.*, 2022). To identify putative FUR-binding boxes in the promoter regions of the transcriptional units identified in this work, the generated motif matrices were used as input for Find Individual Motif Occurrences (FIMO) analysis (<http://meme-suite.org/tools/fimo>) with a cut-off *P*-value of E-04. The FIMO input sequences considered spanned from –500 to +50 bp with respect to the start codon of each gene, considering both the regions directly upstream of the identified genes and those of the first genes of predicted operons they belong to, according to the MicrobesOnline operon information tool (Alm *et al.*, 2005).

Sequence alignment

CLUSTAL OMEGA v.1.2.4 was used to align sequences of interest and generate percent identity matrices (Madeira *et al.*, 2024). Manual alignment for motif visualization purposes was carried out with JALVIEW 2.11.4 (Waterhouse *et al.*, 2009).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed using DNA fragments obtained through PCR, with *Anabaena* genomic DNA as the template and primer pairs designed to amplify sequences 250–350 bp long, centred around predicted FUR-binding boxes in the promoter regions of selected genes (Supporting Information Table S1). Recombinant FurA, Zur and PerR were purified as described previously (Pellicer *et al.*, 2010; Sein-Echaluce *et al.*, 2018; Sarasa-Buisan *et al.*, 2022).

Mixtures of 20 µl final volume containing 50 ng of the corresponding amplified promoter DNA were incubated at room temperature for 15 min with different concentrations of purified protein in binding buffer (10 mM of Bis–Tris–HCl, pH 7.5, 40 mM KCl, 0.1 mg ml^{–1}, BSA, 1 mM of 1,4-dithiothreitol and 5% (v/v) of glycerol), and either 5 µM ZnSO₄ or 100 µM of MnCl₂ to provide the corepressor metal for Zur and

FurA/PerR, respectively. Additionally, 50 ng of a 150-bp-long internal fragment of gene *pkn22* (*ifpkn22*) was included as competitor DNA in each mixture to probe nonspecific binding. After incubation, samples were mixed with a 6× loading buffer (30 mM of Bis–Tris, pH 8, 30% glycerol and 0.05% bromophenol blue), loaded into a nondenaturing 6% polyacrylamide gel and ran at 4°C under a voltage of 90 V for 110 min. Gels were stained with SYBR[®] Safe (Invitrogen) and visualized in a GelDoc 2000 device (Bio-Rad).

Bacterial strains and culture conditions

Strains used in this work were the wild-type (WT) *Anabaena* sp. PCC7120, as well as the FUR protein deregulation variants AG2770FurA, Δ*zur* and EB2770FurC. The Δ*zur* strain is a deletion–insertion mutant with a C.S3 cassette interrupting the *zur* gene (Napolitano *et al.*, 2012), while the remaining three variants contain the shuttle vector pAM2770 harbouring the coding sequences of *furA*, *zur* or *perR*, respectively, under the control of the copper-inducible *petE* (plastocyanin) promoter to drive overexpression of FurA, Zur or PerR, respectively (Gonzalez *et al.*, 2010; Sein-Echaluce *et al.*, 2015; Sevilla *et al.*, 2019).

All cyanobacterial cultures were grown in BG11 media at 28°C under constant illumination of 30 µmol m^{–2} s^{–1} in an orbital shaker at 120 rpm. pAM2770 derivative-containing strains were grown in the presence of 50 µg ml^{–1} of neomycin (Sigma-Aldrich), with 2 µg ml^{–1} of streptomycin and spectinomycin (Sigma-Aldrich) for the Δ*zur* strain. Since overexpression driven by the *petE* promoter has been described to be sufficient under standard BG11 concentrations, no additional copper was added (Gonzalez *et al.*, 2010).

Biofilm growth assays

The biofilm-forming ability of the FUR deregulation *Anabaena* variants compared with the parent strain was evaluated through static growth in uncoated Ibidi µ-Slide eight-well high-chambered coverslips, applying 300 µl per well of cultures adjusted to an OD_{750 nm} of 0.15 from log phase cultures. All cultures were spun down by centrifugation and had their supernatants removed and replaced with fresh BG11 media to equalize culture starting points. Parafilm was used to seal the lids and minimize evaporation. After 10 d of incubation under 7.5 µmol photons m^{–2} s^{–2} light intensity and 28°C, planktonic and loosely adhered cells were removed from the substrate before staining each well with 0.25% (wt/vol) crystal violet (Merck, Darmstadt, German) for 2 min. Excess dye was removed through three subsequent washes with distilled water. Plates were photographed with a Canon EOS 600D camera to assess biofilm biomass through image quantification with the processing software Fiji (Schindelin *et al.*, 2012). The raw integrated density values obtained for each well were normalized to the average of two control wells, which were photographed and processed alongside every plate to account for minor differences between images, and then expressed as a ratio to the average WT normalized value.

Real-time PCR

For each strain, total RNA was prepared from three independent late-log cultures, harvested by centrifugation and processed as previously described (Sarasa-Buisan *et al.*, 2022). Reverse transcription was carried out on 2 µg of total RNA using SuperScript retrotranscriptase (Invitrogen) following the manufacturer's indications. Reaction set-up consisted of 12.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 µl of 25 µM primer mixture and 10 ng of cDNA template in a final volume of 30 µl in nuclease-free water (Ambion, Waltham, MA, USA). Amplification was performed at 60°C for 40 cycles using the QuantStudio 5 system (Applied Biosystems). Primers were designed using the Primer Express 3 software (ThermoFisher), with sequences available in Table S1. Transcript-level normalization was carried out using the *rnpB* housekeeping gene (Vioque, 1992), and relative quantification and expression fold changes were calculated according to the comparative Ct method ($\Delta\Delta C_t$ method) (Livak & Schmittgen, 2001). The fold change threshold for significant transcriptional differences was set up to ≥ 1.5 or ≤ -1.5 .

Results and Discussion

Identification of novel genes potentially involved in the synthesis of EPSs and biofilm formation in *Anabaena* sp. PCC7120

Previous bioinformatic studies based on the search of functional motifs of glycosyl transferases and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of genes potentially participating in different EPS biosynthesis pathways in *Anabaena* led to the identification of a plethora of genes associated with this process (Potnis *et al.*, 2021). Most of them were GTs, genes involved in the Wz pathway and ABC transporters. With the aim to identify further novel genes relevant to biofilm formation in *Anabaena*, we have used a different approach that entails exhaustive mining of the literature related to bacterial EPSs and biofilms in prokaryotes, followed by bioinformatic analysis to search for potential homologues in *Anabaena*. The vast majority of the selected genes showed to be involved in those processed according to *in vivo* approaches (i.e. usually through mutagenesis in the corresponding organism). Although a common rule of thumb is that two sequences are considered homologous if they share > 30% identity across their entire lengths, this criterion often overlooks many homologues. Therefore, hits were filtered using an expectation value (e-value) threshold of $E-03$. Additional factors, such as identity percentage, sequence coverage, and cellular localization, were then considered to select up to four potential *Anabaena* homologues from all the hits returned, with 10 exceptions in which the fourth to seventh homologues showed a similar fit.

This method allowed us to connect 292 genes from *Anabaena*, including 183 that are novel, with genes from other bacteria known to be involved in biofilm formation, which belonged to various functional categories (Table S2). As expected, most genes found were related to sugar metabolism, EPS synthesis and

transport, or linked to type-IV pili machinery and other secretion systems. However, the identification of several proteins related to chemotaxis and its regulation, two-component (TC) systems and orphan response regulators, which are known to modulate biofilm formation in multiple bacteria, including cyanobacteria, greatly expanded the range of processes potentially involved in biofilm formation in *Anabaena*.

Genes from *Anabaena* previously related to EPS synthesis and biofilm formation A compilation of genes encoding *Anabaena* homologues to proteins previously associated with EPS synthesis and biofilm formation in other bacteria is shown in Table S3. These genes are known to be involved in carbohydrate metabolism or EPS synthesis and secretion in *Anabaena* based on published data available and/or database annotations (Bateman *et al.*, 2023; Kanehisa & Goto, 2000). In addition to 75 genes previously reported by Potnis *et al.* (2021), which include seven clusters of GTs, as well as other sugar transferases and genes participating in polysaccharide synthesis and export genes, by using this alternative approach, we have identified 36 additional genes related to carbohydrate metabolism, synthesis of EPS and biofilm formation.

Most of these novel genes are GTs, as well as nucleotidyltransferases including *all3274*, *all4645* and *alr2825*. *alr2825* is part of a cluster spanning from *alr2825* to *alr2841* that contains genes present in the *hep* island, which together with All5341, Alr3699 and All4160 are required for the formation of the HEP layer of the heterocyst (Awai & Wolk, 2007; Wang *et al.*, 2007). Alr3698 (HepB) is also predicted to be a GT whose requirement for the formation of the HEP layer has not been demonstrated, but it shows a 45% identity with WcaL involved in the synthesis of colanic acid in *E. coli* (Wang *et al.*, 2007). Colanic acid is an essential component in the structure of the EPSs that are part of certain bacterial biofilms (Danese *et al.*, 2000).

Several genes have been identified based on their homology to genes from heterotrophic bacteria, such as epimerases *all2853* and *all3509*. All2853 is homologous to Q83VQ2, an epimerase from *Methylobacillus* sp. strain 12S, and to a dehydratase protein from *Xanthomonas axonopodis* pv. citri (Yoshida *et al.*, 2003; Li & Wang, 2011), while *all3509* is homologous to the UDP-glucuronate 4-epimerase Npun_F1376 from *Nostoc punctiforme*, as well as to the corresponding enzyme from *Methylobacillus* sp. strain 12S (Table S3) (Pereira *et al.*, 2009; Yoshida *et al.*, 2003). Similarly, both the Wzc homolog *all5222* and *all0496* (located in the operon *all0496-0495*), which encodes a cyanoexosortase B, show homology with the corresponding proteins from *N. punctiforme* and *Methylobacillus* sp. strain 12S (annotated as Wza) (Yoshida *et al.*, 2003). Additionally, All0495 is homologous to GumB from *X. axonopodis* (XAC2585), which is essential for establishing structured biofilms on either abiotic or biotic surfaces (Rigano *et al.*, 2007).

Novel genes in Table S3 also include the *Anabaena* RNA polymerase sigma factors *sigJ* and *sigF*. Previous studies evidenced that genes *Synpcc7942_1510* from *S. elongatus* sp. PCC7942 (Zedler *et al.*, 2023; Suban *et al.*, 2024) and *sigB* from *Bacillus subtilis* (Nadezhdin *et al.*, 2020), which encode close homologues of the RNA polymerase sigma factor SigJ from *Anabaena*, are involved

in the modulation of genes related to biofilm formation. SigJ was already known to regulate desiccation tolerance and the synthesis of extracellular polysaccharide in this cyanobacterium (Yoshimura *et al.*, 2007). In addition to these sigma factors, and in good concordance with our previous report in *Anabaena* (Olivan-Muro *et al.*, 2023), the transcriptional regulator Zur (FurB) has also been related to the modulation of biofilms in *S. elongatus* (Simkovsky *et al.*, 2022). A recent work has also highlighted a similar role of Zur in this process in *Yersinia pseudotuberculosis*, with reports indicating that the absence of Zur inhibited biofilm formation, echoing findings from *Anabaena* (Olivan-Muro *et al.*, 2023; Gu *et al.*, 2024).

Genes not previously described as involved in carbohydrate metabolism and synthesis of biofilms in *Anabaena* that are implicated in biofilm formation in other prokaryotes Making a biofilm is a multistep, complex process that goes beyond the synthesis and export of EPSs, requiring the adjustment of other metabolic pathways. Therefore, we sought to identify other *Anabaena* transcriptional units potentially relevant for the establishment of biofilms. The information available about genes from other cyanobacteria and bacterial models, which are not directly related to carbohydrate metabolism and biofilm synthesis but are known to be involved in biofilm formation or whose expression is affected in biofilms, has been used for a comprehensive search of orthologues in *Anabaena*. Table S4 summarizes the genes present in the *Anabaena* genome not previously related to the formation and regulation of biofilms in this organism. Among them, we have identified homologues of several methyltransferases (Fisher *et al.*, 2013; Pereira *et al.*, 2009). Although the specific role of the *Nostoc punctiforme* Npun_F1378 and *Synechocystis* sp. PCC6803 Slr1610 methyltransferases in EPS synthesis has not been firmly established, some methyltransferases are known to regulate lipopolysaccharide O synthesis termination (Jansson *et al.*, 1985; Whitfield, 1995; Clarke *et al.*, 2004; Marczak *et al.*, 2019). Some of the identified homologues (i.e. *all2121*, *alr3527*, *all3750*, *alr5370* and *alr2865*) may perform this role in *Anabaena*, although no such cases have been described so far. Furthermore, our approach allowed us to identify a set of alcohol dehydrogenases (Kaleta *et al.*, 2022; Romeu *et al.*, 2022) and several proteins related to the stress response, some of them containing the PRC-barrel domain (Romeu *et al.*, 2022). Most of the corresponding orthologues from the filamentous cyanobacterium *Toxifilum* sp. LEGE 06021 were differentially expressed under biofilm-forming conditions based on hydrodynamic conditions (Romeu *et al.*, 2022). In particular, *all4050* encodes the PRC-barrel domain protein AvaKa that has been reported to be essential for dehydration tolerance in *Anabaena*. Among other stresses, desiccation elicits the production of EPS in cyanobacteria, especially during biofilm development (Sakamoto *et al.*, 2011; Rossi & De Philippis, 2015). Furthermore, a large set of transcriptional regulators and TC systems from *Anabaena* shows homology with regulatory systems that have been reported to modulate the formation of biofilms in other bacteria or respond to shifts to this mode of growth. This group of genes will be discussed later in [Regulatory systems involved in the synthesis of biofilms](#) section.

It is worth noting the occurrence of homologues of pilin biogenesis proteins present in other cyanobacteria that present motility or chemotaxis, as well as in *X. axonopodis* (Table S4) (Li & Wang, 2011; Schatz *et al.*, 2013; Nagar *et al.*, 2017; Conradi *et al.*, 2019; Simkovsky *et al.*, 2022), whose potential roles in *Anabaena*, which lacks pili, deserve further investigation. Inactivation of homologues of type II secretion/type IV pilus assembly systems in *S. elongatus* led to a biofilm-forming mutant of this unicellular cyanobacterium (Nagar *et al.*, 2017). Additionally, biofilm formation in *S. elongatus* requires the genes *pcc7942_1134* and *pcc7942_1133*, along with three nonannotated short genes located upstream of the former, named *ebfG1-3* (Schatz *et al.*, 2013; Parnasa *et al.*, 2016). The products of *pcc7942_1134* (EbfG4) and *ebfG1-3* contain an N-terminal double-glycine motif, which is a characteristic of bacteriocin precursors. Meanwhile, *pcc7942_1133* encodes PteB, a protein that features an N-terminal peptidase C39 domain responsible for cleaving the N-terminal glycine motif. Both genes are found in the same cluster as the peptidase Synpcc7942_1127 (EbfE), required for the processing of enable biofilm formation with a GG-motif (EbfG) precursor proteins, whose secretion is essential for biofilm development (Parnasa *et al.*, 2016, 2019). Although we could not identify any homologues for EbfG1-4 in *Anabaena* through BLASTp, we found four homologues encoding ABC transporter ATP (adenosine triphosphate)-binding proteins, which exhibit a high degree of identity with PteB, namely Alr7014 (57.7% identity), Alr1201 (55.8%), Alr5147 (54.1%) and Alr1927 (31.9%) (Table S4). Additionally, All5219 shows 74% identity to EbfE, which suggests the possibility of a similar biofilm regulatory pathway in *Anabaena*. Considering the short sequence of EbfG1-4, which may hinder homolog identification through BLASTp, a previous bioinformatic study reporting the presence of various gene clusters encoding GG-motif-containing proteins described as potential bacteriocin precursors in different cyanobacteria, including *Anabaena* (Wang *et al.*, 2011), prompted us to further investigate the presence of potential homologues to these small proteins.

Identification of proteins with a GG-secretion motif potentially involved in biofilm formation in *Anabaena*

The protein sequences of putative bacteriocin precursors previously identified *Anabaena* (Wang *et al.*, 2011) were aligned with the *S. elongatus* EbfG1-4 sequences in order to identify the most likely homologues (Fig. 1). Although lower identities were obtained for potential EbfG1-3 homologues (16.18 to 21.43% sequence identity with the best matches for each), EbfG4 showed 30% or greater identity with four *Anabaena* proteins showing GG-secretion motifs belonging to the same cluster: Asr5139, Alr5140, Alr5141 and Asr5142 (Fig. 1b; Table S5).

The genes encoding the most probable homologues from *Anabaena* are arranged in clusters containing several GG-motif-containing proteins next to ABC transporter ATP-binding C39 peptidases (genes encoding Alr7014, Alr1201 and Alr5147 – the three best PteB homologues, in that order) and HlyD-family secretion proteins (Alr5148, Alr7010 and Alr1200 – the three best PCC7942_1132 homologues) (Fig. 1c). A similar



Fig. 1 Identification of potential *Anabaena* sp. PCC7120 homologues to *Synechococcus elongatus* PCC 7942 EbfG1-4. (a) GG-motif alignment of EbfG1-4 and *Anabaena* sp. PCC7120 GG-motif-containing proteins previously identified by Wang *et al.* (2011). (b) Full sequence identity matrix of the potential homologues. The best matches for each EbfG protein are outlined in blue. (c) *Anabaena* sp. PCC7120 clusters most likely to correspond to the *ebfG1-4* cluster based on identity and published data, taking into account *cds* potentially involved in their processing, modification and secretion. Groups of GG-motif-containing proteins displaying a higher identity and genomic proximity to each other are highlighted in the same colour in (a, b). Raw data for (b) can be found in Supporting Information Table S5.

arrangement is present in *S. elongatus*, in which EbfG1-3 are encoded upstream and contiguous to PCC7942_1134 (EbfG4), immediately followed by PteB and the HlyD-family secretion protein (Parnasa *et al.*, 2019). Despite the high identity of Asl2024, All2026 and All2027 with EbfG4, and EbfG3 for All2027, the C39 peptidase and HlyD secretion protein of this cluster do not display a significant homology to the corresponding *S. elongatus* proteins, and, according to KEGG annotation, may instead be related to nitrile metabolism. Therefore, considering identity values and gene context, clusters containing *asr1202*, *asr5139* and *all7013* seem to be the best candidates. The *asr5139-alr5148* cluster is particularly promising, due to its structural likeness to the *S. elongatus* cluster (albeit with a larger number of precursors), high identity of several of its encoded GG-motif-containing proteins to EbfG4 and the greater proximity to the *Anabaena* EbfE homolog, All5219.

Given that EbfG1-3 from *S. elongatus* form amyloid structures, likely contributing to the biofilm matrix (Frenkel *et al.*, 2023), we sought to investigate whether EbfG1-4 homologues in *Anabaena* exhibit similar amyloidogenic potential. Using a suite of five distinct computational prediction algorithms (Figs 2, S1), we identified multiple segments predicted as amyloidogenic hotspots. Considering the inherent variability among prediction methods, we focussed on identifying consensus regions where at least three algorithms concurred (Fig. S1). This analysis revealed a higher prevalence of predicted amyloidogenic regions within

the Asr5139-Alr5146 protein cluster than that of other analysed homologues. Nevertheless, further experimental validation is required to confirm amyloid formation and its functional relevance in *Anabaena* biofilms. Despite the established structural and functional diversity of bacteriocins (Sugrue *et al.*, 2024), the precise roles of many remain unclear. Furthermore, the presence of a GG-motif does not reliably predict bacteriocin activity. Therefore, mutational analyses of these gene groups are crucial to elucidate their specific functions in *Anabaena* and determine how they contribute to the formation of biofilms.

Regulatory systems involved in the synthesis of biofilms

Studies on the modulation of biofilm formation in heterotrophic bacteria and unicellular cyanobacteria have unveiled a significant number of TC systems, regulatory proteins and transcriptional regulators involved in this process. Our search for homologues in *Anabaena* provided valuable clues about the potential participation of novel transcriptional regulators in the control of biofilms in this cyanobacterium. Table 1 shows a summary of the most relevant regulatory systems included in Table S4.

Reported regulators known to affect biofilm formation in other cyanobacteria elicited the identification of several proteins in *Anabaena* containing the GGDEF motif, which is conserved at the catalytic domain of diguanylate cyclase (Ryjenkov *et al.*, 2005). The Sll0821 homologue of All0219 and Alr2306 from



Fig. 2 Predicted amyloidogenic hotspots in the protein sequences of potential *Anabaena* sp. PCC7120 homologues to *Synechococcus elongatus* PCC 7942 EbfG1-4, represented as darker sections. Hotspots were determined based on prediction consensus of at least three of five bioinformatic methods applied (APPNN, Waltz, Aggrescan, ArchCandy and TANGO). Genes belonging to one gene cluster are represented in the same colour. A more detailed representation of each method's results is available in Supporting Information Fig. S1.

Synechocystis sp. PCC6803 encodes the cyanobacteriochrome photoreceptor Cph2 that regulates floc formation in response to blue and green light (Conradi *et al.*, 2019). Besides this, All1219 showed 50% identity with LBDG_02920 (gene *dgc2*) from *Lep- tolyngbya boryanum*. The inactivation of *dgc2* in *L. boryanum* showed that this protein suppresses gliding motility and biofilm formation in this filamentous cyanobacterium (Toida *et al.*, 2023). GGDEF-EAL domain-containing proteins have also been reported to regulate twitching motility and biofilm formation in many other bacteria, such as *Pseudomonas aeruginosa*, *Shewanella* spp. and *Escherichia coli*, as well as other enterobacteriaceae (Huang *et al.*, 2003; Jonas *et al.*, 2009; Sanchez-Torres *et al.*, 2011; Rick *et al.*, 2022), evidencing the importance of the cyclic di-GMP in biofilms.

Several potential regulators identified in this study were homologous to genes located in the *xss* (extracellular sulphated polysaccharide biosynthesis) cluster present in the megaplasmid pSYSM of *Synechocystis* sp. PCC 6803, which spans from *sl15042* to *sl15060* (Maeda *et al.*, 2021). This cluster includes genes involved in the synthesis and translocation of EPS, as the putative transcriptional regulator XssQ, which activates genes encoding sulfo-transferases and some GTs, and the TC system XssR/XssS. *Anabaena* contains several homologues to XssRS components from *Synechocystis* sp. PCC6803 and *X. axonopodis* RbfRS, some of them in operons, such as *alr2278–2280*, *all2875–2874* and *alr3760–3761* (Table 1). In addition, the *Anabaena* genome encodes four potential homologues to *xssQ*, namely *alr1232*, *alr2242*, *alr3789* and *all4929*. These contain NATCH domains, typical of dNTPases, and which are involved in defense signalling pathways and the transcriptional regulation of genes related to environmental stress and cell signalling (Arya & Acharya, 2016). Interestingly, these four genes are contiguous to several TC systems, not yet characterized. *alr1232* is part of a cluster (*alr1229–1233*) encoding a hybrid TC sensor and regulator

(*alr1231*), a TC system (*alr1229–1230*), and a diacylglycerol O-acyl transferase (*alr1233*). Alr1229 shows homology to the TC sensor kinase ColS (XAC3249) and with a chemotaxis protein from *X. axonopodis* pv. citri (XAC1746). The ColR/ColS TC system plays important roles in the formation of bacterial biofilms, synthesis of lipopolysaccharides and tolerance to environmental stresses, including response to peroxide, aromatic xenobiotics and excess of several metals (Ainsaar *et al.*, 2014). In *Xantomonas* spp., the ColRS system is a positive regulator of important virulence genes and essential for biofilm formation. ColRS mutants were defective in biofilm formation and O-antigen biosynthesis and produced less lipopolysaccharides than the WT (Yan & Wang, 2011). The ColRS signalling pathway is well-conserved in pseudomonads and has been identified as a major player in supporting membrane functionality and stress tolerance in *P. putida*, as well as contributing to virulence in *P. aeruginosa* (Ainsaar *et al.*, 2014).

The genome of *Anabaena* contains six homologues to the response regulator (RR) ColR (XAC3250) that seem to play different roles. At the moment, the best characterized is *rpaA* (*all0129*) (37.2% identity with XAC3250). RpaA is essential for the transduction of signals from the *kai* core clock proteins to downstream clock-controlled genes, thereby coupling the internal circadian rhythms with environmental changes, including cell differentiation (Arbel-Goren *et al.*, 2024). Therefore, the circadian clock, via regulators such as RpaA, limits heterocyst formation to certain phases of the circadian cycle, enabling optimal nitrogen fixation by aligning cellular functions with environmental signals. In addition to its autoregulation, *rpaA* is directly controlled by FurA and the master regulator of nitrogen control NtcA (Guío *et al.*, 2025). A bioinformatic search of RpaA-binding boxes unveiled potential binding sites in the promoters of FurC (PerR) and FurA (Arbel-Goren *et al.*, 2024), suggesting the occurrence of a cross-talk between RpaA and FurA. Both

Table 1 Selected *Anabaena* sp. PCC7120 regulatory proteins showing homology to those involved in biofilm formation and exopolysaccharide synthesis in other bacteria.

Identified <i>Anabaena</i> sp. PCC7120 homologues		Bacterial query proteins					
Operon/ORFa	Product	ORF	Microorganism	Product	Identity	E-value	Source
<i>alr0072</i>	TC system RR	XAC3250	<i>Xanthomonas axonopodis</i> pv. Citri	TC system response regulator ColR	36.9%	6.9E-40	Li & Wang (2011)
<i>asr0105-alr0106</i>	Putative regulatory protein	BSU15670	<i>Bacillus subtilis</i>	Extracellular matrix regulatory protein A	72.0%	4.4E-40	Winkelman et al. (2013)
<i>alr0129</i>	TC system response regulator RpaA	XAC3250	<i>Xanthomonas axonopodis</i> pv. Citri	TC system response regulator ColR	37.2%	3.9E-38	Li & Wang (2011)
<i>alr0219</i>	Hypothetical protein with GGDEF-EAL motif	<i>slr0821</i>	<i>Synechocystis</i> sp. PCC6803	Cyanobacteriochrome photoreceptor Cph2	39.3%	5.5E-93	Conradi et al. (2019)
<i>alr1219</i>	Hypothetical protein, GGDEF motif-containing	LBDG_02920	<i>Leptolyngbya boryana</i>	GGDEF motif protein	50.1%	0.0E+00	Toida et al. (2023)
<i>alr1229-alr1231</i>	TC system histidine kinase	XAC3249	<i>Xanthomonas axonopodis</i> pv. Citri	TC system sensor kinase ColS	27.3%	2.3E-19	Li & Wang (2011)
<i>alr1232</i>	Possible transcriptional regulator	<i>slr5058</i>	<i>Synechocystis</i> sp. PCC6803	Transcriptional regulator XssQ	34.4%	1.6E-147	Maeda et al. (2021)
<i>alr1964</i>	TC system response regulator ManR	XAC3250	<i>Xanthomonas axonopodis</i> pv. Citri	TC system response regulator ColR	35.7%	3.9E-45	Li & Wang (2011)
<i>alr2242</i>	Possible transcriptional regulator	<i>slr5058</i>	<i>Synechocystis</i> sp. PCC6803	Transcriptional regulator XssQ	26.3%	1.0E-160	Maeda et al. (2021)
<i>alr2278-alr2280</i>	TC system hybrid sensor and regulator TC system RR	<i>slr5060</i>	<i>Synechocystis</i> sp. PCC6803	TC system sensor histidine kinase XssS	36.6%	0.0E+00	Maeda et al. (2021)
		<i>slr1759</i> XAC0494	<i>Xanthomonas axonopodis</i> pv. Citri	TC system sensor kinase Hik14	35.2%	9.8E-180	Kobayashi et al. (2023)
				TC system sensor kinase Rbfs	33.6%	2.1E-100	Li & Wang (2011)
<i>alr2280</i>	TC system RR	XAC0495	<i>Xanthomonas axonopodis</i> pv. Citri	TC system response regulator Rbfr	27.2%	1.6E-13	Li & Wang (2011)
<i>alr2325</i>	Transcriptional regulator Ancrpb	Q83VR7	<i>Methylobacillus</i> sp. strain 12S	cAMP-binding EPS synthesis regulator Epsk	32.4%	1.2E-10	Yoshida et al. (2003)
<i>alr2428-alr2429</i>	TC system sensor kinase	XAC3250	<i>Xanthomonas axonopodis</i> pv. Citri	TC system response regulator ColR	36.0%	7.1E-41	Li & Wang (2011)
<i>alr2430</i>	Translocation and assembly module TamB	AOA522XHL7	<i>Toxifilum</i> sp. LEGE 06021	Uncharacterized protein	24.5%	2.2E-83	Romeu et al. (2022)
<i>alr2473</i>	Ferric uptake regulation protein Zur	<i>Synpcc7942_0817</i>	<i>Synechococcus elongatus</i> sp. PCC7942	Ferric uptake regulator family protein	64.0%	1.4E-58	Simkovsky et al. (2022)

Table 1 (Continued)

Identified <i>Anabaena</i> sp. PCC7120 homologues		Bacterial query proteins					
Operon/ORFa	Product	ORF	Microorganism	Product	Identity	E-value	Source
all/2875–all/2874	TC system sensor kinase with LOV-domain	slr1759	<i>Synechocystis</i> sp. PCC6803	TC system sensor kinase Hik14	40.3%	0.0E+00	Kobayashi <i>et al.</i> (2023)
		slf5060		TC system sensor histidine kinase XssS	45.3%	0.0E+00	Maeda <i>et al.</i> (2021)
all/2874	TC system response regulator with diguanylate cyclase activity CdgS	XAC0494	<i>Xanthomonas axonopodis</i> pv. Citri	TC system sensor kinase Rbfs	32.5%	1.3E–93	Li & Wang (2011)
		slr1760		TC system response regulator Rre8	53.4%	1.1E–109	Kobayashi <i>et al.</i> (2023)
		slf1673		TC system RR	50.3%	4.2E–56	Kobayashi <i>et al.</i> (2023)
all/2962	Transcriptional regulator	slf5059	<i>Xanthomonas axonopodis</i> pv. Citri	TC system response regulator XssR	49.6%	8.4E–37	Maeda <i>et al.</i> (2021)
		XAC0495		TC system response regulator Rbfr	22.2%	9.3E–10	Li & Wang (2011)
all/3760–all/3761	TC system sensor kinase or hybrid regulator	BSU_24610	<i>Bacillus subtilis</i>	XRE family transcriptional regulator SinR	30.6%	9.4E–06	Kearns <i>et al.</i> (2005)
		PA0048		Transcriptional regulator	33.3%	5.1E–05	Kaleta <i>et al.</i> (2022)
all/3789	Possible transcriptional regulator	slf5060	<i>Pseudomonas aeruginosa</i> PAO 1	TC system sensor histidine kinase XssS	36.3%	2.1E–144	Maeda <i>et al.</i> (2021)
		XAC0494		TC system sensor kinase Rbfs	35.0%	3.0E–106	Li & Wang (2011)
all/4727–all/4726	TC system sensor kinase	slr5058	<i>Synechocystis</i> sp. PCC6803	Transcriptional regulator XssQ	32.0%	3.3E–126	Maeda <i>et al.</i> (2021)
		XAC3249		TC system sensor kinase ColS	26.6%	3.6E–25	Li & Wang (2011)
all/4929	Putative NACHT domain-containing signal transduction protein	XAC3250	<i>Xanthomonas axonopodis</i> pv. Citri	TC system response regulator ColR	31.4%	1.1E–30	Li & Wang (2011)
		slr5058		Transcriptional regulator XssQ	25.4%	7.1E–147	Maeda <i>et al.</i> (2021)
all/5174–all/5172	TC system RR	XAC3250	<i>Xanthomonas axonopodis</i> pv. Citri	TC system response regulator ColR	41.4%	3.3E–44	Li & Wang (2011)
		slf5058		Transcriptional regulator XssQ	31.8%	4.4E–04	Kearns <i>et al.</i> (2005)
as/7034–all/7033	Transcriptional regulator	BSU_24610	<i>Bacillus subtilis</i>	XRE family transcriptional regulator SinR	31.8%	4.4E–04	Kearns <i>et al.</i> (2005)

^aPredicted transcriptional units based on the data available through the MicrobesOnline operon tool. ORF, open reading frame; RR, response regulator; TC, two-component.

FurA and PerR have been related previously to the modulation of nitrogen metabolism, and their overexpression affects heterocyst differentiation (López-Gomollón *et al.*, 2007; Gonzalez *et al.*, 2013; Sarasa-Buisan *et al.*, 2022).

Furthermore, the uncharacterized operon *all4727–4726* encodes two homologues to ColRS from *X. axonopodis* (31.4 and 26.6% identity with almost complete coverage in the alignment). Other two homologues of XAC3250 in *Anabaena* (i.e. *alr2428*,

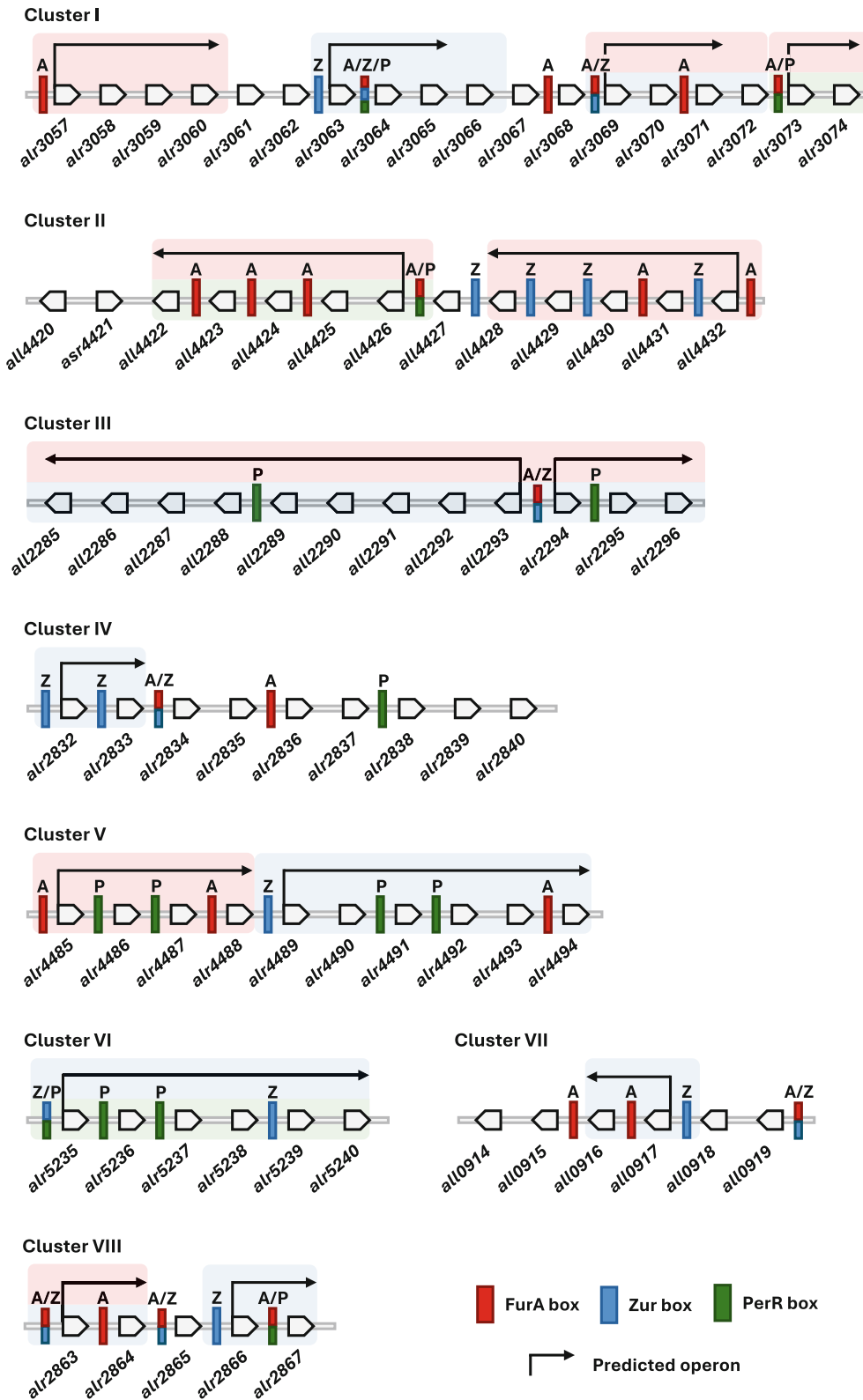


Fig. 3 Distribution of putative ferric uptake regulator (FUR)-binding boxes throughout the eight gene clusters involved in exopolysaccharide synthesis in *Anabaena* sp. PCC7120 described by Potnis *et al.* (2021). Red, blue and green represent Fur or iron uptake regulator (A), zinc uptake regulator (Z) and peroxide response regulator (P) regulation, respectively. Coloured backgrounds indicate the presence of a binding box directly upstream of a predicted operon according to the MicrobesOnline operon tool, indicated by a black arrow spanning the putative transcriptional unit.

Table 2 Summary of transcriptional units for which *in vitro* binding to their promoter was observed for at least one ferric uptake regulator protein through electrophoretic mobility assays in this work.

Operon ^a	Gene	Product	EMSA result ^b		
			FurA	Zur	PerR
<i>all0059</i>		Polysaccharide biosynthesis transport protein	+	–	
<i>asr0105–alr0106</i>	<i>asr0105</i> <i>alr0106</i>	Putative regulatory protein Guanylate kinase		+	
<i>alr0277</i>		RNA polymerase sigma-37 factor SigJ	[+]	[+]	[–]
<i>all0493–all0492</i>	<i>all0492</i>	Acyl phosphate : glycerol-3-P acyltransferase	+		
	<i>all0493</i>	Hypothetical plasma membrane protein			
<i>all0496–all0495</i>	<i>all0495</i>	Polysaccharide biosynthesis/export protein	+		
	<i>all0496</i>	Cyanoexosortase B system-associated protein			
<i>alr0657–alr0658</i>	<i>alr0657</i> <i>alr0658</i>	UDP-glucuronate decarboxylase RfbB UDP-glucose 6-dehydrogenase		+	
<i>alr0776</i>		Hypothetical protein with GT motifs	+		
<i>alr0892–alr0895</i>	<i>alr0892</i> <i>alr0893</i> <i>alr0894</i> <i>alr0895</i>	Hypothetical protein Protease I Possible polyketide cyclase Alcohol dehydrogenase		+ ^d + ^d	
<i>all0917–all0916</i>	<i>all0916</i> <i>all0917</i>	ABC transporter adenosine triphosphate (ATP)-binding subunit ABC transporter permease protein	+	–	
<i>all0919</i>		Colanic acid biosynthesis glycosyltransferase	+	+	
<i>alr1490–alr1491</i>	<i>alr1490</i> <i>alr1491</i>	ABC transporter ATP-binding component Probable ABC transporter permease	–	+	+
<i>alr2242</i>		Possible transcriptional regulator	+	+	+
<i>all2293–all2283</i>	<i>all2283</i>	Antisigma factor antagonist	+	[+] ^e	–
	<i>all2284</i>	Sigma-B activity negative regulator RsbW			
	<i>all2285–all2289</i>	Glycosyltransferases			
	<i>all2290</i>	Similar to PS biosynthesis export protein			
	<i>all2291–all2292</i>	Glycosyltransferases			
	<i>all2293</i>	Probable polysaccharide pyruvyl transferase			
<i>alr2294–alr2296</i>	<i>alr2294</i> <i>alr2295</i> <i>alr2296</i>	Polysaccharide export outer membrane protein Probable GumC LPS synthesis protein Unknown membrane protein			
<i>alr2325</i>		Transcriptional regulator AncrpB			[+] ^f
<i>alr2372–alr2374</i>	<i>alr2372</i> <i>alr2373</i> <i>alr2374</i>	ABC transporter ATP-binding protein ABC-2-type transport system permease protein Unknown protein	+		
<i>all2473</i>		Ferric uptake regulation protein FurB	[+] ^g	[+] ^g	[+] ^g
<i>all2770</i>		Dolichyl-phosphate-mannose synthase	+		
<i>alr2832–alr2833</i>	<i>alr2832</i> <i>alr2833</i>	Putative glycosyltransferase Polysaccharide biosynthesis transport protein		+	
<i>alr2856</i>		Polysaccharide biosynthesis transport protein	+		–
<i>all2874–all2875</i>	<i>all2875</i> <i>all2874</i>	LOV-domain two-component system sensor kinase Two-component system response regulator with diguanylate cyclase activity CdgS		+	
<i>alr2881–alr2882</i>	<i>alr2881</i> <i>alr2882</i>	Putative glycosyltransferase Exopolysaccharide synthesis protein ExoD	+	–	
<i>all2963</i>		Oxidoreductase	+	+	
<i>alr3057–alr3060</i>	<i>alr3057</i> <i>alr3058</i> <i>alr3059</i> <i>alr3060</i>	Probable glycosyltransferase Probable glycosyltransferase Polysaccharide biosynthesis transport protein Unknown protein, WzyC-like	+		
<i>alr3061</i>		Similar to acetyltransferase		+	
<i>alr3069–alr3071</i>	<i>alr3069</i> <i>alr3070</i> <i>alr3071</i>	Probable glycosyltransferase Probable glycosyltransferase Probable glycosyltransferase	+	[–] ^e	
<i>all3184</i>		Ribosome hibernation promoting factor	+	–	
<i>alr3464</i>		Glutamine-fructose-6-P-aminotransferase	+		+
<i>all3509</i>		Nucleotide sugar epimerase	+	+	
<i>alr3756–alr3757</i>	<i>alr3756</i> <i>alr3757</i>	Unknown protein Cellulose synthase catalytic subunit			+
<i>all3853</i>		Group 3 sigma 37-type sigma factor SigF	[+]	[–] ^c	[–] ^c

Table 2 (Continued)

Operon ^a	Gene	Product	EMSA result ^b		
			FurA	Zur	PerR
<i>all4052–all4051</i>	<i>all4052</i>	Transketolase	+	+	
	<i>all4051</i>	PRC-barrel domain protein AvaKc			
<i>all4219</i>		ABC transporter ATP-binding component	+		
<i>all4388</i>		Polysaccharide biosynthesis/export protein		+	+
<i>all4426–all4422</i>	<i>all4426</i>	Probable glycosyltransferase	+		–
	<i>all4425</i>	Unknown protein			
	<i>all4424</i>	Probable glycosyltransferase			
	<i>all4423</i>	Hypothetical protein with GT motifs			
	<i>all4422</i>	UDP-N-acetyl-D-mannosamine transferase			
<i>all4432</i>		Probable exopolysaccharide biosynthesis protein	+	–	–
<i>alr4485–alr4488</i>	<i>alr4485</i>	Transport permease protein	+	–	–
	<i>alr4486</i>	ABC transporter ATP-binding subunit			
	<i>alr4487</i>	Hypothetical protein with glycosyl- and methyltransferase motifs			
	<i>alr4488</i>	Hypothetical protein with glycosyl- and methyltransferase motifs			
<i>all5073</i>		Putative inorganic carbon transporter	+		
<i>asr5139–alr5140</i>	<i>asr5139</i>	Uncharacterized bacteriocin precursor	+	+	+
	<i>alr5140</i>	Uncharacterized bacteriocin precursor			
<i>all5222^h</i>		Polysaccharide biosynthesis transport protein	+	+	+
<i>alr5223^h</i>		Glycosyltransferase	+		+

Full data are included in Supporting Information Table S7.

^aPredicted transcriptional units are based on data provided by the MicrobesOnline operon tool.

^bBinding results obtained from previous works are displayed between brackets.

^cGuío *et al.* (2025).

^dOperon prediction is strong for *alr0892–alr0893* (0.85 pOp) and *alr0894–alr0895* (0.97 pOp), but lower for *alr0893–alr0894* (0.63 pOp). It is possible that *alr0894–alr0895* operate as a single transcriptional unit.

^eOlivan-Muro *et al.* (2023).

^fSarasa-Buisan *et al.* (2023).

^gHernández *et al.* (2004).

^hThe intergenic region between divergent genes *all5222* and *alr5223* is 1144 bp long and was thus divided into three sections. Binding to the middle region may correspond to transcriptional regulation of either or both.

annotated as hybrid sensor and regulator with 36% identity to the RR ColR, and *all5174* with 41.4% identity) are organized in clusters with diverse potential RRs. In the case of *alr2428*, it is followed by genes related to carbohydrate metabolism and transport proteins, such as TamB and the ABC transporter Alr2433. The fifth homolog (*all1964*, 35.7% identity) encodes the RR ManR, part of the TC system involved in Mn²⁺-sensing in *Anabaena* (Huang & Wu, 2004). Finally, the ColR homolog *alr0072* (36.9% identity) has been identified as one of the CopR homologues in *Anabaena* (Giner-Lamia *et al.*, 2012). While the TC system CopRS is essential for copper resistance in *Synechocystis* sp. PCC 6803, the precise functions of the different paralogues present in *Anabaena* remain to be investigated. It is worth noting that as in *rpaA*, the promoter region of *alr0072* contains FurA- and Zur (FurB)-binding sites (Guío *et al.*, 2025).

It is remarkable that some *Anabaena* genes show a high identity with regulatory proteins of phylogenetically distant bacteria (i.e. *X. axonopodis*, *B. subtilis* or *P. aeruginosa*), which are involved in the control of biofilm formation. In particular, the gene coding for the extracellular matrix regulatory protein from *B. subtilis* YlzA (RemA) shows 72% identity with Asr0105 and lies upstream of a guanylate kinases in both organisms. In *B. subtilis*, RemA activates the transcription of *eps* and *tapA-sipW-tasA*

operons that synthesize EPS and TasA amyloid proteins present in the extracellular matrix (Winkelman *et al.*, 2013). These operons are repressed by SinR, which in *B. subtilis* is a master regulator of biofilm formation (Kearns *et al.*, 2005). In *Anabaena*, SinR is represented by homologues *asl7034* (30.6% identity) and *all2962* (31.8% identity). The *all2962* homolog is also similar to the *P. aeruginosa* PA0048 transcriptional regulator (33.3% identity), which plays a role in structured biofilm formation (Kaleta *et al.*, 2022). The conservation of *ylzA* and *sinR* across large evolutionary distances points towards an essential or advantageous nature of these genes to establish biofilms in diverse bacterial lineages. These pathways may either have originated early in bacterial evolution or be conserved due to potential horizontal gene transfer events owing to their effectiveness in promoting colony survival and resilience.

Other *Anabaena* regulatory genes with homology to heterotrophic bacteria are AnCrpA (*alr0295*) and AnCrpB (*alr2325*), both showing identities higher than 30% with the gene Q83VR7 from *Methylobacillus* sp. (strain 12S), annotated as ‘cAMP-binding EPS synthesis regulator’ or EpsK (Yoshida *et al.*, 2003). In *Anabaena*, AnCrpA regulates the expression of gene clusters relevant in nitrogen fixation processes (Suzuki *et al.*, 2007), while the role of AnCrpB seems to be more related to biofilms, since it

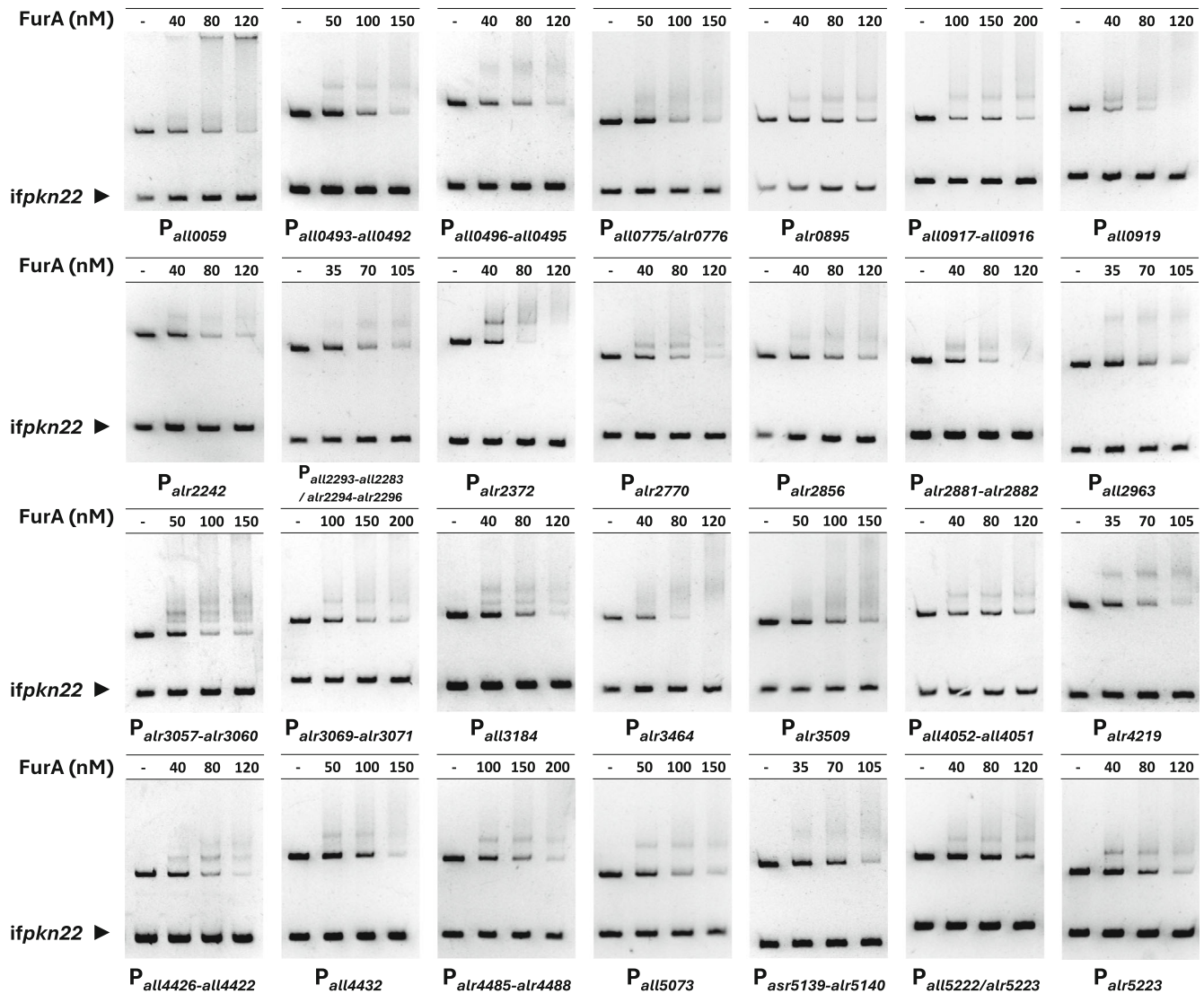


Fig. 4 *In vitro* interaction between Fur or iron uptake regulator (FurA) and the promoter regions of a selection of genes, evaluated through electrophoretic mobility shift assays. All assays were performed with DNA fragments free or incubated along with the indicated increasing concentrations of FurA (nM). An internal fragment of gene *pkn22* (*ifpkn22*) was used as nonspecific competitor DNA.

is upregulated in response to rehydration (Higo *et al.*, 2007). AnCrpB has shown to be regulated by PerR (FurC) (Sarasa-Buisan *et al.*, 2023) and RpaA (Arbel-Goren *et al.*, 2024), unveiling a new layer of regulatory interactions in *Anabaena*.

In summary, these findings identify potential regulatory candidates within the intricate network of regulatory systems in *Anabaena* that may modulate biofilm formation in response to environmental cues such as metal, desiccation and nitrogen availability. However, further genetic validations are needed to confirm their specific roles. Key homologues identified in *Anabaena* appear to integrate signals associated with external metal concentrations, linking environmental stress responses with biofilm dynamics. This regulatory network shares notable conservation with pathways in phylogenetically distant bacteria, suggesting a deep evolutionary advantage in maintaining biofilm-promoting genes across diverse bacterial lineages. Furthermore, the cross-talk

between nitrogen regulators (e.g. NtcA and RpaA) and metal-sensing pathways indicates a coordinated mechanism that adapts biofilm formation to optimize nitrogen fixation and metabolic resilience.

Role of FUR proteins in the control of biofilm formation

The presence of FUR boxes has been detected in the promoter regions of a significant number of genes that have been predicted to play a role in the formation of biofilms. In addition to some genes participating in the general pathway of polysaccharide synthesis, this set includes GTs, genes linked to EPS export pathways, GG-motif containing proteins, the *sigF* RNA polymerase sigma factor and genes encoding several transcriptional regulators, some of them still uncharacterized (Tables S3, S4 and S6). FUR boxes are present in all eight clusters associated with EPS

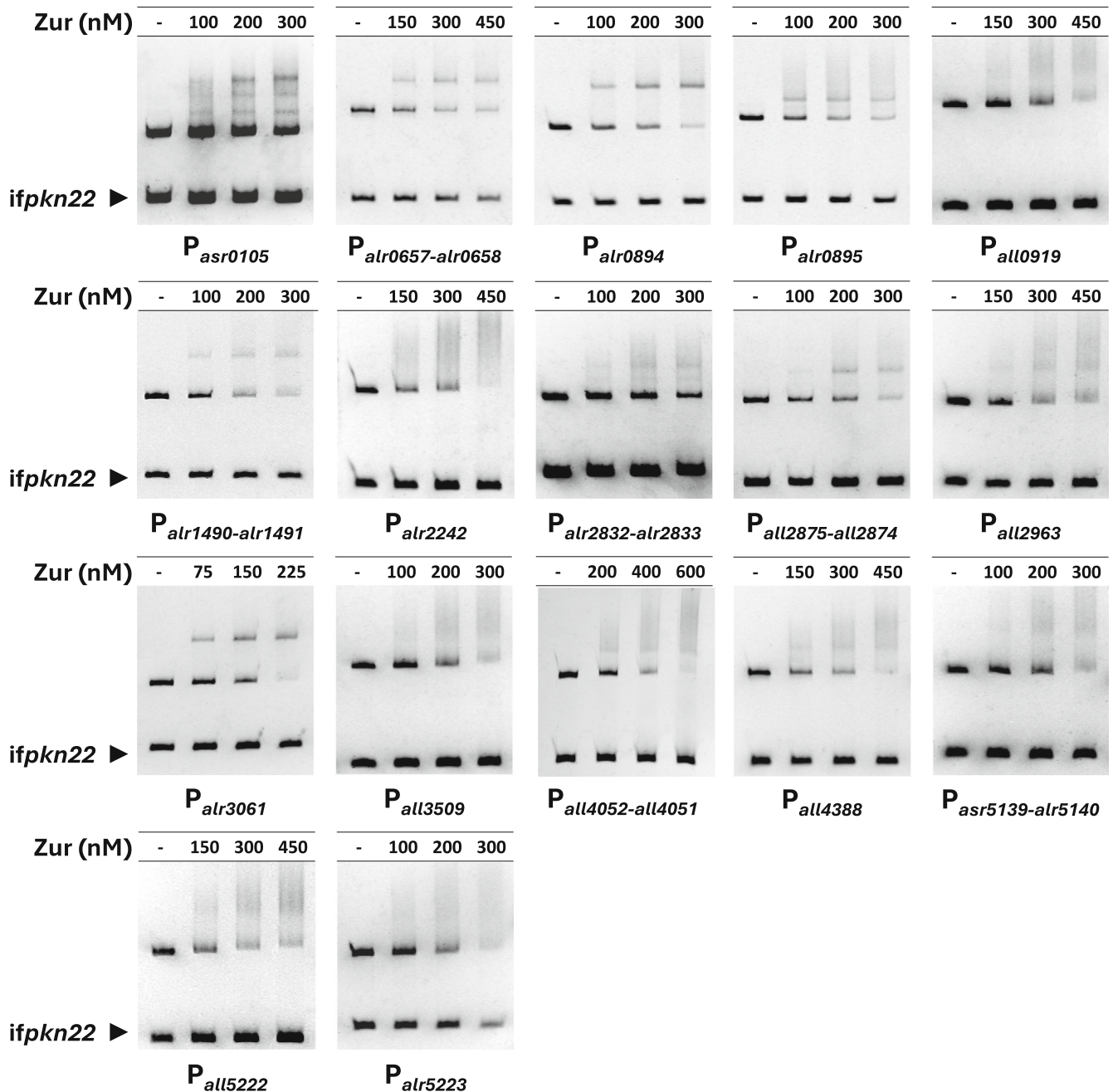


Fig. 5 *In vitro* interaction between zinc uptake regulator (Zur) and the promoter regions of a selection of genes, evaluated through electrophoretic mobility shift assays. All assays were performed with DNA fragments free or incubated along with the indicated increasing concentrations of Zur (nM). An internal fragment of gene *pkn22* (*ifpkn22*) was used as nonspecific competitor DNA.

synthesis and export described previously (Potnis *et al.*, 2021), and some of these genes exhibited binding sequences for more than one FUR paralogue (Fig. 3), suggesting a key role of FUR proteins in carbon and EPS metabolism and, in turn, in the synthesis of biofilms. The number of FUR boxes identified in this study (Tables S3, S4) was larger for FurA (129 binding sites) than for Zur and PerR (78 and 62 binding sites, respectively). These results are in good concordance with our previous transcriptional studies (Gonzalez *et al.*, 2014, 2016), showing the widespread

presence of FurA boxes in the *Anabaena* genome. The presence of Zur boxes in a large number of promoters is also consistent with our previous findings, which demonstrated that Zur not only regulates genes related to zinc homeostasis but also affects genes related to carbon metabolism. Notably, the number of genes involved in carbon metabolism and whose transcription was affected in a *zur*-deficient *Anabaena* was comparable to that of genes associated with zinc and metal homeostasis (Olivan-Muro *et al.*, 2023). In a similar way, the identification of PerR

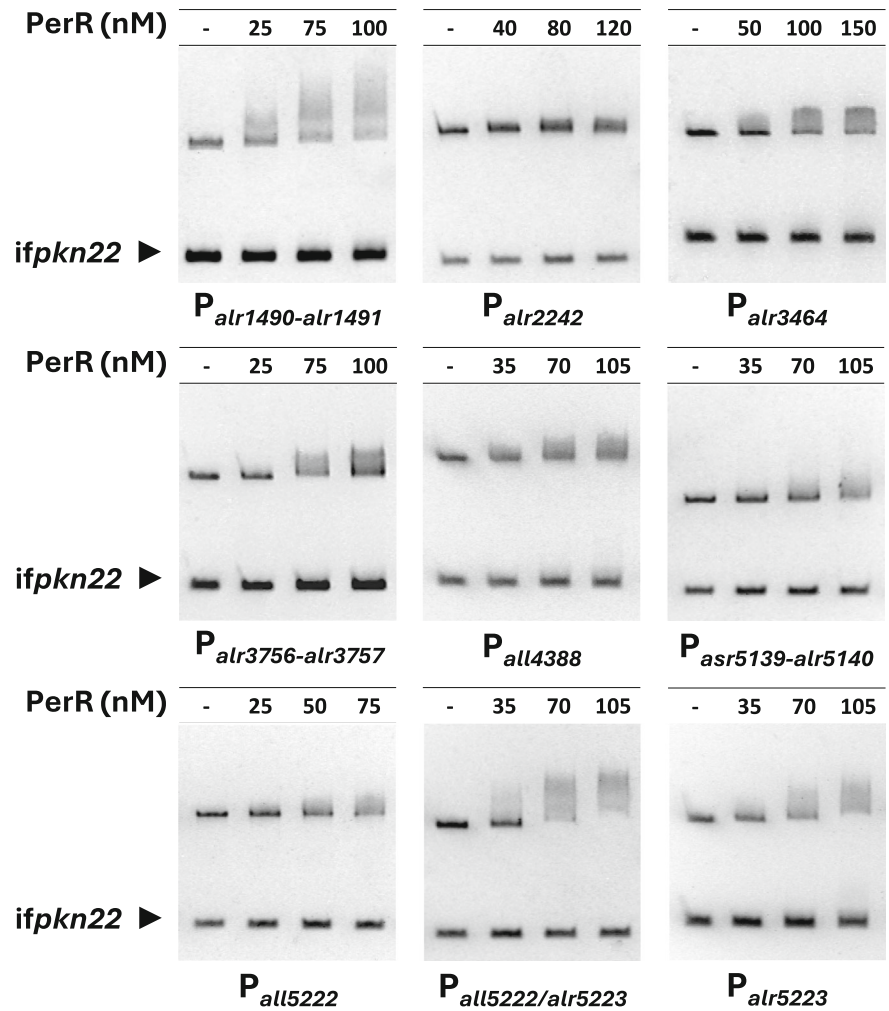


Fig. 6 *In vitro* interaction between peroxide response regulator (PerR) and the promoter regions of a selection of genes, evaluated through electrophoretic mobility shift assays. All assays were performed with DNA fragments free or incubated along with the indicated increasing concentrations of PerR (nM). An internal fragment of gene *pkn22* (*ifpkn22*) was used as nonspecific competitor DNA.

targets unveiled genes playing relevant functions in nitrogen fixation and carbon assimilation processes (Sarasa-Buisan *et al.*, 2023).

A search for direct targets of FUR proteins was carried out by conducting EMSAs to examine the binding of the three FUR paralogues to the promoter regions of genes containing FUR boxes, primarily those related to EPS synthesis and transport. Considering the role of biofilms to maintain hydration (Kato *et al.*, 2004), some genes related to desiccation tolerance, such as *sigJ*, genes *alr0894–0895* (encoding a protein with polyketide cyclase/dehydrase and lipid transport domains, and an alcohol dehydrogenase, respectively), were included in these assays. In addition, the GG-motif-containing proteins *asr1202* and *asr5139* together with a set of genes involved in assembly and export of EPS previously identified (Pereira *et al.*, 2013), as well as some regulators known to modulate biofilm synthesis in other organisms, were tested. The results of these EMSA experiments are included along with the corresponding putative FUR-binding sequences in Table S7. A summary of the positive results obtained with the EMSA is presented in Table 2 and Figs 4–6. EMSAs revealed that the majority (86%) of promoters with predicted FUR boxes analysed in this study exhibited binding

activity (49 of 57 promoters showed a clear band shift). Table S8 shows that this trend aligns with previous analyses using FIMO computational predictions of FUR boxes in the *Anabaena* genome and validation by EMSA on a subset of promoters (Sarasa-Buisan *et al.*, 2022, 2023, 2024; Oliván-Muro *et al.*, 2023; Guío *et al.*, 2025). Conversely, only 23% of promoters without predicted FUR boxes analysed in this study were positive by EMSA. Since these promoters did not display unspecific binding to the DNA used as a negative control, some positive results could be attributed to the stringent cut-off applied to minimize false positives. FUR proteins may bind to some promoters with FUR boxes below this threshold, rendering them undetectable by our computational approach.

To assess *in vivo* influence of FurA, Zur and PerR on the transcription of some potential targets, a selection of genes with diverse functions was used in real time RT-PCR assays conducted on both *Anabaena* WT and FUR-deregulated variants, namely FurA- and PerR-overexpressing strains (Gonzalez *et al.*, 2010; Sevilla *et al.*, 2019) and the Δzur mutant (Napolitano *et al.*, 2012). The complete dataset of results is presented in Table 3. The expression of several genes was affected by the misregulation of both FurA and Zur. These genes encoded the

Table 3 Relative transcription of a selection of genes, determined by Real-Time PCR in AG2770FurA (+FurA), Δ zur or EB2770PerR (+PerR) cells related to the wild-type *Anabaena* sp. PCC7120, accompanied by the summary of electrophoretic mobility assay experiments performed with their promoter sequences.

ORF	Product	EMSA results			Real-Time PCR		
		FurA	Zur	PerR	FurA	Zur	PerR
<i>alr0657</i>	UDP-glucuronate decarboxylase RfBB	nt	+	nt	1.06 ± 0.28	-1.48 ± 0.14	1.24 ± 0.09
<i>alr0895</i>	Alcohol dehydrogenase	+	+	nt	3.74 ± 0.77	29.95 ± 6.31	-1.13 ± 0.31
<i>alr1490</i>	ABC transporter ATP-binding component	- ^a	+	+	1.58 ± 0.29	-1.38 ± 0.30	-2.34 ± 0.61
<i>alr2242</i>	Possible transcriptional regulator	+	+	+	-3.48 ± 1.04	-3.66 ± 0.89	-1.32 ± 0.53
<i>all2293</i>	Probable polysaccharide pyruvyl transferase	+	+	- ^a	-1.94 ± 0.43	-2.32 ± 0.59	-1.08 ± 0.15
<i>alr2294</i>	Polysaccharide export outer membrane protein	+	+	- ^a	-1.43 ± 0.38	-2.47 ± 0.69	1.07 ± 0.28
<i>alr2372</i>	ABC transporter ATP-binding protein	+	nt	nt	1.04 ± 0.13	-1.39 ± 0.14	-1.22 ± 0.25
<i>alr2856</i>	Polysaccharide biosynthesis transport protein	+	nt	- ^a	-1.85 ± 0.25	-1.38 ± 0.18	2.37 ± 0.49
<i>all2875</i>	Two-component system sensor kinase	nt	+	nt	2.56 ± 0.15	-2.76 ± 0.35	3.35 ± 0.65
<i>all2963</i>	Oxidoreductase	+	+	nt	1.25 ± 0.31	2.69 ± 0.73	-1.04 ± 0.09
<i>alr3057</i>	Probable glycosyltransferase	+	nt	nt	-2.05 ± 0.21	-1.36 ± 0.03	-1.02 ± 0.19
<i>alr3061</i>	Similar to acetyltransferase	nt	+	nt	-2.33 ± 0.51	-2.10 ± 0.32	-2.55 ± 0.26
<i>all3184</i>	Ribosome hibernation promoting factor	+	- ^a	nt	-1.04 ± 0.19	-2.02 ± 0.23	1.19 ± 0.26
<i>all3509</i>	Nucleotide sugar epimerase	+	+	nt	-1.10 ± 0.25	-2.18 ± 0.20	-1.48 ± 0.33
<i>all4052</i>	Transketolase	+	+ ^a	nt	2.43 ± 0.70	6.14 ± 0.63	1.66 ± 0.62
<i>all4432</i>	Probable exopolysaccharide biosynthesis protein	+	- ^a	- ^a	-3.31 ± 0.77	-4.96 ± 1.06	-1.77 ± 0.21
<i>alr4485</i>	Transport permease protein	+	- ^a	- ^a	-1.21 ± 0.33	-2.40 ± 0.27	1.31 ± 0.53
<i>asr5139</i>	GG-motif-containing protein	+	+	+	-1.19 ± 0.24	-1.61 ± 0.60	-1.08 ± 0.14
<i>all5222</i>	Polysaccharide biosynthesis transport protein	+	+	+	-3.14 ± 1.31	1.27 ± 0.66	1.20 ± 0.46
<i>alr5223</i>	Glycosyltransferase	+	+ ^a	+	-1.98 ± 0.41	-2.08 ± 0.32	1.49 ± 0.57

Values are expressed as fold change with SD and correspond to three biological and two technical replicates. Values in bold indicate differential expression between the variant strain and the WT; blue background represents concordance with positive EMSA results. Green cells indicate FUR-promoter binding observed for the corresponding promoter, with red for negative results. nt, not tested; does not contain a putative binding box.

^aNo putative binding box predicted within the gene's tested promoter sequence.

probable polysaccharide pyruvyl transferase *all2293*, the putative transcriptional regulator *alr2242*, which is homologous to XssQ from *Synechocystis* sp. PCC6803 (Maeda *et al.*, 2021), the TC sensor kinase gene *all2875* and the alcohol dehydrogenase gene *alr0895*. Overexpression of FurA also resulted in significant changes in the expression of several genes coding for GTs and polysaccharide exporters, whose promoters were confirmed as FurA targets by EMSA (Fig. 4). However, *c.* 41% of genes yielding positive results by EMSA did not exhibit significant transcriptional changes upon FurA overexpression under the tested conditions. It remains plausible that these genes are regulated by FurA under alternative physiological conditions. Conversely, the changes in the expression of the TC sensor kinase gene *all2875* are likely due to an indirect effect, as neither FurA boxes nor FurA binding to the promoter were detected. (Table 3). In the

case of the Δ zur mutant, *c.* 85% of genes with significant transcriptional changes showed positive EMSA results. In general, the deletion of *zur* caused greater variations in the expression of the genes affected, such as *all4432* and the transketolase-encoding *all4052*. A dramatical increase was observed in the transcription of *alr0895*, encoding a zinc-containing alcohol dehydrogenase, much more pronounced in the *zur* deletion mutant than in the FurA variant, in accordance with our previous transcriptomic results and the established role of Zur in the regulation of a set of carbon metabolism-related gene clusters, which include genes involved in desiccation tolerance (Olivan-Muro *et al.*, 2023). This may contribute to the altered ability of Zur-deregulated mutants to tolerate oxidative stress and develop biofilms (Sein-Echaluze *et al.*, 2015; Olivan-Muro *et al.*, 2023).

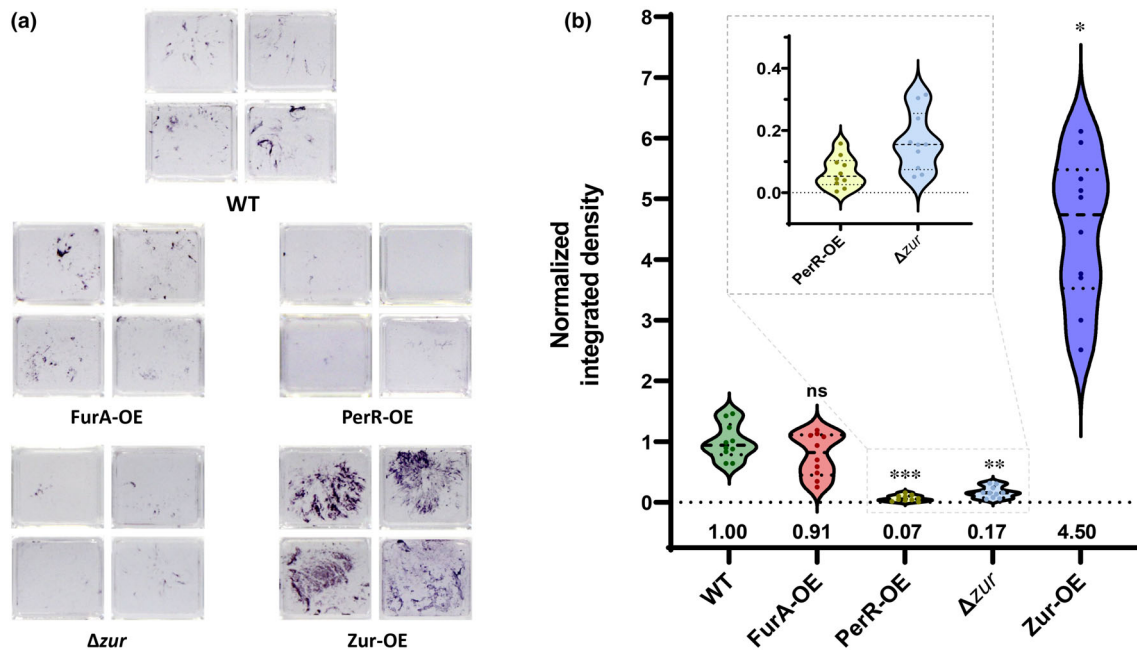


Fig. 7 Impact on biofilm formation of deregulation of the *Anabaena* sp. PCC7120 ferric uptake regulator (FUR) paralogs. Strains used include the wild-type (WT) *Anabaena* and variant strains AG2770FurA (FurA-OE), EB2770FurC (PerR-OE), Δ zur or VCS2770FurB (Zur-OE). OE, overexpressing. (a) Representative crystal violet-stained biofilms grown on Ibidi uncoated eight-well plates. For every strain, each well corresponds to an individual experiment. (b) Image quantification of stained biofilm per well, with integrated density as proxy for biofilm biomass. Values correspond to five independent experiments consisting in two individual wells each, normalized to the average integrated density of the WT strain. Median and quartiles are represented with dashed and dotted lines, respectively. The mean value is included under each dataset. Inset: magnified view of the PerR-OE and Δ zur data. Statistical significance determined through a Kruskal–Wallis test is represented as follows: ns, non significant, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Finally, although several genes were significantly affected by *perR* overexpression, only one in the set of genes tested presented PerR boxes and positive interaction with DNA as tested by EMSA. This gene encoded the ABC transporter protein Alr1490. However, genes *alr2856*, *all2875*, *alr3061*, *all4432* and *alr5144* showed altered transcription levels and negative EMSA results (Table 3). These results point to an indirect regulation by PerR, likely involving ‘secondary’ transcriptional regulators. FUR proteins form complex regulatory networks, affecting gene expression both directly and indirectly, as seen with several genes involved in nitrogen metabolism (Sarasa-Buisan *et al.*, 2022; Guío *et al.*, 2025). Conversely, several genes tested in the *perR* strain that exhibited PerR boxes and resulted positive in EMSA did not show significant transcriptional differences with the WT *Anabaena*. In previous studies (Sarasa-Buisan *et al.*, 2022), we observed that, despite the importance of this regulator in *Anabaena*, many transcriptional changes caused by *perR* overexpression only occur under stress conditions (i.e. nitrogen deficiency). This could explain the absence of changes in q-RT-PCR for several potential target genes that were positive for PerR in EMSA *in vitro* assays. Interestingly, several of the identified proteins whose expression is affected in the FUR variants, including some not previously associated with biofilm synthesis, remain uncharacterized in *Anabaena*, yet may play crucial roles. Examples include *asr0105*, *alr2242* and the TC system *all2875*–*all2874*. Generally, tested genes showing significant transcriptional changes in FurA and Δ zur variants exhibited the same trend.

However, it should be noted that, unlike the Δ zur strain, AG2770FurA and EB2770FurC are FurA- and PerR-overexpressing variants, suggesting that these proteins might play a compensatory role in the regulation of those targets. Our results show a general decrease in the expression of tested genes across all *Anabaena* variants. The observed downregulation in FurA- and PerR-overexpressing variants relative to WT indicates that these proteins act as transcriptional repressors. Conversely, the downregulation in the *zur*-defective mutant points to a role for this protein as an activator. Although Zur seems to work mainly as a repressor in the cyanobacteria (Napolitano *et al.*, 2012; Oliván-Muro *et al.*, 2023), the activation of several genes by this regulator has also been previously demonstrated (Oliván-Muro *et al.*, 2023).

Analysis of biofilm formation in FUR-deregulated variants

To verify the influence of FUR paralogs on biofilm formation, we quantified biofilm production in *Anabaena* WT, Δ zur and overexpressing variants of the three FUR paralogs (Fig. 7). Under standard growth conditions (BG11, 7.5 $\mu\text{mol photons m}^{-2} \text{s}^{-2}$ light intensity and 28°C), significant variations in biofilm formation were observed across the FUR variants tested, evidencing the influence of this family of regulators in the formation of biofilms. Notably, the *zur*-overexpressing strain exhibited a substantial increase in biofilm production, consistent with our previous findings (Oliván-Muro *et al.*, 2023).

Conversely, biofilm formation was significantly impaired in the Δzur - and $\Delta perR$ -overexpressing strains. Although the number of predicted PerR direct targets in biofilm-related genes was smaller than for FurA and Zur, in addition to a potential indirect regulation by PerR of pivotal genes for biofilm formation, it should be considered that overexpression of $\Delta perR$ affects the integrity of *Anabaena* cell envelope (Sarasa-Buisan *et al.*, 2024). The bacterial cell envelope serves as the primary interface between the cyanobacterium and its surroundings, regulating the transport of molecules and mediating interactions with the environment. Given its pivotal role in these fundamental processes, the integrity and functionality of the bacterial cell envelope are likely to have a significant impact on the ability of bacteria to form biofilms. Consistently with the quantitative RT-PCR results (Table 3), and despite the larger number of biofilm-related genes exhibiting FurA boxes, $\Delta furA$ overexpression resulted in a less pronounced increase in biofilm and was more similar to the WT levels. The difference between the *in vitro* EMSA results, which indicated numerous FurA-target biofilm-related genes, and the observed *in vivo* biofilm phenotype suggests that FurA-mediated regulation of these genes may be condition-dependent, reflecting its role as a global regulator responsive to multiple stimuli and possessing a broader regulon than its paralogues. Overall, these results expand our understanding of biofilm synthesis and regulation in nitrogen-fixing cyanobacteria and highlight the role of Fur proteins in shaping biofilm architecture, connecting biofilms with metal and nitrogen homeostasis, and paving the way for future applications in green biotechnology.

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Competing interests

None declared.

Author contributions


IO-M, JG, MFF and ES designed the research. IO-M, JG and GA-T performed the research. IO-M and MFF analysed the data. IO-M, JG and GA-T collected the data. IO-M, JG, MFF and ES interpreted the data. MFF served as site PI. IO-M and MFF wrote the manuscript. ES reviewed and edited the manuscript.

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Data availability

All data supporting the findings of this study are available within the article and its Supporting Information files (Tables S1–S8).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Predicted amyloidogenic hotspots in the protein sequences of potential *Anabaena* sp. PCC7120 homologues to *Synechococcus elongatus* PCC 7942 EbfG1–4.

Table S1 Oligonucleotides used in this work.

Table S2 Query bacterial proteins used in this work based on their relation to biofilm formation or exopolysaccharide (EPS)

synthesis, displaying the open reading frame (ORF) corresponding to their identified *Anabaena* sp. PCC7120 homologues.

Table S3 Genes encoding *Anabaena* sp. PCC7120 homologues to proteins related to exopolysaccharide (EPS) synthesis and biofilm formation in other bacteria, already known to be involved in carbohydrate metabolism or EPS synthesis and secretion based on functional annotation and published data available.

Table S4 Genes encoding *Anabaena* sp. PCC7120 homologues to proteins related to exopolysaccharide (EPS) synthesis and biofilm formation in other bacteria, which have no clear established link to carbohydrate metabolism, EPS synthesis and secretion or biofilm formation based on functional annotation and published data available.

Table S5 Protein sequence identity percentages of the different *Synechococcus elongatus* sp. PCC7942 bacteriocin precursors required for biofilm formation with GG-motif containing proteins identified for *Anabaena* sp. PCC7120 by Wang *et al.* (2011).

Table S6 Putative ferric uptake regulator-binding boxes found upstream of *Anabaena* sp. PCC 7120 GG-motif containing proteins identified by Wang *et al.* (2011).

Table S7 Summary of results for all electrophoretic mobility assays carried out to evaluate promoter–ferric uptake regulator (FUR) protein binding potentially affecting the expression of selected transcriptional units related to exopolysaccharide synthesis or biofilm formation, along with their corresponding putative FUR-binding sequences.

Table S8 Relation between the Find Individual Motif Occurrences (FIMO) prediction of putative ferric uptake regulator-binding boxes in target gene promoters and observation of protein–promoter binding through electrophoretic mobility assays.

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