Biochimie 209 (2023) 61–72

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Lrp as a potential transcriptional regulator involved in stress response in *Haloferax mediterranei*

Laura Matarredona, Mónica Camacho, Vanesa Bautista, María-José Bonete, Julia Esclapez*

Biochemistry and Molecular Biology and Soil and Agricultural Chemistry Department, Biochemistry and Molecular Biology Area, Faculty of Science, University of Alicante, Ap 99, 03080, Alicante, Spain

ARTICLE INFO

Article history:
Received 15 September 2022
Received in revised form
30 November 2022
Accepted 17 January 2023
Available online 25 January 2023

Handling Editor: J.L. Mergny

ABSTRACT

The *Archaea* domain consists of a heterogeneous group of microorganisms with unique physiological properties that occupy a wide variety of niches in nature. *Haloferax mediterranei* is an extremely halophilic archaeon classified in the Phylum Euryarchaeota, which requires a high concentration of inorganic salts for optimal growth. In haloarchaea, transcription factors play a fundamental role in an adequate adaptation to environmental and nutritional changes, preserving the survival and integrity of the organism. To deepen knowledge of the Lrp/AsnC transcriptional regulator family, a *lrp* gene (HFX_RS01210) from this family has been studied. Site-directed mutagenesis has allowed us to identify the TATA-box and two potential sites of the transcriptional factor (TF) to its own promoter and autoregulate itself. Several approaches were carried out to elucidate whether this transcriptional regulator is involved in stresses due to heavy metals and limited nitrogen conditions. Characterization of the *lrp* deletion mutant and the Lrp overexpressed strain, suggests that the level of *lrp* expression depends on the nitrogen source and the presence of cobalt. The most striking results were obtained in the presence of nitrate as a nitrogen source due to the inability of the deletion mutant to grow. All these results confirm that Lrp is a powerful candidate for a regulatory role in the stress response, particularly under N-limiting conditions and the presence of cobalt.

1. Introduction

Saline and hypersaline environments, such as soda lakes, lagoons, salt ponds, salt deposits or salt marshes, are distributed all over the world and have a common characteristic: the total concentration of salts in the water and soil is higher than 3.5% (w/v) [1–4]. Inhabiting these conditions, different microbial ecosystems can be found depending on factors such as light, salt concentration, temperature, percentage of oxygen, or nutrient availability [5–7]. Salt-loving organisms found in *Bacteria*, *Archaea* and *Eukarya* are the most represented microorganisms. Over the time, organisms have survived against a wide range of stressful conditions that alter cellular homeostasis [5]. In *Archaea*, some organisms have developed different strategies that have allowed them to adapt, such as stress proteins, chaperones, thermoprotectants, proteasomes, multicellular structures, and other molecules [7,8]. Transcriptional factors (TFs) are an important mechanism of gene regulation in the stress response. Based on members per genome, the most abundant families of TFs in archaea are Lrp/AsnC, MarR, ArsR, and TrmB [9]. The Lrp/AsnC family may be involved in several functions; some of them are the regulation of genes required in the amino acid metabolism, nutrient transport, stress tolerance, motility, or maintenance processes [10–13]. In the haloarchaeon *Halobacterium salinarum*, it has been shown that TFs from this family are upregulated in response to oxidative stress conditions [14]. This family of TFs can act as global or local transcriptional regulators.

Haloferax mediterranei is a microorganism belonging to the Haloarchaea class that requires high salt concentrations between 20 and 25% NaCl for optimal growth [15]. Regarding metabolism, it is chemoheterotrophic, being able to use a wide variety of carbon sources compared to other halophilic archaea and nitrate, nitrite or even amino acids as nitrogen sources [3,16]. *Hfx. mediterranei* is considered a model organism to be studied extensively because of the extensive knowledge of its metabolism and the molecular biological techniques available as well as the potential

* Corresponding author.
E-mail addresses: lsm69@alu.ua.es (L. Matarredona), camacho@ua.es (M. Camacho), vanesa.bautista@ua.es (V. Bautista), mjbonete@ua.es (M.-J. Bonete), julia.esclapez@ua.es (J. Esclapez).

https://doi.org/10.1016/j.biochi.2023.01.012
0300-9084/

biotechnological applications [17–22]. This haloarchaeon can adapt its metabolism and cope with stressful situations depending on the availability of nutrients and environmental parameters. It has been shown that it can adapt to adverse conditions such as low/high temperatures, low/high salt concentrations, changes in pH, oxidative stress, and the addition of metals (Ni^{2+}, Li^{+}, Co^{2+}, As^{5+}), considered a polyextremophilic valuable microorganism for developing new biotechnological applications [15]. It should also be noted that this haloarchaeon can use different nitrogen sources as ammonium, nitrate, nitrite or amino acids, being ammonium as its preferred source of nitrogen and nitrate, nitrite and aspartate as worse nitrogen source, considering them as N-limiting conditions [23].

The nitrogen (N-cycle) in *Hfx. mediterranei* has been studied in depth [24]. This microorganism is able to carry out the complete denitrification of nitrate (NO_3^-) to molecular nitrogen (N_2) under anaerobic conditions, and also the assimilation of nitrate (NO_3^-) or nitrite (NO_2^-) to ammonium (NH_4^+) which is subsequently incorporated into carbon skeletons during the synthesis of nucleotides, amino acids and other compounds [18]. From a biochemical point of view, it is known in detail all the components of the assimilatory nitrate reduction in *Hfx. mediterranei*. All the enzymes have been purified and characterized and the conditions under which they are activated are known [23,25–28]. Nevertheless, there are still many unknowns about the regulation of its gene expression. Although some proteins related to nitrogen assimilation metabolism were identified with differential expression at the transcriptional level using microarray technology [29], there is no transcriptional regulator of *Hfx. mediterranei* identified to be involved in the gene expression regulation of the nitrogen cycle. For this reason, it is important to continue the search for transcriptional regulators involved in the regulation of this pathway and to elucidate their mechanisms of action at the molecular level. The molecular mechanisms of gene expression regulation in Haloarchaea in terms of nitrogen metabolism remain unknown and there is not much research focusing on determining the function of transcriptional regulators. Nevertheless, previous work analyses Lrp transcriptional regulators from *Hbt. salinarum* [30] which activates the gene expression of the *glnA*, an enzyme involved in ammonium assimilation and amino acid metabolism [25,31,32]. *Hfx. mediterranei* contains 14 *lrp* genes annotated in its genome and one of them is the *lrp* gene (HFX_RS01210) located next to the *glnA* gene (HFX_RS01205) with the same gene-environment as *Hbt. salinarum* and whose sequence is almost identical. Previous studies in *Hfx. mediterranei* have shown that this TF is a tetrameric protein of 67 kDa and showed differential expression at the transcriptional and translational level in the presence of stress conditions such as hydrogen peroxide, cobalt, and arsenic. These results proposed that the Lrp could act as a stress regulator of metabolism [32]. The present work aims to elucidate the possible role of Lrp (HFX_RS01210) in the stress response regulation in nutrient deficiencies including, nitrogen-limiting conditions and the presence of heavy metals.

2. Materials and Methods

2.1. Strains and growth conditions

Escherichia coli DH5α strain was used to clone PCR products and prepare constructions with different vectors, and *E. coli* JM110 was used to prepare unmethylated DNA for efficient transformation of *Hfx. mediterranei*. Both strains were grown aerobically in Luria-Bertani medium with ampicillin (100 μg/mL) at 37 °C.

The *Hfx. mediterranei* R4 (ATCC 33500^T), HM26 (R4 Δ*pyrE2*), and HM26-Δ*lrp* strains were grown in complex medium (Hm-CM) at pH 7.3 containing 20% (w/v) seawater [16] and 0.5% (w/v) yeast extract. The overexpression strain, Lrp-HM26, was grown in minimal media (Hm-MM).

2.2. Site-directed mutagenesis and β–galactosidase assay

The promoter of the *lrp* gene (p.*lrp*) was previously cloned in pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) [32]. This construction was used to perform site-directed mutagenesis in different nucleotides of the promoter region using the modified oligonucleotides summarized in Table S1. The different mutants of p. *lrp* were produced as described in Ref. [33]. After sequencing the positive transformants for each mutation, the constructions were subsequently cloned into the pVA513 halophilic expression vector, kindly provided by Prof. Mike Dyall-Smith (University of Melbourne, Australia). *Hfx. mediterranei* R4 cells were transformed with these constructions as described previously, and the transformants were selected on 0.3 μg/mL of novobiocin agar plates as a screening method. The mutants were characterized in different culture media: Hm-CM, defined medium (Hm-DM) with 20 mM NH_4Cl, and Hm-DM with 0.2 mM $CoCl_2$ by measuring β-galactosidase along the growth curve. The β-galactosidase activity was determined as previously described [32]. All values in the figures are expressed as the mean of three replicates ± the standard deviation.

2.3. DNA-protein interaction analysis

To test the binding affinity of the p. *lrp*, a DNA-protein interaction assay was carried out following the protocol described in Ref. [27], an adapted pull-down assay for Haloarchaea. Three different DNA samples were used in this assay: the p. *lrp* promoter region as the target DNA, and a constitutive promoter from *Hfx. mediterranei* (ferredoxin promoter, p. *fdx*) as a negative control; and a random DNA fragment from *Hfx. mediterranei* (glucose dehydrogenase gene) as a second negative control. The third negative control was a sample without DNA. The biotinylated DNA for each sample was obtained by PCR using the following oligonucleotides labelled with biotin (Table S1). Samples of the elutions were analyzed in an SDS-PAGE with silver staining. Bands present only in the elutions of the p. *lrp* compared with the three negative controls, were analyzed by mass spectrometry; nano-ESI LC-MS/MS analysis was carried out via the Research Technical Services (SSTTI) from the University of Alicante and analyzed with Spectrum Mill Software (Agilent, Santa Clara, CA, USA). Focusing on the Lrp, an assay was performed using cell extracts at 60% (w/v) of *Hfx. mediterranei* R4 grown until mid-exponential phase in Hm-DM with 20 mM NH_4Cl.

2.4. Construction and characterization of HM26-Δlrp deletion mutant

HM26-Δ*lrp* was constructed by the pop-in/pop-out method following the strategy previously described in *Hfx. mediterranei* [34,35]. The oligonucleotides used are shown in Table S1. Genomic organizations (Fig. S1) of the pop-out clones and wild type were confirmed by PCR screening, Southern blot analysis, and Sanger sequencing (Stabvida, Caparica, Portugal). PCR was performed according to the manufacturer's instructions using Supreme NZY-Proof DNA polymerase (NZYtech) and analyzing more than one hundred pop-out clones. For the Southern blot, 3 and 5 μg of genomic DNA were digested with *Aat*II (Thermo Scientific, Waltham, Massachusetts, United States); the pre-hybridization and hybridization were carried out at 65 °C and the chemiluminescent detection was performed as is described in the DIG Application Manual (Roche, Basel, Switzerland).

HM26-Δ*lrp* was phenotypically characterized compared with

HM26 parental strain in different culture media conditions. The detailed culture medium composition is shown in Table 1. All the cultures were inoculated with non-preadapted cells grown in Hm-CM and the initial optical density was set up to 0.02. Three biological replicates were made for each strain and culture condition.

2.5. Analysis of the lrp overexpression effect on the Hfx. mediterranei growth

The *lrp* (HFX_RS01210) was homologously overexpressed in *Hfx. mediterranei* HM26 using Hm-MM as a culture medium [32]. The overexpression strain (Lrp-HM26) and HM26 strain were characterized in different culture media by measuring the optical density at 600 nm wavelength (OD_{600}) at regular intervals (6–8 h depending on the culture's growth rate) until reaching the stationary phase. The culture media used in this assay were described above in Table 1, but the characterization of stress conditions was performed with Hm-DM in the presence of 20 mM ammonium instead of Hm-CM. Three independent biological replicates were performed for each condition to ensure reproducibility, and the HM26 strain was used as a control in each condition. All cultures were inoculated from cells grown in Hm-MM, and the starting OD_{600} of all the cultures was 0.02, and the lag phase, log phase, and stationary phase were statistically analyzed and compared among groups. Doubling time (*d.t.*) was calculated as previously [15].

3. Results and discussion

3.1. Mutagenesis and characterization of the p.lrp using β-galactosidase as a reporter gene

According to previous studies, the possible elements of the p. *lrp* promoter region have been manually identified, a possible TATA box and BRE element (B recognition element) sequence, basic elements for the transcription of the gene [36–38]. In addition, by analyzing the sequence, a possible *lrp*-type regulator binding site has also been found, which would be involved in the autoregulation of its expression [37,38]. To confirm the function of these elements, three identified consensus sequences have been mutated by site-directed mutagenesis into three different sites resulting in Mut-1, Mut-2 and TATA-box (Fig. 1).

The three mutants of p. *lrp* were cloned into pVA513, creating the constructions pVA-p. *lrp* (Mut-1), pVA-p. *lrp* (Mut-2) and pVA-p. *lrp* (TATA). These constructions were transformed in *Hfx. mediterranei* R4 and then, the transformants were characterized in four culture media by measuring the specific activity of *bgaH* gene from *Hfx. lucentense* which is used as the reported gene. Fig. 2 shows the growth of the transformants by measuring their optical density at 600 nm; and their *bgaH*-specific activity in different culture media. To test whether these mutated areas may be involved in regulating the expression of the regulator five culture media were assayed: i) defined medium with ammonium (nitrogen source) as a rich medium, ii) nitrate as a nitrogen-limited medium, iii) in the presence of cobalt, iv) in the presence of nickel and v) complex medium. Different nitrogen sources were used because this regulator could be involved in the regulation of glutamine synthetase (GS), and it recognized *in vitro* the promoter of assimilative nitrate reductase [27]. On the contrary, activity was measured in the presence of cobalt and nickel to study the effect of two divalent metals [15]. There are changes in the expression of the transcriptional regulator under these conditions [32] and thus, check if the mutated areas may be involved in the molecular mechanisms of gene expression regulation.

In all the analyzed cultures media, Mut-1, Mut-2 and TATA-box transformants showed the same growth profile as the control (p.*lrp* without mutation); no significant differences were detected, and all cultures present an exponential increase in growth until reaching maximum growth in the stationary phase with optical densities around 4 in the case of ammonium and complex media except with cobalt; defined medium containing cobalt reach to an optical density around 1.5 only. Concerning the characterization of the TATA-box mutant, no β-galactosidase specific activity was detected under any of the analyzed conditions; values were close to zero throughout the growth. The identified TATA-box sequence in *Hfx. mediterranei* "TATT" was similar to "TTWT" the one proposed by Brenneis et al. as a TATA-box consensus sequence located at position −27/-28 in halophilic archaea [36]. Based on these results, it appears that the TATA-box of this transcriptional regulator has been identified; this sequence plays a key role in the promoter region. On the other hand, the β-galactosidase specific activity (U/mg) profile of the Mut-1 and Mut-2 changes along the time presenting significant differences compared to p. *lrp*, suggesting that this region "AAAGACCTTTCCGCTTAAT" could be a promising regulation zone to bind its own transcriptional regulator as in *Pyrococcus furiosus* [37,39]. The β-galactosidase specific activity experienced an abrupt and rapid decrease during all the growth profiles in Mut-1 and Mut-2, regardless of the growth phase in complex medium, defined medium with ammonium or nitrate as nitrogen source, and in the presence of nickel. In the N-rich medium, the highest specific activity values were recorded when the culture reached the stationary phase in both the control (0.513 U/mg) and mutant 1 (0.1984 U/mg). In a complex medium, the highest values were obtained at mid-exponential phase with 0.513 U/mg for the control and 0.181 U/mg for Mut-1; the β-galactosidase specific activity decreased almost 3-fold. Mutant 2 in these two media did not vary its activity, having a constant value close to 0.07 U/mg along the growth curve. Similar results were obtained with nitrate as a nitrogen source; the control reached 0.5 U/mg of

Table 1
Culture media used in the construction and characterization of the *lrp* deletion mutant (HM26-Δ*lrp*).

Generation of the *lrp* deletion mutant	
Description	**Composition**
Pop-in	Hm-MM
Pop-out	Hm-CM in the presence of 50 mM MOPS and 750 μg/mL 5-FOA.
Phenotypic characterization of the HM26-Δ*lrp* and HM26	
Description	**Culture media**
Different nitrogen source	Hm-DM in the presence of 20 mM ammonium (N-rich conditions) or nitrate (N-limiting conditions). 0.3 μg/mL novobiocin was added.
Complex medium	Hm-CM
Oxidative stress[a]	Hm-CM cultures were grown to OD_{600} of 0.8 (mid-exponential phase) before adding 8 mM of H_2O_2.
Metal stress[a]	Hm-CM cultures containing 0.4 mM $NiSO_4$; 2 mM Na_2HAsO_4; 0.2 mM $CoCl_2$; and 0.5 M LiCl.

[a] The addition of hydrogen peroxide and metals was performed as [15]. All the cultures media used in the characterization are described in Ref. [32].

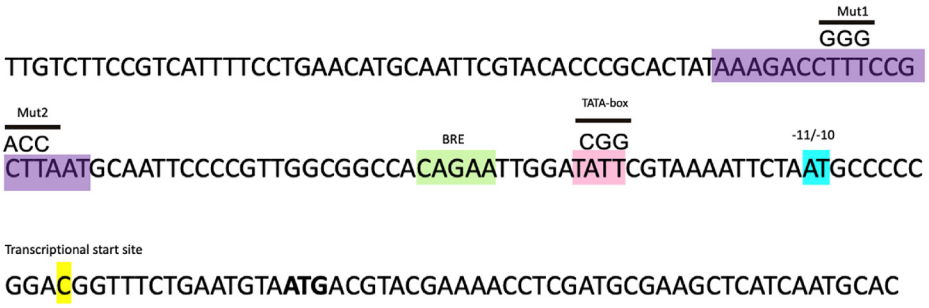

Fig. 1. Identification of possible elements of the p. *lrp* promoter region from *Hfx. mediterranei*. In purple, the binding motif; in green, the BRE-element; in dark pink, the TATA-box; in blue, the −11/-10 motif; in yellow, the transcriptional start site; and in bold, the ATG of the *lrp* gene. The modifications of the mutants are also shown.

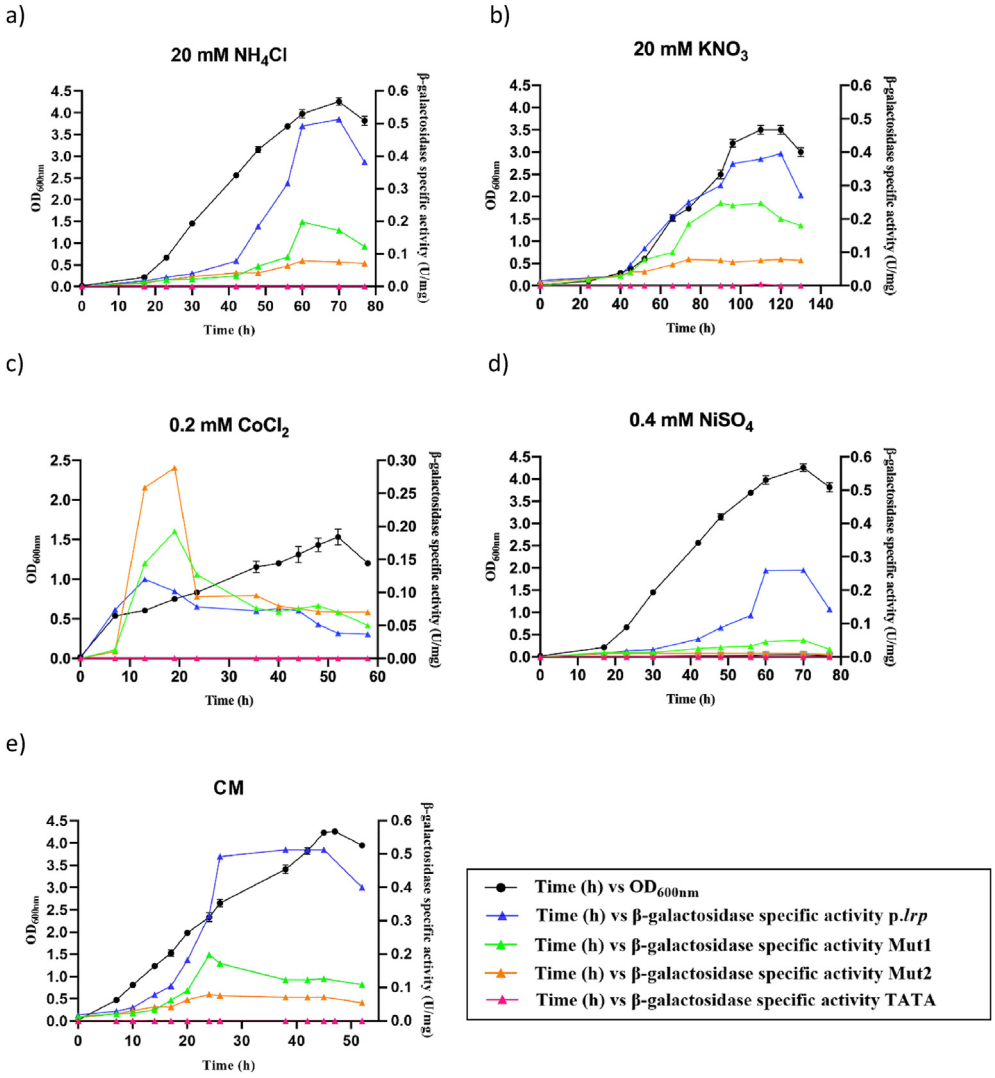

Fig. 2. Cell growth and β-galactosidase specific activity were determined for *Hfx. mediterranei* p. *lrp*, p. *lrp* (Mut-1), pVA-p. *lrp* (Mut-2) and pVA-p. l*rp* (TATA) in different culture media. (a) defined medium with 20 mM NH_4Cl as nitrogen source, (b) defined medium with 20 mM KNO_3 as nitrogen source, (c) defined medium with 0.2 mM Co^{2+}, (d) defined medium with 0.2 mM Ni^{2+}, and (e) complex medium.

specific activity, Mut-1 0.392 U/mg, and Mut-2 0.07 U/mg. In the presence of nickel, the control achieved a specific activity of almost 0.3 U/mg while Mutant 1 and Mutant 2 had an activity of less than 0.05 U/mg. However, in the presence of cobalt, the profile obtained is totally different, both mutants showed lower values of specific activity at the beginning of growth compared to the control. Measuring specific activity in the culture medium with cobalt, both mutants 1 (0.193 U/mg) and 2 (0.289 U/mg) showed higher values of specific activity at the beginning of growth compared to the control (0.119 U/mg). Consequently, it appears that the mutated

zones must be involved in the union of the regulator; nonetheless, the molecular mechanism of the regulatory process is still unknown.

3.2. Pull-down assay

To find possible transcriptional regulators of the p. *lrp* promoter, a DNA-protein pull-down assay was performed using extracts of *Hfx. mediterranei* R4 in Hm-DM and the presence of cobalt. A recently optimized method using extracts from halophilic archaea was followed. Three negative controls were used in this pull-down assay (p.*fdx* promoter, a fragment of the glucose dehydrogenase gene and without DNA). The constitutive ferredoxin promoter was used to identify unspecific recognition of the promoter sequences; a fragment glucose dehydrogenase gene was used to test the unspecific union of a protein to any DNA fragment, and the negative control without DNA allowed us to discard unspecific protein binding to DNA magnetic beads complex [27]. The two elution fractions were analyzed via SDS-PAGE and silver staining; the protein band profiles of the sample (p.*lrp*) were compared with the three controls (Fig. 3) to identify possible proteins that may join the promoter region.

Under these conditions, different bands of unspecific bound proteins were observed in the three control samples. Although four bands were selected to be analyzed by mass spectrometry (Agilent 1100 Series LC/MSD Trap SL) to identify which protein it corresponds to, three of them showed inconclusive results. Only the band of approximately 16 kDa was identified in the first elution of the pull-down assay in Hm-DM, which corresponds to a transcriptional regulator of the Lrp/AsnC family, particularly, it is the Lrp under study (HFX_RS01210) (Table 2).

Based on all these results, it is known that via *in vitro* this transcriptional regulator recognized its own promoter p.*lrp*. Therefore, there is evidence of the autoregulation of this protein. It is not surprising because transcriptional regulators of this family tend to self-regulate in other species such as *Bacillus subtilis* [40], *Pyr. furiosus* [39] *or Agrobacterium tumefaciens* [41]. Focusing on nitrogen metabolism, Lrp recognizes the p.*nas* (assimilatory nitrate reductases promoter) in *Hfx. mediterranei* and could be involved in the regulation of its gene expression [27] as well as an Lrp from *Hbt. salinarum* recognized the promoter of the *glnA* enzyme [30]. The mechanism of regulation *in vivo* remains unknown. That is further evidence that this regulator may be involved in the regulation of nitrogen metabolism genes, in addition to other genes.

3.3. Characterization of HM26-Δlrp deletion mutant

Analyzing the gene-environment revealed that this transcriptional regulator is located downstream of the glutamine synthetase gene (*glnA*; HFX_RS01205), an enzyme that plays a fundamental role in ammonium assimilation and amino acid metabolism [24]. In addition, previous studies have shown that *lrp* could be involved in the stress response of *Hfx. mediterranei* since its expression change in the presence of hydrogen peroxide, cobalt, and arsenic [32]. To elucidate the role of *Hfx. mediterranei* Lrp transcriptional regulator in response to different stresses, HM26-Δ*lrp* was constructed by the pop-in/pop-out strategy. Two different deletion mutants have been constructed. The first attempt was to delete the complete *lrp* gene sequence (Fig. 4); however, after analyzing more than a hundred colonies, all the emerging pop-out clones had the wild-type version with the parental HM26 genotype. The *glnA* and *lrp* genes share the promoter region in opposite directions and the potential *glnA* binding site "ATGACGTACGAAAACCTCG" was partially deleted in the complete deletion mutant. Although there is no clear evidence, one possible interpretation could be that in the construction of this deletion mutant an essential element for *glnA* expression has been removed and for this reason, it was not possible to obtain the complete deletion mutant. Subsequently, a second deletion mutant was constructed in which only 364 nucleotides of 459 were deleted, keeping the first 97 nucleotides of the gene (Fig. 4). In this partial-mutant, many colonies of the pop-out clones were analyzed by PCR screening and Southern blot (Fig. S2); successfully, some of them had the deleted version. Consequently, the whole gene sequence cannot be removed because it negatively affects *glnA* expression, an essential enzyme in the assimilatory pathway of the nitrogen cycle in *Hfx. mediterranei* [25].

The phenotypic characterization of partial *lrp* deletion mutant from *Hfx. mediterranei* HM26 was performed using culture media with different nitrogen sources such as ammonium or nitrate. In addition, stress conditions were also tested as it is known that in other archaeal domain microorganisms, this family of transcriptional regulators can participate in multiple physiological processes acting as global regulators. Previous studies analyzing the role of this Lrp transcriptional factor showed significant differences in expression under different stress conditions using assays such as promoter characterization and protein level determination by

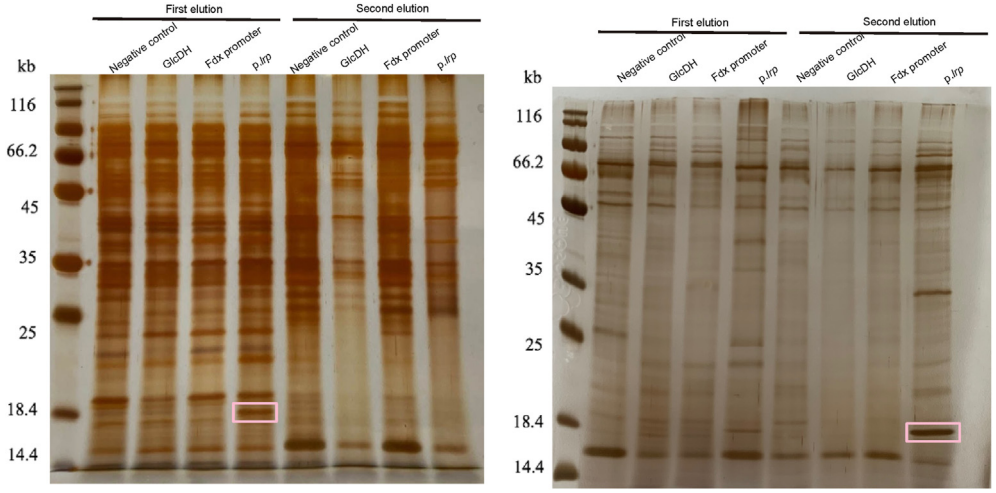

Fig. 3. Silver staining of the pull-down assay with protein extracts in (a) Hm-DM and (b) Hm-DM with 0.2 mM cobalt using different biotinylated DNA fragments (p.*fdx* promoter; glucose dehydrogenase, GlcDH; and p. *lrp* promoter) and without DNA. The protein band surrounded in pink was selected for analysis by mass spectrometry.

Table 2
Mass spectrometry analysis of pull-down sample in Hm-DM.

Spectra	Distinct peptides	Distinct summed MS/MS Search Score	%AA Coverage	Total Protein Spectral Intensity	Species	NCBI Database	Protein Name
11	5	89.86	45	1.59×10^8	*Hfx. mediterranei* ATCC 33500	HFX_RS01210	Lrp/AsnC family transcriptional regulator

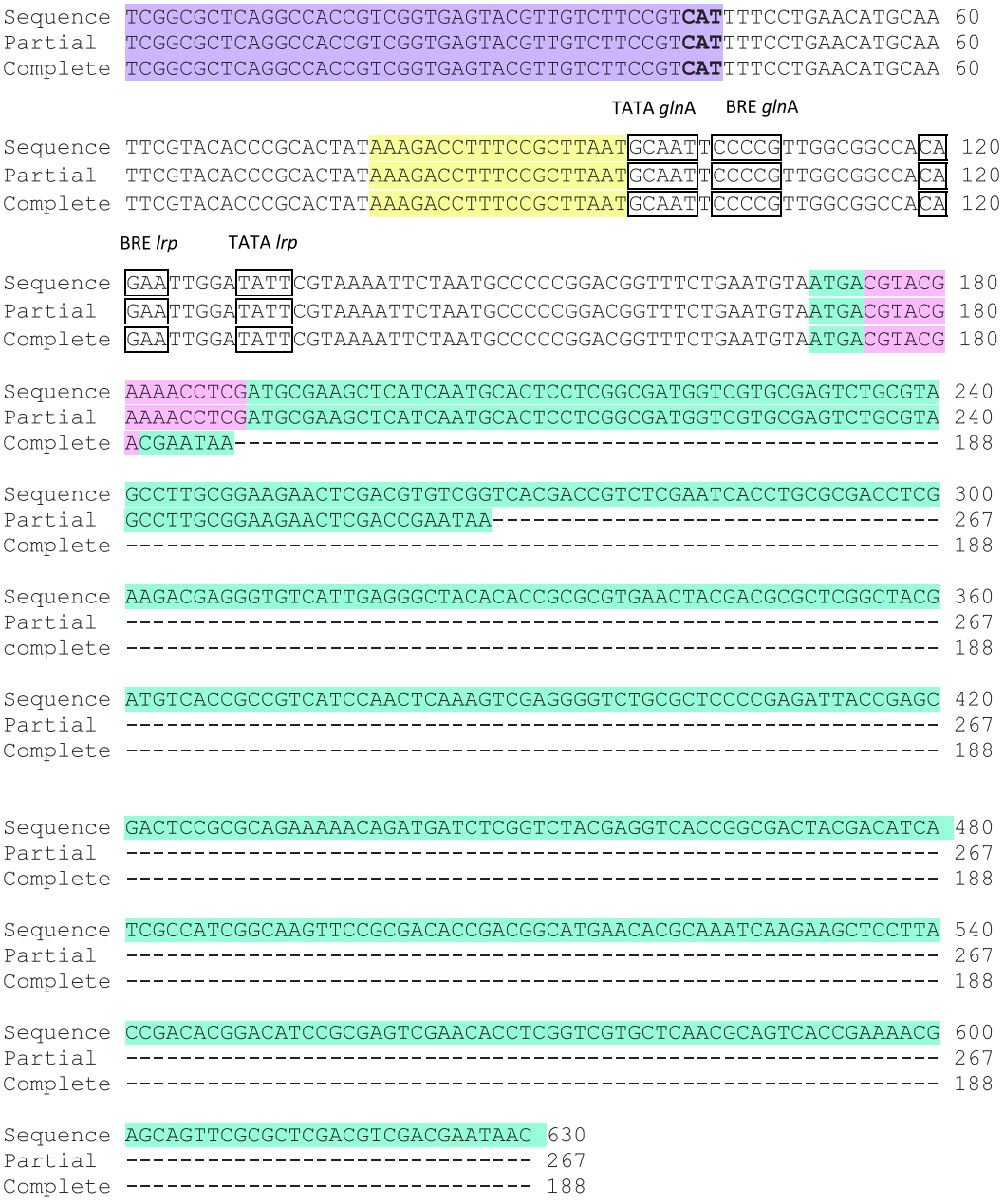

Fig. 4. Sequence alignment of the original sequence in the *Hfx. mediterranei* genome, the partial deletion mutant, and the complete deletion sequence. In purple: part of the glutamine synthetase gene; in green: the *lrp* transcriptional regulator gene; gaps in both mutants represent the deleted nucleotides; in yellow: potential *lrp*-type regulator binding site in p. *lrp* region; in pink: potential *lrp*-type regulator binding site in p. *glnA* region.

Western blot [32]. In all the tested conditions strains HM26 (control) and HM26-Δ*lrp* (partial deletion mutant) were grown in triplicate and cell growth was quantified by measuring the optical density at 600 nm at regular intervals (Fig. 5). Therefore, to analyze if the deletion mutant substantially impacted cell growth, the three phases (lag phase, log phase, and stationary phase) of the growth curve were compared with the control (HM26) grown in the same media, where the most striking difference was found in growth under N-limiting conditions (nitrate as nitrogen source). Slight growth differences have also been observed in the culture media

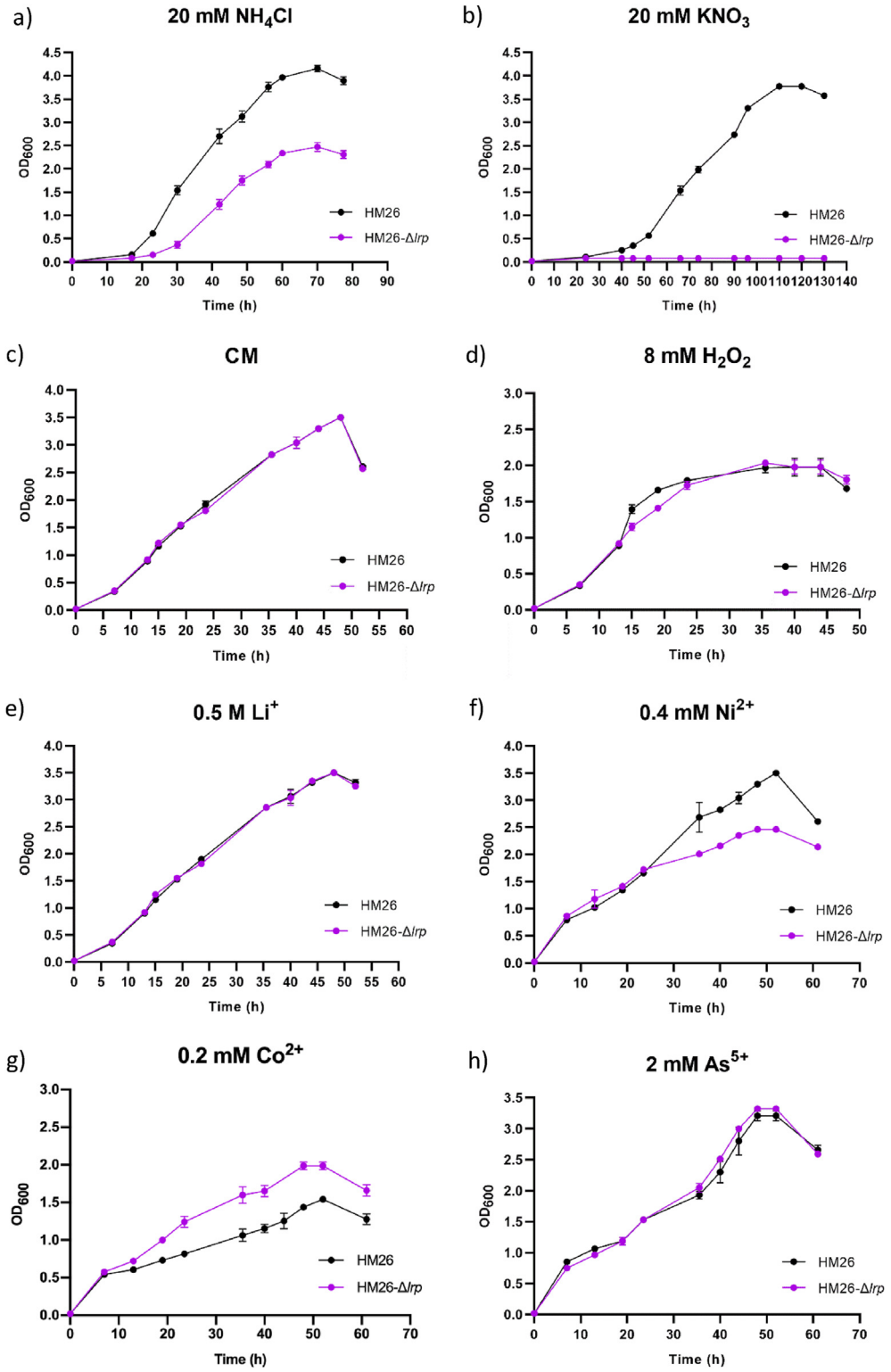

Fig. 5. Growth of *Hfx. mediterranei* HM26 and HM26-Δ*lrp* under different conditions. (a) 20 mM NH_4Cl; (b) 20 mM KNO_3; (c) CM; (d) 8 mM H_2O_2; (e) 0.5 M Li^+; (f) 0.4 mM Ni^{2+}; (g) 0.2 mM Co^{2+}; (h) 2 mM As^{5+}. Data are based on three independent replicates. Plotted values are the mean of triplicate measurements, and error bars represent ± SD.

containing ammonium (non-limiting nitrogen conditions), cobalt, and nickel.

As can be seen in Fig. 5a, there was a significant difference in the growth curve between HM26-Δ*lrp* and HM26 under non-limiting nitrogen conditions (ammonium as nitrogen source), highlighting that the deletion mutant could not grow over 2.5 of optical density, and the growth was considerably less than the control. Changing to N-limiting conditions (nitrate), surprisingly, no growth was detected under the medium with 20 mM of KNO_3 (Fig. 5b); the partial deletion strain was not able to grow using nitrate as a

nitrogen source, although the wild strain can grow under these conditions reaching 3.7 values of OD_{600}. Consequently, these results reveal that the Lrp transcriptional regulator, in some unknown way, is involved in N-limiting conditions.

In addition, it seems that Lrp transcriptional regulators play an essential role in coordinating the metabolism in response to environmental shifts. For this reason, the characterization of the HM26-Δ*lrp* and HM26 was also carried out using different culture media with external stressors such as hydrogen peroxide and some metals. It was used the complex medium as the standard medium for the stress conditions as the growth of this strain (HM26-Δ*lrp*) in the presence of ammonium as a nitrogen source is affected, but the wild-type strain HM26 and the deleted one, HM26-Δ*lrp*, resulted having the same growth profile in complex medium (Fig. 5c). Analyzing the results, no significant differences have been found in the presence of 8 mM of H_2O_2 or 2 mM Na_2HAsO_4 between HM26-Δ*lrp* and HM26 (Fig. 5d and h). These results were expected since it is known that under these conditions there was no expression of Lrp [32]. Unexpectedly, in the presence of 0.5 M LiCl (Fig. 5e), both strains followed the same growth trend. These growth results were not as expected since Lrp showed transcriptional and translational expression with lithium. According to these results, this transcriptional regulator would not be directly involved in response to lithium stress and its expression changes may be due to other factors. In contrast to these results, the deletion mutant showed differential growth concerning HM26 in the presence of divalent metals (Fig. 5f and g). In the presence of nickel, delayed growth of the HM26-Δ*lrp* strain in compared to the HM26 strain was observed. Nonetheless, the opposite is true in the presence of cobalt. Remarkably, the HM26-Δ*lrp* strain achieved a higher stationary phase than the control in the presence of cobalt. These results are in agree with previous studies in which Lrp expression was detected in the presence of low concentrations of cobalt [32].

As can be seen in Table 3, the growth kinetics were also analyzed in all the tested conditions. The HM26-Δ*lrp* reached the worse results under N-limiting conditions (20 mM KNO_3), underscoring the importance of this transcriptional regulator concerning the assimilative nitrate pathway for *Hfx. mediterranei*. Furthermore, even though HM26-Δ*lrp* showed growth in media with 20 mM NH_4Cl, significant differences were detected regarding doubling time. In the presence of hydrogen peroxide, cells of both strains grew much slower and reached lower optical density values than in complex media without stress. However, no significant differences were detected in the growth kinetics. When cobalt was added to the culture media, as discussed before, although the HM26-Δ*lrp* reached higher optical densities, the growth kinetics resulted not to be significant. Even though in most of the tested conditions, data between the strains was insignificant, doubling time values were promising and delightful because most of them were around 1 h.

Table 3
Doubling time for *Hfx. mediterranei* HM26 and HM26-Δ*lrp* under different culture conditions.

Condition	Strain	d.t. (h)*	*p*-Value	significance
20 mM NH_4Cl	HM26	2.8 ± 0.2	<0.001	***
	HM26-Δ*lrp*	14.2 ± 0.4		
20 mM KNO_3	HM26	8.2 ± 0.5	<0.001	***
	HM26-Δ*lrp*	–		
		–		
CM	HM26	1.07 ± 0.02	>0.999	Ns
	HM26-Δ*lrp*	1.03 ± 0.01		
8 mM H_2O_2	HM26	5.0 ± 0.3	>0.999	Ns
	HM26-Δ*lrp*	4.1 ± 0.2		
0.5 M Li^+	HM26	1.21 ± 0.03	>0.999	Ns
	HM26-Δ*lrp*	1.09 ± 0.04		
0.4 mM Ni^{2+}	HM26	1.04 ± 0.04	>0.999	Ns
	HM26-Δ*lrp*	0.82 ± 0.08		
0.2 mM Co^{2+}	HM26	1.49 ± 0.08	>0.999	Ns
	HM26-Δ*lrp*	1.123 ± 0.04		
2 mM As^{5+}	HM26	0.97 ± 0.02	>0.999	Ns
	HM26-Δ*lrp*	1.15 ± 0.01		

Ns: $p > 0.05$; ***$p \leq 0.001$. **Hfx. mediterranei* growth was followed by measuring its optical density at 600 nm, doubling times are calculated as described in the Materials and Methods section.

In previous work, it was deduced that the Lrp expression was not directly dependent on the nitrogen source, considering both the characterization of the *lrp* promoter region and the protein expression profile because the expression level did not change. In contrast, the most significant changes were found under stress conditions concluding that it may function as a transcriptional regulator in response to stress [32]. Although Lrp is expressed using ammonium or nitrate as a nitrogen source, that is to say, in N-limiting or N-rich conditions, its mechanism of action must be ligand-dependent. Different effector molecules of the Lrp regulator can induce conformational changes that lead to a decrease or increase in DNA binding affinity and subsequently modulate Lrp expression as a function of the environment. After analyzing the results from the characterization of the *lrp* deletion mutant, it has been confirmed that its expression profile clearly changes in response to the nitrogen source. It is not unreasonable to think that this transcriptional regulator is involved in the complex regulation of the *glnA* gene expression as in a previous study in *Hbt. salinarum* [30]. In that study, the Lrp activates the gene expression of the *glnA* gene, influences the peptide and phosphate transport, and participates in the central intermediary metabolism acting as a global transcriptional regulator [30]. These data from the characterization of the deletion mutant together with previous studies in which Lrp recognizes *in vitro* the promoter of the assimilative nitrate reductase [27] support the idea that Lrp can be considered a transcriptional regulator of the nitrogen cycle under N-limiting conditions.

3.4. Characterization of homologous overexpression of pTA1992.lrp in Hfx. mediterranei HM26

The construction pTA1992. *lrp* was previously used to overexpress the Lrp in *Hfx. mediterranei* HM26 strain using Hm-MM as culture media to elucidate its quaternary structure [32]. In this work, phenotypic characterization of the overexpressing strain (Lrp-HM26) was performed by analyzing its growth compared to the growth of the parental strain, *Hfx. mediterranei* HM26, in minimal medium and other culture media under different stress conditions (N-limiting conditions, presence of hydrogen peroxide and metals). The culture conditions used are the same as in the characterization of the deletion mutant, but a minimal medium in which glucose is exchanged as a carbon source for casamino acids has also been tested. The growth curves of the overexpression strain and parental strain HM26 were very similar (Fig. 6c) in minimal medium, in the presence of hydrogen peroxide (Fig. 6d), lithium (Fig. 6e) as well as nickel (Fig. 6f). Both strains showed identical adaptation time and similar growth in the exponential phases. Furthermore, statistical analysis of the growth parameters in these culture media revealed no significant differences between the growth of the parental strain HM26 and the overexpression strain Lrp-HM26 (Table 4). These results resemble those previously shown for the deletion mutant; under these culture media conditions (except nickel), no significant differences were found between the mutant and the control strain. Interesting results were obtained from an N-rich source (ammonium). Thanks to the characterization of the mutant previously discussed, it was known that in the absence of the *lrp* gene, *Hfx. mediterranei* had slower growth reaching lower optical densities in the presence of ammonium as a

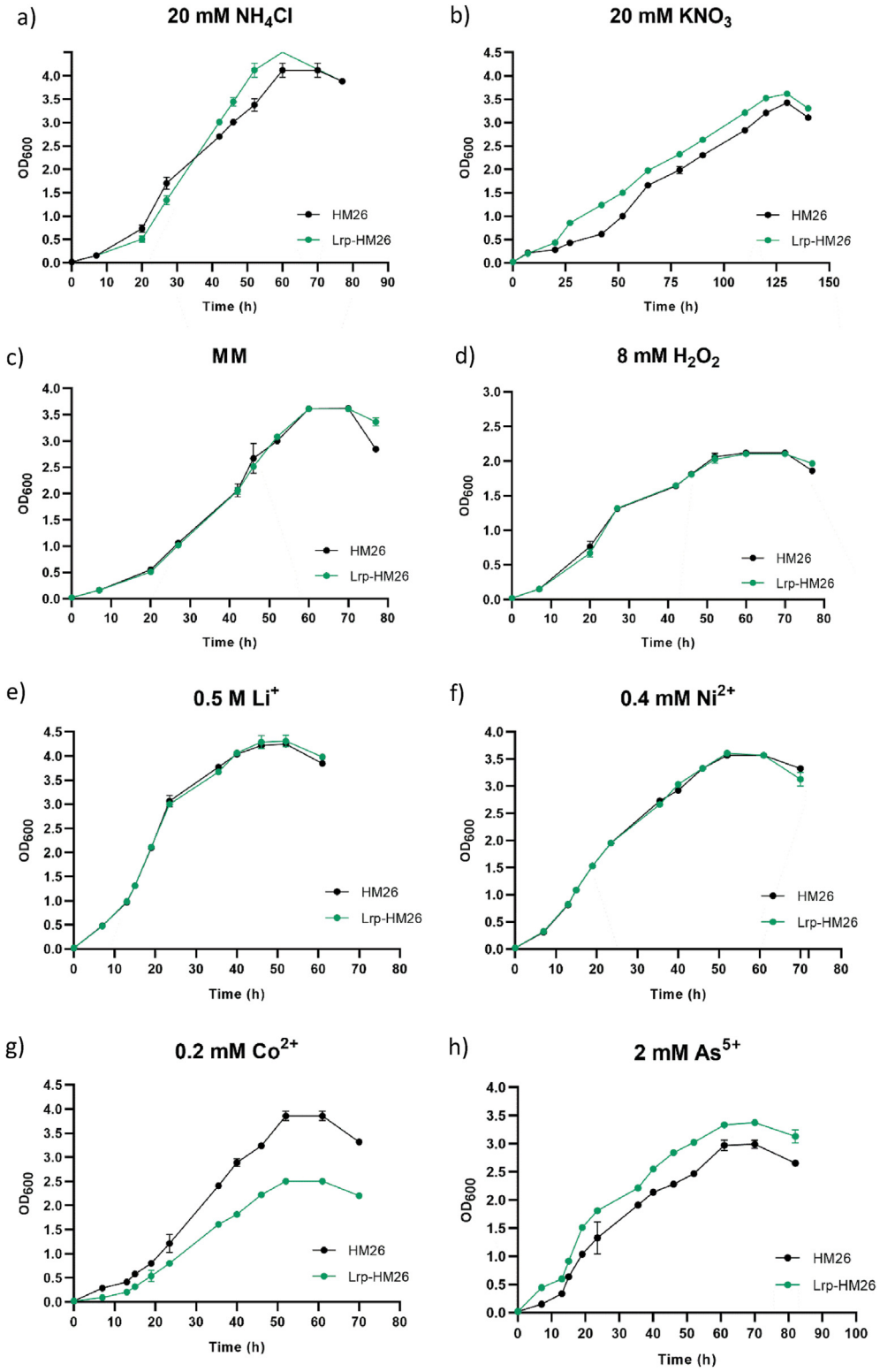

Fig. 6. Growth of *Hfx. mediterranei* HM26 and Lrp-HM26 under different conditions. (a) 20 mM NH_4Cl; (b) 20 mM KNO_3; (c) MM; (d) 8 mM H_2O_2; (e) 0.5 M Li^+; (f) 0.4 mM Ni^{2+}; (g) 0.2 mM Co^{2+}; (h) 2 mM As^{5+}. Data are based on three independent replicates. Plotted values are the mean of triplicate measurements, and error bars represent ± SD.

nitrogen source. On the contrary, when the Lrp was overexpressed, the Lrp-HM26 strain grew significantly faster, reaching higher optical densities from the mid-exponential phase onwards under this condition (Fig. 6a). In addition, statistical analysis of the growth parameters determined that there were significant differences in growth between the parental HM26 strain and the Lrp-HM26 strain (Table 4). Focusing on the N-limiting condition (nitrate), revealing results were found (Fig. 6b). Based on the knowledge that the mutant cannot to grow using nitrate as a nitrogen source, the opposite effect has been observed when Lrp is overexpressed. The

Table 4
Doubling times for *Hfx. mediterranei* HM26 and the Lrp overexpressed strain Lrp-HM26 under different conditions.

Condition	Strain	d.t. (h)*	*p*-Value	Significance
20 mM NH_4Cl	HM26	2.1 ± 0.1	0.006	**
	Lrp-HM26	4.6 ± 0.1		
20 mM KNO_3	HM26	5.2 ± 0.2	0.003	**
	Lrp-HM26	2.2 ± 0.1	–	–
MM	HM26	3.05 ± 0.09	>0.999	Ns
	Lrp-HM26	3.47 ± 0.18		
8 mM H_2O_2	HM26	4.3 ± 0.2	>0.999	Ns
	Lrp-HM26	4.5 ± 0.3		
0.5 M Li^+	HM26	3.2 ± 0.2	>0.999	Ns
	Lrp-HM26	3.10 ± 0.03		
0.4 mM Ni^{2+}	HM26	4.0 ± 0.1	>0.999	Ns
	Lrp-HM26	3.8 ± 0.2		
0.2 mM Co^{2+}	HM26	3.4 ± 0.1	0.005	**
	Lrp-HM26	6.43 ± 0.01		
2 mM As^{5+}	HM26	1.29 ± 0.06	>0.999	Ns
	Lrp-HM26	0.87 ± 0.01		

Ns: $p > 0.05$; **$p \leq 0.01$. **Hfx. mediterranei* growth was followed by measuring its optical density at 600 nm, doubling times are calculated as described in the Materials and Methods section.

Lrp-HM26 strain shows a higher growth profile throughout the growth curve and a lower doubling time than the HM26 strain under this condition. In the presence of cobalt, it was also found clear differences in the growth of the two strains since the Lrp-HM26 strain showed less growth, reaching lower optical density values in the stationary phase (Fig. 6g). Focusing on analyzing the doubling time for *Hfx. mediterranei* HM26 and the Lrp overexpressed strain Lrp-HM26 under different conditions, significant differences in growth were detected since the Lrp-MH26 doubling time was almost three times higher with 6.43 h than the overexpression strain. The last condition analyzed was the presence of arsenic (Fig. 6h). As can be seen, the Lrp-HM26 strain has a higher growth reaching a higher stationary phase. However, no significant differences are found when analyzing the doubling time (Table 4).

These results are in agree with those shown in the previous section as *Hfx. mediterranei* showed higher growth in the presence of cobalt when the *lrp* gene was deleted. With all these results, it could be believed that cobalt may have some effect on the transcriptional mechanism of the *lrp* gene in different ways: i) Lrp could be involved in the transcriptional regulation of genes related to cobalt metabolism, ii) cobalt could act as a ligand of Lrp and induce changes in its quaternary structure, iii) cobalt could induce the expression of Lrp and, in turn, this TF repress the expression of other genes.

Different works support these hypothesis, for example, some studies link metals such as cobalt and nickel to control the expression of TFs (activators or repressors) involved in metal stress and homeostasis such as in *Agrobacterium tumefaciens* [42] and *E. coli* [43]. In *Bacteria*, there is a crystal structure of a transcriptional regulator belonging to the DtxR family in *Corynebacterium diphtheriae* that is complexed with cobalt [44]. The natural ligand for this family of TFs is ferrous iron but *in vitro* nickel and cobalt are also used as activators [45]. Focusing in Haloarchaea, a previous study showed that a TF from the DtxR family can use heavy metals, including cobalt, nickel and manganese, as ligands in *Hbt. salinarum* [46]. Regarding to the possible role of Lrp in Co metabolism, *Hfx. mediterranei* genome has annotated genes related to this pathway such as hydrolases, cobalt transporter and methyltransferases (E6P09_RS18645; E6P09_RS11870; E6P09_RS04215; E6P09_RS18590; E6P09_RS18655) and it is known that this microorganism is capable to uptake cobalt from the culture medium [15]. Studying the promotor regions of these genes, one potential *lrp* binding site was found in the cobalt-factor II c (20)-methyl transferase gene (E6P09_RS18655) and two other possible binding regions have been identified in E6P09_RS04215 and E6P09_RS18645. Therefore, the Lrp regulator could be related to genes involved in cobalt metabolism although future work is needed to confirm this hypothesis.

As shown the results obtained, although cobalt and nickel are divalent metals, they have different effects on *Hfx. mediterranei* Lrp. Several studies linked nickel, cobalt and other divalent metals to transcriptional regulators expression, even there are examples describing different effects of nickel and cobalt in the expression of genes involved in metal pumping [42].

4. Conclusions

This study tries to elucidate the role of the Lrp (HFX_RS01210) transcriptional regulator in the regulatory stress response in *Hfx. mediterranei*. Site directed mutagenesis of p. *lrp* region allowed us to identify the TATA box and a potential TF binding site to its own promoter. In addition, different approaches evidence the hypothesis that this transcriptional regulator would bind to this consensus-binding site sequence region to autoregulate itself at transcriptional level. Construction of *lrp* deletion mutant was laborious, the complete gene sequence could not be deleted as all the pop-out clones analyzed had the parental genotype, so a partial mutant was made in which part of the start of the sequence was not deleted. Characterization of partial mutant clear evidenced that the *lrp* is involved in the response to N-limiting conditions and the presence of cobalt. That is the first evidence that removing the *lrp* gene, *Hfx. mediterranei* does not grow in the presence of nitrate as a nitrogen source, so it must regulate some essential genes for this pathway. To conclude, the Lrp regulator can autoregulate its expression level and there is evidence that relates this TF with the expression regulation of the assimilatory pathway of the nitrogen cycle of *Hfx. mediterranei,* specifically acting as activator of *glnA* and *nasA* genes. In addition, it has been evidenced the possible involvement of Lrp in response to metal stress conditions in *Hfx. mediterranei*, for example, to the presence of cobalt. Although more work is needed to elucidate the molecular mechanisms of the Lrp expression, this work is an excellent starting point to extend knowledge about nitrogen availability and metal stress response in Haloarchaea.

Funding

This work was supported by Programa Propio para el Fomento de la I + D + I del Vicerrectorado de Investigación y Transferencia de Conocimiento" of the University of Alicante (VIGRO-016).

Author contributions

L.M., J.E. and M.-J.B. conceived and designed the experiments; L.M. performed most of the experiments; L.M. and J.E. analyzed the data; M.-J.B., and J.E. supervised the experimental work; M.C., resources; L.M. wrote initial draft preparation; L.M., V.B., M.C., M.-J.B., and J.E. reviewed and edited the manuscript; M.-J.B., project administration. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that the research was conducted without any commercial or financial relationships that could be constructed as a potential conflict of interest.

Acknowledgments

We thank Mike Dyall-Smith and Thorsten Allers for providing pVA513 and 555 pTA1992 vectors, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2023.01.012.

References

[1] B. Zafrilla, R.M. Martínez-Espinosa, M.A. Alonso, M.J. Bonete, Biodiversity of Archaea and floral of two inland saltern ecosystems in the Alto Vinalopó Valley, Spain, Saline Syst. 6 (2010) 10, https://doi.org/10.1186/1746-1448-6-10.

[2] A. Oren, Molecular ecology of extremely halophilic Archaea and Bacteria, FEMS Microbiol. Ecol. 39 (2002) 1–7, https://doi.org/10.1111/j.1574-6941.2002.tb00900.x.

[3] A.Ş. Andrei, H.L. Banciu, A. Oren, Living with salt: metabolic and phylogenetic diversity of archaea inhabiting saline ecosystems, FEMS Microbiol. Lett. 330 (2012) 1–9, https://doi.org/10.1111/j.1574-6968.2012.02526.x.

[4] P. DasSarma, M.D. Capes, S. DasSarma, Comparative genomics of *Halobacterium* strains from diverse locations, in: S. Das, H.R. Dash (Eds.), Microb. Divers. Genomic Era, Elsevier, Ámsterdam, 2019, pp. 285–322, https://doi.org/10.1016/b978-0-12-814849-5.00017-4.

[5] E. Conway de Macario, A.J.L. Macario, Molecular biology of stress genes in methanogens: potential for bioreactor technology, Adv. Biochem. Eng. Biotechnol. 81 (2003) 95–150, https://doi.org/10.1007/3-540-45839-5_4.

[6] E. Conway de Macario, A.J.L. Macario, Stressors, stress and survival: overview, Front. Biosci. 5 (3) (2000) 780–786, https://doi.org/10.2741/A550.

[7] M. Madigan, K.S. Bender, D.H. Buckley, W.M. Sattley, D.A. Stahl, Brock Biology of Microorganisms, fifteenth ed., Pearson, Madrid, 2017.

[8] A.J.L. Macario, M. Lange, B.K. Ahring, E. Conway de Macario, Stress genes and proteins in the archaea, Microbiol. Mol. Biol. Rev. 63 (4) (1999) 923–967, https://doi.org/10.1128/MMBR.63.4.923-967.1999.

[9] E. Perez-Rueda, R. Hernandez-Guerrero, M.A. Martinez-Nuñez, D. Armenta-Medina, I. Sanchez, J.A. Ibarra, Abundance, diversity and domain architecture variability in prokaryotic DNA-binding transcription factors, PLoS One 13 (4) (2018), e0195332, https://doi.org/10.1371/journal.pone.0195332.

[10] J.M. Calvo, R.G. Matthews, The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*, Microbiol. Rev. 58 (3) (1994) 466–490, https://doi.org/10.1128/mr.58.3.466-490.1994.

[11] A.B. Brinkman, T.J.G. Ettema, W.M. de Vos, J. van der Oost, The Lrp family of transcriptional regulators, Mol. Microbiol. 48 (2) (2003) 287–294, https://doi.org/10.1046/j.1365-2958.2003.03442.x.

[12] N. Song, Y. Cui, Z. Li, L. Chen, S. Liu, New targets and cofactors for the transcription factor LrpA from *Mycobacterium tuberculosis*, DNA Cell Biol. 35 (4) (2016) 167–176, https://doi.org/10.1089/dna.2015.3040.

[13] C.A. Ziegler, P.L. Freddolino, The Leucine-responsive Regulatory Proteins/Feast-Famine Regulatory Proteins: an ancient and complex class of transcriptional regulators in bacteria and archaea, Crit. Rev. Biochem. Mol. Biol. 56 (4) (2021) 373–400, https://doi.org/10.1080/10409238.2021.1925215.

[14] C.L. Plaisier, F.-Y. Lo, J. Ashworth, A.N. Brooks, K.D. Beer, A. Kaur, M. Pan, D.J. Reiss, M.T. Facciotti, N.S. Baliga, Evolution of context dependent regulation by expansion of feast/famine regulatory proteins, BMC Syst. Biol. 8 (2014) 122, https://doi.org/10.1186/s12918-014-0122-2.

[15] L. Matarredona, M. Camacho, B. Zafrilla, G. Bravo-Barrales, J. Esclapez, M.-J. Bonete, The survival of *Haloferax mediterranei* under stressful conditions, Microorganisms 9 (2021) 336, https://doi.org/10.3390/microorganisms9020336.

[16] F. Rodriguez-Valera, G. Juez, D.J. Kushner, *Halobacterium mediterranei* spec, nov., a new carbohydrate-utilizing extreme halophile, Syst. Appl. Microbiol. 4 (1983) 369–381, https://doi.org/10.1016/S0723-2020(83)80021-6.

[17] B. Lledó, R.M. Martínez-Espinosa, F.C. Marhuenda-Egea, M.J. Bonete, Respiratory nitrate reductase from haloarchaeon *Haloferax mediterranei*: biochemical and genetic analysis, Biochim. Biophys. Acta Gen. Subj. 1674 (2004) 50–59, https://doi.org/10.1016/j.bbagen.2004.05.007.

[18] J. Torregrosa-Crespo, R.M. Martínez-Espinosa, J. Esclapez, V. Bautista, C. Pire, M. Camacho, D.J. Richardson, M.J. Bonete, Anaerobic metabolism in *Haloferax* genus: denitrification as case of study, in: Advances Microbial Physiology, vol. 68, Academic Press, Amsterdam, 2016, pp. 41–85, https://doi.org/10.1016/bs.ampbs.2016.02.001.

[19] M. Rodrigo-Baños, I. Garbayo, C. Vílchez, M.J. Bonete, R.M. Martínez-Espinosa, Carotenoids from haloarchaea and their potential in biotechnology, Mar. Drugs 13 (9) (2015) 5508–5532, https://doi.org/10.3390/md13095508.

[20] T. Mainka, N. Mahler, C. Herwig, S. Pflügl, Soft sensor-based monitoring and efficient control strategies of biomass concentration for continuous cultures of *Haloferax mediterranei* and their application to an industrial production chain, Microorganisms 7 (12) (2019) 648, https://doi.org/10.3390/microorganisms7120648.

[21] Y. Zhao, X. Zhuang, S. Ahmad, S. Sung, S.Q. Ni, Biotreatment of high-salinity wastewater: current methods and future directions, World J. Microbiol. Biotechnol. 36 (2020) 37, https://doi.org/10.1007/s11274-020-02815-4.

[22] C. Nájera-Fernández, B. Zafrilla, M.J. Bonete, R.M. Martínez-Espinosa, Role of the denitrifying Haloarchaea in the treatment of nitrite-brines, Int. Microbiol. 15 (3) (2012) 111–119, https://doi.org/10.2436/20.1501.01.164.

[23] J. Esclapez, G. Bravo-Barrales, V. Bautista, C. Pire, M. Camacho, M.J. Bonete, Effects of nitrogen sources on the nitrate assimilation in *Haloferax mediterranei*: growth kinetics and transcriptomic analysis, FEMS Microbiol. Lett. 350 (2014) 168–174, https://doi.org/10.1111/1574-6968.12325.

[24] M.J. Bonete, R.M. Martínez-Espinosa, C. Pire, B. Zafrilla, D.J. Richardson, Nitrogen metabolism in haloarchaea, Saline Syst. 4 (2008) 9, https://doi.org/10.1186/1746-1448-4-9.

[25] V. Rodríguez-Herrero, G. Payá, V. Bautista, A. Vegara, M. Cortés-Molina, M. Camacho, J. Esclapez, M.J. Bonete, Essentiality of the *glnA* gene in *Haloferax mediterranei*: gene conversion and transcriptional analysis, Extremophiles 24 (3) (2020) 433–446, https://doi.org/10.1007/s00792-020-01169-x.

[26] L. Pedro-Roig, M. Camacho, M.J. Bonete, Regulation of ammonium assimilation in *Haloferax mediterranei*: interaction between glutamine synthetase and two GlnK proteins, Biochim. Biophys. Acta 1834 (1) (2013) 16–23, https://doi.org/10.1016/J.bbapap.2012.10.006.

[27] S. Pastor-Soler, M. Camacho, V. Bautista, M.J. Bonete, J. Esclapez, Towards the elucidation of assimilative *nasABC* operon transcriptional regulation in *Haloferax mediterranei*, Genes 12 (2021), https://doi.org/10.3390/genes12050619.

[28] R.M. Martínez-Espinosa, F.C. Marhuenda-Egea, M.J. Bonete, Assimilatory nitrate reductase from the haloarchaeon *Haloferax mediterranei*: purification and characterisation, FEMS Microbiol. Lett. 204 (2001) 381–385, https://doi.org/10.1016/s0378-1097(01)00431-1.

[29] J. Esclapez, C. Pire, M. Camacho, V. Bautista, R.M. Martínez-Espinosa, B. Zafrilla, A. Vegara, L.A. Alcaraz, M.J. Bonete, Transcriptional profiles of *Haloferax mediterranei* based on nitrogen availability, J. Biotechnol. 193 (2015) 100–107, https://doi.org/10.1016/j.jbiotec.2014.11.018.

[30] R. Schwaiger, C. Schwarz, K. Furtwängler, V. Tarasov, A. Wende, D. Oesterhelt, Transcriptional control by two leucine-responsive regulatory proteins in *Halobacterium salinarum* R1, BMC Mol. Biol. 11 (2010) 40, https://doi.org/10.1186/1471-2199-11-40.

[31] R.M. Martínez-Espinosa, J. Esclapez, V. Bautista, M.J. Bonete, An octameric prokaryotic glutamine synthetase from the haloarchaeon *Haloferax mediterranei*, FEMS Microbiol. Lett. 264 (1) (2006) 110–116, https://doi.org/10.1111/j.1574-6968.2006.00434.x.

[32] L. Matarredona, M. Camacho, M.J. García-Bonete, B. Esquerra, B. Zafrilla, J. Esclapez, M.J. Bonete, Analysis of *Haloferax mediterranei* lrp transcriptional regulator, Genes 12 (2021) 802, https://doi.org/10.3390/genes12060802.

[33] J. Esclapez, C. Pire, V. Bautista, R.M. Martínez-Espinosa, J. Ferrer, M.J. Bonete, Analysis of acidic surface of *Haloferax mediterranei* glucose dehydrogenase by site-directed mutagenesis, FEBS Lett. 581 (5) (2007) 837–842, https://doi.org/10.1016/j.febslet.2007.01.054.

[34] G. Bitan-Banin, R. Ortenberg, M. Mevarech, Development of a gene knockout system for the halophilic archaeon *Haloferax volcanii* by use of the *pyrE* gene, J. Bacteriol. 185 (3) (2003) 772–778, https://doi.org/10.1128/JB.185.3.772-778.2003.

[35] L. Pedro-Roig, C. Lange, M.J. Bonete, J. Soppa, J. Maupin-Furlow, Nitrogen regulation of protein-protein interactions and transcript levels of GlnK PII regulator and AmtB ammonium transporter homologs in Archaea, MicrobiologyOpen 2 (5) (2013) 826–840, https://doi.org/10.1002/mbo3.120.

[36] M. Brenneis, O. Hering, C. Lange, J. Soppa, Experimental characterization of *cis*-acting elements important for translation and transcription in halophilic archaea, PLoS Genet. 3 (12) (2007) e229, https://doi.org/10.1371/journal.pgen.0030229.

[37] K. Yokoyama, S.A. Ishijima, L. Clowney, H. Koike, H. Aramaki, C. Tanaka, K. Makino, M. Suzuki, Feast/famine regulatory proteins (FFRPs): *Escherichia coli* Lrp, AsnC and related archaeal transcription factors, FEMS Microbiol. Rev. 30 (1) (2006) 89–108, https://doi.org/10.1111/j.1574-6976.2005.00005.x.

[38] E. Peeters, C. Wartel, D. Maes, D. Charlier, Analysis of the DNA-binding sequence specificity of the archaeal transcriptional regulator Ss-LrpB from *Sulfolobus solfataricus* by systematic mutagenesis and high resolution contact probing, Nucleic Acids Res. 35 (2) (2007) 623–633, https://doi.org/10.1093/nar/gkl1095.

[39] A.B. Brinkman, I. Dahlke, J.E. Tuininga, T. Lammers, V. Dumay, E. De Heus, J.H.G. Lebbink, M. Thomm, W.M. de Vos, J. van der Oost, An lrp-like transcriptional regulator from the archaeon *Pyrococcus furiosus* is negatively autoregulated, J. Biol. Chem. 275 (49) (2000) 38160–38169, https://doi.org/10.1074/jbc.M005916200.

[40] C. Beloin, R. Exley, A.L. Mahé, M. Zouine, S. Cubasch, F. Le Hégarat, Characterization of LrpC DNA-binding properties and regulation of *Bacillus subtilis lrpC* gene expression, J. Bacteriol. 182 (16) (2000) 4414–4424, https://doi.org/10.1128/jb.182.16.4414-4424.2000.

[41] K. Cho, S.C. Winans, The *putA* gene of *Agrobacterium tumefaciens* is transcriptionally activated in response to proline by an Lrp-like protein and is not autoregulated, Mol. Microbiol. 22 (5) (1996) 1025–1033, https://doi.org/10.1046/j.1365-2958.1996.01524.X.

[42] T. Dokpikul, P. Chaoprasid, K. Saninjuk, S. Sirirakphaisarn, J. Johnrod, S. Nookabkaew, R. Sukchawalit, S. Mongkolsuk, Regulation of the cobalt/nickel efflux operon *dmeRF* in *Agrobacterium tumefaciens* and a link between the iron-sensing regulator RirA and cobalt/nickel resistance, Appl. Environ.

Microbiol. 82 (15) (2016) 4732–4742, https://doi.org/10.1128/AEM.01262-16.
[43] D. Blaha, S. Arous, C. Blériot, C. Dorel, M.A. Mandrand-Berthelot, A. Rodrigue, The *Escherichia coli* metallo-regulator RcnR represses *rcnA* and *rcnR* transcription through binding on a shared operator site: insights into regulatory specificity towards nickel and cobalt, Biochimie 93 (3) (2011) 434–439, https://doi.org/10.1016/j.biochi.2010.10.016.
[44] E. Pohl, R.K. Holmes, W.G.J. Hol, Crystal structure of a cobalt-activated diphtheria toxin repressor-DNA complex reveals a metal-binding SH3-like domain, J. Mol. Biol. 292 (3) (1999) 653–667, https://doi.org/10.1006/jmbi.1999.3073.
[45] T.J. McGenity, R.T. Gemmell, W.D. Grant, H. Stan-Lotter, Origins of halophilic microorganisms in ancient salt deposits, Environ. Microbiol. 2 (3) (2000) 243–250, https://doi.org/10.1046/j.1462-2920.2000.00105.x.
[46] M. Martinez-Pastor, W.A. Lancaster, P.D. Tonner, M.W.W. Adams, A.K. Schmid, A transcription network of interlocking positive feedback loops maintains intracellular iron balance in archaea, Nucleic Acids Res. 45 (17) (2017) 9990–10001, https://doi.org/10.1093/nar/gkx662.