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Note

A proteome-wide screen to identify transcription factors interacting with the *Vibrio cholerae rpoS* promoter

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A B S T R A C T

We describe a proteomic approach to identify transcription factors binding to a target promoter. The method's usefulness was tested by identifying proteins binding to the *Vibrio cholerae rpoS* promoter in response to cell density. Proteins identified in this screen included the nucleoid-associated protein Fis and the quorum sensing regulator HapR.

Isolation of regulatory mutants has played a major role in increasing our understanding of the regulation of gene expression (Shuman and Silhavy, 2003). There are cases, however, in which the above approach can encounter significant obstacles. For instance, the methods used to genetically manipulate model organisms are not always equally effective in less studied microorganisms; some regulatory mutations can be highly pleiotropic, often be deleterious, unstable and difficult to select. In addition, cases have been reported in which the phenotypic expression of a mutation is masked by redundancy or superimposed interaction with other regulators (Russell et al., 2018). Finally, some regulatory mutants may escape selection if expression of their gene products is restricted to a narrow time window during bacterial growth or permissive culture conditions for their expression are not met. Here we describe an alternative protein-to-gene approach to identify regulatory proteins binding to a target promoter that circumvents the above obstacles.

A typical example of a gene subject to complex multilevel regulation is *rpoS*, encoding the alternative RNA polymerase sigma S subunit (RpoS), also known as the general stress response regulator (Hengge, 2011). RpoS plays a critical role in the survival of bacteria in hostile environments by activating the expression of multiple genes in response to environmental stressors (Hengge, 2011). In *V. cholerae*, the general stress response is enhanced by quorum sensing (Joelsson et al. 2007; Wang et al., 2011). However the mechanism of this regulation remains unknown. Thus, we decided to use the *rpoS* promoter to validate our proteome-wide screen and identify transcription factor interacting with it in response to population cell density.

For this study, *V. cholerae* mutants were derived from the O1 serogroup El Tor biotype strain C7258ΔlacZ (Silva et al., 2008). Strains were grown in Luria-Bertani (LB) medium or tryptic soy broth (TSB) with agitation at 37 °C to stationary phase. Culture media were supplemented with ampicillin (Amp, 100 μg/mL), kanamycin (Km, 25 μg/mL), polymyxin B (PolB, 100 units/mL) or L-arabinose (0.2%) as required. Chromosomal deletions of *fis* and/or *hapR* were created in strain C7258ΔlacZ by allelic exchange as described previously (Wang et al., 2011; Wang et al., 2012; Wang et al., 2014). Briefly, genomic DNA flanking the *fis* locus was amplified using the Advantage 2 PCR kit (Takara Bio USA, Inc.) and primer combinations 5′-GCCTCTAGAATG CGTAAAATGCGC/5′GAAGTCGACATTCGGTCTAGCTCT and 5′/GCAG TCGACGAGCGACTTTTTTGT/5′-GAAGCATGCTGCACAAAAGAGGCA. The 5′ and 3′ sequences flanking the *fis* locus were sequentially cloned as *Xba*I-*Sal*I and *Sal*I-*Sph*I fragments into plasmid pUC19. Then, a Km resistance gene was retrieved from plasmid pUC4K (Taylor and Rose, 1988) as a *Sal*I fragment and inserted within the *fis* flanking chromosomal sequences. The resulting *fis* deletion/insertion was cloned in the suicide vector pCVD442 (Donnenberg and Kaper, 1991) and the resulting plasmid was introduced into strains C7258ΔlacZ or AJB51ΔlacZ (C7258Δ*lacZ*Δ*hapR*) (Silva et al., 2008) by conjugal transfer from *E. coli* strain S17-1λpir (de Lorenzo et al., 1993). Exconjugants were selected in LB medium containing Amp and PolB and deletion mutants AJB91 (Δ*fis*) and AJB92 (Δ*fis*Δ*hapR*) were obtained by sucrose selection as described previously (Wang et al., 2012; Wang et al., 2014; Wang et al., 2015). The suicide vector pCVD-RpoS-LacZ (Wang et al., 2014) was used to integrate an *rpoS-lacZ* fusion into the chromosomal *rpoS* locus by conjugal transfer from *E. coli* S17-1λpir to strains C7258ΔlacZ (wild type, Wt), AJB51ΔlacZ (Δ*hapR*) AJB91 (Δ*fis*) and AJB92 (Δ*fis*Δ*hapR*).

Based on RNA-Seq data (Wang et al., 2015) ((http://www.ncbi.nlm.nih.gov/geo/), we amplified a DNA fragment encoding the *rpoS* promoter with primers 5'-CCGTGGCTGTGGCTTCAAG (biotinylated) and 5'-CTCATAGCGGCCTCCCCCT. The biotinylated promoter probe

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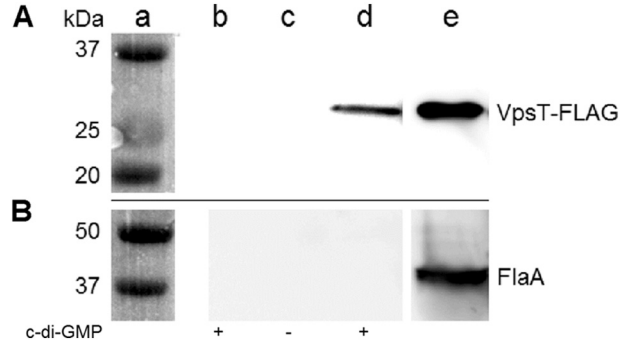

Fig. 1. Pull-down of DNA-binding protein VpsT using a biotinylated *rpoS* promoter probe. Strain HX22 containing plasmid pAT1662 was grown in LB medium and VpsT expression was induced by addition of L- arabinose (0.2%). Pull-down reactions consisted of 100 µg of streptavidin-coupled dynabeads; 2.5 µg biotinylated *rpoS* promoter probe; HX22 lysate containing 1.5 mg total protein in 0.5 mL and *c*-di-GMP (10 µM). A. The presence of VpsT in the lysate and pull-down fractions was determined by western blot using the anti-FLAG M2-peroxidase monoclonal antibody. B. The blot was also probed with mAb 3E1 against FlaA, a protein not expected to bind to DNA. Lane a, molecular weight standard; lane b, empty dynabeads plus *c*-di-GMP control reaction; lane c, dynabeads bound-*rpoS* promoter DNA without addition of *c*-di-GMP; lane d, dynabeads bound-*rpoS* promoter DNA plus *c*-di-GMP; lane e, initial bacterial lysate.

spanned nucleotides −688 to +5 relative to the *rpoS* start codon. To identify transcription factors responsive to cell density, we conducted our assay with strain C7258ΔLuxO (Sultan et al., 2010) locked in high cell density mode and strain AJB51ΔLacZ (Δ*hapR*) locked in low cell density mode (Miller et al., 2002). A mixture containing 100 µg of streptavidin-coupled dynabeads and 2.5 µg of biotinylated promoter DNA in 20 µL was incubated for 90 min at room temperature. The dynabeads-DNA complex was washed to remove unbound DNA and resuspended in 500 µL of a bacterial lysate containing 1.5 mg of total proteins. The mixture was incubated for 90 min at room temperature and the dynabeads-DNA-protein complexes were captured and washed 5 times with 500 µL of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M NaCl. Pulled-down proteins were eluted by resuspension and boiling 5 min in Laemmli sample buffer. Samples were loaded and run 1 cm into Mini-Protean TGX precast gels (BioRad), stained with Coomassie brilliant blue and processed for nanoLC-tandem mass spectrometry using an AB Sciex 5600 TripleTOF mass spectrometer. The mass spectrometry data was processed for protein identification using an in-house protein Pilot 4.5 search engine (SCIEX), the UniProt protein databases and a trypsin digestion parameter. A no-DNA control was conducted in parallel to each pull-down experiment. For preparing *V. cholerae* lysates, strains were grown in LB medium to stationary phase. Cells were harvested, resuspended in lysis buffer (20 mM HEPES pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.1% Tween 20, 30 mM KCl, 75 mM NaCl) plus Roche's cOmplete protease inhibitor cocktail) and disrupted by sonication. The cleared lysates were adjusted to 3 mg/mL of total proteins with lysis buffer. Finally, 500 µL of the lysates were incubated with 6 µg of calf thymus DNA for 10 min prior to addition of the streptavidin-coupled dynabead-DNA promoter probe.

To test the *rpoS* promoter probe, we used strain HX22 (Wang et al., 2014), which expresses the transcription factor VpsT tagged with the FLAG epitope. VpsT binds to the *rpoS* promoter in a *c*-di-GMP-dependent manner to a region located 40 bp upstream of the start codon (Wang et al., 2014). To induce VpsT-FLAG expression, strain HX22 was transformed with plasmid pAT1662 which expresses the diguanylate cyclase VCA0956 from the *araBAD* promoter (Tischler and Camilli, 2004). Strain HX22 containing pAT1662 was grown in LB medium in the presence of 0.2% L-arabinose, the cells were collected, and lysates were prepared as described above. The biotinylated *rpoS* promoter probe was reacted with the lysates, and VpsT present in the soluble and pulled-down fractions was detected by western blot using the BM chemiluminescence western blot kit (Roche Applied Sciences) and anti-FLAG M2-peroxidase monoclonal antibody (Sigma-Aldrich) as described previously (Ayala et al., 2018). As show in Fig. 1, VpsT could be detected in the pull-down fraction only in the lysate supplemented with 10 µM *c*-di-GMP. We also investigated the presence of flagellin FlaA, a protein not expected to bind to any DNA, using the monoclonal antibody 3E1 (Rasmussen et al., 2011). As expected, flagellin FlaA, could be detected only in the lysate (Fig. 1).

We then used the above pull-down conditions and LC/MS/MS to identify proteins interacting with the *rpoS* promoter in the Δ*luxO* and Δ*hapR* mutant lysates. The proteins identified in this assay are shown in Table 1. Hits were defined as proteins identified by (*i*) two or more matching peptides (95%) in both replicas of the Δ*luxO* and/or the Δ*hapR* lysates and (*ii*) two or more matching peptides (95%) compared to the minus DNA control. Regulatory proteins identified in the screen included the nucleoid-associated proteins HU, Fis, H-NS, HapR and transcription activators HexR, CysB and SorC. We considered Fis and HapR for further study because both proteins participate in *V. cholerae* quorum sensing (Miller et al., 2002; Lenz and Bassler, 2007).

To validate the Fis and HapR mass spectrometry hits, we conducted electrophoresis mobility shift assays (EMSA). Purification of regulatory protein HapR has been described previously (Wang et al., 2011). To purify *V. cholerae* Fis, we amplified the *fis* open reading frame (ORF)

Table 1
Mass spectrometry identification of proteins interacting with the *rpoS* promoter.

Tag ID[a]	Gene	Description	Peptide (95%) [a]		Peptide (95%)[a]	
			Δ*luxO*	Control	Δ*hapR*	Control
VC0273	*hupA*	DNA binding protein HU alpha subunit	4/5	1/1	4/4	2/0
VC0290	*fis*	Nucleoid associated protein Fis	2/2	0/0	1/0	0/0
VC0583	*hapR*	Hemagglutinin/protease regulatory protein	3/3	0/0	0/0	0/0
VC0766	*xseA*	Exodeoxyribonuclease 7 large subunit	10/12	0/0	9/11	0/0
VC1130	*hns*	Nucleoid associated protein H-NS	15/25	2/1	14/10	1/1
VC1148	*hexR*	Glucose repressor HexR for enter-Doudoroff pathway, RpiR family	15/17	0/0	13/16	0/0
VC1730	*topA*	DNA topoisomerase I	5/9	0/0	4/8	0/0
VC1907	*cysB*	Cys regulon transcriptional activator CysB	4/8	0/0	3/3	0/0
VC2096	*seqA*	Negative regulator of initiation of replication	31/42	0/0	28/34	0/0
VC2430	*parC*	DNA topoisomerase IV subunit A	2/3	0/0	0/0	0/0
VCA0283		Uncharacterized protein	3/4	0/0	1/2	0/0
VCA0322		Uncharacterized protein	10/9	0/0	9/7	0/0
VCAO447		Methyltransferase/DNA for RVC repeated sequence	4/5	0/0	2/3	0/0
VCA0621	*sorC*	Transcriptional activator, SorC family	17/17	0/0	12/13	0/0
VCA1050		Phage antirepressor KilAC domain protein	2/3	0/0	1/2	0/0

[a] The data represents the values for the peptide (95%) parameter detected in two independent experiments (experiment 1/experiment 2).

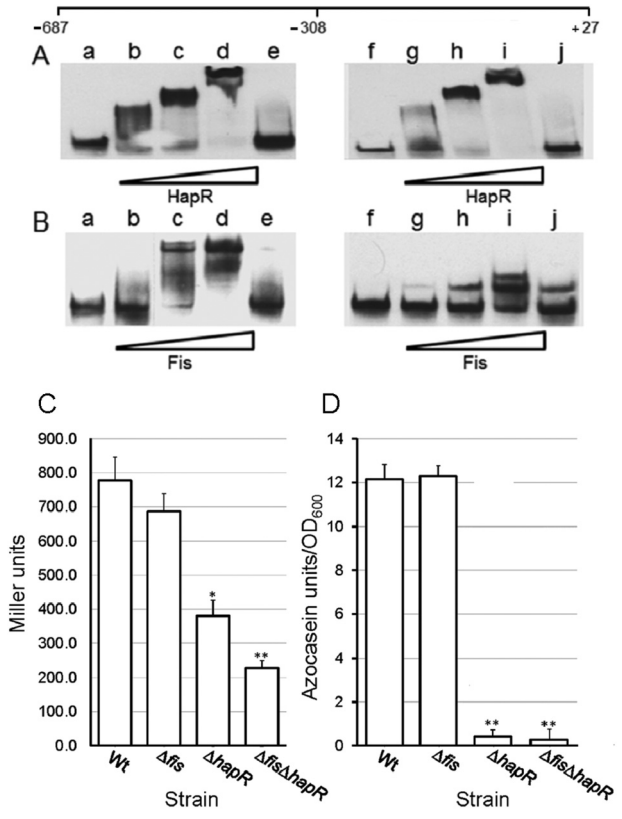

Fig. 2. Validation of Fis and HapR interaction with the *rpoS* promoter. Two DNA fragments were amplified spanning nucleotides −687 to −308 (fragment I) and − 309 to +27 (fragment II) relative to the *rpoS* start codon as indicated above the gel images. A. Binding of purified HapR to DNA fragments I (left) and II (right). The DIG-labeled DNA fragments were treated as follows: lanes a and f, no protein; lanes b and g, 150 nM HapR; lanes c and h, 250 nM HapR; lanes d and i, 350 nM HapR; lanes e and j, 350 nM HapR plus a 200-fold excess of the corresponding unlabeled DNA probe. B Binding of purified Fis to DNA fragments I (left) and II (right). Lanes a and f, no protein; lanes b and g, 30 nM Fis; lanes c and h, 80 nM Fis; lanes d and i, 150 nM Fis; lanes e and j, 150 nM Fis plus a 200-fold excess of the corresponding unlabeled DNA probe. C. Strains C7258ΔLacZ (Wt), AJB91 (Δ*fis*), AJB51ΔLacZ (Δ*hapR*) and AJB92 (Δ*fis*Δ*hapR*) containing a *rpoS-lacZ* fusion integrated into the chromosomal *rpoS* locus were grown in LB medium to stationary phase and β-galactosidase activity was measured as an indicator of promoter activity. D. Strains C7258ΔLacZ (Wt), AJB91 (Δ*fis*), AJB51ΔLacZ (Δ*hapR*) and AJB92 (Δ*fis*Δ*hapR*) were grown to stationary phase in TSB and production of HA/protease measured using an azocasein assay. Bars represent the average of at least three independent experiments. Error bars denote the STDEV. Symbols, *, $p < .05$, ** $p < .01$ (unpaired *t*-test).

with primers 5′-GGAATTCCATATGTTCGAACAAAATCT and GTTAAGC TCTTCAGCAGTTCATGCCGTATTTTTT. The amplified fragment was digested with restriction enzymes *Nde*I and *Sap*I and ligated into similarly digested pTXB-1 (New England Biolabs) to generate pTXB1-Fis. Fis was then expressed from the T7 promoter in *E. coli* ER2566 and purified using an Impact™ kit (New England Biolabs) following the same procedure used to prepare HapR (Wang et al., 2011). EMSA were conducted using the second-generation digoxigenin (DIG) gel shift kit (Roche Applied Sciences) as previously reported (Wang et al., 2012; Wang et al., 2014; Wang et al., 2015). DNA binding was conducted in 20 mM HEPES, (pH 7.6), 1 mM EDTA, 10 mM $(NH_4)_2 S0_4$, 1 mM DTT, 0.2% Tween 20 (*w/v*), 30 mM KCl, 50 ηg/mL poly d[I−C], 50 ηg/mL poly d[A-T], 5 ηg/ml poly L-lysine. Protein-DNA complexes were separated by electrophoresis in 5% Tris-borate-EDTA polyacrylamide gels and transferred to nylon membranes, and DNA was visualized using an anti-DIG Fab fragment-AP conjugate, followed by chemiluminescence detection. For EMSA, the DNA sequence located upstream of the *rpoS* ORF was split in two regions comprising nucleotides −687 through −308 and − 309 through +27 with reference to the *rpoS* start codon. These regions were amplified with primer combinations 5′-CCGTGGC TGTGGCTTCAAG / 5-TACGGTTCCATCTGCAGTAGC and 5′-GTGTATT CGGGCAACGCA/5′-TTTGGTTACGGTATTG, respectively. In Fig. 2AB, we confirmed that both Fis and HapR interact with DNA sequences located upstream the *rpoS* ORF. Several Fis binding motifs with top ranking scores could be identified within this region using the Virtual Footprint software (http://www.prodoric.de/vfp/vfp_promoter.php). We further examined the effect of Fis and HapR on *rpoS* expression using an integrated *rpoS-lacZ* fusion expressed from *rpoS* native transcription and translation signals, as well as the production of HA/protease in wild type and mutant strains. Production of HA/protease encoded by *hapA* is strongly dependent on RpoS and HapR (Jobling and Holmes, 1997; Yildiz and Schoolnik, 1998; Benitez et al., 2001; Silva and Benitez, 2004). Expression of β-galactosidase activity was measured as described in (Miller, 1972) and expressed in Miller units. Expression of HA/protease in cell-free supernatants was measured using an azocasein-degradation assay as described previously (Benitez et al., 2001). One azocasein unit was defined as the amount of enzyme producing an increase of 0.01 optical density units at 442 nM per h. As shown in Fig. 2CD, deletion of *fis* had no effect on *rpoS* and *hapA* expression while the *hapR* mutant expressed diminished *rpoS* and negligible HA/protease. Although the general stress response was reported to be positively modulated by quorum sensing (Joelsson et al. 2007), binding of HapR (or a protein regulated by HapR) to *rpoS* promoter sequences was not demonstrated. Here we show that HapR binds to DNA sequences located upstream from the *rpoS* ORF suggesting that quorum sensing regulation of *rpoS* expression is direct. Fis has been reported to negatively regulate *rpoS* expression in *E. coli* and *S. enterica* (Hirsch and Elliott, 2005b; Hirsch and Elliott, 2005a). In *V. cholerae,* expression of Fis declines sharply when bacteria transit from low to high cell density (Lenz and Bassler, 2007). In rich laboratory media, *V. cholerae* enters quorum sensing mode prior to stationary phase. On this basis, Fis would not be expected to have a strong effect in the regulation of fully induced *rpoS* expression. Instead, we suggest that Fis could contribute to the down-regulation of *rpoS* expression during exponential growth. The effect of other DNA binding proteins identified in our screen on RpoS expression remains to be examined. These factors, however, could represent new regulatory inputs contributing to modulate RpoS levels in response to complex and changing environmental variables.

In summary, we describe a broadly applicable proteomic approach to identify regulatory DNA-binding proteins. The utility of this methodology was demonstrated by identifying proteins interacting with the *V. cholerae rpoS* promoter. Both known (*i.e.,* VpsT), as well as new regulators interacting with *rpoS* promoter sequences, (HapR, Fis, HexR, CysB and SorC) were identified. Additional transcription factors may be searched for by using different culture conditions to prepare bacterial lysates and/or supplementing lysates with ligands (*i.e.*, cAMP, *c*-di-GMP), predicted to modulate protein-DNA binding activities. Moreover, methodology can be easily adapted to other nucleotide sequence motifs in which protein-DNA interactions drive biological function. Lastly, the method can be useful in cases where genetic screens are difficult to implement, or the phenotype of a regulatory mutant is masked by redundancy, superimposed interactions with other regulators, or compensatory mechanisms.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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