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Research Article

Expression, purification and DNA-binding activities of two putative ModE proteins of *Herbaspirillum seropedicae* (Burkholderiales, Oxalobacteraceae)

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Abstract

In prokaryotes molybdenum is taken up by a high-affinity ABC-type transporter system encoded by the *modABC* genes. The endophyte β-Proteobacterium *Herbaspirillum seropedicae* has two *modABC* gene clusters and two genes encoding putative Mo-dependent regulator proteins (ModE1 and ModE2). Analysis of the amino acid sequence of the ModE1 protein of *H. seropedicae* revealed the presence of an N-terminal domain containing a DNA-binding helix-turn-helix motif (HTH) and a C-terminal domain with a molybdate-binding motif. The second putative regulator protein, ModE2, contains only the helix-turn-helix motif, similar to that observed in some sequenced genomes. We cloned the *modE1* (810 bp) and *modE2* (372 bp) genes and expressed them in *Escherichia coli* as His-tagged fusion proteins, which we subsequently purified. The over-expressed recombinant His-ModE1 was insoluble and was purified after solubilization with urea and then on-column refolded during affinity chromatography. The His-ModE2 was expressed as a soluble protein and purified by affinity chromatography. These purified proteins were analyzed by DNA band-shift assays using the *modA2* promoter region as probe. Our results indicate that His-ModE1 and His-ModE2 are able to bind to the *modA2* promoter region, suggesting that both proteins may play a role in the regulation of molybdenum uptake and metabolism in *H. seropedicae*.

Key words: Herbaspirillum seropedicae, *ModE1* protein, *ModE2* protein.

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Introduction

Molybdenum (Mo) is an essential trace element, which is part of the catalytic site of important enzymes such as nitrate reductase, nitrogenase and xanthine oxidase (Hille, 1996). Molybdenum is predominantly found in nature as the MoO_4^{2-} oxyanion, and is the only second-row transition metal required by most living organisms. Because the concentration of molybdenum in the environment is low, the majority of prokaryotes have high-affinity molybdate ABC-type uptake systems coded by the *modABC* genes which must be able to distinguish between molybdate and chemically similar oxyanions such as sulfate, phosphate and vanadate (Corcuera *et al.*, 1993; Self *et al.*, 2001). In *Escherichia coli* the expression of the *mod* operon is regulated by the ModE protein in response to the intracellular molybdenum concentration. The ModE-molybdate complex binds with high affinity to the *modABC* promoter region and represses its transcription (Anderson *et al.*, 1997; McNicholas *et al.*, 1998b) and can also enhance the transcription of some operons coding for molybdoenzymes and proteins of the molybdopterin biosynthesis pathway (Anderson *et al.*, 2000; Self *et al.*, 2001).

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It is known that *E. coli* ModE is a homodimer with a helix-turn-helix (HTH) DNA-binding motif at the N-terminal domain and a molybdate-binding site at the C-terminal DiMop domain, which is made up of two sub-domains in tandem that bind two molybdate ions per dimer at the domain interfaces (Hall *et al.*, 1999; Schüttelkopf *et al.*, 2003). Structural data have revealed that *E. coli* ModE discriminates between oxyanions based on size and charge, with the C-terminal domain undergoing a conformational change induced by the ligand which results in an alteration of the surface of the dimer and is a molecular switch regulating the recruitment of the partner proteins necessary for the positive regulation of transcription (Gourley *et al.*, 2001). The structures of *E. coli* ModE in its liganded on holo-state and unliganded off apo-state have been determined by Schüttelkopf *et al.* (2003), a major change between the two states being the relative orientation of the two HTH motifs at the N-terminal which suggests that this conformational change enhances the DNA binding activity of the ModE-molybdate complex.

Several Bacteria and Archaea encode proteins that contain the DNA-binding domain characteristic of ModE, but completely lack a molybdate-binding domain and cannot bind molybdate, although their HTH-containing domains are probably capable of binding DNA (Studholme and Pau, 2003). The microaerobic endophytic diazotroph *Herbaspirillum seropedicae* (Burkholderiales, Oxalobacteraceae) associates with several Gramineae of economic interest (Baldani *et al.*, 1986; Young, 1992). While *E. coli* contains only a single copy of molybdenum ABC-type transport system, *H. seropedicae* has two distinct clusters encoded by *modABC*-like genes in its genome sequence. In addition *H. seropedicae* has two genes (*modE1* and *modE2*) coding for two ModE-like regulator proteins. Sequence analysis has revealed that the *modE1* product has a typical ModE domain architecture, with an HTH motif at the N-terminal domain and a DiMop domain with two Mop subdomains at the C-terminal (Schultz *et al.*, 1998 and Letunic *et al.*, 2006), while the *modE2* product shows only the HTH motif related to DNA binding. Similar structures have been observed in homologous proteins of other bacteria (Studholme and Pau, 2003).

In this paper we describe how we have over-expressed and purified both ModE1 and ModE2 proteins and analyzed their DNA-binding activity at the *H. seropedicae modA2B2C2* promoter region.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The *E. coli* strains and plasmids used in this study are given in Table 1. Cells were grown in Luria broth (LB) or LB-agar and, when required, kanamycin was added at a concentration of 50 μg mL^{-1}. All chemicals were at least analytical grade and were purchased from Merck Biosciences (Germany) or Sigma-Aldrich (Germany) unless otherwise stated. Mo-free solutions were prepared using activated charcoal-treated water as described by Schneider *et al.*, (1991).

Cloning of *modE1* and *modE2*

All manipulations were performed as described in Sambrook *et al.* (1989). We used *H. seropedicae* genomic DNA as the template for the polymerase chain reaction (PCR) amplification of *modE1* with the oligonucleotide *Nde*I-modE1R (5'CATCGGCAAGCATATGAGTACC3'), *Bam*HI-modE1F (5'GGTGTCAGGATCCCAGAATG3') and *modE2* with the oligonucleotide *Nde*I-modE2R (5'GTACAATCACGCATATGAATG3'), *Bam*HI-modE2 (5'GCAGGCGGATCCGGTATTTG3'), the primers containing *Nde*I or *Bam*HI restriction sites (underlined) for further manipulations. The PCR products containing *modE1* (874 bp) and *modE2* (415 bp) were directly cloned into the pCR®2.1 TOPO plasmid vector (Invitrogen, USA) giving the plasmids pCR2.1modE1 and pCR2.1modE2, respectively. The plasmids pCR2.1modE1 and pCR2.1modE2 were then digested with *Nde*I and *Bam*HI (MBI Fermentas, Germany) and the fragments containing *modE1* and *modE2* were purified using QIAquick gel extraction kit (Qiagen) and inserted into the expression vector pET-28a(+), resulting in the pET28amodE1 and pET28amodE2 plasmids. The inserts of these two plasmids were sequenced using DYEnamic ET Terminator Cycle Sequencing Kit (GE HealthCare) in an ABI PRISM 377 DNA Sequencer to confirm that no mutation was introduced during the amplification procedure. The pET28amodE1 plasmid expressed ModE1 and the pET28amodE2 plasmid expressed ModE2 as the polyhistidine-tag (His-tag) fusion proteins His-ModE1 and His-ModE2. These plasmids were introduced into *E. coli* strain BL21(DE3) by electrotransformation for protein expression and purification.

Over-expression and purification of *H. seropedicae* His-ModE1 and His-ModE2

Cells carrying the pET28amodE1 or pET28amodE2 plasmids were grown aerobically in 250 mL of LB medium at 37 °C to an optical density of approximately 0.3 measured at λ = 600 nm in a photospectrometer and 0.5 mM of

Table 1 - Bacterial strains and plasmids.

Strain/plasmid	Characteristics*	Source
E. coli BL21(DE3)	Host for expression of His-ModE1 and His-ModE2 proteins	Novagen, Darmstadt, Germany
H. seropedicae SMR1	Wild type, Nif^+, Sm^R	Souza *et al.*, 2000†
HS05-EG-048-B06	1.5 kbp insert of *H. seropedicae* genome in pUC18R cloning vector, Ap^R	Genopar Consortium
pET-28a(+)	High-copy His-tag expression vector; Km^R	Novagen, Darmstadt, Germany
pCR®2.1 TOPO	TA cloning vector, *lacZ*α fragment, Km^R, Ap^R	Invitrogen, Carlsbad, USA
pCR2.1modE1	pCR®2.1 derivative carrying *mod1* PCR product	This study
pCR2.1modE2	pCR®2.1 derivative carrying *modE2* PCR product	This study
pET28amodE1	pET-28a(+) derivative carrying *Nde*I-*Bam*HI *modE1* gene from *H. seropedicae*	This study
pET28amodE2	pET-28a(+) derivative carrying *Nde*I-*Bam*HI *modE*2 gene from *H. seropedicae*	This study

*Km, kanamycin; Ap, ampicillin. †Microbiology 146:1407-1418.

isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression of the recombinant proteins. After 3 h incubation at 37 °C the cells were harvested and stored frozen at -80 °C until extraction and purification of the His-ModE1 and His-ModE2 proteins. To purify His-ModE1 we re-suspended induced cells in 20 mL of lysis buffer (0.05 M Tris-HCl pH 8, 0.5 M NaCl, 0.01 M EDTA, 1 mM phenylmethyl-sulfonylfluoride (PMSF), 200 μg mL^{-1} lysozyme and 10% (w/v) glycerol), kept on ice for 30 min and disrupted by sonication using an Ultrasonic processor XL (Heat Systems) at 40% maximum intensity for five thirty--second bursts. The crude extract was centrifuged at 12,000 *g* for 15 min at 4 °C and the supernatant (S1) stored and the pellet resuspended by gentle agitation for one hour on ice in 20 mL of wash buffer (2 M urea, 0.05 M Tris-HCl pH 8, 0.5 M NaCl and 0.5% (w/v) Triton X-100) and then re-sonicated as described above, this process being repeated twice two produce two further supernatant fractions (S2 and S3). The washed inclusion bodies were resuspended in 20 mL of solubilization buffer (8 M urea, 0.5 M NaCl and 0.05 M Tris-HCl pH 8) and centrifuged as described above to remove remaining aggregates which could act as nuclei for aggregation during refolding. The supernatant (S4) containing the solubilized His-ModE1 was loaded onto a 5 mL HiTrap Chelating HP (GE Healthcare) charged with Ni^{2+} and equilibrated with solubilization buffer, 10 volumes of which was used to the column. For His-ModE1 refolding, the column was washed with 20 volumes of buffer A (0.05 M Tris-HCl pH 8 and 0.5 M NaCl) with an 8 M to 0 M decreasing linear gradient of urea at a flow rate of 2 mL min^{-1} and then with 5 volumes of the same buffer before eluting the on-column refolded His-ModE1 protein using 10 volumes of buffer A with a linear gradient of imidazole increasing to 1 M. The 0.28 M to 0.4 M imidazole fractions containing His-ModE1 were pooled and dialyzed against buffer A containing 50% (w/v) glycerol for 20 h at 4 °C and the purified protein stored at -80 °C in 100 μL aliquots. To purify His-ModE2 we resuspended the induced cells in 20 mL of lysis buffer (0.05 M Tris-HCl pH 8.0, 0.5 M NaCl and 10% (w/v) glycerol) and disrupted by sonication and centrifuged as described above. After centrifugation the supernatant, containing soluble His-ModE2, was loaded onto a HiTrap Chelating Ni^{2+} affinity column (GE Healthcare) equilibrated with buffer A and 10 volumes of buffer A with an increasing linear gradient of imidazole up to 1 M was used to elute the His-ModE2 protein at an imidazole concentration of between 0.32 M and 0.36 M. The fractions containing the protein were pooled and glycerol added to a final concentration of 50% (w/v) and the His-tagged ModE2 stored at - 80 °C.

We analyzed the expressed proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970), the gels being stained with Coomassie brilliant blue R-250. Molecular weight markers (GE Healthcare) were run along with the proteins. Protein quantification was performed as described by Bradford (1976) using bovine serum albumin as standard. The purity of His-ModE1 and His-ModE2 proteins was estimated by densitometric analysis using an UVP (Inc. Upland, CA USA) transilluminator.

DNA band shift assay

The shotgun library of the *H. seropedicae* genome project provided the HS05-EG-048-B06 plasmid carrying the *modA2* promoter region. Following digestion of the plasmid with *Bam*HI from the cloning vector and *Nhe*I, the 340 bp DNA fragment was extracted from a 0.8% (w/v) agarose gel and cleaned using the QIAquick Gel Extraction kit (Qiagen).

For the DNA binding assays we used the Klenow enzyme (New England Biolabs) to label the 340 bp DNA fragment with 10 mCi mL^{-1} [α^{32}P]-dCTP according to method of random primer labeling (Sambrook *et al.*, 1989) and cleaned the product with a PCR clean-up kit (Qiagen). The 10 μL reaction mixtures contained 5 nM of [^{32}P]-end-labelled 340 bp DNA and 3 μg mL^{-1} of heterologous herring DNA in binding buffer (0.05 M KCl, 0.01 M Tris-HCl pH 8.0, 7 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 10% (w/v) glycerol) (McNicholas *et al.*, 1997), the reactions being started by the addition of 100-800 nM of His-ModE1 or His-ModE2 (Figure 4A) and incubated at room temperature for 10 min. After incubation, reactions were loaded on a 4% non-denaturing polyacrylamide gel (19:1) in TBE buffer (Sambrook *et al.*, 1989). Electrophoresis was carried out at 60 V for 2 h at 4 °C. The gels were dried on a GD-1 slab gel dryer and visualized using a Storm 820 Phosphorimager (GE Healthcare).

Results and Discussion

The *H. seropedicae* sequences obtained by us are available under the following GenBank accession numbers: Z54207 for *nifHDK*; AF088132 for *nifENXorf1orf2*; EF666057 for *fdxAnifQmodA1B1C1*; AY007317 for *modE1*; M60319 for *nifA*; and EF666058 for *modE2modA2B2C2*.

Genetic organization of *H. seropedicae modABC* genes

Genes coding for a putative ABC-type molybdate transport system (*modA1B1C1*) were previously found downstream from the *nifHDKENXorf1orf2fdxAnifQ* genes in *H. seropedicae* (Klassen *et al.*, 1999). A similar organization was observed in the phototrophic purple bacterium *Rhodobacter capsulatus (Wang et al., 1993)*. In *H. seropedicae*, the *modA1B1C1* gene cluster probably forms a single operon with the *nif* genes under the control of the *nifH* promoter (Machado *et al.*, 1996; Klassen *et al.*, 1999) (Figure 1A). A *modE*--like gene, named *modE1*, is also located in the *nif* region downstream from the *nifA* gene and

24.5 kbp downstream from the *modA1* gene. Our *H. seropedicae* genome sequencing identified a second related *mod* cluster, named *modA2B2C2*, located in a distinct region of the genome, probably forming a single operon with another *modE*-like gene, *modE2*, immediately upstream and transcribed divergently from *modA2*. The organization of this gene cluster is more closely related to that seen in most other microorganisms, including *E. coli*, *Haemophilus influenzae*, *Azotobacter vinelandii* and *R. capsulatus* (Grunden and Shanmugam, 1997). Two ModE-like proteins have been found in some organisms (Studholme and Pau, 2003) but this organization has not been studied in detail. The DNA of *R. capsulatus* codes for two similar but functionally non-identical Mo-dependent regulators (MopA and MopB proteins), which have overlapping function in the Mo-dependent repression of *modABC*, *anfA* (coding for an ABC-type high-affinity Mo transport system), *morAB* and *modC* (*morABC* genes code for a putative ABC-type transport system of unknown function). However, Wiethaus *et al.* (2006) reported that the Mo-dependent activation of the *mop* gene (coding for a putative Mo-binding protein) is performed by MopA protein but not by MopB.

ModE1 protein

We isolated *modE1* from the *H. seropedicae* genome project shotgun genomic library and sequence analysis confirmed that this gene is 810 bp long and has high similarity to *modE* from *Burkholderia xenovorans* LB400 (GenBank YP_553832; 80% identity) and *Burkholderia vietnamiensis* G4 (GenBank YP_001115213; 70% identity). The protein we deduced from the *modE1* produced a protein containing 269 amino acids and a calculated molecular mass of 28 kDa. Our *in silico* analysis revealed an N-terminal domain (residues 28 to 88) characteristic of molybdenum binding proteins (Protein families (Pfam) accession number 00126) and which contains a HTH motif probably involved in DNA-binding (McNicholas *et al.*, 1998b; Studholme and Pau, 2003). At the C-terminal region of the ModE1 protein we found two transport-associated OB (DiMop) domains (residues 129 to 192 and 201 to 264) (Pfam 03459) similar to a molybdate-binding domain (MopI and MopII subdomains) (Figure 1B) (Schultz *et al.*, 1998; Letunic *et al.*, 2006). This domain organization of the *H. seropedicae* ModE1 is similar to the *E. coli* ModE proteins (McNicholas *et al.*, 1998b; Hall *et al.*, 1999; Schüttelkopf *et al.*, 2003; Studholme and Pau, 2003). To analyze the *H. seropedicae* ModE1 protein we constructed a plasmid to over-express ModE1 as a fusion to a His-tag sequence. Although several procedures were tested (data not shown), the His-ModE1 protein was found to be in an insoluble form (Figure 2A). Modifications of the induction temperature, use of lactose and modification of the lysis buffer composition and or the salt concentration did not improve the solubility of His-ModE1, but even so the expressed protein represented over 40% of the total cell protein content as determined by SDS-PAGE densitometry. Due to the difficulty in obtaining soluble *H. seropedicae* His-ModE1 we developed a procedure for solubilization of the inclusion bodies and refolding of the denatured protein. The soluble fraction after cell sonication showed a very faint His-ModE1 band, indicating that the majority of the protein was insoluble (Figure 2A). The use of sonication to lyse the cells in the presence of lysozyme produced a pellet with a minimum contamination of intact cells or cell debris and sheared genomic DNA. The insoluble protein was then solubilized and semi-purified with urea and affinity chromatography, the His-ModE1 protein being eluted with an imidazole gradient in a soluble form with an overall yield of 4% and a purity of up to 98% as determined by densitometric analyses of Coomassie-stained SDS-PAGE gels (Figure 2A). The stored frozen imidazole aliquots were shown to be stable for several months and no precipitated His-ModE1 protein was observed. The His-ModE1 protein was tested for DNA-binding using the *H. seropedicae modA2* promoter region. We also tested the protein refolding procedure using dialysis to decrease the amount of urea, however, protein precipitation was observed (data not shown). Since the mobility of column-bound proteins is restricted, the formation of protein aggregates is less prone to occur when the denaturing agent is decreased.

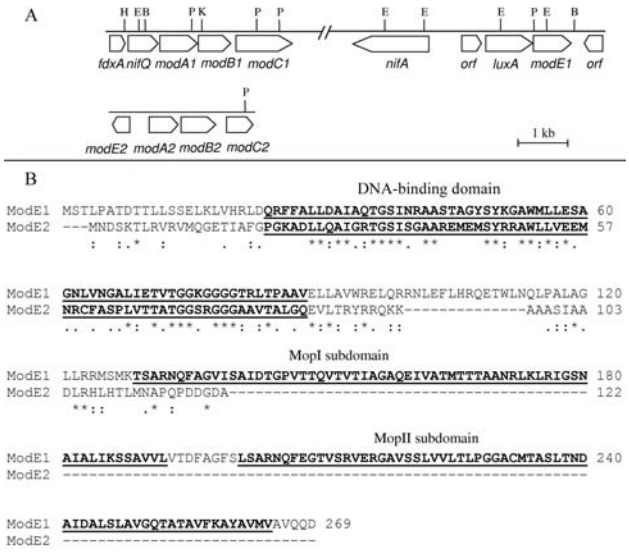

Figure 1 - Panel A shows the physical and genetic maps of the *modA1B1C1*, *modE1*, *modA2B2C2* and *modE2* regions. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I and P, *Pst*I. Panel B shows the amino acid alignment of *H. seropedicae* ModE1 and ModE2 proteins. DNA binding domain, and MopI and MopII (OB) domains for molybdate binding are indicated in bold and underlined. Alignment was produced by the Clustal W program and identification of the motifs was obtained by the SMART program (Schultz *et al.*, 1998; Letunic *et al.*, 2006). (*) indicates identical amino acids; (:) indicates highly similar amino acids; (.) indicates amino acids with low similarity.

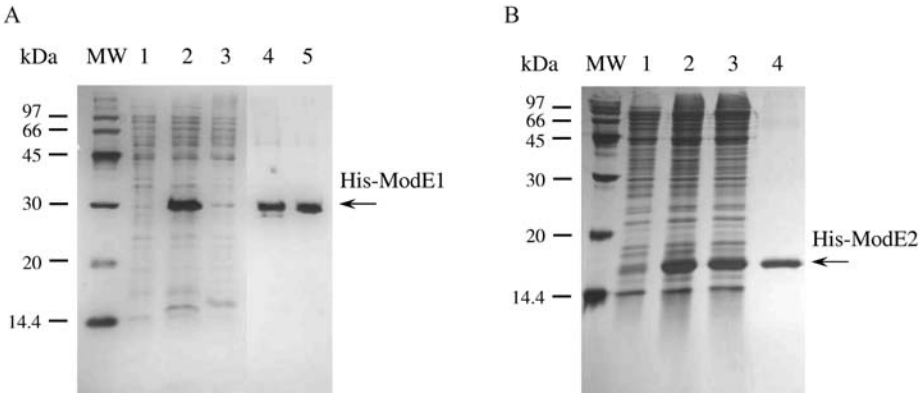

Figure 2 - Electrophoresis (15% SDS-PAGE) of the over-expression and purification of the *H. seropedicae* His-ModE1 and His-ModE2 proteins. Panel A shows His-ModE1 lanes: lane 1, total cellular protein (TCP) of non-induced *E. coli* BL21(DE3) carrying pET28amodE1; lane 2, TCP of *E. coli* BL21(DE3) carrying pET28amodE1 induced by 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h at 37 °C; lane 3, soluble fraction of the crude extract (S1); lane 4, sample after solubilization with 8 M urea (S4); and lane 5, purified fraction of His-ModE1. Panel B shows His-ModE2 lanes: lane 1, TCP of non-induced *E. coli* BL21(DE3) carrying pET28amodE2; lane 2, TCP of *E. coli* BL21(DE3) carrying pET28amodE2 induced by 0.5 mM IPTG for 3 h at 37 °C; lane 3, soluble fraction of the crude extract; and lane 4, purified fraction of His-ModE2. Molecular weight (MW) markers in kDa). Arrows indicate the His-tag proteins. Proteins were stained with Coomassie brilliant blue R-250.

ModE2 protein

In our study we confirmed that the *H. seropedicae modE2* is 372 bp long and codes for a protein made up of 122 amino acids and with a predicted size of 13 kDa. This protein has a DNA-binding domain made up of 21 to 85 amino acids residues (Pfam 00126) (Schultz *et al.*, 1998; Letunic *et al.*, 2006) which is similar to the N-terminal domain of the molybdenum-binding protein of *Methylobacillus flagellatus* KT (GenBank YP_546401; 53% identity), *Novosphingobium aromaticivorans* DSM 12444 (GenBank YP_497784; 60% identity), and *Methylococcus capsulatus* str. Bath (GenBank YP_113805; 51% identity). However, ModE2 does not show a molybdate-binding domain, a domain that is also missing in similar proteins of other organisms (Studholme and Pau, 2003). When we compared ModE1 and ModE2 we found that these proteins have low sequence identity (14%) and similarity (16%) as shown in Figure 1B, with the highest identity shared in the HTH domain. In contrast to His-ModE1, the His-ModE2 protein over-expressed in *E. coli* BL21(DE3) carrying the plasmid pET28amodE2 was mostly in a soluble form (Figure 2B). This solubility difference may be because of the lower content of hydrophobic amino acids in His-ModE2. We purified His-ModE2 from the soluble fraction of the crude extract using standard Ni^{+2} affinity chromatography, with 50% (v/v) glycerol being added to the pooled affinity chromatography protein fractions because precipitation occurred when high concentrations of His-ModE2 were dialyzed. The overall His-ModE2 protein yield was 10.4% and its purity was 98% as determined by densitometric analyses of Coomassie-stained SDS-PAGE gels (Figure 2B).

DNA-binding activity of His-ModE1 and His-ModE2

The ModE proteins regulate the transcription of *modABC* genes in response to molybdenum availability in *E. coli* and this regulation is based on the binding of the ModE-molybdate complex to a conserved binding sequence in the promoter region of the target genes (Grunden *et al.*, 1996; Anderson *et al.*, 1997; McNicholas *et al.*, 1997; Grunden *et al.*, 1999; Self *et al.*, 2001). Furthermore, ModE also seems to regulate the expression of other genes involved with molybdenum (Anderson *et al.*, 2000; McNicholas *et al.*, 1998a; Tao *et al.*, 2005).

Sequence analysis of the *H. seropedicae modE2-modA2* intergenic region revealed that it contains the TATAT-N_7-TATAT motif, very similar to the ModE-protected regions of the *modA* operator/promoter DNA after DNase I footprinting in *E. coli* (Anderson *et al.*, 1997; McNicholas *et al.*, 1997; Grunden *et al.*, 1999), indicating that this sequence is the target for ModE1 and/or ModE2 proteins. In a variety of organisms the TATAT-N_7-TATAT motif sequence is similar to the operator/promoter DNA sequence of operons known to be under molybdate control and repressed by ModE-molybdate or its homologs (Gourley *et al.*, 2001; Self *et al.*, 2001). The probable *H. seropedicae* ModE binding site overlaps the -10 region of the putative σ^{70}-dependent *modA2* promoter, as also reported in the *E. coli modE-modABC* regulating region (Anderson *et al.*, 1997; Grunden *et al.*, 1999), further suggesting that these genes are regulated by a ModE-like protein (Figure 3). To further investigate this we used a 340 bp *H. seropedicae modA2* promoter region fragment and the DNA band-shift assay to test the binding of His-ModE1 and His-ModE2 proteins to target DNA (Figure 4). Both purified His-ModE1 and His-ModE2 proteins bound to the *modA2* promoter region in the presence of excess (3 $\mu g.mL^{-1}$) unlabelled heterologous herring DNA, as revealed by a decrease in the migration rate of the [^{32}P]-labelled *modA2* promoter DNA fragment (Figure 4A). The ratio of protein-bound DNA was dependent on the protein

concentration (Figure 4A) and 600 nM of purified His-ModE1 and His-ModE2 bound to 100% of the DNA in the system (Figures 4B and C). Also, with the increasing concentration of unlabelled *modA2* promoter region, the binding of the labeled DNA was diluted out, indicating that both proteins are able to bind to the *modA2* promoter (Figures 4B and C). The presence of the DNA-protein complex in the well may suggest that a high molecular mass complex is formed, although, since the complex is disrupted by unlabelled homologous DNA, it is unlikely to be a result of protein-DNA precipitation. Molybdate did not interfere in the DNA-binding under our experimental conditions, since similar DNA binding was observed when we added 1 mM sodium molybdate (data not shown). The lack of molybdate-dependent binding in our experiments may have been due to the presence of trace amounts of molybdenum in the chemicals and solutions used for protein purification, which could be sufficient to saturate His-ModE1. A similar effect was observed in the binding of purified *E. coli* ModE to the *moaA* promoter region (McNicholas *et al.*, 1997). Since DNA-binding was observed in the presence of herring DNA and the dilution-out of the DNA-protein complex occurred using unlabelled competitor DNA, our results indicate that *H. seropedicae* His-ModE1 was on-column refolded to an active form that recognized and bound the *H. seropedicae modA2* promoter region. The over-expressed and purified His-ModE2 protein also recognized and bound the same DNA fragment.

Overview

In this study the fusion proteins ModE1 and ModE2 were not only expressed and purified but also assayed for their *in vitro* DNA-binding activities. Both proteins were able to bind the *modA2* promoter, suggesting involvement in the regulation of the transcription of *modA2B2C2* genes in *H. seropedicae*. Additional studies involving the charac-

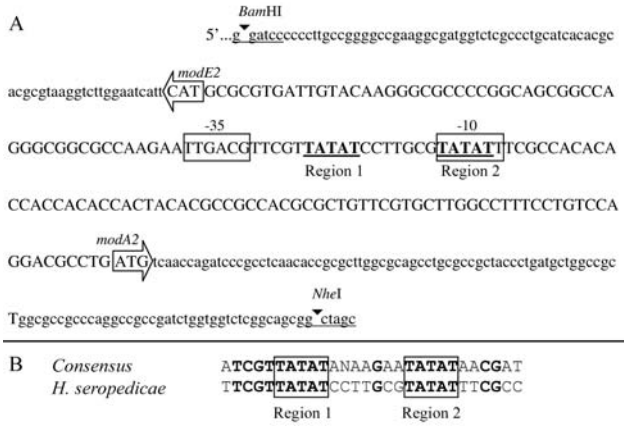

Figure 3 - Panel A shows the nucleotide sequence of the 340 bp DNA fragment containing the *H. seropedicae modE2-modA2* intergenic region. Large arrows indicate the transcription direction of *modE1* and *modA2*. The -10/-35 type promoter region of *modA2* are shown in boxes and the predicted ModE-binding sites (regions 1 and 2) are in bold and underlined. Panel B shows the nucleotide alignment of a consensus sequence for ModE-binding. The base 5 of the consensus presented the same degree of conservation for nucleotides A and T among the sequences used for the alignment, with 'N' indicating any nucleotide (Studholme and Pau, 2003; BMC Microbiol. 3, doi: 10.1186/1471-2180-3-24).

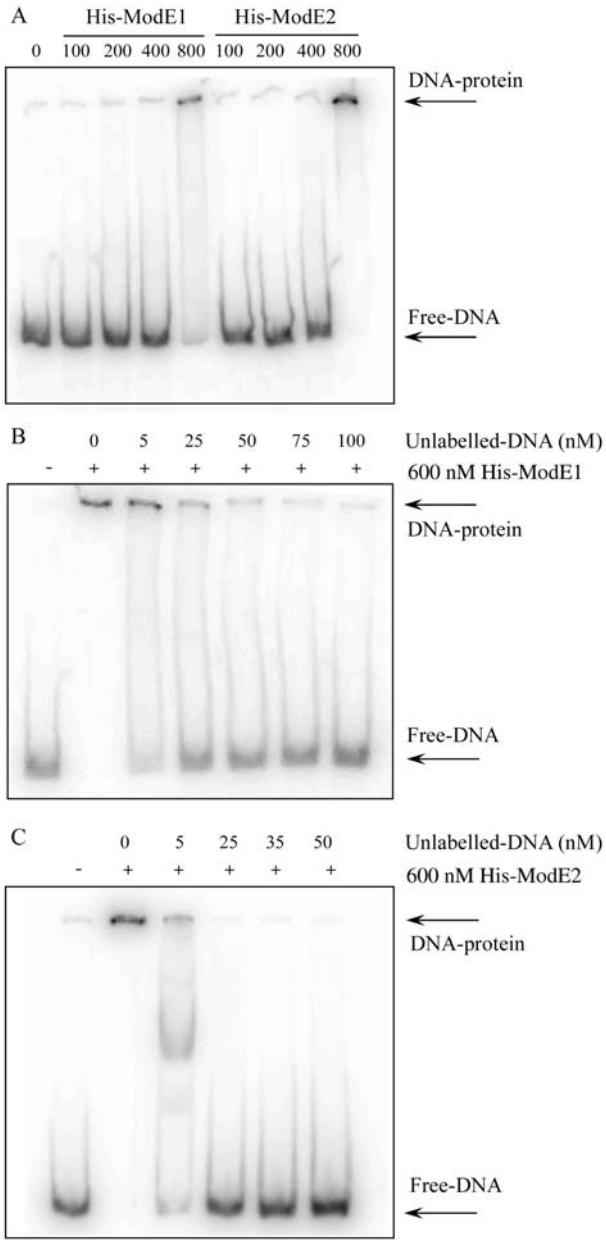

Figure 4 - DNA band-shift assay with the *H. seropedicae* His-ModE1 and His-ModE2 proteins and a 340 bp DNA fragment containing the *modA2* promoter region. Experiments were carried out as described in the Material and Methods. Panel A shows the amount (nM) of purified His-ModE1 and His-ModE2 incubated with 5 nM of [^{32}P]-labeled *modA2* promoter region in the presence of 3 μg/mL of herring DNA. Panel B shows the amount (nM) of unlabeled competitor DNA added to a reaction mixture containing 600 nM of purified His-ModE1 and 5 nM of [^{32}P]-labeled *modA2* promoter region. Lane (-) shows sample containing only the [^{32}P]-labeled DNA. Panel C shows the amount (nM) of unlabeled competitor DNA incubated with 600 nM of purified His-ModE2 and 5 nM of [^{32}P]-labeled promoter region of *modA2*. Lane (-) shows sample containing only the [^{32}P]-labeled DNA. Arrows indicate the migration of free-DNA or DNA bound to protein. Autoradiograms were obtained using Storm 820 Phosphorimager.

terization of *modE1* and *modE2* mutants aimed at elucidating their physiological roles in the regulation of molybdate uptake will contribute to a better understanding of this process in *H. seropedicae*.

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Internet Resources

H. seropedicae Genome Sequencing Project, http://www.genopar.org/.

Simple Modular Architecture Research Tool (SMART), http://smart.embl-heidelberg.de/

Protein Family (Pfam), http://www.sanger.ac.uk/.

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