Transcriptional control of the $F_0F_1$-ATP synthase operon of *Corynebacterium glutamicum*: SigmaH factor binds to its promoter and regulates its expression at different pH values

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Summary

*Corynebacterium glutamicum* used in the amino acid fermentation industries is an alkalophilic microorganism. Its $F_0F_1$-ATPase operon (*atpBEFHAGDC*) is expressed optimally at pH 9.0 forming a polycistronic (7.5 kb) and a monocistronic (1.2 kb) transcripts both starting upstream of the *atpB* gene. Expression of this operon is controlled by the SigmaH factor. The sigH gene (*sigH*) was cloned and shown to be co-transcribed with a small gene, *cg0877*, encoding a putative anti-sigma factor. A mutant deleted in the *sigH* gene expressed the *atpBEFHAGDC* operon optimally at pH 7.0 at difference of the wild-type strain (optimal expression at pH 9.0). These results suggested that the SigmaH factor is involved in pH control of expression of the $F_0F_1$ ATPase operon. The SigmaH protein was expressed in *Escherichia coli* fused to the GST (glutathione-S-transferase) and purified to homogeneity by affinity chromatography on a GSTrap HP column. The fused protein was identified by immunodetection with anti-GST antibodies. DNA-binding studies by electrophoretic mobility shift assays showed that the SigH protein binds to a region of the *atpB* promoter containing the sigmA recognition sequence (−35)TTGGAT…18nt…GTTA(−10).

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(extracytoplasmic function) family (Engels et al., 2004). SigH has been reported to play a role in the expression of genes involved in heat-shock and oxidative stress but its role in the control of other genes or operons remains obscure (see Discussion).

Initial studies in our group suggested that the $F_0F_1$-ATPase operon may be under control of the SigmaH (SigH) factor (Barriuso-Iglesias et al., 2006). It was, therefore, important to study the expression of the $F_0F_1$-ATPase operon in a C. glutamicum mutant defective in sigH as compared with its response to pH changes in the wild-type parental strain. Furthermore, it was also important to analyse the possible SigH binding to the $atpBEFHAGDC$ promoter in this microorganism.

Results

Deletion of the sigH gene causes a drastic change in the pH response of the $F_0F_1$-ATPase operon

In the C. glutamicum genome, the sigH gene (621 nt, access No. BX 927147.1, Kalinowski et al., 2003) is preceded upstream by an open reading frame (ORF) encoding a hypothetical protein, and is followed in the same orientation by a small gene encoding a putative anti-sigma factor (cg0877), also named rsrA or rshA. The sigH and cg0877 genes are separated by only three nucleotides. Downstream of cg0877 and separated by 232 bp in the complementary strand, is located the whiB1 gene (also named whcE, Kim et al., 2005b) that encodes a transcriptional factor.

As reported previously, C. glutamicum is an alkaliphilic microorganism and the $F_0F_1$-ATPase operon is expressed optimally at pH 9.0 (Barriuso-Iglesias et al., 2006). Since a putative SigH binding site was found in the promoter of the $atpBEFHAGDC$ operon encoding the $F_0F_1$-ATPase complex, we decided to study expression of this operon in the wild-type C. glutamicum ATCC 13032 and in a $\Delta sigH$ mutant derived from it (Engels et al., 2004).

Northern analysis of the transcripts formed from the $atpBEFHAGDC$ operon in the wild-type strain were made using the probes named B, B1, B2 and D, internal to $atpB$ (probes B, B1 and B2) and $atpD$ genes respectively. The size of the transcripts (7.5 and 1.2 kb) was the same in the wild type and the $\Delta sigH$ mutant, but the pattern of response to pH was clearly different (Fig. 1). The best results were obtained with probes B, B2 and D. Using probes B or B2 we observed two transcripts of 7.5 and 1.2 kb respectively, as described for the wild-type (Barriuso-Iglesias et al., 2006). With probe D, only the 7.5 kb transcript was found. The large 7.5 kb transcript corresponds to a polycistronic transcript of the $atpBEFHAGDC$ operon, whereas the short 1.2 kb transcript obtained with probes B or B2, but not with probe D, corresponds to a monocistronic mRNA of the first gene of the operon.

Interestingly, whereas maximal expression of the $F_0F_1$-ATPase operon in the wild-type strain was observed at pH 9.0 with any of the three probes (Barriuso-Iglesias et al., 2006), optimal expression of this operon in the $\Delta sigH$ mutant occurred at pH 7.0 (Fig. 1). These results suggest that expression of the $F_0F_1$-ATPase operon is under the control of the ECF $\sigma^H$ factor.

Transcriptional organization of the sigH-cg0877 region

SigH is encoded by a 621 nt ORF and is very closely linked to cg0877 (3 nt separation). In order to study the transcriptional response of sigH to pH stress, we analysed the transcription of sigH using a sensitive RNA–RNA hybridization procedure with an antisense RNA probe (see Experimental procedures). The 615 nt RNA probe obtained was named probe H (Fig. 2A).

Hybridizations with total RNA of the wild-type C. glutamicum grown at pH 6.0, 7.0 and 9.0 using the same amount of total RNA, showed a single 0.9 kb transcript that appears to correspond to the bi-cistronic transcript of sigH and cg0877 [Correction added on 28 January 2013 after first online publication: the text in parenthesis referring to 16S RNA probe has been deleted from the above statement,]. The expression of these genes was clearly higher at alkaline pH (9.0) than at lower pH 7.0 (Fig. 2C).

The possible joint transcription of sigH and cg0877 (encoding a putative anti-sigma factor) was supported by the lack of an identifiable separate promoter for cg0877 and by the finding of a putative strong transcriptional terminator downstream of cg0877 formed by an inverted repeat sequence of 72 nt, with a calculated $\Delta G$ of $-40 \text{kcal mol}^{-1}$ (Fig. 2B). This putative transcriptional terminator belongs to the type I, or Mycobacterium type (Unniraman et al., 2002), and is located 87 nt downstream of cg0877 stop codon (see Discussion).

Identification of the SigH-encoded protein

A very high expression of sigH was obtained in Escherichia coli cultures at 37°C following addition of IPTG (0.1 mM). However, under these conditions most of the SigH protein was insoluble (inclusion bodies, Fig. 3A). The best condition to obtain soluble SigH protein was growing the culture at 25°C before and after induction with a 0.3 mM IPTG concentration (Fig. 3B). A band of the expected size (49 kDa) for the GST–SigH protein was observed in extracts of the induced cultures as compared with the non-induced ones (Fig. 3C). The identity of the induced protein was confirmed by immunodetection with anti-GST antibodies (Fig. 3D).

The fused GST–SigH protein was purified from the soluble fraction of E. coli transformants containing the pGEX–SigH construction using GST-Trap HP columns, as
After washing with 15 ml of PBS buffer, the GST–SigH protein was eluted with 10 mM reduced glutathione in 50 mM Tris-HCl buffer pH 8.0. The fractions were collected, analysed by SDS-PAGE and tested with antibodies against GST. As shown in Fig. 3D, almost all of GST–SigH protein (reactive with anti-GST antibodies) was found in fractions 5–8 of the 20 collected fractions. In this way, about 500 mg of pure GST–SigH protein was obtained per 100 ml of E. coli culture. The nature of the purified protein was identified by peptide mass fingerprinting as described in Experimental procedures.

The purified GST–SigH binds to the atpBEFHAGDC operon promoter

Binding of the purified GST–SigH protein to the promoter of the atpBEFHAGDC operon was tested by the electrophoretic mobility shift assay (EMSA). For this purpose, the $f_0f_1$ operon promoter was cloned as a 357 bp DNA fragment using a high fidelity DNA polymerase (Platinum® Pfx, Invitrogen) and oligonucleotides PxB-U and PxB-D2 as primers (Table 1). The amplified DNA fragment was purified by filtration through GFX PCR DNA and gel band purification kit (GE Healthcare) and
cloned in pGEM<sup>®</sup>-T Easy (this construction was named pGEM-P<sub>f0f1</sub>). Promoter DNA-binding assays were performed with the purified GST–SigH protein in buffers A and B, differing essentially in the magnesium salt utilized and in the presence of polyethylene glycol (PEG) in buffer B. Results of the binding experiments are shown in Fig. 4. It is observed that there is a good binding of the GST–SigH protein, but not of the separate GST protein used as control, to the labelled F<sub>0</sub>F<sub>1</sub>-ATPase promoter, in buffer A; however, binding was barely visible in buffer B (not shown), indicating that some of the components of this buffer (most likely PEG) are unfavourable for the protein-DNA interaction. The intensity of the retarded band was proportional to the amount of GST–SigH protein used (25–100 pmol) and the labelled band was not formed when the reaction was supplemented with additional unlabelled probe. These results show that SigH binds to the promoter of the atpBE-FHAGDC operon and, therefore, participates in the pH control of this operon in <i>C. glutamicum</i>. The used DNA fragment contains the consensus sequence GGAT...18nt...GTTA in the −35 to −10 region of the promoter.

**Discussion**

Sigma factors are very important auxiliary components of the RNA polymerase since they determine the choice of promoters and, therefore, the genes to be expressed under specific physiological conditions. A survey of the sigma promoters in 19 corynebacterial species has been conducted...
made (Pátek and Nešvera, 2011). The σA housekeeping genes sigma factor, which belongs to the σ3 class, is similar to the σ70 of *E. coli* and *Mycobacterium tuberculosis* (Oguiza et al., 1996). The alternative sigma factors σB, σE, σH and σM are present in different corynebacteria and appear to be involved in expression of genes for several important processes. Thus, σB is involved in the response to several stress conditions and in the expression of genes required for the transition of the exponential growth phase to the stationary phase (Halgasova et al., 2002; Larisch et al., 2007; Ehira et al., 2008). The σE factor (for the so-called extracytoplasmic factors) has a role on the expression of genes in response to the cell-wall damage (Pátek et al., 2003). The σH is involved in the response to heat stress (Barreiro et al., 2009; Ehira et al., 2009; Busche et al., 2012). σM controls the expression of a few genes involved in the control of disulfide stress (Nakunst et al., 2007).

It is noteworthy that sigH is involved in the control of the expression of the genes encoding two other sigma factors, namely σM (Nakunst et al., 2007) and σB (Ehira et al., 2008), in addition to be required for the expression of its own gen (Kim et al., 2005a).

One of the best known SigH-dependent systems is that of *B. subtilis* that may serve as a model for other Gram-positive bacteria. In *B. subtilis*, SigH (encoded by the *spo0H* gene) is the first in a cascade of five sigma factors that lead to reprogramming the bacterial metabolism to form heat-resistant endospores (Carter and Moran, 1986; Dubnau et al., 1988). After entering the stationary phase, derepression of *spo0H* (encoding the SigH factor) several proteins involved in sporulation and development of competence are formed (Grossman, 1995; Fujita and Sadaie, 1998).

It is interesting that in *B. subtilis* cultures grown at low pH values, the genes regulated by SpoOH (including the own *spo0H*) are expressed only at basal levels, whereas increased levels of SpoOH (SigH) are formed following addition of compounds that maintain higher pH values (Cosby and Zuber, 1997). The increased levels of SpoOH lead to high expression of *spo0A*, a key regulator of the late differentiation steps. Spo0A in its phosphorylated

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**Fig. 3.** Overexpression of SigH as a GST–SigH fused protein in *E. coli*. A. SDS-PAGE gel of the GST–SigH overexpression at 37°C. B. SDS-PAGE gel of the GST–SigH overexpression at 25°C and different IPTG concentrations. Control: pGEX–SigH without induction; Sp: supernatant (soluble fraction); P: pellet (insoluble fraction). Molecular size markers, in kDa, are indicated on the left side of panel A. Arrows show the GST–SigH fusion protein overexpression. C. Purification of the GST–SigH fusion protein by affinity chromatography on Glutathione Sepharose. Samples were analysed in 12.5% acrylamide SDS-PAGE gels. Sp-C/Sp-I: Control or induced supernatant. MW: Molecular size markers, in kDa. 3–8: Elution fractions from the affinity chromatography. D. Immunodetection of the GST–SigH fusion protein by Western blot analysis with anti-GST antibodies. Sp-C/Sp-I: 20 μg of non-induced and induced supernatants. MW: Molecular size markers (in kDa). 4–8: 5 μl of the affinity chromatography eluted fractions.
form represses formation of AhrB, which controls negatively the transcription of spo0H, thus feedback regulating the levels of Spo0H. This circuit is extremely important in the control of sporulation and its relation with pH changes.

The role of sigH has been also studied in some detail in Mycobacterium tuberculosis, where it is induced by heat-shock or by treatment with diamines (Graham and Clark-Curtiss, 1999; Raman et al., 2001). Under reducing conditions in the cell, the expression of sigH is regulated negatively by an anti-sigma factor protein named RshA (Song et al., 2003) by protein–protein interaction. Using a M. tuberculosis sigH null mutant (Manganelli et al., 2002) it was observed that several other transcriptional factors are under control of SigH including SigB and SigH itself, in addition to enzymes involved in redox metabolism (thioredoxin and thioredoxin–oxidoreductase) and enzymes of cysteine and molybdopterin biosynthesis and the chaperones Hsp70 and ClpB.

In S. coelicolor, the sigH gene is linked to the ushX gene encoding an anti-sigma factor. Both genes are co-transcribed. As in M. tuberculosis, the SigH–UshX complex, in response to external stress signals, dissociates releasing the sigma factor that binds to the RNA polymerase and recognizes the sigH-dependent transcription of ushX.

Table 1. Strains, plasmids and oligonucleotides used in this work.

| Bacterial strains/plasmids/oligonucleotides | Relevant characteristics | Source/reference |
|-------------------------------------------|--------------------------|-----------------|
| E. coli DH5α                               | φ80lacZΔM15, recA1, endA1, gyrAB, thi-1, hsdR17(r−, m−), supE44, relA1, deoR, Δ(lacZYA-argF) U169, phoA | Hanahan (1983) |
| C. glutamicum ATCC 13032                    | Wild type               | American Type Culture Collection |
| C. glutamicum ΔsigH                         | In frame deletion of the sigH gene | Abe et al. (1967) |
| Plasmid                                    |                          | |
| pGEM®-T Easy Vector                        | AmpR, cloning vector     | Promega Corporation, Madison, USA. |
| pGEM-TB                                    | pGEM®-T Easy + 750 bp internal fragment of atpB | Barruso-Iglesias et al. (2006) |
| pGEM-TB1                                   | pGEM®-T Easy + 275 bp internal fragment of atpB | Barruso-Iglesias et al. (2006) |
| pGEM-TB2                                   | pGEM®-T Easy + 350 bp internal fragment of atpB | Barruso-Iglesias et al. (2006) |
| pGEM-TD                                    | pGEM®-T Easy + 850 bp internal fragment of atpD | Barruso-Iglesias et al. (2006) |
| pGEM-TH                                    | pGEM®-T Easy + 615 bp internal fragment of sigH | This work |
| pGEM-Pf0f1                                 | pGEM®-T Easy + Promoter region of F0F1 operon | This work |
| pGEM-TsigH                                 | Vector system for protein expression | GE Healthcare |
| pGEX–SigH                                  | pGEM®-T Easy + C. glutamicum sigH gene | This work |
| Oligonucleotides used as primers           |                          | |
| SIGH-U:                                    | 5′-GAAAACCCGACCCGGCCA-3′ | This work |
| SIGH-D:                                    | 5′-CTCCGAAATTTCTCTATGT-3′ | This work |
| Forward:                                   | 5′-GTAAACGGACGGCCAGT-3′ | This work |
| Reverse:                                   | 5′-GGGCGCCGCTGATACCATC-3′ | This work |
| 16S-3:                                    | 5′-GGGCGCCGCTGATACCATC-3′ | This work |
| 16S-5:                                    | 5′-GGGCGCCGCTGATACCATC-3′ | This work |
| EXH-U:                                    | 5′-AAAGGATCCCATGCTGTA-3′ | This work |
| EXH-D:                                    | 5′-GGGCGCCGCTGATACCATC-3′ | This work |
| 5′-pGEX:                                   | 5′-GGGCGCCGCTGATACCATC-3′ | This work |
| 3′-pGEX:                                   | 5′-GGGCGCCGCTGATACCATC-3′ | This work |
| PxB-U:                                    | 5′-AGTGGATCCCATGCTGTA-3′ | This work |
| PxB-D2:                                   | 5′-GGGCGCCGCTGATACCATC-3′ | This work |

Fig. 4. Electrophoretic mobility-shift assays of the f0f1 promoter region using increasing concentrations (25, 50 and 100 pmol) of the GST–SigH protein. The assays were performed in buffer A as indicated in Experimental procedures. Lane G, control with pure GST protein (150 pmol). Lane P, probe without protein. Lane D, reaction diluted with excess (500-fold) unlabelled probe. Note the formation of one DNA–protein complex (arrow) at increasing protein concentration.
promoters (Kormanec et al., 2000; Sevcikova and Kormanec, 2002; Kormanec and Sevcikova, 2002a,b).

Much less is known about the role of SigH in Corynebacteria. Five of the seven putative sigma factors in the genome of C. glutamicum, SigC, SigD, SigE, SigH and SigM belong to the ECF subclass (Engels et al., 2004). sigH of C. glutamicum encodes a small protein of 206 amino acids that is closely linked to a downstream ORF (cg0877) separated by only 3 bp that encodes a putative anti-sigma factor (named RsrA) that may play a role similar to UshX in S. coelicolor. Interestingly, downstream of sigH-rsrA in opposite orientation and separated by a putative strong transcriptional terminator (Fig. 2A and B) is located the whiB1 gene, also named whcE, that encodes another putative transcriptional factor (Kim et al., 2005b).

As described in this work, purified SigH protein binds to the promoter of the atpBEFHAGDC operon. Expression of the sigH gene responds to the pH of the culture, being clearly higher at pH 9.0 than at neutral or slightly acid pH values. In this way, SigH mediates pH control of the F0F1-ATPase operon in C. glutamicum, which was previously reported to be optimal at about pH 9.0 (Barriuso-Iglesias et al., 2006). A sigH null mutant shows optimal expression of the atp operon at neutral pH in contrast to the wild-type strain. SigH has been reported to modulate its own expression in C. glutamicum that is optimal in the stationary phase of growth (Kim et al., 2005a).

SigH may participate in the control of cellular processes through other sigma factors. Halgasova and colleagues (2002) reported that in C. glutamicum subsp. flavum, SigH regulates the expression of sigB under situations of heat-, cold- or acid-stress. This observation correlates well with our own observation of SigH response to acid or basic pH values.

In addition to the control of the F0F1-ATPase operon, SigH has been reported to play an important role in the control of several types of stress in C. glutamicum. We observed that SigH regulates in a positive manner the expression of the dnaK operon that encodes a chaperone involved in heat-shock (Barreiro et al., 2004) and the expression of the heat-shock regulators HspR and CigR under conditions of severe heat-stress (Barreiro et al., 2004; 2009; Engels et al., 2004; Ehira et al., 2009). SigH also regulates the expression of whcE, the regulator of the thioredoxin system (Kim et al., 2005b) and the clpC and clpP1P2 that encode heat-shock proteases (Engels et al., 2004). In summary, SigH exerts the upper level of control of genes involved in the stress response to unfavourable nutritional or environmental conditions. Up to 47 regulatory interactions have been described to be mediated by SigH (Baumbach et al., 2006; 2007). A summary of those interactions is shown in Fig. 5 taking into account the results presented in this article, and those reported by other authors (Halgasova et al., 2002; Barreiro et al., 2004; Engels et al., 2004; Kim et al., 2005a,b; Barriuso-Iglesias et al., 2006).

Experimental procedures

Bacterial strains, plasmids, and culture conditions

The bacterial strains, plasmids and oligonucleotides used in this work are listed in Table 1. Corynebacterium glutamicum ΔsigH was kindly provided by Jens Schweitzer and Michael Bott (Jülich, Germany). Escherichia coli was grown in Luria–Bertani (LB) broth (Sambrook and Russell, 2001) at 37°C. Corynebacterium glutamicum strains were grown in trypticase soy broth (TSB) at 30°C and at different pH conditions, in flasks. Escherichia coli transformants were selected in presence of ampicillin (100 μg ml⁻¹) kanamycin (50 μg ml⁻¹) or chloramphenicol (25 μg ml⁻¹). Corynebacterium glutamicum transformants were selected on media with kanamycin (30 μg ml⁻¹) or chloramphenicol (12 μg ml⁻¹).

DNA isolation and manipulation

Escherichia coli plasmid DNA was obtained by alkaline lysis. Total C. glutamicum DNA was prepared as described by Martin and Gil (1999). DNA manipulations were performed as reported by Sambrook and Russell (2001). DNA fragments were isolated from agarose gels using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Escherichia coli cells were transformed by standard methods (Sambrook and Russell, 2001), whereas C. glutamicum cells were transformed by electroporation (van der Rest et al., 1999).

RNA extraction

Total RNA from corynebacteria grown at different pHs (OD₆₀₀ 3.5–4.0) was extracted essentially as described by Eikmanns and colleagues (1994), except that the cell pellet obtained after centrifugation was frozen with liquid nitrogen and kept at −70°C until RNA extraction (Barreiro et al., 2001). The RNA concentration was determined spectrophotometrically by the absorbance at 260 nm.

Northern hybridizations

Denaturing RNA electrophoresis was performed in 0.9% agarose gels in MOPS buffer (20 mM MOPS, 5 mM sodium acetate; 1 mM EDTA pH 7.0) with 17% (v/v) formaldehyde. RNA (30–40 μg) was dissolved in denaturing buffer (50% formamide, 20% formaldehyde, 20% MOPS (5x) with 10% DYE [50 μg] glycerol, 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 1 mM EDTA) (Sambrook and Russell, 2001) and 1% ethidium bromide). RNA probes for atp1, atpB, atpD and sigH were labelled with digoxigenin and Northern hybridizations were performed following the instructions of the DIG Northern Starter Kit manufacturer (Roche Applied Science). The hybridization temperature was 68°C.

Preparation of RNA probes

For transcriptional analysis of the sigH gene a RNA probe was prepared as follows: a 615 bp fragment internal to sigH...
was cloned by PCR in pGEM®-T Easy. The sigH fragment was excised with the Apal endonuclease and placed under control of the SP6 RNA polymerase promoter in vector pGEM®-T Easy.

**Protein expression and purification**

To study the possible binding of the SigH protein to the promoter region of the *atpBEFHAGDC* operon, it was necessary to obtain an active sigmaH protein. The *sigH* gene was overexpressed in *E. coli* using the pGEX-2T expression system (GE Healthcare). *sigH* was amplified as a 660 bp PCR fragment from total DNA of *C. glutamicum* with Platinum® pfx DNA polymerase, using the ExH-U/ExH-D primers (Table 1), and cloned into pGEM®-T Easy vector (Promega). The insert was excised by digestion with BamHI and EcoRI (the restriction sites were in the upstream and downstream primers respectively) and was cloned into pGEX-2T expression plasmid (GE Healthcare). The new pGEX–SigH plasmid was introduced in *E. coli* DH5α. The correct integration of the insert was established by sequencing. To obtain the fusion protein, *E. coli* cells were grown in LB medium in an orbital shaker (250 r.p.m.) to an OD_{600} of 0.6 and under the four following conditions: (i) culture growth at 37°C before and after IPTG induction, (ii) growth at 25°C prior to induction and at 37°C following IPTG addition, (iii) growth at 25°C prior to induction and at 30°C following IPTG addition and (iv) growth at 25°C before and after IPTG addition. These four conditions were combined with increasing IPTG concentrations from 0.1 to 0.3 mM.

Cells were harvested by centrifugation, washed twice with NaCl 0.9% and lysed by sonication. The soluble fraction was separated by centrifugation and the protein was purified in an ÄKTA-FPLC using a Glutathione Sepharose 4B column (GE Healthcare). The protein was eluted with 10 mM reduced glutathione (in 50 mM Tris-HCl, pH 8.0) following the manufacturer’s recommendations and conserved in 40% glycerol at -80°C before use. Protein concentrations were determined with the Bradford reagent (Bio-Rad).

**Peptide mass fingerprint**

The protein band of interest were manually excised from stained gels, placed in an Eppendorf tube and washed twice with double distilled water. The proteins were digested following the method of Havlis and colleagues (2003) and processed for further analysis as described previously (Jami et al., 2010). The samples were analysed with a 4800 Proteomics Analyzer matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrometer (AB Sciex). All MS spectra were internally calibrated using peptides from the trypsin auto digestion. The analysis by MALDI-TOF/TOF mass spectrometry produced peptide mass fingerprints, and the peptides observed were collected and represented as a list of monoisotopic molecular weights with a signal-to-noise (S/N) ratio greater than 20 using the 4000
Series Explorer v3.5.3 software (AB Scien). For protein identification, Mascot generic files were automatically created and used to interrogate a non-redundant protein database (NCBI) using a local license of Mascot v 2.2.04 from Matrix Science through the Global Protein Server v 3.6 (AB Scien).

Electrophoretic mobility shift assays (EMSA)

DNA-binding tests were performed by EMSA. The DNA fragments were labelled at both ends with digoxigenin using the DIG Oligonucleotide 3’-End Labelling Kit, 2nd Generation (Roche Applied Science). The binding reaction was performed in two reaction buffers: buffer A) 10 mM Tris-HCl pH 8.0, 0.4 mM MgCl2, 10 mM KCl, 0.2 mM DTT, 1.6 mM glutathione and 0.01% Nonidet P40 (Sola-Landa et al., 2005); buffer B) 25 mM Tris-acetate pH 8.0, 8 mM Mg-acetate; 10 mM KCl; 1 mM DTT and 3.5% w/v PEG 2005; buffer B) 25 mM Tris-acetate pH 8.0; 8 mM Mg-acetate; 10 mM KCl; 1 mM DTT and 3.5% w/v PEG 2005 (Wigneshwararaj et al., 2002). The binding reaction also contained 50 µg mL⁻¹ poly[d(I-C)]. The samples were loaded onto a 5% polyacrylamide (29:1) native gel in 0.5x TBE buffer. After electrophoresis (5 h, 80 V), DNA was electroblotted onto a nylon membrane in 0.5x TBE buffer (1 h, 200 mA). The DNA was fixed by UV cross-linking and loaded onto a 5% polyacrylamide (29:1) native gel in 0.5x TBE buffer. After electrophoresis (5 h, 80 V), DNA was electroblotted onto a nylon membrane in 0.5x TBE buffer (1 h, 200 mA). The DNA was fixed by UV cross-linking, detected with anti-digoxigenin antibodies and developed by chemiluminescence with the CDP-Star TM reagent (Roche Applied Science).

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Conflict of interest

None declared.

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