Cloning and characterization of the 5′-flanking region of the oxalate decarboxylase gene from *Flammulina velutipes*

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The oxalate-degrading enzyme, oxalate decarboxylase (OXDC), was purified and characterized from *Flammulina velutipes*, a basidiomycetous fungus [Mehta and Datta (1991) J. Biol. Chem. **266**, 23548–23553]. The cDNA cloning and analyses revealed that *OXDC* transcription was induced by oxalic acid. However, in this report, we show that *OXDC* transcription is induced by low pH, not by oxalate. To understand the regulatory mechanism of *OXDC* expression, we have cloned and analysed a 580-bp genomic fragment from the 5′-flanking region of the *OXDC* gene. Sequence analysis showed the presence of several eukaryotic transcription factor binding motifs within the −580 bp of the upstream region. Electrophoretic-mobility-shift assays with partially purified cell extracts revealed specific binding of a factor in acid-induced, but not in uninduced, extracts. Furthermore, DNase I protection assays using the partially purified fraction from oxalic acid-induced extract revealed a footprint of a 13-bp sequence 5′GCGGGGTCGCCGA3′, termed low pH responsive element (LPRE), corresponding to the −287 to −275 bp region of the *OXDC* promoter. Our results suggest that in *F. velutipes* cells, activation of *OXDC* transcription in response to low pH is mediated by the binding of a novel transcription factor through the LPRE site in the *OXDC* promoter.

Key words: low pH responsive element, oxalic acid, pH regulated promoter.

INTRODUCTION

Micro-organisms capable of growing over a wide range of pH require (i) an efficient pH homoeostatic system and (ii) regulatory mechanisms which ensure that those molecules exposed to the environment (such as certain permeases, small metabolites and extracellular enzymes) are only produced under respective pH conditions. pH homoeostasis is the process whereby a cell maintains a relatively constant intracellular pH (pH_i) over a broad range of external pH (pH_o) values. The basis for this phenomenon is apparent modulation of primary cellular proton pumps [1–3]. These processes are under post-translational control, since pH homoeostasis functions normally in the presence of protein synthesis inhibitors [4]. In many micro-organisms, pH homoeostasis, by modulation of gene expression, involves the sigma factor of RNA polymerase [5–10]. As far as a genetic control is concerned, in a number of cases, acid-induced gene expression functions to decrease the acidity of bacterial products in acidic environments. For example, expression of lysine decarboxylase (cadA) and arginine decarboxylase (adi) in *Escherichia coli* are induced at low external pH [11]. The products of the reactions catalysed by these enzymes are decarboxylated amino acids and CO_2. Concomitant with the production and excretion of these molecules is an increase in the pH of the media due to the consumption of protons during decarboxylation [12]. In *Oxalobactor formigenes* and other organisms that degrade oxalate, the initial step in catabolism is the transport of substrate into the cell. In addition, there is a decarboxylation that yields CO_2 along with corresponding C_{n-1} monocarboxylate. It is suggested that the inward transport of divalent oxalate is coupled to the outward movement of its product, the formate, and this electrogenic antiport forms the basis for energy coupling in these organisms [13]. However, deaminases are expressed under alkaline conditions [14] and lactate dehydrogenase (ldh) activity is increased during fermentation, resulting in enhanced production of neutral end products for the maintenance of the homoeostatic condition [15–16].

Environmental acidic pH also plays an important role in regulating the expression of the pathogenesis genes of a variety of plant and animal pathogens, including *Salmonella typhimurium*, *Staphylococcus aureus*, *Vibreo cholerae*, *Erwinia amylovora*, and *Agrobacterium tumefaciens* [17–21]. pH regulation of gene expression in *Aspergillus nidulans* is mediated by the zinc-finger-containing transcription factor PacC. In alkaline culture conditions, this factor is converted into its truncated functional form in response to the ambient pH signal transduced by the products of six genes. This in turn activates the transcription of those genes required at alkaline pH and represses those whose expressions are exercised at low pH [22–26]. Rim1p, a PacC homologue from *Saccharomyces cerevisiae*, regulates the expression of genes involved in sporulation and invasive growth [27]. Whereas in *Candida albicans* growth, morphogenesis and virulence are regulated by the expression of pH-regulated gene 1 at alkaline to neutral pH [28] and pH-regulated gene 2 at acidic pH [29], so far the transcription factor(s) which is involved in the regulation of these genes is unknown. The mechanism of gene regulation by ambient pH is an intriguing subject of biological importance.

Oxalate decarboxylase (OXDC) was earlier reported from the wood-rotting fungus *Flammulina velutipes* (previously known as

Abbreviations used: BA, *Bst*EII–*Ava*I fragment; BM, *Bst*EII–*Msc*I fragment; EMSA, electrophoretic-mobility-shift assay; LPRE, low pH responsive element; LPRF, low pH responsive factor; OXDC, oxalate decarboxylase.

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Collybia velutipes) and shown to be induced at the transcriptional level by oxalic acid [30]. In this report, we show that the induction of OXDC is pH dependent. Furthermore, using electrophoretic-mobility-shift assay (EMSA) and DNase I footprinting, we demonstrate that acid-induced cells contain a LPRF (low pH responsive factor) that specifically binds to a 13-bp sequence (5′-GCGGGGTCGCCGA-3′) through a major groove of DNA.

EXPERIMENTAL

Northern analysis of OXDC

Total cellular RNA was isolated from 15-day-old mycelia using Tripure reagent (Boehringer Mannheim). RNA was denatured with glyoxal, separated on a formaldehyde/1.5 % (w/v) agarose gel (20 μg/lane), as described [31], and capillary blotted on to the Gene Screen Plus membrane as instructed in the manual. Filters were probed with a [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) labelled gene-specific DNA fragment.

Cloning of the OXDC 5′-flanking region

Genomic DNA from *F. velutipes* was isolated as described earlier [30] and the DNA was sheared using a 26-gauge needle. The extent of shearing was controlled such that the genomic fragments had an average size of 5.0 kb. The sheared DNA was used for construction of a genomic library in λgt11, and the library was screened [31] using a 1.2 kb cDNA clone of *OXDC* [32]. Five positive clones were obtained. A 2.6 kb *Eco*RI fragment was excised from clone-2 and subcloned into pTZ18U (US Biochemicals, Cleveland, OH, U.S.A.) which yielded pSC24.

DNA sequence analysis

The initial 500-bp sequence of pSC24 was determined by the dideoxy chain-termination method [33] on double stranded DNA using a Sequenase 2.0 kit (US Biochemicals) and reverse sequencing primers. To sequence the whole upstream region, it was subcloned with an engineered *Bgl*II site downstream of ATG, so that the same deletions could be utilized for a translational fusion with *LacZ* for promoter analysis. To engineer a *Bgl*II site, PCR was carried out using primers 012 and 03 (Table 1). The reactions were done with Vent exo$^-$ polymerase, under conditions recommended by the manufacturer (New England Biolabs), and amplification was carried out by an initial five cycles (1 min at 94 °C, 1 min at 40 °C and 1 min at 72 °C) followed by 25 more cycles (1 min at 94 °C, 1 min at 54 °C and 1 min at 72 °C). The PCR product was digested with *Nsi*I and rest of the 1.8-kb upstream region was isolated from pSC24 by digesting with *Hin*dIII and *Nsi*I. Both the fragments were subcloned into pBluescript II (Stratagene) at a *Sma*I site to yield pBOD. pBOD was digested with *Bam*HI, re-ligated and screened for reverse orientation, which was named pBODRO. Plasmids pBOD and pBODRO were subjected to unidirectional deletions to generate overlapping subclones [34] and sequenced. Computer-assisted sequence analysis and comparisons were done using the programs of the PC/Gene sequence analysis package (Intelligenetics Inc., Mountain View, CA, U.S.A.).

Table 1 Oligonucleotides and their sequences used in this study

Oligonucleotide	Sequence	Position
DE-1	5′-CGAGCAGCCATGGACGTCGTTCTC-3′	−1479 to −1455
DE-5	5′-TATTACCGGTCATATCTAATAATGC-3′	−448 to −472
03	5′-CTCGATCCTTTACTCGAAG-3′	−533 to −515
04	5′-CCGAGGTAACATAACGTGA-3′	−260 to −242
06	5′-TTGAGGTACCGGTCGCAGTT-3′	113 to 94
012	5′-TTAGATCTTGGTTGAACATCGGAATGC-3′	19 to −8
OP1	5′-GTGGACCCGTGACCGTAC-3′	−143 to −160
OP2	5′-CAATATTGAAGCGGTGGAC-3′	−298 to −317
OP3	5′-CGGCTATTAGAGGTCAAGGTC-3′	−391 to −411
OP4	5′-CTCGGGAGTTCACGGTC-3′	−343 to −327
OP5	5′-CCATACCCTACACCACATC-3′	−630 to −612

Primer extension analysis

A 20 nucleotide antisense primer O6 (Table 1) was synthesized corresponding to the cDNA region 117–136 bp down-stream of the translation initiation codon. Primer was radiolabelled with T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech). Labelled primer (1 ng) was added to 0.5 μg of total RNA and the reaction was carried out by rTth polymerase (PerkinElmer) as instructed in the manual. After completion of the reaction, samples were extracted with phenol/chloroform and precipitated with ethanol. The extension products were dissolved in a denaturing dye solution and analysed on a 6 % (w/v) polyacrylamide/urea gel. The size was determined by comparison with a DNA sequencing ladder.

Construction of promoter-β-galactosidase fusion constructs

Different deletion subclones of pBOD were digested with *Hin*dIII and *Bgl*II, and the resulting inserts were purified after resolving on 1.0 % (w/v) agarose gel. Inserts were ligated with *Hin*dIII- and *Bam*HI-digested pSZ211 yeast-*E. coli* shuttle vector [35] and recombinant plasmids were used to transform *S. cerevisiae* strain AH22 by lithium acetate [36]. β-Galactosidase assay was monitored as described [37]. To determine the levels of *OXDC-LacZ* gene expression a 1.0 ml fresh overnight culture of the yeast transformants were used to inoculate 10 ml of minimal medium (synthetic dextrose) without leucine. Two sets of cells were allowed to grow until D_{600} reached 1.0, followed by the addition of oxalic acid (14 mM) to one set of cells and then both sets were grown for another 2 h. Since all transformants used in this study contained multiple plasmid copies, precautions were taken to avoid errors: (i) cell culture of a similar density was used for the enzyme assay; (ii) six different transformants from the same construct were assayed.

Preparation of cell extract

The culture of *F. velutipes* [30] was induced with 14 mM oxalic acid, 14 mM potassium oxalate and 14 mM HCl for 2 h. Mycelia discs were washed with Milli Q water, ground in liquid nitrogen and resuspended in homogenizing buffer [50 mM Hepes/KOH, pH 7.5, 400 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 10 % (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol and 1 mM PMSF]. Cell suspension was lysed with glass beads (0.45–0.5 mm) in cold, using a Braun MSK mechanical disintegrator. Cell debris was pelleted at 10000 ***g*** for 10 min at 4 °C and the extract was clarified by ultra-centrifugation at 100000 ***g*** for 2 h at 4 °C. The supernatant was decanted and precipitated by the addition of saturated ammonium sulphate (up to 50 % saturation). Proteins were recovered by centrifugation at 10000 ***g*** for 20 min at 4 °C. The supernatant was subjected to a 50 %–80 % ammonium sulphate precipitation. The pellet was resuspended in binding buffer [20 mM Hepes/KOH, pH 7.5, 40 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol and 5 % glycerol]. Ammonium sulphate was eliminated by short dialysis in the cold against three changes of binding buffer. Protein concentration in the extract

was measured using the Bio-Rad assay reagent with bovine γ-globulin as the standard.

EMSA

OXDC promoter was digested with various restriction enzymes (see Figure 4) and the resulting DNA fragments were purified by agarose gel electrophoresis. The promoter fragments were generated by PCR with combination of different primers (Table 1). PCR was carried out by Vent exo$^-$ DNA polymerase (New England Biolabs) for 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min using pBOD as a template. All DNA fragments were purified by agarose gel electrophoresis and end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech), using T4 polynucleotide kinase (New England Biolabs), and separated from free nucleotide by a Sephadex G-50 spin column. EMSA was performed with [γ-^{32}P]ATP-labelled fragments of the *OXDC* promoter that were incubated with cell extract in binding buffer and 2 μg (2000-fold excess) of poly dA-dT at 4 °C for 15 min. Protein–DNA complexes and free DNA were fractionated on 5% polyacrylamide gels in Tris/glycine buffer (50 mM Tris/HCl, pH 8.3, 380 mM glycine and 1 mM EDTA) at 4 °C and visualized by autoradiography.

Inhibition with major- and minor-groove-binding drugs

The 490-bp *Bst*EII–*Msc*I fragment (BM) of the *OXDC* promoter was incubated for 30 min at 25 °C with varying concentrations of distamycin A (10 μM to 1.0 mM), actinomycin D (10 μM to 1.4 mM) and Methyl-Green (50 μM to 1.25 mM), drugs that specifically interact with the minor or major groove of the DNA double helix [32,33]. The DNA/drug mix was then analysed for binding by EMSA.

South-Western blotting analysis

Aliquots of cell extract (40 and 80 μg) from uninduced cells and from those induced with oxalic acid, was electrophoresed on an 8% (w/v) polyacrylamide gel. The protein samples were electroblotted on to a nitrocellulose filter. The membrane was incubated in blocking buffer [5% (w/v) nonfat dry milk, 25 mM Hepes, pH 7.6, 1 mM EDTA and 1 mM dithiothreitol] for 2 h and then incubated with binding buffer containing [γ-^{32}P]ATP-labelled BM probe for 2 h, followed by brief washing [10 mM Tris/HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA] and visualized by autoradiography.

DNase I footprinting analysis

The DNase I footprinting was performed as described earlier [40]. The DNA probes were obtained by restriction digestion [BM and *Bst*EII–*Ava*I fragment (BA)] and PCR amplification with end-labelled primers OP5 and OP1. Binding reactions were performed as described for EMSA in a total volume of 20 μl with DNA fragments containing 40000 c.p.m. After 15 min incubation at 4 °C, 5 μl of binding buffer with 1 mM $MgCl_2$ and 0.6 unit of DNase I were added followed by incubation at 25 °C for 3 min. The reaction was terminated with 70 μl of DNase I stop buffer (71% ethanol, 50 μg/ml tRNA and 5%-satd ammonium acetate). The samples were precipitated and resuspended in 10 μl of loading buffer [98% (v/v) formamide, 0.002% Xylene Cyanol, and 0.002% Bromophenol Blue]. Analysis of DNase I product was carried out by electrophoresis on 8% polyacrylamide/urea gel. Dideoxy sequencing reactions using labelled primer OP1 was run in parallel to locate sequence positions and protected regions.

RESULTS

Transcription of *OXDC* is induced by low pH

We have shown that *OXDC* expression in *F. velutipes* is induced by oxalic acid and that this induction involves regulation at the transcriptional level previously [30]. Since the addition of oxalic acid also lowers the pH of the medium from 5.5 to 3.0, it was of interest to determine whether this induction is mediated by the substrate oxalic acid and/or low pH. Two different pH values have been used for this analysis: pH 5.5 containing either potassium oxalate or no addition (as uninduced) and pH 3.0 containing either HCl or oxalic acid. Total RNA was prepared from the mycelia treated for 2 h and used for Northern blot analysis using *OXDC* cDNA as a probe. *OXDC* mRNA was detected only in presence of oxalic acid (pH 3.0) or HCl (pH 3.0) whereas, no signal was observed in the case of potassium oxalate (pH 5.5) and medium (pH 5.5) used as uninduced control (Figure 1). These results suggest that the expression of *OXDC* is induced by low pH and can occur in the absence of exogenous oxalate.

Cloning and characterization of *OXDC* promoter

Using 1.2 kb *OXDC* cDNA clone [30], a λgt11 genomic library of *F. velutipes* was screened and five positive clones were identified. Restriction mapping of the clones generated 2.6 kb insert from clone 1, 2 and 4, whereas clone 3 gave 2.0 and 0.9 kb inserts; clone 5 gave 1.3 and 0.33 kb inserts. Clone 2 was subcloned and sequenced for further characterization. Figure 2 shows the nucleotide sequence of the *OXDC* promoter that spans a region of 0.58 kb, while remaining upstream sequence has a significant similarity with the C-5 sterol desaturase (results not shown). Analysis of the promoter sequence revealed several transcriptional factor binding motifs e.g. Sp1 motif (-572 to -577 bp), C/EBP (-432 to -440), and an AP-1 motif (-274 to -282 bp). The 5′-CAAT-3′ sequence was found at position -174 to -178 and a TATA element is present at -39 to -44 bp. Transcription start site of the *OXDC* gene was mapped as an adenine residue at -11 position with respect to ATG (results not shown). These data are in complete agreement with

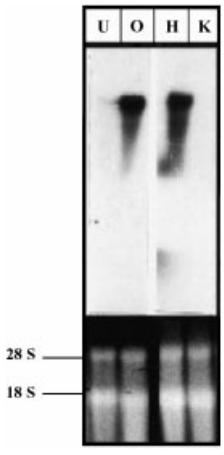

Figure 1 Low pH activates transcription of the *OXDC* gene in *F. velutipes*

Northern blot analysis of total RNA was performed using a full-length cDNA probe for *OXDC*. The bottom panel shows ethidium bromide staining of the 28 S rRNA to show consistency of loading samples. The levels of *OXDC* mRNA increases only in oxalic acid- and HCl-treated samples (lane O and H respectively). Uninduced (lane U) and potassium oxalate-treated (lane K) samples did not show any transcript.

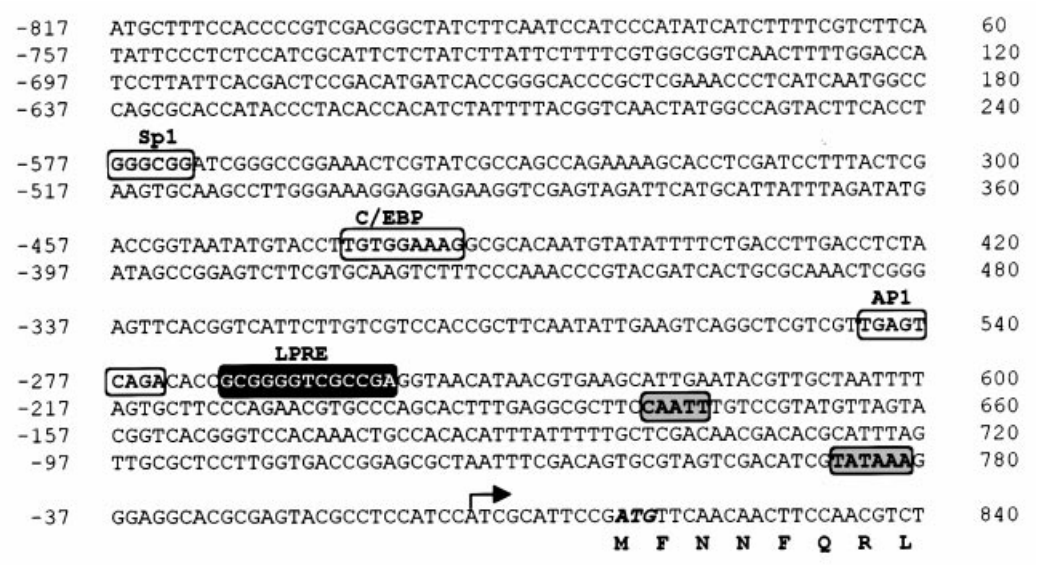

Figure 2 Nucleotide sequence of the *OXDC* promoter and its 5′-flanking region

The number of the left column pertains to the first nucleotide location in each lane with respect to ATG (in bold italic) as +1. The transcription initiation site of the *OXDC* gene is marked with an arrow. Putative *cis*-acting motifs such as, Sp1, AP1 and C/EBP elements are boxed; TATA and CAAT boxes are marked by grey boxes. The putative low pH responsive element (LPRE) sequence is marked by a black box.

the 5′ end determined by 5′-RACE [32]. *In vivo* promoter analysis was carried out using overlapping promoter fragments as *OXDC-LacZ* translation fusion in *S. cerevisiae* (AH22). The results did not show any induction in response to oxalic acid and low pH, while it was active at basal level (results not shown).

Identification of *trans*-acting factor(s) that bind to the *OXDC* promoter

EMSAs were carried out in order to determine if a specific *trans*-acting factor binds to the promoter to activate *OXDC* transcription in response to low pH. The extracts were prepared from mycelia that were induced with oxalic acid, HCl, potassium oxalate and from uninduced cells as control. Initially, we assayed the BM probe in EMSA using the 50 %-satd ammonium sulphate fraction and the 50–80 %-satd fraction. The results showed that 50–80 %-satd ammonium sulphate-cut protein fractions from oxalic acid- and HCl-induced cells were capable of specific gel retardation of the BM probe (Figure 3A). Therefore, the subsequent analyses were done using this protein fraction, hereafter referred to as partially purified protein. To determine the optimal amount of the protein, a dose–response experiment was carried

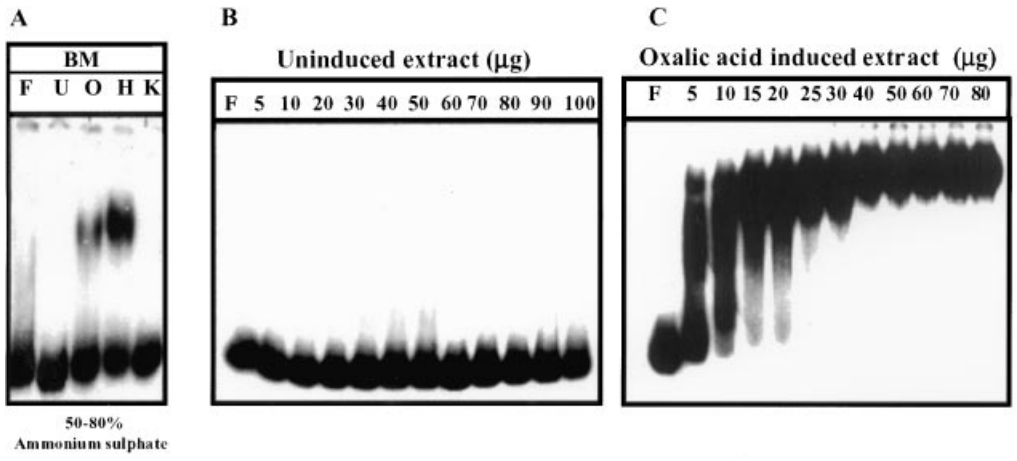

Figure 3 *OXDC* promoter binds with a protein factor from only oxalic acid- and HCl-induced extracts

(**A**) EMSAs wherein the binding reaction was carried out with the BM probe (506 bp) using partially purified (50–80 %-satd ammonium sulphate precipitated protein fraction) protein from uninduced (lane U), oxalic acid-induced (lane O), potassium oxalate-induced (lane K) and HCl-induced (lane H) mycelia. In a separate set of experiments, the binding reaction was carried out with 2 ng of BM probe and increasing amounts of protein from uninduced (**B**) and oxalic acid-induced (**C**) extracts. Lane F represents the free probe.

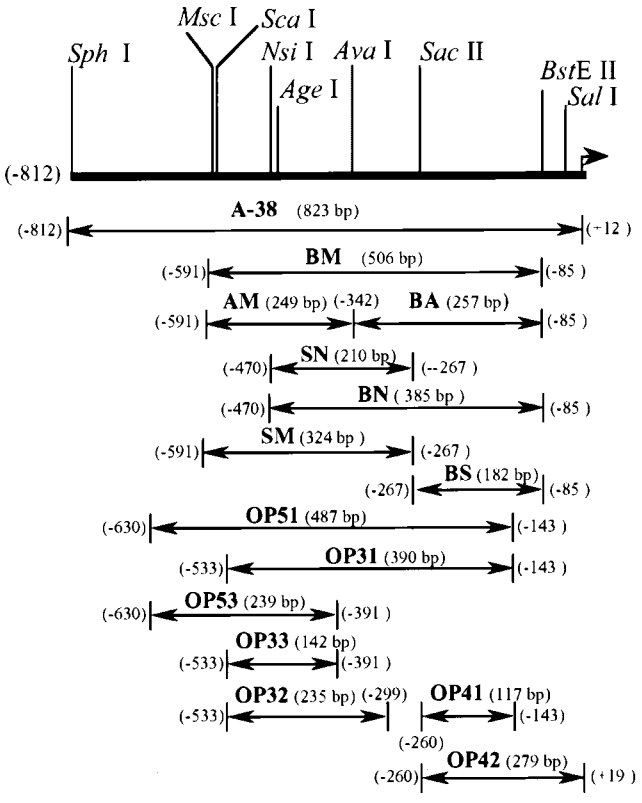

Figure 4 Physical map of the *OXDC* promoter

Probes encompassing different regions of the promoter used in EMSAs are shown along with their size in bp and their respective position along the promoter at arrowheads. These probes were generated by restriction digestion and PCR amplification as described in the Experimental section.

out. A fixed amount of the BM probe was combined with increasing amounts of partially purified proteins from uninduced (5–100 μg, Figure 3B) and oxalic acid-induced (5–80 μg, Figure 3C). The results showed that the BM probe was retarded to a single slow-migrating band when oxalic acid-induced fraction was used. In addition, the probe was progressively and quantitatively shifted as the amount of protein was increased displaying good dose–response (Figure 3C). Inspection of lanes 5, 10, 15 and 20 seems to show the presence of multiple shifted species. In an attempt to confirm that the BM probe results in a single complex, the EMSA was carried out at lower protein concentrations. A single retarded complex was seen even at the concentration of 1.25 μg (results not shown). We determined that at 40 μg input of induced fraction, the binding was at near saturation. However, the same probe was not retarded when the uninduced fraction was used even at the highest dose of 100 μg (Figure 3B). Similarly, HCl-induced extract also showed a dose–response and saturation profile as observed in oxalic acid-induced extract (results not shown). These results showed that a protein or a protein complex from acid-induced cells, but not from uninduced cells, specifically recognized the BM probe.

The EMSA was carried out with various deletions of the BM probe to determine the minimal sequence required for the factor binding. A series of truncations of the largest fragment A38 were constructed (Figure 4) using restriction digestion or PCR and binding reactions were carried out with oxalic acid-induced partially purified protein fraction. The results showed that the probe A38 and OP51 were retarded in the presence of induced protein fraction but not with uninduced fraction (results not shown), consistent with the retardation of the BM probe (Figure 3A). However, the probe OP31 resulted in a specific retarded band both in uninduced and induced protein fraction (results not shown). The binding was specific since a 100-fold excess of cold OP31, when used as a competitor, could abolish the complex formation, whereas a 100-fold excess of cold pUC19 could not do so (results not shown). Since BM/OP51 resulted in a single retarded complex under only induced conditions, retardation with OP31 can be explained as follows: the retardation could be due to the presence of a small molecular mass protein(s), both in uninduced as well as in induced conditions, which may be displaced by structural distortion when probed with the larger fragments BM or OP51. Upon induction this structural distortion favours the binding of the uninducible factor to LPRE. Further truncation from both 5′ and 3′ ends spanning -533 to -143 and the fragments that bisected the BM and OP51 probe did not show any binding (results not shown). Interestingly, none of the other smaller fragments analysed were able to function in EMSA (results not shown). Thus, we conclude that the minimal functional segment in factor binding spans -533 to -143 of the *OXDC* promoter. In order to establish the specificity of the binding, the BM probe along with 40 μg of oxalic acid-induced protein fraction was assayed in the presence of a 1000-fold excess of non-specific pUC19 DNA or a 20-fold and 50-fold excess of cold BM DNA. We observed that the binding was not impaired in the presence of non-specific competitor pUC19 DNA (results not shown). However, the binding was quantitatively abrogated in the presence of 20- and 50-fold excess of cold competitor BM DNA (results not shown). This showed that the binding of the protein(s) was specific to the BM probe DNA. However, OP51 can also compete for the binding site when used as a cold competitor (results not shown), whereas other promoter fragments could not effect the complex formation.

Binding does not require divalent cations and is resistant to high ionic strength

In order to determine the nature of interaction involved in complex formation, the binding reaction was carried out in presence of divalent cations. DNA binding gradually decreased as a function of salt concentration and was abolished only at salt concentrations as high as 2.5 M KCl (Figure 5A). This suggests that ionic interactions may be only marginally involved in complex formation, whereas binding in the presence of different concentrations of EDTA remained unaffected, and the complex was stable, even at a concentration of 100 mM EDTA (Figure 5B), demonstrating that divalent cations are not required for the complex formation.

Protein factor interacts with the *OXDC* promoter through the major groove of the DNA molecule

EMSA was carried out to determine whether the factor binding to DNA element interacts through the major or the minor groove of the DNA double helix. Binding reactions were carried out in the presence of actinomycin D and distamycin A, which specifically bind to the minor groove [38], and methyl-green that interacts with the major groove [39]. Incubation of BM probe alone with actinomycin D decreases its mobility, while incubation with methyl-green and distamycin A did not affect the mobility of the free probe. The DNA–protein complex formation was not

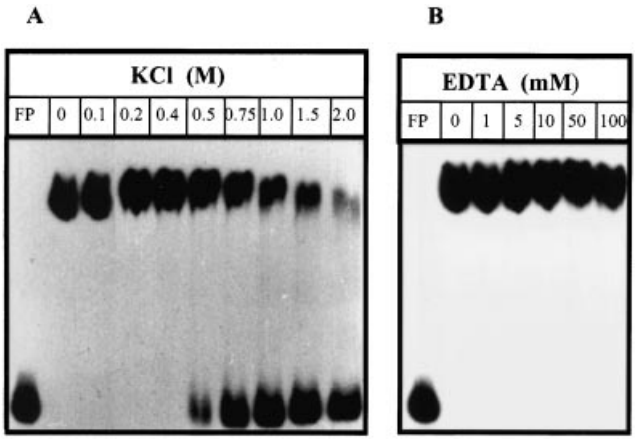

Figure 5 Complex formation does not require divalent cations and is resistant to high ionic strength

(**A**) EMSAs wherein the binding reactions were carried out with the BM probe and 40 μg of induced extract at KCl concentrations ranging from 0.1 to 2.0 M. (**B**) EMSAs carried out in the absence of EDTA (lane 2) and in the presence of EDTA at concentrations ranging from 1 to 100 mM using the BM probe and 40 μg of induced extract. Lane 1 represents the free probe.

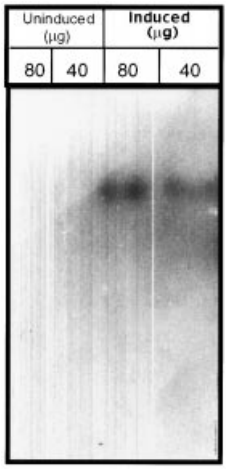

Figure 7 South-Western analysis

Protein (40 and 80 μg) from uninduced and oxalic acid-induced extracts were fractionated through an 8% native-polyacrylamide gel. Proteins were blotted on to a nitrocellulose membrane and probed with the labelled BM DNA fragment in binding buffer.

affected when the probe was incubated in actinomycin D or distamycin A (Figures 6A and 6B). In contrast, the complex formation was inhibited by methyl-green in a dose-dependent manner (Figure 6C). These results clearly demonstrate that protein factor interacts with DNA through the major groove.

South-Western analysis

The South-Western analysis [11] was used as an independent assay for the identification of the *trans*-acting factor. We used partially purified proteins, 40 and 80 μg of each, from uninduced and oxalic acid-induced cell extracts using the BM probe. The analysis using SDS/PAGE did not show any binding, whereas native PAGE showed a discrete band with induced cell extract but not with uninduced extract (Figure 7). The absence of DNA binding in SDS/PAGE could be due to either the irreversible denaturation of proteins or the protein which binds to the DNA being a multi-protein complex, like CCAAT-binding protein ('CBF') which is a heterotrimeric protein, and the DNA-binding domain is formed by the contribution of all three polypeptides [41].

Inducible factor protects a 13-bp sequence of the *OXDC* promoter

DNase I footprint assay was used to determine the sequence bound by the inducible *trans*-acting factor. End-labelled BM and OP51 were used as the sense and antisense strand probe, incubated with partially purified extracts from oxalic acid-

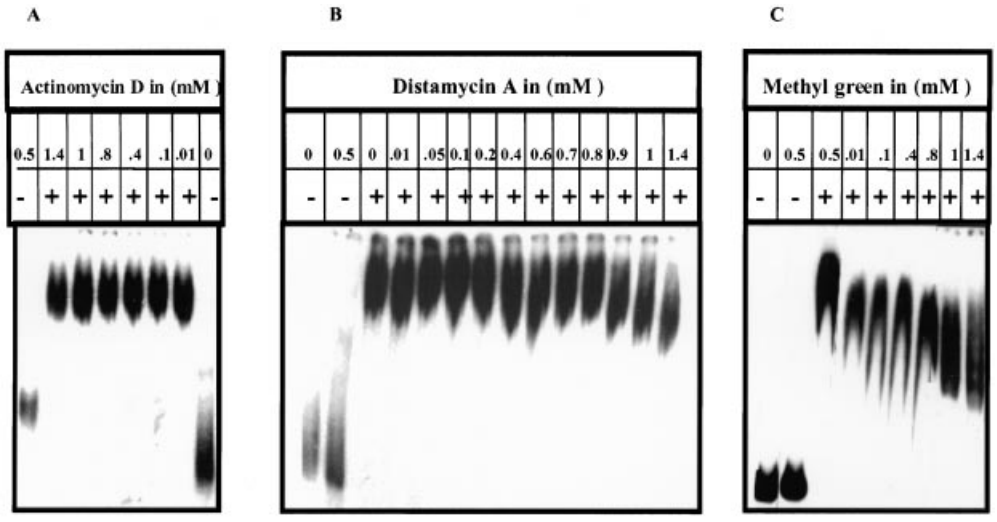

Figure 6 Factor interacts with the *OXDC* promoter through the major groove of DNA

Assays wherein the binding reaction was carried out with BM probe alone and increasing amounts of actinomycin D (**A**), distamycin A (**B**) and Methyl-Green (**C**). Partially purified protein from oxalic acid-induced extract was added subsequently and the complex thus formed was analysed. The presence (−) and absence (+) of protein extract in the reactions are indicated.

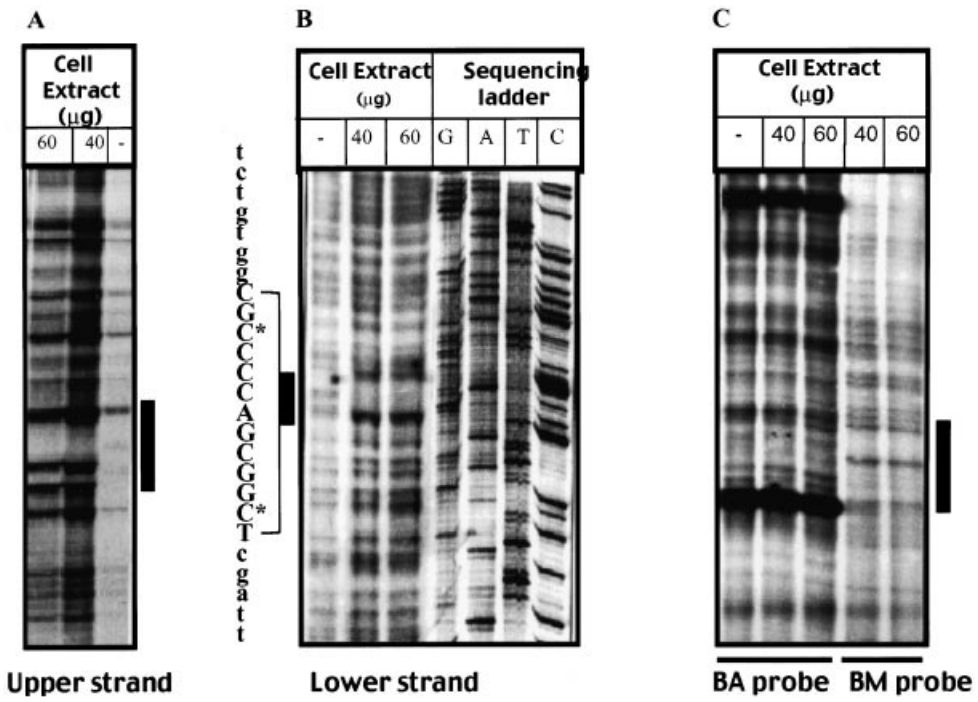

Figure 8 DNase I footprinting analysis of the *OXDC* promoter

(**A**) DNase I protection assay of the BM probe (upper strand) in presence of 40 and 60 μg of induced extract and without extract (lane —). (**B**) Footprint wherein partially purified protein from oxalic acid-induced extract was incubated with the labelled lower strand of the OP51 DNA fragment. Lanes G, A, T and C indicate the sequence ladder of G, A, T and C reactions respectively. Sequencing was carried out by labelled OP1 primer with vent exo$^-$ DNA polymerase and pBOD as template. DNase I protected regions are shown as solid boxes and their respective sequence is schematically presented. DNase I hypersensitive sites are marked with an asterisk. (**C**) DNase I protection assay with the BM and BA probes. The first three lanes at the left side represent the ladder from the BA probe while the last two lanes represent the ladder from the BM probe.

induced cells and then treated with DNase I. As a control, no extract was added to one of the reactions to serve as free DNA. The results showed a 13-bp sequence with three hypersensitive sites in the upper strand (Figure 8A) and two hypersensitive sites in the lower strand (Figure 8B). Comparison of the adjacent sequence data revealed that the protected sequence was 5′-GCGGGGTCGCCGA-3′ spanning −287 to −275 of the *OXDC* promoter. Importantly, this segment of the promoter is within the fragments that bound the inducible factor in the EMSA (Figure 3). In order to confirm that the factor can protect the site from DNase I in BA probe, which also contains the target site but is shorter in size, a DNase I protection assay was performed. It did not give any protection (Figure 8C) which is consistent with the data where the BA probe failed to show any complex formation. These data strongly suggest that the induced factor protects a 13-bp site only in the BM and OP51 probes. A thorough search of this sequence in the transcription factor motif database yielded no match suggesting that this might be a novel motif.

DISCUSSION

Our results provide the evidence that low environmental pH stimulates *OXDC* mRNA expression in *F. velutipes*. It has been shown that the pH of the media increases due to the consumption of protons during decarboxylation for the maintenance of homoeostatic conditions [2]. Similarly, *F. velutipes* may also have evolved the mechanism to combat this stress by enhancing the expression of the *OXDC* gene. In this study, we have isolated and characterized the genomic clones containing the 5′-flanking region of *OXDC* to identify the transcriptional control elements. *In vivo* analysis was carried out in *S. cerevisiae* because the transformation system in *F. velutipes* is not available. The promoter showed only basal level activity and there is no oxalate and/or low pH responsive transcription. The absence of oxalic acid or low pH responsiveness in *S. cerevisiae* could be due to the absence of respective transcription factor or lack of similar signalling component(s) at low pH.

An important step towards understanding the molecular mechanism of oxalic acid- and/or low pH-dependent regulation is the identification of *trans*-acting regulatory proteins. We have identified a protein complex from partially purified cell extract, which specifically binds to 506 bp (BM) and 487 bp (OP51) fragments. The complex formation occurs only with the HCl- or oxalic acid-induced protein extracts, whereas uninduced and potassium oxalate-induced extracts did not show any binding. DNA fragments containing deletions on either side of the OP51 region and sub-fragments of BM did not show any binding, moreover they could not abolish the complex formation when used as a competitor.

The failure of sub-fragments in complex formation may be due to the co-operative protein–protein interaction and stabilization of the protein–DNA complex. However, involvement of DNA structure (e.g. base composition, length of DNA and anisotropy), apart from defined sequence elements in complex formation, cannot be ruled out [42–44].

Characterization of the protein–DNA complex revealed several striking features of the interaction. Although there was a slight concentration-dependent decrease in complex formation in the presence of salt, the complex was observed even at 2 M KCl,

suggesting that the interaction is specific and ionic interactions may play a marginal role in complex formation. Moreover, using dephosphorylated cell extract did not affect the binding (results not shown). *In vitro* binding inhibition with the major-groove-binding drugs demonstrates that binding proteins interact with the target site through the major groove of DNA. The majority of site-specific DNA-binding proteins predominately recognize the specific sequences through the major groove by hydrogen binding with the bases and electrostatically interacting with the phosphate groups. However, there are examples, such as TBP (TATA-binding protein), T cell receptor α-gene enhancer and the high mobility group 1 (Y) protein which binds specifically to the minor groove of DNA and induce bending [45–47]. Furthermore, DNase I protection assays revealed a footprint of a 13-bp sequence 5′-GCGGGGTCGCCGA-3′, corresponding to the -287 to -275 region of the *OXDC* promoter, though the footprint windows appeared to be compressed. This could be due to the unfavourable distance of the end-labelled end of OP51 (125 bp) and BM (185 bp) fragments from the mapped LPRE. In addition, the probe fragment in both the cases was about 500 bp and thus the compression in the footprint is not unlikely. Indeed, footprint windows in DNase I protection assays with DNA probes of approx. 500 bp, which occurs around 200 bp away from the labelled ends, are often difficult to detect [48].

These results suggest that the *F. velutipes OXDC* gene was induced by acidic pH and not by exogenous oxalate. Interestingly, recently it was shown that the oxalate decarboxylase enzyme in *B. subtilis* is also induced by acidic pH but not by oxalate [49], indicating that the regulation of the *OXDC* gene is evolutionarily conserved. The promoter region of the *F. velutipes OXDC* gene revealed an acid-inducible DNA binding factor(s) and a novel *cis*-element recognized by this factor. The binding of this factor is observed only in acid pH, thus behaving as a regulated transcription factor. The results all together suggest that the acid-inducible factor is a novel transcription factor. Our interest would be to clone and characterize the low pH-inducible transcription factor and determine the other genes that are regulated by this factor.

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