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Molecular characterisation of *meaB*, a novel gene affecting nitrogen metabolite repression in *Aspergillus nidulans*

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Abstract Mutations within the *meaB* gene elicit the inappropriate expression of several activities subject to nitrogen metabolite repression in *Aspergillus nidulans* and also have some unrelated phenotypic effects. We have cloned *meaB* and isolated a full length cDNA clone. Northern analysis has shown that *meaB* expression is not subject to nitrogen metabolite repression. *meaB* encodes a novel protein of 418 amino acids and contains a significantly high number of S/TPXX motifs, a motif common in transcriptional regulatory proteins. We have sequenced three mutations within *meaB*, two of which have an identical phenotype to that produced by gene disruption.

Key words: Nitrogen metabolite repression; *meaB*; *areA*; (*Aspergillus nidulans*)

1. Introduction

The utilisation of nitrogen sources in the environment by the filamentous fungus *Aspergillus nidulans* involves a strictly regulated and co-ordinated pattern of gene expression [1]. *A. nidulans* is capable of utilising a wide range of nitrogen containing compounds as sole nitrogen source, including ammonium, nitrate, nitrite, purines, amides and most amino acids. In the presence of ammonium or L-glutamine the expression of those activities required for the uptake and utilisation of other nitrogen sources is prevented, a phenomenon known as nitrogen metabolite repression [2–6]. Only in the absence of a repressing nitrogen source are the genes encoding these activities expressed [1].

Nitrogen metabolite repression is mediated by the activity of a positive *trans*-acting gene *areA* [6,7]. Null mutants of *areA* are characterised by their inability to grow on nitrogen sources other than ammonium and L-glutamine [2,6]. The product of this gene, AREA, is a member of the GATA family of DNA binding proteins [8–10], capable of binding at promoter sequences containing the core motif -GATA- to direct gene expression [11,12]. *areA* activity is regulated, in response to the nitrogen state of the cell, by two independent mechanisms. The first mechanism involves the 3′ UTR of the transcript, which affects AREA levels via transcript stability [13]. Disruption of this region leads to increased transcript stability under repressed conditions and results in a partially derepressed phenotype characterised by the inappropriate expression of activities normally subject to nitrogen metabolite repression. A similar phenotype results from either disruption of the last 12 C-terminal amino acids or specific amino acid substitutions in the DNA binding domain, possibly via altered protein-protein interactions [13]. Glutamine appears to be the key metabolic effector in nitrogen metabolite repression [1].

In *Neurospora crassa* regulation of nitrogen metabolism is achieved via the AREA homologue NIT2 [14,15], which is negatively regulated by NMR, the product of the negative regulatory gene *nmr* [16]. NMR interacts with the DNA binding domain and C-terminus of NIT2 and this disrupts DNA binding in vitro. Disruption of *nmr* results in the general derepression of activities subject to nitrogen metabolite repression within the cell, as does deletion of the C-terminal domain of *nit-2* [17]. AREA and NIT2 show perfect conservation of their last nine amino acids, together with 98% identity over their DNA binding domains [9]. Since these two domains are important for both the NMR-NIT2 interaction [17] and correct modulation of AREA function [13], AREA might well be regulated by an NMR-like protein in *A. nidulans*. Indeed, *nit-2* can complement an *areA*$^-$ phenotype, and shows partial regulation [18].

Methylammonium can induce nitrogen metabolite repression in the wild type and is toxic, its toxicity being reversed by ammonium and mutations increasing the conversion of the nitrogen source to ammonium [1,19,20]. Two mutations at a single locus, *meaB6* and *meaB20*, were selected as conferring resistance to 100 mM methylammonium when 10 mM nitrate was provided as a nitrogen source, through derepression of nitrate reductase [6,19,21,22]. A third mutation at this locus, *meaB100*, was selected as a supressor of the *areA*^{r}*-130* mutation on nitrate and nitrite [5]. In addition to nitrate reductase, these mutations result in the derepression of a number of other activities subject to nitrogen metabolite repression including nitrite reductase, xanthine dehydrogenase and urate oxidase [21].

The general nitrogen derepressed phenotypes of *nmr*$^-$ mutants and *meaB*$^-$ mutants, together with the similarity of nitrogen metabolite repression in *Neurospora* and *Aspergillus* have given rise to speculation that *nmr* and *meaB* may be isofunctional and homologous [19]. However, *meaB* mutants also show additional pleiotropic effects [19] not seen in *nmr* mutants (Polley and Caddick unpublished). These effects include reduced utilisation of L-arginine, L-aspartic acid, L-ornithine, L-proline, and L-threonine as carbon sources by *meaB6* strains, whilst all *meaB* mutations affect the toxicity of amino acid analogues. In complete medium *meaB6* and *100* mutants are resistant to *p*-fluorophenylalanine, whilst *meaB20* strains show variation in their phenotypes, according to the batch of complete medium used, ranging from hypersensitive to resistant, presumably in response to fluctuations in the phenylalanine content of the medium [19]. In minimal media *meaB20* strains are hypersensitive to this analogue. All three mutations also result in hypersensitivity to nitrite toxicity which is non-additive to that caused by mutations at the

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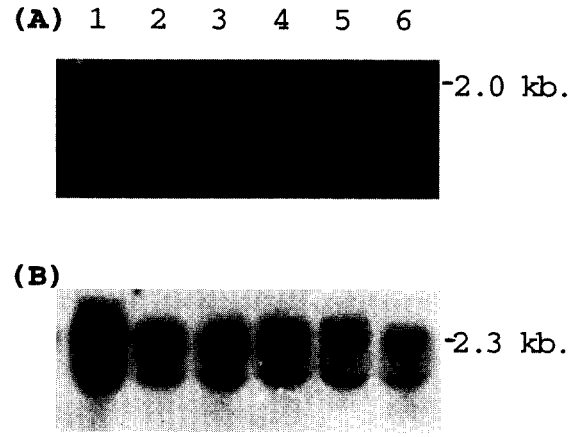

Fig. 1. *meaB* transcript levels during growth under derepressing and repressing nitrogen regimes in *meaB6*, *−20* and *−100* mutants and a *meaB*$^+$ strain. Total RNA was separated on a 1% denaturing formaldehyde agarose gel and probed with *meaB* (A) and actin (B) specific probes. The size of the transcript was determined using Boehringer RNA molecular weight markers. Lanes: 1, *meaB6* derepressed; 2, *meaB20* derepressed; 3, *meaB100* derepressed; 4, *meaB6* derepressed; 5, *meaB*$^+$ repressed; 6, *meaB*$^+$ derepressed.

ninA locus [4]. Given that *ninA* probably affects nitrite efflux [5] one possible role for MEAB is that it affects the flow of metabolites across membranes, and possibly their compartmentalisation within the cell [19]. Mutation of the *meaB* gene product could lead to altered gradients of metabolites within the cell, affecting protein synthesis and nitrogen metabolism within the cell.

In this article we report the cloning and sequencing of *meaB* together with its characterisation at a molecular level. *meaB* encodes a novel protein product which is not homologous with NMR, but might be a new transcription factor.

2. Materials and methods

2.1. Strains and growth conditions

The following *A. nidulans* strains were derived from strains provided by Arst [19]: *pabaA1* (wild type), *yA2 argB2 meaB6*, *yA2 inoB2 meaB6*, *biA1, inoB2 meaB20*, *biA1 meaB100*. Media and supplements used were those of Cove [23], whilst mycelial suspensions were grown overnight in liquid culture and assayed for nitrate reductase activity as described by Platt et al. [13].

2.2. General molecular genetic techniques

Standard molecular techniques were performed as per Sambrook et al. [24]. *A. nidulans* total RNA was isolated according to the method of Sokolovsky et al. [25], whilst total genomic DNA was prepared as described by Raeder and Broda [26]. Transformation of *A. nidulans* with foreign DNA was performed using the method of Tilburn et al. [27]. Alkaline and northern transfers were performed using Zeta Probe (Bio-Rad) according to the manufacturers instructions, as were stringent hybridisation procedures. Bluescript (Stratgene) was used for subcloning, whilst pILJ16 [28], containing the *argB* gene, was used to complement *argB2* in co-transformation experiments.

Computer analysis of protein sequences was performed using the PROSITE program, whilst homology searches were performed using the TFASTA and BLITZ programs. All programs were made available via the EBI.

3. Results and discussion

3.1. Cloning meaB

The hypersensivity to nitrite toxicity of all *meaB* mutants [19] formed the basis of our selection procedure. An *A. nidulans* total genomic cosmid library [29], was screened to identify cosmids capable of restoring growth on 30 mM nitrite plus 10 mM ammonium in a *meaB6* strain. The library was ordered into 33 pools of 8 clones, each clone specific to chromosome III [29]. A single pool gave higher than background levels of colonies resistant to nitrite toxicity. Each of the eight clones from the pool were individually used to co-transform, together with pILJ16, *argB2 meaB6* strains. *argB*$^+$ transformants were selected and tested for *meaB* phenotype. A single cosmid W02 E01 restored wild-type growth at high frequency. W02 E01 was digested with a range of restriction endonucleases and the complete digests used for co-transformation in place of the intact cosmid. Digestion of the cosmid with *Kpn*I, *Eco*RV, *Bam*HI or *Xho*I did not prevent it from rescuing *meaB*$^+$ activity at a high frequency, indicating that none of these enzymes cuts within an esssential region of *meaB*, allowing it to function when inserted heterologously [30]. A single 5.2 kb *Kpn*I fragment, capable of restoring *meaB* activity at high level, was subcloned into Bluescript. cDNAs cross-hybridising to this region and capable of restoring *meaB*$^+$ activity were identified in a 24 h *A. nidulans* expression library constructed by Aramayo and Timberlake [31]. The cDNAs included a clone with an insert approx. 2 kb in size which northern analysis has shown to be the approximate size of the full length transcript (Fig. 1). This insert was fully sequenced, together with several partial cDNA clones and the genomic copy.

3.2. Sequence and predicted protein product of meaB

The *meaB* transcript contains a single long open reading frame which extends through nucleotides +1 to 1698 (Fig. 2) and encodes a putative protein of 418 amino acids. The putative initiation codon lies within a favourable initiation context (70% match to consensus sequence TCACAATGGC) [32]. Within the transcribed region, just upstream of the putative translation start site, lies an out of frame ATG situated in a far less favourable initiation context (only 40% identity to consensus sequence), which does not fit the consensus sequence identified as preventing downstream initiation ('leaky scanning') in *A. nidulans* [33]. Motif searches with the PROSITE program revealed no major functional structural motifs within the protein, although it does contain 12 potential phosphorylation sites and four potential glycosylation sites.

BLITZ searches of the SWISS-PROT database revealed 26% identity with the largest subunit of RNA polymerase II (RPBhI) of *Arabidopsis* over the protein's entire length. This region of identity maps mainly to the carboxy-terminal domain (CTD) of RPBhI, a region composed of a heptapeptide

Table 1
Nitrate reductase activity in strains with different *meaB* alleles

Allele	Nitrogen derepressed	Nitrogen repressed
wild type	118 ± 6	4 ± 1
*meaB*Δ	86 ± 10	23 ± 3
meaB6	83 ± 5	25 ± 4
meaB20	89 ± 10	18 ± 2

Cells were grown in liquid culture for 16 h at 37°C in inducing repressed (20 mM NO_3^-, 40 mM NH_4^+) or inducing derepressed (20 mM NO_3^-) conditions. Three replica trials were used for each condition, and three replica readings taken for each trial. Activities are given as nmol nitrite produced/min per mg soluble protein.

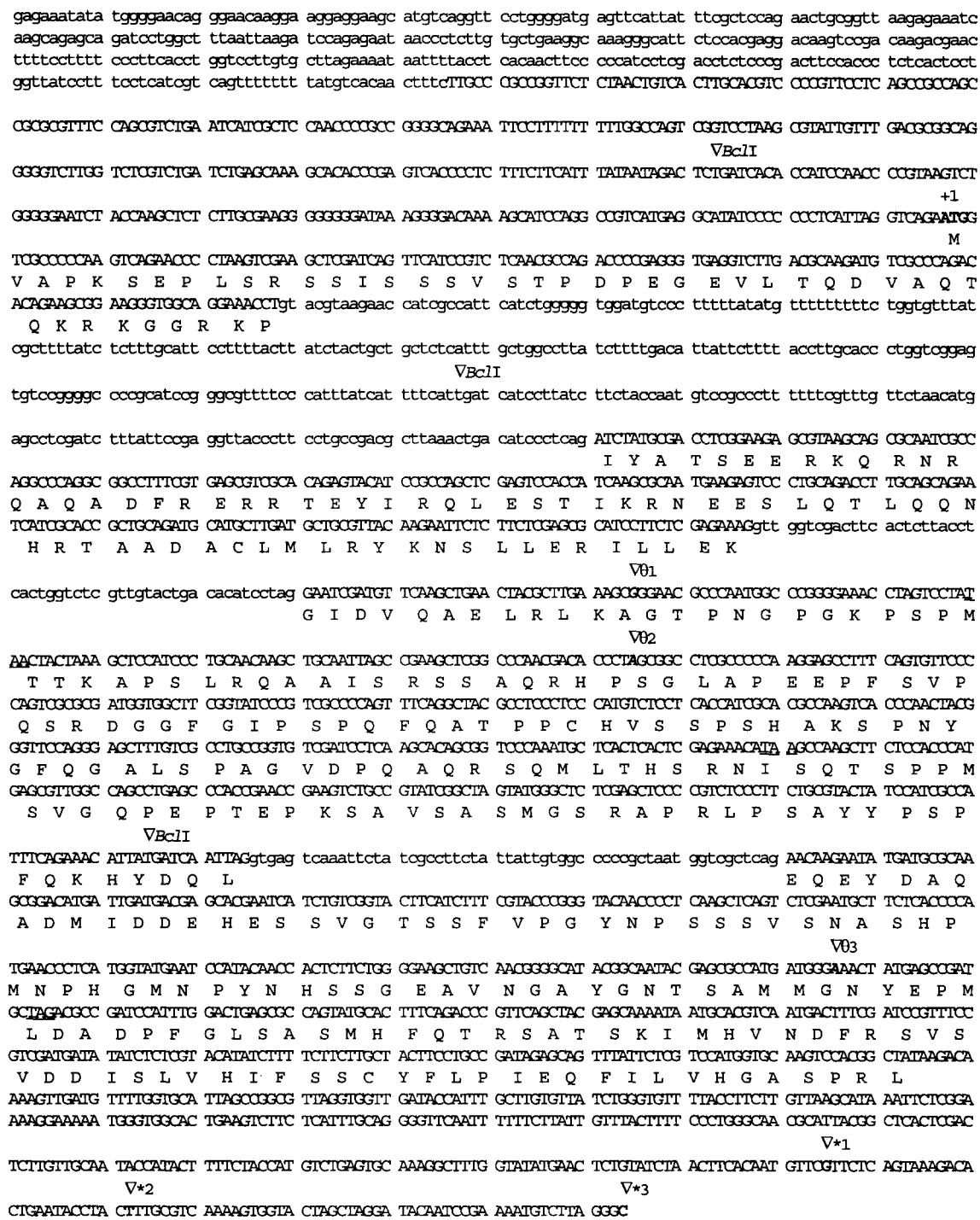

Fig. 2. Nucleotide sequence of *meaB* together with its flanking regions. The ATG translation start codon is labelled +1, whilst the predicted protein sequence is shown beneath the DNA sequence. Nucleotides of interest are denoted in bold lettering. Polyadenylation sites are surmounted by ▽*, whilst termination codons brought in line by the frame shift mutations are underlined. ▽θ1 marks the guanine deleted in *meaB100* strains resulting in termination of the transcript at nucleotide 794. The frame shift results in the following novel sequence at the C-terminus after residue 128: ERPMARGNLVL. ▽θ2 marks the adenine deleted in *meaB6* strains resulting in termination of the transcript at nucleotide 1076. The frame shift results in the following novel sequence at the C-terminus after residue 161: AASPPRSLSVFPSRAMVASVSRRPSFRLPLPPMSPHHRTPSHPTTGSRELCRLPVSILKHSGPKCSLTRET. ▽θ3 marks the adenine deleted in *meaB100* strains resulting in termination of the transcript at nucleotide 1500. The frame shift results in the following novel sequence at the C-terminus after residue 350: TMSRC. The *Bcl*I sites shown were used to construct pMB19. Introns and non-transcribed regions are listed in lower-case text whilst upper-case letters denote the transcript.

(YTPTSPK) repeat [34,35] and thought to be involved in regulating the RNA polymerase II activity via specific localisation of the protein to discrete subnuclear domains [36,37]. MEAB contains no repeats of this nature, and much of the residue identity results from the serine-rich sequences of the two proteins. There are, however, 11 S/TPXX motifs in

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MEAB    RKQRNRQAQADFRERRTEYIRQLESTIK
        |  || ||  || ||  ||::||   |
Cin5p   RAAQNRSAQKAFRQRREKYIKNLEEKSK
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Fig. 3. Sequence comparison of MEAB and Cin5p, a putative regulatory protein in yeast. The two sequences show 45% identity and 56% similarity over a 28 amino acid region.

MEAB which have been identified as occurring at high frequency in transcriptional control proteins [38]. The frequency of S/TPXX motifs in MEAB is 26×10^{-3}, which is comparable to known regulatory proteins and far higher than that found in general proteins [38]. Random reassortment of the amino acid sequence of MEAB predicts that only six such sequences should be found. A TFASTA search revealed 45% identity to a 31 amino acid region of Cin5p (Fig. 3), a putative yeast regulatory protein (accession number u16780.gb_pl, Hoyt, M.A., unpublished). No specific function has been reported for this region of Cin5p.

Pairwise alignment of MEAB and NMR has revealed no significant similarity, with the two proteins, showing at best only 17% identity over a 90 amino acid stretch. The number of S/TPXX motifs in the protein sequence together with its similarity to RPBhI and a putative yeast transcription factor Cin5p does raise the possibility that MEAB is actually a DNA binding protein, unlike NMR which binds directly with NIT2 at the DNA binding domain and C-terminal domain [17].

Sequencing of five different cDNAs has revealed the existence of at least three polyadenylation sites within the 3′ region of the sequence, although there is an absence of the polyadenylation signal AATAAA. This sequence, whilst common in higher eukaryotes, is absent from many filamentous fungal genes, and 3′ transcript heterogeneity is also a common feature of many fungal genes [32].

Comparison of the cDNA and genomic sequences revealed the existence of three introns; the first intron is 332 nucleotides which is unusually large for *A. nidulans* [32]. Elimination of all three introns by homologous intergration, using a partial *meaB* cDNA clone had no obvious effect on phenotype (data not shown). It is therefore unlikely that any of the three introns have any overt functional significance. The 5′ and 3′ splice sites are well conserved, all three introns conforming to the consensus sequence GTAY/AGTT/C and ACAG, respectively [32].

3.3. Expression of meaB

Northern blot experiments have shown that *meaB* expression is largely independent of nitrogen metabolite repression, yielding a single RNA transcript of approx. 2.0 kb (Fig. 1). The amount of *meaB* mRNA was relatively low, approx. 5% of the level of γ-actin mRNA [39], which is consistent with the very low codon bias of the gene. Total mRNA extracted from *meaB*$^-$ strains showed no change in the level or size of transcript produced, suggesting that *meaB* is not autoregulated.

3.4. Characterisation of meaB6, 20 and 100 mutations and deletion of the meaB gene

Using the *argB2* co-transformation system, internal PCR fragments of *meaB* were used to identify regions of the gene capable of restoring function in *meaB6, 20* and *100* strains. A series of 300 bp PCR fragments was made, and three fragments identified as spanning the various mutations were amplified from the respective mutant strains and sequenced.

All three are frame shift mutations resulting from single base pair deletions, leading to truncation of the *meaB* product (Fig. 2). *meaB100* and *6* cause translation to terminate at residues 140 and 233, respectively, whilst *meaB20*, which confers the weakest derepression of the three mutations, causes termination at residue 356. This proves that we have successfully cloned *meaB*, and suggests that the N-terminal 356 residues retain some residual function.

Deletion of *meaB* was achieved using the construct pMB19 which contains a modified version of the 5.2 kb *Kpn*I fragment identified in *3.1* cloned into Bluescript. A *Bcl*I digest was used to delete the *meaB* sequence between nucleotides −124 and 1220 in this fragment (see Fig. 2). The deleted region includes the putative translation initiation codon. The *argB2* gene was amplified from the vector pILJ16 using primers with

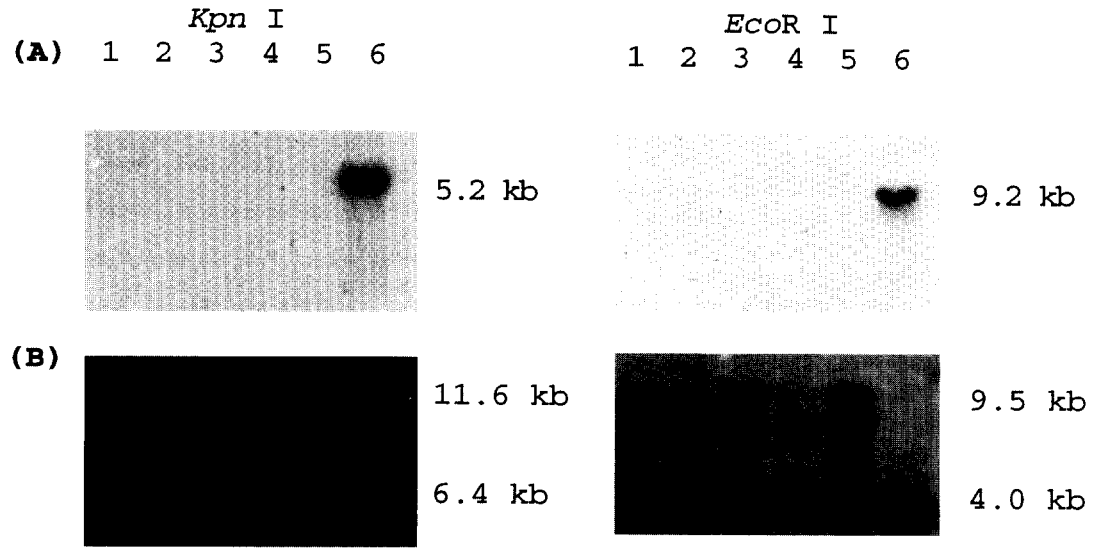

Fig. 4. Southern analysis of genomic DNA recovered from *meaB* mutants generated by gene disruption with pMB19. Sizes are indicated in kb. DNA was probed with a 300 bp segment of *meaB* contained entirely within the region deleted in pMB19 (A) and the *argB* gene (B). Lanes: 1–5, *meaB* mutants; 6, wild-type control.

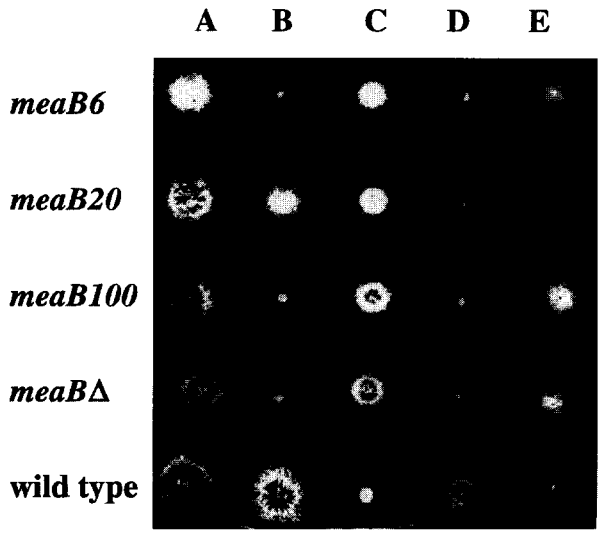

Fig. 5. Phenotypic characterisation of *meaB* mutants produced by gene disruption. Growth is shown after 48 h on minimal media plus: 10 mM NH_4^+ (A); 10 mM NH_4^+, 100 mM ClO_3^- (B); 10 mM NO_3^-, 50 mM methylammonium (C); 10 mM NH_4^+, 30 mM NO_2^- (D); and on complete medium plus 2 mg ml^{-1} DL-*p*-fluorophenylalanine (E).

flanking *Bam*HI restriction sites, which were used to ligate the gene into the reconstituted *Bcl*I site. The new construct was linearised and used to transform an $argB^-$ strain. $argB^+$ $meaB^-$ progeny were purified and characterised by Southern analysis (Fig. 4) to check for appropriate single copy integration and gene replacement, before complete phenotypic analysis. *meaB*Δ strains show an identical phenotype to *meaB6* and *meaB100* mutants in both plate tests (Fig. 5) and nitrate reductase assays (Table 1), confirming that these two frame shift mutations are null mutations in *meaB*.

4. Conclusion

We have cloned and sequenced the *meaB* gene together with the three previously isolated mutations: *meaB6*, *meaB100* and *meaB20*. The fact that MEAB and NMR are not homologous is consistent with the further pleiotropic effects conferred by the *meaB* mutations that are not seen in either *N. crassa* nmr^- mutants or displayed by nitrogen metabolite derepressed *areA* mutants [19]. If MEAB and NMR were functionally equivalent, i.e. if the major function of MEAB were to negatively regulate AREA, then $meaB^-$ mutants should have phenotypes closely resembling growth under derepressing conditions instead of the more pleiotropic phenotypes observed.

Our derived sequence for the *meaB* product reveals a novel protein and does not refute the idea that MEAB affects cell compartmentalisation since the proposed sequence shows no convincing similarity to any known proteins and lacks any major structural motifs, other than S/TPXX. If MEAB is a transcriptional regulatory protein, which would be consistent with the high frequency of S/TPXX motifs, then how such a DNA binding protein could act within the cell to affect processes as diverse as amino acid uptake and/or metabolism, nitrite efflux and nitrogen metabolite repression is unclear. The activity of MEAB cannot be restricted to AREA however, since null mutations in AREA are not fully epistatic to $meaB^-$ mutations [19].

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Note added in proof

The EMBL accession number for *meaB* is X98065.