FacB, the *Aspergillus nidulans* activator of acetate utilization genes, binds dissimilar DNA sequences

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The *facB* gene is required for acetate induction of acetamidase (*amdS*) and the acetate utilization enzymes acetyl-CoA synthase (*facA*), isocitrate lyase (*acuD*) and malate synthase (*acuE*) in *Aspergillus nidulans*. The *facB* gene encodes a transcriptional activator with a GAL4-type Zn(II)₂Cys₆ zinc binuclear cluster DNA-binding domain which is shown to be required for DNA binding. *In vitro* DNA-binding sites for FacB in the 5' regions of the *amdS*, *facA*, *acuD* and *acuE* genes have been identified. Mutations in *amdS* FacB DNA-binding sites affected expression of an *amdS*–*lacZ* reporter *in vivo* and altered the affinity of *in vitro* DNA binding. This study shows that the FacB Zn(II)₂Cys₆ cluster binds to dissimilar sites which show similarity in form but not sequence with DNA-binding sites of other Zn(II)₂Cys₆ proteins. Sequences with homology to FacB sites are found in the 5' regions of genes regulated by the closely related yeast Zn(II)₂Cys₆ protein CAT8.

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Zn(II)₂Cys₆ binuclear cluster

Introduction

Acetate metabolism has been studied in a number of fungi including *Neurospora crassa*, *Coprinus cinereus*, *Saccharomyces cerevisiae* and *Aspergillus nidulans* (e.g. Flavell and Fincham, 1968a,b; Casselton and Casselton, 1974; Armitt *et al.*, 1976; Fernández *et al.*, 1992; Schöller and Schüller, 1994). Acetate is activated by acetyl-CoA synthase (ACS) to acetyl-CoA, which is metabolized via the anaplerotic glyoxylate bypass of the tricarboxylic acid cycle comprising isocitrate lyase (ICL), which converts isocitrate to glyoxylate and succinate, and malate synthase (MAS), which converts glyoxylate and acetyl-CoA to malate (Kornberg, 1966).

The *facB* gene of *A.nidulans* encodes the major regulator of genes involved in acetate utilization. Recessive loss-of-function mutations in *facB* lead to resistance to fluoroacetate, reduced growth on medium containing acetate as a sole carbon source and reduced levels of activities required for acetate utilization, i.e.ACS (encoded by *facA*), ICL (encoded by *acuD*), MAS (encoded by *acuE*), phos-

phoenol pyruvate carboxy kinase (PEPCK) and fructose-1,6-bisphosphatase (FBP) (Apirion, 1965; Armitt *et al.*, 1976; Hynes, 1977; Kelly, 1980). In addition, *facB* mutations affect acetate induction of acetamidase (encoded by *amdS*) and NADP-linked isocitrate dehydrogenase (NADP-IDH) (Hynes, 1977; Kelly and Hynes, 1982; Todd *et al.*, 1997b). These pleiotropic effects have led to the proposal that *facB* is a positively acting regulatory gene involved in acetate induction (Hynes, 1977; Kelly, 1980).

The *cis*-dominant *amdI9* mutation, a single base pair transition at –210 relative to the translational start point of *amdS*, results in a 10-fold increased acetate induction of acetamidase (Hynes, 1975; Hynes *et al.*, 1988). *facB* recessive loss-of-function mutations are epistatic to the *amdI9* mutation (Hynes, 1977). Multiple copies of a plasmid carrying the *amdS* gene with the *amdI9* mutation, but not a wild-type *amdS* plasmid, resulted in reduced growth on acetate medium (Kelly and Hynes, 1987), indicating that the *amdI9* mutation results in increased affinity for, and therefore titration of, the *facB* gene product. Hynes *et al.* (1988) localized regions of 5' *amdS* involved in titration to –219 to –111 by the transformation of an *amdI9* strain with plasmids containing subcloned fragments. Multiple copies of the cloned *facB* gene reversed the titration of the *facB* gene product (Katz and Hynes, 1989).

The *facB* gene encodes a protein of 867 residues which contains features characteristic of transcriptional activators. Near the N-terminus is a Zn(II)₂Cys₆ (or C6 zinc) binuclear cluster DNA-binding motif (residues 24–51), followed by a linker region (residues 52–66) and a series of five leucine zipper-like heptad repeats (residues 67–101) possibly involved in dimerization (Todd *et al.*, 1997a). The Zn(II)₂Cys₆ cluster DNA-binding motif as well as the linker region of FacB shows high similarity with those of the *S.cerevisiae* CAT8 and SIP4 proteins. The CAT8 protein is thought to be the central regulator of gluconeogenesis (Hedges *et al.*, 1995) while SIP4 is involved in carbon metabolism via its interaction with SNF1 (Lesage *et al.*, 1996).

The GAL4 Zn(II)₂Cys₆ DNA-binding motif consists of six conserved cysteine residues, arranged CX₂CX₆CX₆CX₂CX₆C, which form two α-helical structures separated by a proline-associated loop and coordinates two zinc(II) ions to form a cloverleaf shaped structure (Pan and Coleman, 1990, 1991; Gardner *et al.*, 1991; Baleja *et al.*, 1992; Kraulis *et al.*, 1992; Marmorstein *et al.*, 1992). Zn(II)₂Cys₆ DNA-binding domains interact with DNA-binding sites which are similar in both structure and sequence, consisting of conserved terminal trinucleotides, usually in a symmetrical configuration, spaced by an internal variable sequence of defined length, e.g. GAL4 and LAC9 bind to CGGN₁₁CCG, PPR1 and UaY bind to CGGN₆CCG, and PUT3 binds to CGGN₁₀CCG (Carey

et al., 1989; Siddiqui and Brandriss, 1989; Halvorsen *et al.*, 1990, 1991; Roy *et al.*, 1990; Gödecke *et al.*, 1991; Marmorstein *et al.*, 1992; Suárez *et al.*, 1995; for a review, see Todd and Andrianopoulos, 1997).

Here we show that the FacB Zn(II)2Cys6 cluster is required for *in vitro* DNA binding. We have identified FacB-binding sites 5' to *amdS* and genes of acetate utilization and analysed the effects of binding site mutations. The FacB Zn(II)2Cys6 domain binds to two disparate classes of DNA-binding site which are similar in form but dissimilar in sequence to DNA-binding sites of other Zn(II)2Cys6 proteins. Sequences with homology to FacB DNA-binding sites are found in the CAT8-dependent UAS 5' of the *S.cerevisiae* *FBP1* gene, indicating that the CAT8 Zn(II)2Cys6 cluster may bind DNA-binding sites similar to that of FacB.

Results

FacB binds to multiple regions of the *amdS* promoter

N-terminal FacB residues 4–417 were expressed in *Escherichia coli* as a fusion protein with the *E.coli* maltose-binding protein (MBP) (see Materials and methods). Electrophoretic mobility shift assays (EMSAs) with the –268 to +22 region of the *amdS* promoter (Figure 1A), which is required for *facB*-dependent activation of *amdS* expression (Hynes *et al.*, 1988), showed that the MBP–FacB(4–417) fusion protein but not MBP alone can bind to this DNA fragment (Figure 1B). A single major DNA–protein complex was evident at all concentrations of MBP–FacB tested. A minor faster migrating complex was also evident at all concentrations, while at higher concentrations of MBP–FacB an additional, lower mobility, complex was observed. The fixed stoichiometry between the fast migrating complex and the major complex suggests that the former most likely represents a minor degradation product of MBP–FacB.

Extracts from cells expressing the shorter MBP–FacB(4–142) fusion protein (see Materials and methods) exhibited the same DNA-binding specificity for representative DNA probes used in EMSAs as those from cells expressing MBP–FacB(4–417) (data not shown). Thus, residues 4–142, which contain the Zn(II)Cys6 cluster and adjacent heptad repeat region, are sufficient for DNA binding. Consistent with this, the *in vitro*-generated Zn(II)Cys6 cluster mutation *facBR40G41*, which abolishes *in vivo* *facB* function (Todd *et al.*, 1997a), was introduced

into the MBP–FacB fusion protein construct and found to result in loss of MBP–FacB binding to both 5:6 and 31:32 oligonucleotides in EMSAs (Figure 2C).

In three non-overlapping regions of the *amdS* promoter, –647 to –578, –268 to –151 and –147 to +22, specific DNA binding by the FacB fusion protein was

Fig. 1. FacB binds to multiple regions of the *amdS* promoter. (A) The *amdS* 5' regulatory region showing relevant restriction sites and their nucleotide coordinates relative to the translational initiation site (+1). Probes used for EMSAs are shown below with their end coordinates. (B) EMSA using the *amdS* promoter region from –268 to +22, encompassing at least two FacB-binding sites, with increasing concentrations of protein extract. Reactions contained no added protein extract (lane 1), extract expressing the MBP protein (lanes 2–3 with 6 and 60 µg, respectively) or extract expressing MBP–FacB fusion protein (lanes 4–10 with 0.4, 0.75, 1.5, 3, 6 and 60 µg, respectively). (C) Promoter-scanning EMSA using fragments encompassing the *amdS* 5' regulatory region. Each probe is denoted by its end coordinates shown below each set of three lanes, which represent no added protein extract (no extract), extract containing the MBP protein and extract containing the MBP–FacB fusion protein. The amount of extract used was 6 µg, except for reactions with the –647 to –578 probe where 30 µg of extract was used.

observed (Figure 1C). Binding to the -647 to -578 fragment was weaker than that for the other *amdS* probes as five times the amount of extract was required to achieve a similar proportion of retarded probe. For the remaining two regions, -577 to -405 and -405 to -219, no binding by MBP-FacB could be detected, although it is possible but unlikely that this was obscured by the FacB-independent binding noted for the -577 to -405 probe. These data, together with the results from Figure 1B, clearly demonstrate the presence of at least three independent FacB DNA-binding sites in 5' *amdS*. The -647 to -578 fragment contains a sequence with homology to a sequence within the -268 to -151 fragment that has been noted previously (Hynes *et al.*, 1988). Weak binding to this fragment contributes little to *amdS* expression since deletions lacking this site but retaining sequences within the -268 to -151 and -147 to +22 fragments retain *facB*-dependent regulation (T.G.Littlejohn and M.J.Hynes, unpublished data).

To localize further the binding sites for MBP-FacB protein in the -268 to +22 region of 5' *amdS*, specific competition mobility shift assays were performed (Figure 2A). Competition for DNA binding was observed for the double-stranded oligonucleotides 5:6 and 31:32 which span -219 to -199 and -134 to -105, respectively. The double-stranded oligonucleotide 10:11 spanning -185 to -151 did not compete for FacB binding. EMSAs using the 5:6 and 31:32 double-stranded oligonucleotides as probes clearly showed that these sequences contained FacB-binding sites (Figure 2B).

In vivo and *in vitro* analysis of wild-type and mutant FacB DNA-binding sites in 5' *amdS*

Cis-acting 5' *amdS* mutations (Table I) were investigated for their effects on *amdS-lacZ* levels *in vivo* and on *in vitro* MBP-FacB binding. The *amdI9* mutation, a T→C transition at -210, has been found to result in increased responses to acetate induction (Hynes, 1975, 1977; Hynes *et al.*, 1988) and is contained within the 5:6 oligonucleotide which binds MBP-FacB (see above). The *amdI9* mutation resulted in 4-fold acetate induction compared with 1.3-fold induction for the wild-type *amdS* promoter, and this increased induction was FacB dependent (Figure 3A).

Fig. 2. FacB binds to a single binding site in a sequence-specific manner and requires the Zn(II)Cys6 binuclear cluster. (A) Specific competition of FacB binding. EMSA using the *amdS* 5' regulatory region from -268 to +22 as a probe. Binding was performed with 6 µg of MBP-FacB fusion protein extract in the presence of increasing amounts of unlabelled competitor DNAs. Specific competitor DNAs were the unlabelled probe (-268 to +22), the wild-type *amdS* region identified by the *amdI18* mutation (31:32) and the *amdI9* mutant region (5:6). Non-specific competitor control was the oligonucleotide pair spanning the CCAAT motif in the *amdS* promoter (10:11). Reactions contained no competitor, 6.25-, 12.5-, 25-, 50- and 75-fold molar excess of competitor to probe (lanes 1–6), or no competitor, 11-, 22-, 43- and 86-fold molar excess of competitor to probe (lanes 7–11, 12–16 and 17–21). (B) EMSA using the oligonucleotide pairs 5:6, 31:32 and 10:11 as probes with either no added protein extract (no extract), 30 µg of extract containing the MBP protein or 30 µg of extract containing the MBP-FacB protein. (C) EMSA using the wild-type MBP-FacB protein or a mutated MBP-FacB fusion (MBP-FacB R40G41). The latter has the two adjacent cysteine residues in the fourth cysteine position of the Zn(II)2Cys6 binuclear cluster DNA-binding motif mutated. EMSA probes were the 5:6 and 31:32 oligonucleotide pairs, and reactions contained no extract or 6 µg of the MBP-FacB fusion extracts.

Comparative EMSAs using wild-type and *amdI9*-carrying -268 to -115 fragments as probes showed that a greater proportion of probe was retarded by MBP-FacB when the *amdI9* mutation was present than with wild-type sequences (Figure 4). Densitometry showed that this difference in affinity was ~7.5-fold over a range of protein

Table I. 5' *amdS* mutations affecting FacB binding

Mutation		Sequence ^a	
Wild-type	-222	GTTCTGCAGCTTTCCTTGGCCCGTAA	-197
<i>amdI9</i>	-222	GTTCTGCAGCTTcCCTTGGCCCGTAA	-197
Wild-type	-130	CACCATCCGCTCCCCGGGATCAATG	-105
<i>amdI18</i>	-130	CACCATCCGCTCaCCCCGGGATCAATG	-105
<i>amdI15</i>	-130	CACCATCCGCTCCCCcgggacccgGGGATCAATG	-105
<i>amdI41</i>	-130	CACCATCCGCTCCCacatggatcctaGtGATCAATG	-105

^aMutations affecting FacB binding are shown with coordinates relative to the translational startpoint. Substitution and insertion mutations are in lower case and bold. The *amdI9* mutation is a single base pair change of T to C at -210 (Hynes *et al.*, 1988). The *amdI18* mutation is a single base change of C to A at -118 (Corrick *et al.*, 1987) and the *amdI15* mutation is a 10 bp insertion of a *Bam*HI linker between bases -115 and -114 (Littlejohn and Hynes, 1992). The *amdI41* mutation is a 10 bp insertion between bases -115 and -114 and base substitutions at -116, -115 and -113.

<i>amdS-lacZ</i> gene replacement	Strain	<i>facB</i> genotype	β -galactosidase uninduced	β -galactosidase induced	Fold induction
A					
wildtype	MH6707	<i>facB</i> ⁺	33.4 (4.2)	41.5 (6.9)	1.2
	MH6974	<i>facB101</i>	17.8 (3.3)	25.6 (5.3)	1.4
19	MH3408	<i>facB</i> ⁺	32.4 (0.5)	129.5 (7.4)	4.0
-210	MH5077	<i>facB101</i>	20.0 (2.6)	32.7 (3.9)	1.6
I18	MH6927	<i>facB</i> ⁺	33.8 (3.2)	63.8 (3.6)	1.9
-118	MH6961	<i>facB101</i>	28.2 (5.2)	43.3 (5.9)	1.5
19 I15	MH5788	<i>facB</i> ⁺	18.7 (1.2)	56.9 (2.6)	3.0
-210 -117					
19 I41	MH6440	<i>facB</i> ⁺	12.8 (0.9)	22.1 (1.9)	1.7
-210 -117					
I93 I18	MH6122	<i>facB</i> ⁺	0.6 (0.0)	5.8 (0.8)	9.7
-182 -151 -118					
B					
5:6	MH4839	<i>facB</i> ⁺	3.4 (0.8)	7.4 (1.6)	2.2
-647 -117					
31:32	MH4981	<i>facB</i> ⁺	2.3 (0.4)	4.8 (0.3)	2.1
-647 -117					
31:32	MH5287	<i>facB</i> ⁺	0.5 (0.1)	0.5 (0.0)	1.0
-647 -117					

Fig. 3. *Cis*-acting mutations in 5' *amdS* define two distinct FacB sites of action *in vivo*. (A) β -Galactosidase activities of the *amdS-lacZ* fusion gene with the wild-type or mutant promoter under uninduced and induced growth conditions (see Materials and methods). The *amdS* promoter of these constructs is depicted showing the *amdI9* (I9), *amdI18* (I18), *amdI15* (I15), *amdI41* (I41) and *amdI93* (I93) mutations and their nucleotide coordinates relative to the translation initiation site. The strain designation for each of these gene replacement transformants is indicated, as is the *facB* genotype. β -Galactosidase-specific activities as $A_{420\text{ nm}}/10^3/\text{min}/\text{mg}$ of soluble protein are shown for each strain under uninduced (0.1% glucose) and induced (0.1% glucose, 50 mM sodium acetate) conditions, with standard errors indicated in parentheses. (B) β -Galactosidase activities of the *amdS-lacZ* fusion gene as described in (A). The *amdS* promoter of these constructs is depicted showing the mutant deleted for sequences from -647 to -117 and two replacement constructs containing the 5:6 or 31:32 oligonucleotide pair in this deleted promoter.

concentrations (data not shown). Thus, the *amdI9* mutation resulted in an increased affinity of binding by MBP-FacB *in vitro*.

Two *in vitro*-generated mutations, *amdI15* and *amdI41*, with 10 bp insertions of different sequence between -115 and -114 (Table I) in an *amdI9*-containing promoter, resulted in reduced levels of reporter gene expression (Figure 3A). The *amdI41* mutation, but not the *amdI15* mutation, also reduced acetate induction to <2-fold. Binding by MBP-FacB to -147 to +22 fragments from *amdI15* and *amdI41* was reduced compared with that for wild-type (Figure 4), indicating that these mutations reduced affinity for MBP-FacB *in vitro*.

Fig. 4. FacB binds to mutant *amdS* promoters with different affinities. (A) EMSA comparing the *amdI9* mutation in the upstream FacB-binding site and the *amdI18*, *amdI15* and *amdI41* mutations in the downstream FacB-binding site with the wild-type sites. The probes used are indicated beneath each set of three lanes, with the coordinates relative to the translation start of *amdS* shown below this. The three lanes represent no added protein extract (no extract), 6 μg of extract containing the MBP protein and 6 μg of extract containing the MBP-FacB fusion protein. (B) The *amdS* 5' region showing the translation initiation site (ATG) and the location of the *amdI9* and *amdI18* single base substitutions and the *amdI15* and *amdI41* insertion mutations. The coordinates relative to the translational initiation site are shown below these sites. The two regions used as probes in (A) are shown with their end coordinates marked.

The *amdI18* mutation, a C→A substitution at -118, results in elevated *amdS* expression under a number of growth conditions but has not been found to be dependent on the *facB* gene (Hynes, 1978; Corrick *et al.*, 1987). Since it is located within the 31:32 oligonucleotide which binds to MBP-FacB, it was tested in the *in vivo* assay. The *amdI18* mutation increased *amdS-lacZ* expression by <2-fold under limiting glucose plus acetate-inducing conditions even in the presence of the *facB101* mutation, indicating that the effects of this mutation were independ-

Fig. 5. DNase I protection footprinting of wild-type and mutant FacB-binding sites. DNase I protection footprints of the *amdS* wild-type (A), *amdI9* mutant (B) and *amdI18* mutant (C) promoters from –268 to +22 for the coding and non-coding strands are shown. Footprinting using 75 µg of extract containing only the MBP protein or containing the MBP–FacB fusion protein are shown for each probe. A diagrammatic representation of the promoter is shown flanking the footprinted pair of strands such that coordinates denote the two FacB-binding sites in these probes while open rectangles represent the sites evident in the footprinting reactions. The mutations defining *amdI9* and *amdI18* are presented. All coordinates are relative to the translation initiation start site.

ent of *facB* (Figure 3A). *In vitro* binding of MBP–FacB to wild-type and *amdI18*-containing –147 to +22 fragments revealed similar levels of binding (Figure 4).

The *amdI93* deletion of –182 to –151 decreases the spacing between the FacB-binding sites in the –219 to –199 and –134 to –105 regions by 31 nucleotides (Hynes *et al.*, 1988). The *amdI93* deletion results in greatly reduced *amdS*–*lacZ* levels due to removal of the sites of action for the positively acting proteins AnCF and AmdR (Hynes *et al.*, 1983; Littlejohn and Hynes, 1992; Papagiannopoulos *et al.*, 1996). However, increased acetate induction of 9.7-fold was observed (Figure 3A). Thus, the relative proximity of FacB-binding sites in the *amdS* promoter may be important for acetate induction.

To test whether a single FacB-binding site was sufficient for acetate induction of *amdS*–*lacZ* expression, the oligonucleotide pairs 5:6 (–219 to –199) and 31:32 (–134 to –105) were used to replace –646 to –118 sequences in *amdS*–*lacZ* gene replacement derivatives. Each of these oligonucleotides failed to confer acetate induction on the *amdS*–*lacZ* reporter (Figure 3B), suggesting that two binding sites are required for acetate induction by FacB of the *amdS* gene.

FacB binds to dissimilar sequences in 5' *amdS*

DNase I footprinting of the wild-type promoter from –268 to +22 identified a single protected region from –140 to –105 on the coding strand and –143 to –104 on the non-coding strand (Figure 5A) overlapping sequences defined by the 31:32 oligonucleotide (–134 to –105). Footprinting of a –268 to +22 fragment derived from the *amdI18* mutant promoter identified exactly the same

protected regions as those for the wild-type (Figure 5C). In contrast, footprinting of the *amdS* promoter derived from the *amdI9* mutant identified a protected region from –221 to –192 on the coding strand and –227 to –191 on the non-coding strand, encompassing the *amdI9* T→C transition at –210 (Figure 5B). Thus, *in vitro*, MBP–FacB binds preferentially at –221 to –192 in the presence of the *amdI9* mutation. The reason for this preferential binding *in vitro* is not clear but may reflect a lack of sensitivity in the footprinting such that only the strongest sites are detected.

Missing contact interference footprinting using the wild-type *amdS* probe showed that removal of bases from –128 to –111 on the coding strand and –130 to –117 on the non-coding strand significantly affected the ability of the fusion protein to bind DNA (Figure 6A). Similarly, when the *amdI9* probe was used, bases in the region from –213 to –200 on the coding strand and –213 to –199 on the non-coding strand were important for binding (Figure 6B). Immediately outside these two regions, small effects on binding were evident. These results are consistent with results of the DNase I footprinting and EMSAs, defining these two dissimilar sequences as *bona fide* FacB DNA-binding sites. Moreover, both of these sites are functional *in vivo* (see above).

Competition experiments (data not shown) revealed that the MBP–FacB binding to labelled 5:6 could be competed by unlabelled 31:32 and, conversely, binding to labelled 31:32 was competed by unlabelled 5:6. These data and the requirement for an intact Zn(II)2Cys₆ cluster for binding to the 5:6 and 31:32 oligonucleotides (see above; Figure 2C) indicate that the Zn(II)2Cys₆ DNA-binding

Fig. 6. Missing contact footprinting of the two FacB-binding sites. Missing contact footprints of the *amdS* wild-type (A) and *amdI9* mutant (B) promoters from -268 to +22 for the coding and non-coding strands using the MBP-FacB fusion protein are shown at the top. Sequences and nucleotide coordinates encompassing the footprinted region are shown to the right of each footprint, with bases having a major effect on FacB binding marked by a filled circle and those with a minor effect by an open circle. Lanes corresponding to reactions using DNA modified by depyrimidination (Y) or depurination (R) are marked. Lanes from the FacB bound (B) and free (F) fractions are also marked. Densitometric plots derived from the missing contact footprints are shown below these footprints. The sequence of the footprinted region is shown along the inner x-axes for both strands, while nucleotide coordinates are shown on the outer x-axes. The y-axis (ordinate) represents a relative scale of the ratio of free probe to bound probe, corrected for loading differences, such that modified nucleotides which reduce binding by FacB to this probe will have a larger positive value. All coordinates are relative to the translation initiation start site of *amdS*.

domain binds both types of dissimilar FacB DNA-binding site.

FacB binds multiple regions in the promoters of the co-regulated *facA*, *acuD* and *acuE* genes

To identify additional FacB DNA-binding sites, promoter-scanning EMSAs on the *acuD*, *facA* and *acuE* 5' regions were performed (Figure 7). For 5' *acuD*, two overlapping fragments and an adjacent fragment showed strong binding by FacB (-844 to -414, -414 to -202 and -465 to -176), thereby identifying at least two FacB sites (Figure 7B). In addition, the -465 to -176 *acuD* probe resulted in a major fast migrating complex as well as a slower migrating complex suggestive of at least two binding sites in this region. DNase I footprinting of this fragment identified three protected regions from -449 to -422, -333 to -305 and -295 to -269 on the coding strand, and -452 to -423, -333 to -305 and -292 to -266 on the non-coding strand (Figure 8). The central protected site showed homology to the -213 to -199 *amdS* site, while the other two sites showed homology to the -130 to

-111 *amdS* site. Most of the *facB*-dependent acetate induction has been localized to the -414 to -202 region of the *acuD* promoter (Bowyer *et al.*, 1994; De Lucas *et al.*, 1994). Two of the protected regions in 5' *acuD*, -333 to -305 and -292 to -269, were affected by the deletion of bases -347 to -285, which resulted in an 80% reduction of acetate-induced levels of an *acuD-lacZ* fusion gene (Bowyer *et al.*, 1994). Thus, at least one of these FacB DNA-binding sites is functional *in vivo*.

For 5' *facA*, two partially overlapping fragments showed strong binding by FacB (-1265 to -962 and -1127 to -677) while a third exhibited weak binding (-481 to -168) in EMSAs, thereby identifying at least two FacB sites (Figure 7A). For 5' *acuE*, two partially overlapping fragments showed strong binding by FacB (-1010 to -659 and -684 to -404) while a third exhibited weak binding (-404 to -228), thereby identifying at least two FacB sites (Figure 7C). DNase I footprinting was performed on the -684 to -404 region of 5' *acuE* (Figure 8). A single protected region was evident from -645 to -614 on the coding strand and from -650 to -612 on the non-coding strand. The site showed

Fig. 7. FacB binds to distinct sites in the promoters of the genes of the glyoxylate bypass. Promoter-scanning EMSA of the *facA* (A), *acuD* (B) and *acuE* (C) 5' regulatory regions. End-labelled probes were generated by PCR or as restriction fragments (see Materials and methods). Probes used are indicated beneath each set of three lanes by the end coordinates relative to the translational start site. Each set of three lanes represents no added protein extract (no extract), 6 μ g of extract containing the MBP protein and 6 μ g of extract containing the MBP-FacB fusion protein. A diagrammatic representation of the *facA*, *acuE* and *acuD* 5' regulatory regions with the relevant restriction sites and their nucleotide coordinates relative to the translational initiation site (ATG) is shown. The position of probes used for EMSAs is shown, with FacB-specific DNA binding indicated as strong (+), weak (+/-) and undetectable (-).

homology to the -130 to -111 *amdS* site. These DNA-binding analyses of 5' *acuD*, *facA* and *acuE* clearly support the proposal of direct transcriptional control by FacB, and the sites of action for these genes fall into the two distinct classes of FacB-binding sites defined for the *amdS* promoter.

Discussion

FacB DNA-binding sites identified by DNase I footprinting with MBP-FacB fall into two classes according

to sequence (Table II). Class A FacB-binding sites show sequence homology to the binding site at *amdS* -125 to -111, while class B FacB sites show sequence homology to the -219 to -203 *amdI9* sequence, both of which contact MBP-FacB in missing contact interference assays. The class A and class B consensus sequences derived for footprinted sites are TCC/GN₈₋₁₀C/GGA and GCAGNTNNCCN₁₋₂GCG, respectively. Both class A and class B sites show imperfect rotational symmetry. Sequences of fragments which showed MBP-FacB-specific binding in EMSAs, but

Table II. FacB-binding sites

Origin	Position ^a	Sequence ^b
Class A FacB-binding sites		
<i>amdS</i>	-125 to -111	TCCGCTCCCCCG-GGA
<i>amdI18</i>	-125 to -111	TCCGCTCACCCG-GGA
<i>acuD</i>	-289 to -276	TCC-CGCTGAGC-CGA
<i>acuD</i>	-442 to -427	TCCTTTAGACCGAGGA
<i>acuE</i>	-257 to -244	TCC-ATCCTTCC-TGA
<i>acuE</i>	-638 to -624	TCGGGCGTGCGG-GGA
<i>acuE</i>	-808 to -793	TCCTTTCCTGCTAGCA
<i>acuE</i>	-1331 to -1318	TCC-AAGGGTGA-GGA
<i>facA</i>	-464 to -450	TCGCCATATAAG-GGA
<i>facA</i>	-654 to -641	TCC-CAGGCTGG-GGA
<i>facA</i>	-1081 to -1066	TCGTCTGAAATTCGGA
<i>facA</i>	-1382 to -1367	TCGCCTGTTTCAGGGA
Class A consensus		TCC/G N₈₋₁₀ C/GGA
Class B FacB-binding sites		
<i>amdS</i>	-217 to -203	GCAGCTTTCCTT-GGC
<i>amdI9</i>	-217 to -203	GCAGCTTCCCTT-GGC
<i>amdS</i>	-606 to -592	GCAGATTTCCTT-GGC
<i>acuD</i>	-326 to -313	GCA-GGTCTCCC-GGC
<i>acuE</i>	-569 to -554	GCCACTCTGCATCTGC
<i>acuE</i>	-795 to -781	GCAGTGACTCAC-CGC
<i>facA</i>	-914 to -899	GCATGGCCGAGTCTGC
Class B consensus		GCA/C N₈₋₁₀ T/GGC

^aPosition relative to the translational startpoint.

^bThe sequences of FacB DNA-binding sites demonstrated by DNase I footprinting () and predicted within fragments that bound MBP-FacB(4-417) in EMSA. Sites are aligned according to homology to the dissimilar binding sites identified by missing contact interference footprinting at *amdS* -125 to -111 (class A binding sites) and at *amdS* -217 to -203 (class B binding sites). The terminal trinucleotides are shown in bold.

were not subjected to footprinting analysis, were scanned for regions of homology to class A and class B sites. While none of these fragments contained sequences identical to any of the identified sites, all contained sites which conform to either class A or class B FacB-binding site consensus sequences, with inverted terminal TCC/G (class A) or GCC/A (class B) trinucleotide repeats spaced by 8-10 nucleotides of variable sequence (Table II). The *S.cerevisiae* HAP1 Zn(II)2Cys6 protein also binds to different sequences via a single DNA-binding domain (Pfeifer *et al.*, 1987), binding to the CGGN₆CGG sequence of UAS_{CYC1} and to the CGCN₆CGC sequence at UAS_{CYC7} (Zhang and Guarente, 1994), although a more extended and complex consensus sequence has been proposed recently (Ha *et al.*, 1996).

Although the DNA-binding sites for relatively few Zn(II)2Cys6 proteins have been characterized, most binding sites consist of terminal trinucleotides separated by an internal spacer sequence. The terminal trinucleotides are in an inverted orientation in all cases, except for HAP1 and MAL63 DNA-binding sites where they are found as direct repeats (Zhang and Guarente, 1994; Sirenko *et al.*, 1995). Everted terminal trinucleotide repeats have been proposed for LEU3- and PDR3-binding sites (Hellauer *et al.*, 1996). The sequence composition of the internal spacer varies considerably for DNA-binding sites for a particular protein and also for different proteins (Baum *et al.*, 1987; Vashee *et al.*, 1993). The length of the internal spacer sequence varies greatly between sites for different Zn(II)2Cys6 proteins but shows only small variation for binding sites for a particular Zn(II)2Cys6 protein (Vashee *et al.*, 1993).

Fig. 8. DNase I protection footprints of representative regions of the (A) *acuE* and (B) *acuD* promoters for the coding and non-coding strands are shown. Probes represent the *acuD* promoter region from -465 to -176 and the *acuE* promoter region from -684 to -404. Footprinting reactions used 75 µg of extract containing only the MBP protein or containing the MBP-FacB fusion protein. A representation of each promoter is shown flanking the footprinted pair of strands such that open rectangles denote the sites evident in the footprinting reactions. All coordinates are relative to the translation initiation start sites of *acuD* and *acuE*.

Fig. 9. Positions of FacB-binding sites. The positions of predicted and demonstrated (by DNase I footprinting) FacB-binding sites identified within fragments from the 5' regions of *amdS*, *acuD*, *acuE* and *facA* that bound MBP-FacB in EMSAs are shown aligned to their respective translational startpoints. Class A FacB-binding sites are shown as open boxes and class B FacB-binding sites as closed boxes. Relative affinities are indicated as weak (W), medium (M) and strong (S).

The imperfect rotational symmetry of the FacB DNA-binding sites suggests that FacB may bind DNA as a homodimer such that the Zn(II)2Cys6 domain of each monomer subunit contacts the terminal triplets. Folding of the linker region of each monomer may space the Zn(II)2Cys6 domains of the FacB dimer and permit their interaction with terminal trinucleotides spaced 8–10 nucleotides apart, and thereby provide DNA-binding specificity. FacB residues 4–142, which contain the Zn(I)2Cys6-linker-heptad repeat region, are able to dimerize in a yeast two-hybrid assay (R.B.Todd, H.Davidson, M.A.Davis, A.Andrianopoulos and M.J.Hynes, unpublished data). Homodimerization has been demonstrated for other Zn(II)2Cys6 proteins (Carey *et al.*, 1989; Marmorstein *et al.*, 1992; Zhang *et al.*, 1993; Marmorstein and Harrison, 1994; Remboutsika and Kohlhaw, 1994; Fu *et al.*, 1995; Sirenko *et al.*, 1995).

Unlike most other Zn(II)2Cys6 DNA-binding sites which have terminal CGG trinucleotides, those of FacB sites consist of two possible sequences: TCC/G (class A sites) and GCC/A (class B sites). LEU3 and QA-1F DNA-binding sites contain GCC and GGG/A terminal triplets, respectively (Baum *et al.*, 1987; Geever *et al.*, 1989; Remboutsika and Kohlhaw, 1994). PDR3 and NIT-4 DNA-binding sites, like FacB class A sites, may contain TCC terminal triplets (Katzmann *et al.*, 1994; Delahodde *et al.*, 1995; Fu *et al.*, 1995).

MBP-FacB binding affinity is different for sites with identical terminal trinucleotides but different internal sequences (Figure 9). However, there is no clear correlation between the sequence of the internal region and affinity for MBP-FacB binding. The internal spacers have no obvious sequence bias which has been observed in DNA-binding sites for the HAP1 and LAC9 proteins (Halvorsen *et al.*, 1991; Zhang and Guarente, 1994). Thus, the composition of the entire spacer unit or sequence combinations within a particular internal spacer may dictate affinity for the FacB protein. The class B site at -217 to -203 and the class A site at -125 to -111 of *amdS* are of identical lengths, with internal spacers of nine nucleotides. The *amdI9* and *amdI18* single base substitutions are in the central nucleotide of the internal spacer of the -217 to -203 (class B) and -125 to -111 (class A) *amdS* sites,

respectively. The *amdI9* mutation, but not the *amdI18* mutation, significantly affects affinity of FacB binding.

The *amdI15* and *amdI41* mutations are 10 bp insertions into the -125 to -111 (class A) binding site in 5' *amdS* which reconstitute FacB DNA-binding sites but convert TCCN₉GGA sites to TCCN₁₀GGA sites. The *amdI41* mutation also alters the sequence composition of the internal spacer. The *amdI15* and *amdI41* mutations result in a similar decreased affinity for FacB compared with the wild-type site, indicating that different sequence composition in the internal spacer region does not necessarily alter DNA-binding affinity. DNA-binding affinity for sites in the *acuD*, *acuE* and *facA* 5' regions does not correlate strictly with internal spacer length, probably because sequence composition also determines the affinity of a DNA-binding site for FacB. FacB residues in the linker region, which are predicted to follow the DNA backbone along the internal spacer region, may provide DNA-binding specificity by direct contact with DNA in addition to that provided by the positioning of the Zn(II)2Cys6 cluster. Residues within the linker of GAL4 contact phosphate groups on the DNA backbone (Marmorstein *et al.*, 1992). However, in PPR1, residues within the linker are not involved in direct interactions with DNA (Marmorstein and Harrison, 1994).

Three *in vitro* FacB DNA-binding sites were identified in the *amdS* 5' region (Figure 9). The data presented here suggest that both the -125 to -111 (class A) and -217 to -203 (class B) sites may be necessary for *facB*-dependent expression of *amdS*. The *amdI9* mutation in the -217 to -203 FacB-binding site results in increased acetate induction of *amdS* expression, correlating with increased affinity of MBP-FacB protein for fragments containing the *amdI9* mutation. The *amdI15* and *amdI41* mutations affect the -125 to -111 FacB-binding site and, although the *amdI15* mutation has little effect on response to acetate induction of *amdS-lacZ*, the *amdI41* mutation results in reduced acetate induction. The sequence of the binding site has been shown to affect the transcriptional activity of bound HAP1 in *S.cerevisiae* (Ha *et al.*, 1996). Neither the -125 to -111 nor the -217 to -203 sites are sufficient for acetate induction of *amdS* as the 5:6 (-217 to -203 site-containing) and 31:32 (-125 to -111 site-containing) oligonucleotides fail to confer acetate induction. Thus, binding to both sites or interaction between these sites may be necessary for acetate induction. The increased acetate induction observed in the presence of the *amdI93* deletion, which reduces the relative spacing between these sites, is consistent with an interaction between these sites. FacB DNA-binding sites of both classes were detected in each of the 5' regions of *acuD*, *acuE* and *facA* (Figure 9). In the case of *acuD*, deletion of bases -347 to -285 reduces *facB*-dependent acetate induction by 80% (Bowyer *et al.*, 1994), correlating with two *in vitro* FacB DNA-binding sites [at -289 to -276 (class A) and -326 to -313 (class B)]. Overall, there is no obvious pattern of spacing or occurrence of weak or strong sites of either class in these promoters, and the functional relevance of *in vitro* FacB DNA-binding sites identified in the 5' regions of the *facA* and *acuE* genes remains to be determined.

Sequences with homology to class A and class B FacB DNA-binding sites are found in the 5' regions of *N.crassa*

acu-5, *acu-8* and *acu-9*, and in the *Penicillium chrysogenum* *facA* acetate utilization genes and the *S.cerevisiae* carbon metabolic genes *ACS1*, *FBP1*, *MLS1*, *ICL1*, *PCK1*, *ACH1* and *CAT5* (Rogers *et al.*, 1988; Connerton *et al.*, 1990; Lee *et al.*, 1990; Marathe *et al.*, 1990; Sandeman *et al.*, 1991; Hartig *et al.*, 1992; Mercado and Gancedo, 1992; Gouka *et al.*, 1993; Martinez-Blanco *et al.*, 1993; Schöler and Schüller, 1993; Kratzer and Schüller, 1995; Proft *et al.*, 1995b). Therefore, these species may have a regulatory gene involved in acetate metabolism which is homologous to the *A.nidulans* *facB* gene. In *S.cerevisiae*, the *CAT8* gene is a good candidate for a structural and functional *facB* homologue. FBP, ICL and PEPCK levels are reduced in *cat8* mutants (Hedges *et al.*, 1995). The *CAT8* protein shows high similarity with *FacB*, particularly in the Zn(II)2Cys6, linker and heptad repeat regions, and these proteins share a close phylogenetic relationship (Todd and Andrianopoulos, 1997; Todd *et al.*, 1997a). Therefore, it is likely that these proteins interact with similar DNA sequences. *CAT8*-dependent derepression has been shown for *CAT5* (Proft *et al.*, 1995b) and localized in the 5' regions of *FBP1*, at UAS_{1FBP1} and UAS_{2FBP1} (Niederacher *et al.*, 1992; Hedges *et al.*, 1995), and *PCK1*, at UAS_{1PCK1} and UAS_{2PCK1} (Mercado and Gancedo, 1992; Proft *et al.*, 1995a,b). Sequences in 5' *CAT5* and within UAS_{1FBP1} and UAS_{2FBP1} show homology to *FacB* class A DNA-binding sites. Derepression-specific *in vitro* binding by *S.cerevisiae* proteins with sequences derived from UAS_{1FBP1} and UAS_{2FBP1} of *FBP1* has been demonstrated (Niederacher *et al.*, 1992; Schöler and Schüller, 1994; Vincent and Gancedo, 1995). However, evidence for direct interaction of *CAT8* with DNA-binding sites has not been reported. It will be of interest to compare the DNA-binding specificity of *CAT8* with that of *FacB*. It will also be important to determine the role of the closely related Zn(II)2Cys6 protein SIP4 (Lesage *et al.*, 1996) in the regulation of these genes.

Materials and methods

Strains and media

Generally, plasmids were amplified in *E.coli* NM522 (Gough and Murray, 1983). Plasmids for double-stranded sequence analysis were prepared from either XL1-Blue (Bullock *et al.*, 1987) or DH5 α F' (Hanahan, 1983). Bacterial extracts for use in DNA-binding reactions were prepared from JPA101 (Amrad Pharmacia Biotech).

Aspergillus nidulans *amdS-lacZ* gene replacement strains containing *amdS* 5' mutations were constructed by the method of Davis *et al.* (1988). The *amdI9*, *amdI15*, 5:6 and -646 to -118 deletion gene replacements were constructed using the previously described pLIT1, pLIT14, pLIT73 and pLIT23 plasmids, respectively (Littlejohn and Hynes, 1992). The *amdI41* mutation was generated by the method of Kunkel (1985), using pLIT14 as a template and the mutagenic primer MH41 (Table III). The wild-type and *amdI18* gene replacements were generated using the plasmids pCSC5-4 (Davis *et al.*, 1988) and I18SmaSalCSC5-2, respectively. I18SmaSalCSC5-2 was constructed by swapping the *SmaI-SalI* fragment from the cloned *amdI18* allele (Corrick *et al.*, 1987) with that of pCSC5-4. Gene symbols have been described previously (Clutterbuck, 1974).

Aspergillus media and growth conditions were as described by Cove (1966). Nitrogen sources were added at a final concentration of 10 mM and carbon sources at 1% (w/v), except for acetate medium which contained 50 mM sodium acetate as the sole carbon source. Genetic manipulations were carried out using techniques described by Clutterbuck (1974). β -Galactosidase assays were performed as described (Davis *et al.*, 1988).

Preparation of labelled DNA probes

Probes for use in EMSAs were labelled by end-filling 5' overhangs with the Klenow fragment of DNA polymerase I (Promega Corp.) and [α -³²P]dATP (3000 Ci/mmol; Bresatec) or prepared from plasmid templates using standard PCR conditions with one labelled primer and an unlabelled primer. Primers were end-labelled using T4 polynucleotide kinase (Promega Corp.) and [γ -³²P]dATP (4000 Ci/mmol; Bresatec), as recommended by the supplier (Promega Corp.), and purified by electrophoresis on a 4% non-denaturing polyacrylamide gel in 1× TBE. The following PCR probes were generated using the following primer pairs: *acuD* -465 to -176, ACD1 and ACD2; *acuD* -506 to -176, ACD3 and ACD2; *amdS* -268 to +22, MH51 and MH52; *amdS* -268 to -151, MH51 and MH11; *amdS* -147 to +22, MH39 and MH52. The *amdS* -268 to -115 probe was generated by PCR using end-labelled MH51 and unlabelled MH52 and subsequent digestion with *SmaI*. *amdS* plasmids used as templates were pAMD25 (wild-type) (M.J.Hynes, unpublished), I18SmaSalCSC5-2 (*amdI18*) and 41CSLIT14-6 (*amdI41*) (this study), and pLIT1 (*amdI9*) and pLIT14 (*amdI15*) (Littlejohn and Hynes, 1992). *acuD* probes were generated from pICL1, which contains the entire *acuD* gene on a 4.9 kb *EcoRI* fragment in pUC7 (kindly provided by Dr G.Turner; Gainey *et al.*, 1992; J.Brown and G.Turner, unpublished). Primers used to generate these probes are shown in Table III. All other PCR probes were generated from subcloned fragments using the T3 and T7 primers. Oligonucleotide probes for use in EMSAs were made double-stranded (a mixture of 1 μ g of each oligonucleotide in 10 μ l of H₂O was heated at 68°C for 10–15 min and allowed to anneal at 25°C for 20–30 min) and labelled by end-filling using the Klenow fragment of DNA polymerase I (Promega Corp.) and [α -³²P]dATP (3000 Ci/mmol; Bresatec).

Bacterial expression of fusion proteins

Fragments of the *facB* gene were expressed as MBP fusion proteins using the pMAL system (Riggs, 1990; New England Biolabs). The pMALCR1 and pMALC2 vectors contain the MBP-encoding *malE* gene fused via a polylinker to the *lacZ* α gene. All expression constructs were generated by subcloning an *EcoRI-BamHI* (+8 to +422) or *EcoRI-XbaI* (+8 to +1410) DNA fragment from the *facB* coding region into the pMAL polylinker. The resultant plasmids were digested separately at the unique *EcoRI* site, end-filled and religated to put the *facB* gene in the same translational reading frame as *malE*. The pFBMAL1 construct contained the 1.2 kb *EcoRI-XbaI* fragment from the *facB* cDNA in the *EcoRI* and *XbaI* sites of pMALCR1. The pFBMAL1 construct contained a 1.2 kb *EcoRI-XbaI* reconstructed cDNA fragment from the Zn(II)2Cys6 mutant allele *facBR40G41* (Todd *et al.*, 1997a) in pMALCR1. The pFBMAL2 construct contained the intronless 0.4 kb *EcoRI-BamHI* *facB* fragment in the *EcoRI* and *BamHI* sites of pMALC2.

Expression plasmids were transformed into *E.coli* JPA101 or NM522. An overnight culture was diluted 1 in 100 in 2× YT plus 0.2% glucose plus 50 μ g/ml ampicillin, and grown at 37°C with aeration to OD₆₀₀ = 0.4. Expression of the fusion proteins was induced by addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to 0.3 mM (final concentration) for 3 h at 37°C. Cells were harvested and crude extracts were produced, as described in the pilot experiment of the pMAL Protein Fusion and Purification System (New England Biolabs) manual. The soluble fraction was dialysed against 2× 800 ml of 1× binding buffer (-Mg²⁺) (25 mM HEPES-KOH pH 7.6, 40 mM KCl, 1 mM EDTA, 50% glycerol) at 4°C prior to use in EMSAs. Protein concentrations were determined using the Bio-Rad Protein Assay reagent (Bio-Rad). Induced fusion proteins of the predicted sizes [~87 kDa for MBP-FacB(4–417) and MBP-FacBR40G41, and 57 kDa for MBP-FacB(4–142)] were visible by SDS-PAGE in total crude cell extracts from induced cells harbouring the expression plasmids (data not shown). The relative level of induced expression of MBP-FacBR40G41 mutant protein was comparable with that of the corresponding expressed wild-type fusion protein.

Electrophoretic mobility shift assays

Binding reactions comprised protein extract, labelled DNA probes, 1–2 μ g of poly(dIdC) and 1× binding buffer (-Mg²⁺) or 1× binding buffer (+Mg²⁺) (25 mM HEPES-KOH pH 7.6, 40 mM KCl, 1 mM EDTA, 50% glycerol, 5 mM MgCl₂) in 20 μ l volumes. For competition experiments, unlabelled DNA fragments or double-stranded oligonucleotides were included in the reactions. Reactions usually used 6–30 μ g of protein extract (except where indicated) and were carried out in 1× binding buffer (-Mg²⁺). Binding reactions were incubated for 20–30 min at 25°C. Type II gel loading dye was added prior to

Table III. Oligonucleotides used in this study

Oligonucleotide	Sequence
ACD1	5'-TGGCAAGTTGCCCTAAGGC-3'
ACD2	5'-ATCAAGAATCACAGGCCG-3'
ACD3	5'-CCAAAATTCAAGGCTCGG-3'
MH5	5'-GATCTGCAGCTTCCCTTGGCCCGT-3'
MH6	5'-GATCACGGGCCAAGGGAAGCTGCA-3'
MH10	5'-GATCGCCAGCCAATCACCAGCTAGGCACCAGCTAAACCC-3'
MH11	5'-GATCGGGTTTAGCTGGTGCCTAGCTGGTGATTGGCTGGC-3'
MH31	5'-GATCAACACCATCCGCTCCCCGGGATCAATG-3'
MH32	5'-GATCATTGATCCCGGGGAGCGGATGGTGTG-3'
MH39	5'-CTAATTAGTCTCTTATCAACACCATCCG-3'
MH40	5'-CGGATGGTGTGATAAGAGACTAATTAG-3'
MH41	5'-TTGATCACTAGGATCCAGATGGGAGC-3'
MH51	5'-GAGGATCCGTTAGCATGGGCTGATAGC-3'
MH52	5'-GTGGATCCGTTCTTCCCAGGATTGAGGC-3'

electrophoresis on non-denaturing 4% polyacrylamide gels in 1× TBE at 100–150 V in an ice bath or at 4°C.

DNase I footprinting assays

DNase I protection footprinting assays were performed using the protocol of Andrianopoulos and Timberlake (1994). Sequencing standards were generated using the Sequenase kit (United States Biochemical Corp.) and the primer used in PCR to generate the probe.

Missing contact interference assays

Missing contact interference assays were done according to the protocol of Brunelle and Schleif (1987). End-labelled probes were chemically modified to remove pyrimidine or purine bases, and used in EMSAs with protein extracts from *E. coli* JPA101 expressing MBP-FacB(4–417). The binding reactions were carried out in 1× binding buffer (–Mg²⁺) at 25°C for 20 min and electrophoresed through 1.2% agarose in 0.5× TBE at 20 V, 4°C. The amount of protein added to each reaction mix was calibrated by EMSA to give approximately equal proportions of bound and free probe. Bound and free probes were localized by autoradiography of intact gels at 4°C, excised and recovered by electroelution. After phenol/chloroform/isoamylalcohol extraction and ethanol precipitation, the DNA was cleaved with 1 M piperidine at 90°C for 30 min (Maxam and Gilbert, 1980). The cleavage products were subjected to three lyophilizations, resuspended in formamide gel loading dye and electrophoresed on 5% denaturing polyacrylamide gels.

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