Molecular Characterization and Analysis of the *acrB* Gene of *Aspergillus nidulans*: A Gene Identified by Genetic Interaction As a Component of the Regulatory Network That Includes the CreB Deubiquitination Enzyme

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ABSTRACT

Mutations in the *acrB* gene, which were originally selected through their resistance to acriflavine, also result in reduced growth on a range of sole carbon sources, including fructose, cellobiose, raffinose, and starch, and reduced utilization of ω-amino acids, including GABA and β-alanine, as sole carbon and nitrogen sources. The *acrB2* mutation suppresses the phenotypic effects of mutations in the *creB* gene that encodes a regulatory deubiquitinating enzyme, and in the *creC* gene that encodes a WD40-repeat-containing protein. Thus AcrB interacts with a regulatory network controlling carbon source utilization that involves ubiquitination and deubiquitination. The *acrB* gene was cloned and physically analyzed, and it encodes a novel protein that contains three putative transmembrane domains and a coiled-coil region. AcrB may play a role in the ubiquitination aspect of this regulatory network.

THE *acrB2* mutation was isolated in the filamentous fungus *Aspergillus nidulans* as a spontaneous resistant sector on complete medium containing acriflavine in a genetic screen with the joint aims of understanding acriflavine toxicity and obtaining extra tools for genetic mapping (ROPER and KAFER 1957). A strain containing the *acrB2* mutation also showed greater resistance to the presence of crystal violet and malachite green than that showed by the wild-type strain, and the *acrB2* mutant allele is recessive to the wild-type allele (ROPER and KAFER 1957; ARST 1981). Like *acrB* mutations, the *creD34* mutation also confers resistance to acriflavine (KELLY and HYNES 1977; ARST 1981). The *creD34* mutation was identified as a suppressor of the effects of the *creC27* mutation, and it was also shown to suppress the effects of the *creB15* mutation (KELLY and HYNES 1977). Mutations in *creB* and *creC* result in altered regulation of carbon metabolism. The *creB* gene encodes a functional deubiquitinating enzyme (LOCKINGTON and KELLY 2001) and the *creC* gene encodes a protein that contains five WD40-repeat motifs and a proline-rich region (TODD *et al.* 2000). CreB and CreC have been shown to be present in a complex *in vivo* (LOCKINGTON and KELLY 2002), and thus the CreB/CreC complex is part of the deubiquitination aspect of a regulatory ubiquitination pathway that is required for correctly regulated carbon metabolism.

Protein stability is a key regulatory mechanism in the control of metabolism, the cell cycle, cell growth and development, and disease. Selective degradation or stabilization of intracellular proteins by ubiquitin-dependent pathways is essential for correct regulation of many cellular processes. Ubiquitin is covalently attached to substrate proteins by a protein complex usually including an activating enzyme, a conjugating enzyme, and a protein ligase. A number of conjugating enzymes and protein ligases are in the cell, and various combinations confer substrate specificity on the system. The addition of four or more ubiquitins targets the substrates for destruction via the 26S proteasome, and free ubiquitin is recycled. The addition of fewer ubiquitins can alter function or target the protein to the endosomes. This process can be opposed by the action of deubiquitinating enzymes, which remove ubiquitin from specific substrates, thus stabilizing them. Perhaps the best-studied example of a regulatory deubiquitinating enzyme in a multicellular eukaryote is the *Drosophila melanogaster* Fat facets deubiquitinating enzyme, which is required for correct eye development (WU *et al.* 1999). Genetic analysis has revealed that the critical substrate of Fat facets in the eye is Liquid facets (CADAVID *et al.* 2000; CHEN and FISCHER 2000; CHEN *et al.* 2002). Liquid facets is related to mouse epsin (CHEN *et al.* 2002), which acts as a cargo-selective endocytic clathrin adaptor protein, which regulates clathrin-coat assembly (OLDHAM *et al.* 2002).

Since the role in the cell of the CreB/CreC complex is to remove ubiquitin from specific substrates, then

TABLE 1

Aspergillus nidulans **strains**

Strain	Genotype	Origin
wt	*biAl; riboB2*	Derived from PATEMAN *et al.* (1967)
creB1937	*yA1 pabA1; creB1937; riboB2*	LOCKINGTON and KELLY (2001)
creC27	*biA1; creC27*	HYNES and KELLY (1977)
creD34	*creD34; riboB2*	Derived from KELLY and HYNES (1977)
acrB2Y	*yA2; acrB2; choA1*	FGSC869
acrB2G	*biA1; acrB2; riboB2*	Derived from *acrB2Y*
acrB14	*pabaA1; acrB14; pantoB100*	Supplied by H. N. Arst
acrB15	*pabaA1; acrB15*	Supplied by H. N. Arst
creB15 creD34	*creB15 creD34; riboB2 niiA4*	Derived from KELLY and HYNES (1977)
creC27 creD34	*creC27 creD34; riboB2 niiA4*	Derived from KELLY and HYNES (1977)
creB1937 acrB2	*yA⁻ pabaA1; creB1937 acrB2*	This work
creC27 acrB2	*yA2; creC27 acrB2; choA1*	This work
creD34 acrB2	*creD34 acrB2; niiA4*	This work

Gene symbols are described in CLUTTERBUCK (1993, 1997). FGSC, Fungal Genetics Stock Center.

suppressors of the phenotypic effects of mutations in *creB* or *creC* may define components of the specific regulatory ubiquitination system for these substrates. The phenotypic similarities between *acrB2* and *creD34* led us to analyze whether the *acrB2* mutation is also a suppressor of the phenotypes due to *creB* and *creC* mutations, which would indicate a role for AcrB in a regulatory ubiquitination pathway that is required for correct regulation of carbon metabolism. Here we demonstrate that mutations in *acrB* result in altered utilization of various carbon sources in *A. nidulans*. In addition, we show that the effects of the *acrB2* mutation are epistatic to those due to the *creB* and *creC* mutations, and thus the *acrB* gene product is likely to play a role in the regulatory ubiquitination/deubiquitination network that includes the CreB/CreC complex. Thus, to further understand the role of AcrB, we have cloned and physically analyzed the *acrB* gene.

MATERIALS AND METHODS

***A. nidulans* strains, media, growth conditions, and manipulations:** The 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 where otherwise indicated. Genetic manipulations were carried out using techniques described by CLUTTERBUCK (1974). The *A. nidulans* strains used in this study are shown in Table 1. Gene symbols are as described in CLUTTERBUCK (1993, 1997). The *acrB* mutant strains were selected as spontaneous resistant sectors in the presence of acriflavine (ROPER and KÄFER 1957). A strain containing the *acrB2* mutation was obtained from the Fungal Genetics Stock Center, and strains containing the *acrB14* and *acrB15* mutations were obtained from H. N. Arst.

Molecular methods: Standard molecular techniques were as outlined in SAMBROOK *et al.* (1989). *A. nidulans* genomic DNA was prepared according to LEE and TAYLOR (1990). The *A. nidulans* cosmid library used is described in BRODY *et al.* (1991).

Plasmid c24F6-14 was generated by a partial *Eco*RI digestion

of cosmid SW23H06 followed by ligation to recircularize the products of digestion. *Cla*I subclones from c24F6-14 [p4AcrB (6-kb insert), p7AcrB (10-kb insert), and p10AcrB (2-kb insert)] were generated in the pBCSK⁺-based plasmid pCB1004 (CARROLL *et al.* 1994). p4AcrBSacIΔ is a *Sac*I-generated deletion of p4AcrB. p4AcrBKpnIΔ is a *Kpn*I-generated deletion of p4AcrB.

A. nidulans was transformed according to the method of TILBURN *et al.* (1983). Transformants from cotransformation experiments were selected using the *riboB⁺* selectable marker plasmid pPL3 (OAKLEY *et al.* 1987) on media lacking riboflavin. Complementation of the *acrB2* mutant phenotype was scored by morphology on complete medium and resistance to the presence of 0.001% acriflavine added to complete media.

The *acrB* gene was sequenced using a combination of custom-designed oligonucleotide primers. Double-stranded DNA template was prepared using the Promega Wizard + SV miniprep system following the manufacturer's instructions. Sequencing reactions were as outlined in the Applied Biosystems (Foster City, CA) dye terminator system instructions and were analyzed at the Institute of Medical and Veterinary Science sequencing facility (Adelaide, Australia). Sequence analysis was performed using the Australian National Genomic Information Service facility or BioNavigator.

Intron positions were determined by the PCR amplification of *acrB* cDNA segments from DNA prepared from an *A. nidulans* cDNA library (kindly provided by S. Osmani) using custom oligonucleotides as primers and standard PCR conditions and cycle parameters. These products were purified and directly sequenced using the same primers used for amplification. The primer pairs used were acrB1 (5′-AGTCTTGGCTG CTATCCG-3′; −58 to −40) and KBF (5′-GACACGCCACCA TTCGTC-3′; +329 to +312), KBB (5′-GCGAAGGGAAATG CAGAC-3′; +280 to +297) and KB (5′-ATGGGCTAAATCGA GAGC-3′; +726 to +709), AK (5′-GCAATGGATGGTTTC TGC-3′; +646 to +663) and SF (5′-CTCGCCTCGCCAATA GAT-3′; +1617 to +1600), KF (5′-CGCGTGGATACAGAA GCG-3′; +1066 to +1083) and AS (5′-CAATCCTCTGCCTTG GCGG-3′; +2194 to +2177), SB (5′-AAGAGGCTACCGC CGCTC-3′; +2057 to +2074) and SBB (5′-GCGAGCGGTGG CTTGGCA-3′; +2641 to +2624), and SBF (5′-CTTGGGCA CATTGGCGTC-3′; +2601 to +2618) and acrB5 (5′-AACCAG TCACTACACGGA-3′; +3249 to +3232).

The transcription start point was determined by direct sequencing of the nested PCR product and five subcloned prod-

wild type creD34 acrB2G acrB14 acrB15

a. Glucose + ammonium

b. Complete + acriflavine

c. Glucose + ammonium + molybdate

d. Fructose + ammonium

e. Trehalose + ammonium

f. Glucose + fluoroacetamide
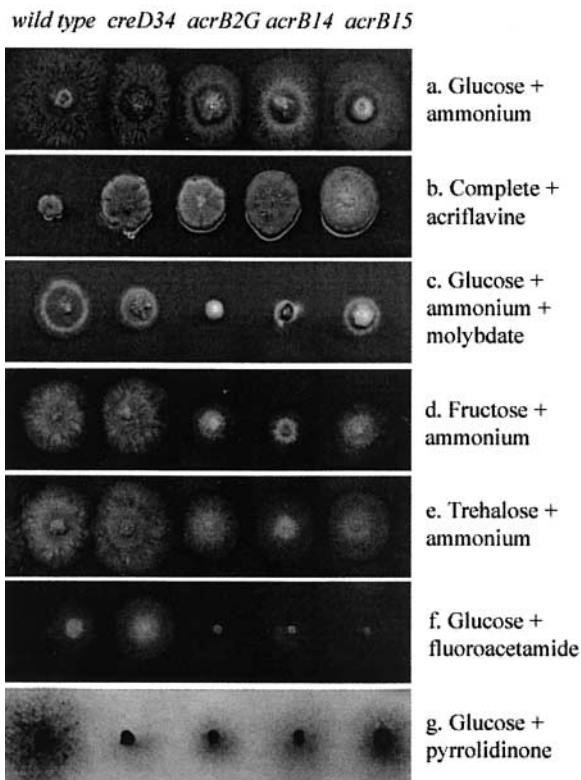
g. Glucose + pyrrolidinone

FIGURE 1.—Phenotypic heterogeneity among strains bearing *acrB* mutant alleles. Strains containing *acrB* mutant alleles, *acrB2* (strain *acrB2G*), *acrB14*, and *acrB15*, were grown at 37° for 2–3 days, along with a strain containing *creD34* and a wild-type strain, on the following media: (a) 1% D-glucose plus 10 mM ammonium L-tartrate, (b) complete medium plus 0.001% acriflavine, (c) 1% D-glucose plus 10 mM ammonium L-tartrate plus 11 mM molybdate, (d) 1% D-fructose plus 10 mM ammonium L-tartrate, (e) 1% D-trehalose plus 10 mM ammonium L-tartrate, (f) 1% D-glucose plus 10 mg/ml fluoroacetamide, and (g) 1% D-glucose plus 10 mM 2-pyrrolidinone.

ucts of 5′ rapid amplification of cDNA ends (5′ RACE) performed on total RNA from D-glucose-grown mycelium. 5′ RACE was performed using the Ambion (Austin, TX) First Choice RLM-RACE kit, following the manufacturer's instructions.

Sequences of mutant alleles were determined by direct sequencing of PCR products derived from genomic DNA of the relevant mutant strain. These were sequenced using the same procedures as for intron determination. Direct sequencing of *acrB* PCR products from wild-type DNA was undertaken for comparison.

RESULTS

Phenotypic analysis of *acrB2*, *acrB14*, and *acrB15*: As selected, the three *acrB* mutant strains, *acrB2*, *acrB14*, and *acrB15*, were resistant to the presence of acriflavine in complete medium, with similar levels of resistance between *acrB* mutant strains and the *creD34* strain (Figure 1). Since the *creD34* mutation leads to sensitivity to molybdate, the three *acrB* mutant strains were tested and found to be more sensitive than the wild-type strain

to the presence of 11 mM molybdate in synthetic complete medium, but the alleles were heterogeneous, with the *acrB2* strain being the most sensitive to the presence of molybdate and the *acrB15* strain showing lesser sensitivity. Thus the effects of the *acrB* mutant alleles when the toxic compounds acriflavine and molybdate are added to the medium are the same as those for *creD34* and the opposite to the effects of *creB* and *creC* mutant alleles, which confer increased sensitivity to acriflavine and resistance to molybdate added to complete medium (HYNES and KELLY 1977; KELLY and HYNES 1977; ARST 1981).

When tested for their ability to utilize various carbon sources, *acrB* mutant strains were found to have pleiotropic phenotypes with respect to carbon source utilization. They show decreased ability to utilize a number of different sugars as sole carbon sources in comparison to both the wild type and the *creD34* mutant strain, including fructose, cellobiose, raffinose, and starch (Figure 1; Table 2). In general, the *acrB14* strain showed a slightly more extreme phenotype than that showed by the *acrB2* and *acrB15* strains. The reduced growth on various sugars may indicate a failure of uptake or a failure to express genes encoding enzymes required for their utilization. The ability to utilize other alternate carbon sources such as quinate and glycerol was only slightly affected by the *acrB* mutations, with the exception of ethanol where the *acrB* mutants grew considerably less well than wild type (Table 2).

Test medium containing 10 mM allyl alcohol in the presence of glucose was used to investigate the expression of the alcohol dehydrogenase encoded by *alcA*. All the *acrB*-containing strains, like the wild-type and *creD34*-containing strains, were resistant, indicating that they showed correct glucose repression of *alcA*. The *acrB* mutant strains were hypersensitive on medium containing glucose plus 10 mg/ml fluoroacetamide, indicating an increased level of the acetamidase expressed from the *amdS* gene compared to the wild-type strain, which is the opposite of the phenotype conferred by the *creD34* mutation. The lack of growth of the *acrB* mutant strains on medium containing glucose plus 10 mM acrylamide as a nitrogen source indicates that this increased level of *amdS* expression is not due to constitutive expression, as acrylamide is a substrate of the acetamidase but not an inducer of *amdS* (HYNES and PATEMAN 1970). Further, wild-type growth of the *acrB* mutant strains on acetamide as a carbon, nitrogen, or carbon and nitrogen source indicates that the *amdS* gene is fully inducible. Thus there is a defect in the glucose-mediated repression of *amdS* but no other effect on expression.

Growth on media containing the ω-amino acids 10 mM β-alanine, 10 mM GABA, or 10 mM pyrrolidinone as nitrogen sources was significantly decreased in the *acrB* mutant strains compared to the wild-type strain, as was the case for a strain containing *creD34*. Similarly, growth of the mutant *acrB* strains on media containing

TABLE 2

Phenotypic analysis of *acrB* mutant strains

Growth condition	Wild type	creD34	acrB2G	acrB14	acrB15
Complete	10	9	9	9	9
Glucose	10	10	9	9	9
Cellobiose	10	10	5	5	5
Fructose	10	10	4	3	4
Galactose	8	8	4	3	4
Lactose	8	8	4	4	4
Maltose	10	10	6	5	6
Raffinose	10	10	6	6	6
Starch	10	10	8	8	8
Sucrose	10	10	7	7	7
Trehalose	10	10	6	5	6
Xylose	10	10	7	7	7
Acetate	9	10	6	5	8
Ethanol	10	10	5	4	5
Glucuronate	8	8	7	7	7
Glycerol	10	10	9	9	9
Succinate	3	3	3	3	2
Quinate	10	10	9	9	9
Acriflavine	1	9	9	10	10
Molybdate	10	5	2	3	4
Fluoroacetamide	4	5	0	0	0
2.5 mM allyl alcohol	10	10	10	10	10
10 mM allyl alcohol	9	9	9	9	9
β-Alanine	2	1	2	1	2
GABA	8	8	2	2	2
Pyrrolidinone	8	8	2	3	1
Glutamate	8	8	4	4	4
Proline	8	8	4	4	5
Threonine	2	2	2	2	2
β-Alanine + glucose	10	5	6	6	6
GABA + glucose	10	8	5	5	5
Pyrrolidinone + glucose	10	1	3	5	7
Glutamate + glucose	10	10	9	9	9
Proline + glucose	10	10	10	10	10
Threonine + glucose	10	4	10	7	10
Glucose + acetamide	10	9	10	9	10
Acetamide	5	5	4	4	4
Acetamide + [NH$_4$]$_2$T	5	5	4	4	4
Glucose + acrylamide	1	1	1	1	1

For full genotypes, see Table 1. Plates were incubated at 37° for 2 or 3 days. Carbon sources were added to carbon-free medium containing 10 mM ammonium L-tartrate at 1.2% (acetate), 1% (D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, lactose, D-maltose, D-raffinose, starch, succinate, sucrose, D-trehalose, and D-xylose), 0.5% (D-glucuronate and ethanol) or 50 mM [(D)-quinate]. Molybdate was added to synthetic complete medium at 11 mM. Fluoroacetamide was added to 1% D-glucose medium at 10 mg/ml. Allyl alcohol was added to synthetic complete medium at 2.5 or 10 mM. Acetamide, β-alanine, GABA (γ-amino-n-butyric acid), L-glutamate, L-proline, 2-pyrrolidinone, and L-threonine were added at 10 mM to 1% glucose medium and at 50 mM when added to medium without another carbon source. Ammonium L-tartrate and acrylamide were added at 10 mM. The overall score assigned to each colony was derived from the colony size, the hyphal density, and the amount of conidiation. For each medium, a score was assigned to define the wild-type growth, and the mutant strains were scored relative to the wild-type score. Scores are not comparable between media. Each plate test was performed on at least three separate occasions.

50 mM GABA and 50 mM pyrrolidinone acting as both a carbon and a nitrogen source was also decreased compared to wild type and the *creD34* mutant strain, but this effect was not obvious on media containing 50 mM β-alanine. The *acrB* mutant alleles affected growth to various degrees on media that had 10 mM pyrrolidinone as a nitrogen source, with the *acrB15* strain having strong, almost wild-type-like growth, compared to the intermediate growth of the *acrB14* strain and the weaker growth of the *acrB2* strain, and this heterogeneity between alleles indicates that they do not all represent complete loss-of-function alleles.

Growth on media containing other amino acids as nitrogen sources, such as 10 mM proline and 10 mM glutamate, was unaffected by the *acrB* mutations. However, decreased utilization of 50 mM proline and 50 mM glutamate when they were used as both carbon and nitrogen sources was observed for the *acrB* mutant strains in comparison to the wild-type strain.

Partial suppression by *acrB2* of *creB* and *creC* mutant phenotypes: The *creD34* mutation was identified as a suppressor of the hypersensitivity to fluoroacetamide conferred by the *creC27* mutation. The effects of the *creD34* mutation are pleiotropic in that it also leads to suppression of the effects of *creC27* on other enzymes subject to carbon catabolite repression, such as alcohol dehydrogenase I, but it does not suppress the effects of *creC27* that are apparent under derepressing conditions, such as the poor growth on D-quinate medium (KELLY and HYNES 1977; KELLY 1980). The *creD34* mutation suppresses not only some of the phenotypes conferred by the *creC27* mutation, but also some of the phenotypic effects of the *creB15* mutation, and, weakly, of the *creA204* mutation (KELLY and HYNES 1977; KELLY 1980). Similarities between the phenotypes of *creD* and *acrB* mutant strains led us to test whether the effects of mutation in *acrB* are also epistatic for some of the phenotypes conferred by *creB* and *creC* mutations.

The presence of the *acrB2* mutation reversed the sensitivity conferred by the *creB1937* and *creC27* mutations to the presence of acriflavine in complete media (Figure 2c). The *creD34 acrB2* double mutant strain was even more resistant to the presence of acriflavine than was either of the individual mutant strains, indicating an additive effect. The resistance of the *creB1937* and *creC27* strains to the presence of molybdate was also reversed in the relevant *acrB2* double mutant strains and to a greater degree than that in the *creD34* double mutant strains (Figure 2e). The *acrB2* mutation also reversed the toxic effects of allyl alcohol added to D-glucose medium in *creC27* and *creB1937* backgrounds, the same phenotypic suppression previously seen for the *creD34* mutation (Figure 2f).

The *acrB2* mutation results in reduced growth compared to wild type on sole carbon sources such as fructose, maltose, starch, cellobiose, ethanol, and acetate, a phenotype not seen for the *creD34* mutation (Figure 1; Table 2). This reduced growth is also seen in the *acrB2creB1937* and *acrB2creC27* double mutant strains compared to the single *creB1937* and *creC27* mutant phenotypes, respectively (Figure 2g). The inability of the *creB1937* and *creC27* mutant strains to utilize D-quinate as an alternate carbon source was partially suppressed by the *acrB2* mutation in the double mutant strains, and this effect was more pronounced than that in the *creD34* double mutant strains (Figure 2h). Finally, the *acrB2creB1937* and *acrB2creC27* strains grew as poorly as the *acrB2* strain on glucose medium containing 10 mM pyrrolidinone as the nitrogen source (Figure 2i).
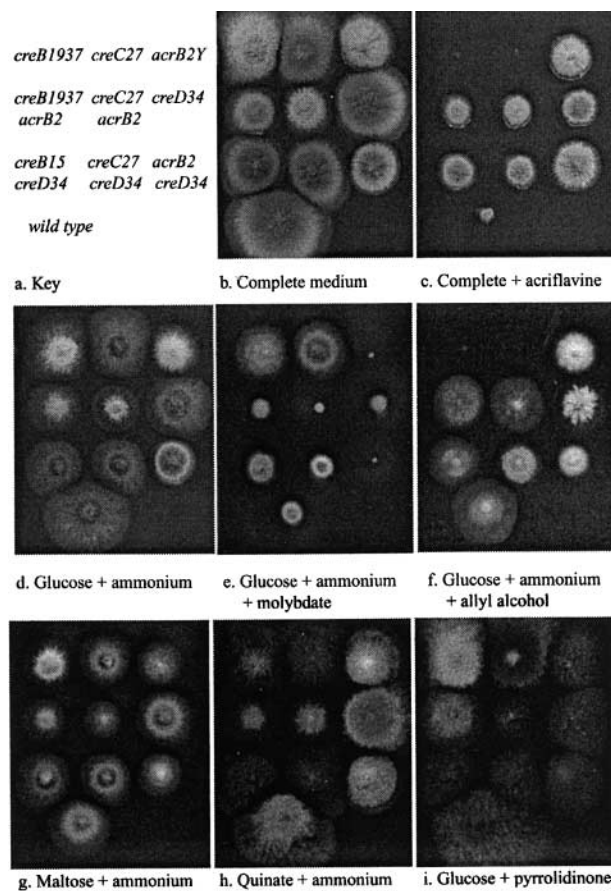

FIGURE 2.—Epistatic effects of the *acrB2* mutation on *creB* and *creC* mutant phenotypes. (a) Key to strain placement. Indicated strains were grown at 37° for 2–3 days on the following media: (b) complete medium, (c) complete medium plus 0.001% acriflavine, (d) 1% D-glucose plus 10 mM ammonium L-tartrate, (e) 1% D-glucose plus 10 mM ammonium L-tartrate plus 33 mM molybdate, (f) 1% D-glucose plus 10 mM ammonium L-tartrate plus 10 mM allyl alcohol, (g) 1% D-maltose plus 10 mM ammonium L-tartrate, (h) 50 mM (D)-quinate plus 10 mM ammonium L-tartrate, and (i) 1% D-glucose plus 10 mM 2-pyrrolidinone.

Molecular cloning of *acrB*: We took advantage of the close genetic linkage of *acrB* and *creB* (CLUTTERBUCK 1997) to clone the *acrB* gene. The bacterial artificial chromosome (BAC) clone that contains *creB*, BAC4B1 (LOCKINGTON and KELLY 2001), was tested for complementation of the *acrB2* mutation in a transformation assay testing for the reversal of the resistance to acriflavine in complete medium conferred by the *acrB2* mutation. This BAC clone was found to complement the *acrB2* phenotype. Cosmid clones have been ordered within this BAC (LOCKINGTON and KELLY 2001) and the cosmids containing the *creB* gene, SL10A08 and RW18E04 (LOCKINGTON and KELLY 2001), were tested and failed to complement the *acrB2* mutation in the transformation assay. The cosmids flanking these cosmids, SL27D09 and SL19A07, which also hybridized to BAC4B1 (LOCKINGTON and KELLY 2001), were also

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CTTGACAGTAGCTGCCTGTAATGTCAGGCCATAAAGTTTCTGCCTTAGGCAATTTTGAGGATGTTTCCAATCTTTAGGTCGTTTC    -349
GTCTTTTTCGAACCAGGTCGTCTCTTCCTCTCCTTCTTCCCGCCTCTACACTCCCCCACGCCACGTCTTATCCCCTTCACTCTCCCC    -262
        #
CTCTATCTTATTATTCGAATTCTTCCCCTCTTTGCTCTGTGTATTCTCATATCTACTTGTTTCTTACGTTTCTCCCTTTTTGTCATT    -175
CATTCATTTTAACTGCAGAGACCCTGGCCTCGAATCCAGTCTTTTCCAAACTCCCTTCCGCCCTTTCCCGCGAACTTTTTTTGGAAC     -88
TGCCATCGACTTCATTTCCACCTGGTCCAAGTCCTTGGCTGCTATCCGCCAAGCCGACTCGGACCTGGGTGTTGGGTTCCCTCGTCC      -1
ATGCCACGTTCATCGGCTACGGCTAGGAAGAGTCATAGCAATCGACAAGACCATGGCGGCGGCGGTTCTGGGAAGAAGCCAAGCAAA      87
  1   M  P  R  S  S  A  T  A  R  K  S  H  S  N  R  Q  D  H  G  G  G  S  G  K  K  P  S  K
CAAAAGTCAAGTGGGCATTTAAACGCAACATACAACGGTACTGCTGGATCCGAAACTGGACCCGTCTTCGCAGGTCGACTGGCCATCG     174
 30   Q  K  S  S  G  H  L  N  A  T  Y  N  G  T  A  G  S  E  T  G  P  S  S  Q  V  D  W  P  S
CATCGTTCTGGAGACCAATCGATCGCAGCAGCCGCAGCCAAATCTAACGGACCGGTCGATAGCTTGAAAGCGGACACCAACGGACGT     261
 59   H  R  S  G  D  Q  S  I  A  A  A  A  A  K  S  N  G  P  V  D  S  L  K  A  D  T  N  G  R
GGTTATCCGGGCGGATATGCGAAGGGAAATGCAGACATGTCTTACGGGCAGACGAATGGTGGCGTGTCGCCGAATGGTGGACTCGCC     348
 88   G  Y  P  G  G  Y  A  K  G  N  A  D  M  S  Y  G  Q  T  N  G  G  V  S  P  N  G  G  L  A
GGGCCCGGCTTCACGTCGTACGGATAAGTCGGTCACTGGGACCAAGAGGACAACTTCGAATGCGTCGGTGAATCCGTTCCAGCTGGCA     435
117   G  P  A  S  R  R  T  D  K  S  V  T  G  T  K  R  T  T  S  N  A  S  V  N  P  F  Q  L  A
TCCACCATTCTCCGATCGTGTCCAATGTACGACACTATCGCCATCTTGATCTTTCTGCTTCAGCTCCCGCCTATGGTTCTCACTTTG     522
146   S  T  I  L  R  S  C  P  M  Y  D  T  I  A  I  L  I  F  L  L  Q  L  P  P  M  V  L  T  L
GTTCAATTCTTGTTTGCGTCCTTGACATTTATGCCTCCCAGCGGCACCGCTTCTGGATCCTTCACCTCCAACTTCGATATTTTCCAG     609
175   V  Q  F  L  P  A  S  L  T  F  M  P  P  S  G  T  A  S  G  S  F  T  S  N  F  D  I  F  Q
                 (T→T)=acrB14
GGACCCGCCGGAACCCCCTCGCTCGGTACCATGATTGCAATGGATGGTTTCTGCCTGCTTGTATGGGGCCTCTTTATGTGGACGTGG     696
204   G  P  A  G  T  P  S  L  G  T  M  I  A  M  D  G  F  C  L  L  V  W  G  L  F  M  W  T  W
GCCCAGAATTTTGCTCTCGATTTAGCCCATGTCCAGGTTGCCATCACTCTGGGCGGTGGAGGTGCAGGGAAAAATGGTGGTGTCAAT     783
233   A  Q  N  F  A  L  D  L  A  H  V  Q  V  A  I  T  L  G  G  G  G  A  G  K  N  G  G  V  N
GCGCTCTGCGTCGGTATTGTTCTGATTCTGCATCTCATACGCAGCAAAGGAATACAGGATTTTGTCGTCGGCCATCTTGTTTCAGCA     870
262   A  L  C  V  G  I  V  L  I  L  H  L  I  R  S  K  G  I  Q  D  F  V  V  G  H  L  V  S  A
AAAATCATTAGCCCCGATTTACTGTCGCATTATTCTTACCTCATGCCCGCCGAATTCAAGCGCACCGAATCGCAATCATCCCCGAGT     957
291   K  I  I  S  P  D  L  L  S  H  Y  S  Y  L  M  P  A  E  F  K  R  T  E  S  Q  S  S  P  S
TGGATCCGGAGCCTGCTTGCTGTACATATTCTGGCCCAGGCGGGTACTGCGATGGCGAGGCGATCGATGACTAAAAATAGGACCCCG    1044
320   W  I  R  S  L  L  A  V  H  I  L  A  Q  A  G  T  A  M  A  R  R  S  M  T  K  N  R  T  P
GCCCCATCACGATCAGGCAAACGCGTGGATACAGAAGCGTCTGCCGGCTCACAAACCCAGATCGACTCGGCGTTCGAATCCGCGGCC    1131
349   A  P  S  R  S  G  K  R  V  D  T  E  A  S  A  G  S  Q  T  Q  I  D  S  A  F  E  S  A  A
AGCGTTTCTTCCTATCTAGGCCCCGACGGGCAGATTATCACTGCCGCGCATAAGGACGGCAGGGATCGTTTGATATCGGCAAAAAAA    1218
378   S  V  S  S  Y  L  G  P  D  G  Q  I  I  T  A  A  H  K  D  G  R  D  R  L  I  S  A  K  K
CGACGAAGGCAGGCAAATCAAGTCAGGAGCCGGCAACCTTTTTGGGCTGCATGGCAAGCACAAAAGTCACGGTCATGAGGGAGTAT    1305
407   R  R  R  Q  A  N  Q  V  R  S  R  Q  P  F  W  A  A  L  A  S  T  K  V  T  V  M  R  E  Y
GAACATTCTAGGGCCTTGTCAAAAACTGCTAGAGGACTTGCTACGACGGAGGCGGATCTTCAAGGCGTTTCTTTGGACGATGGACTT    1392
436   E  H  S  R  A  L  S  K  T  A  R  G  L  A  T  T  E  D  D  L  Q  G  V  S  L  D  D  G  L
GTTTGGATTACGTATGTGGATAGCTCGACGATTAAGTTTGCAGCTGGGGATTTTGCGTCTTCGGACGACCATTCCGCGTCAGGTGTC    1479
465   V  W  I  T  Y  V  D  S  S  T  I  K  F  A  A  G  D  F  A  S  S  D  D  H  S  A  S  G  V
TGCGAAGCAGGCCGTGTGAGCAGCAGGGATGCGGAGCCGTTTTACGTCTGCGTCAATGGTGCGCCATGGGCAACGGTGGTCATCACT    1566
494   C  E  A  G  R  V  S  S  E  D  A  E  P  F  Y  V  C  V  N  G  A  P  W  A  T  V  V  I  T
AAAGAGCATGATCCTTCAAAAGCGTCTAATACAATCTATTGGCGAGGCGAGATATCAGGTCTTGCACCCAATTGCGCGTATACTTGC    1653
523   K  E  H  D  P  S  K  A  S  N  T  I  Y  W  R  G  E  I  S  G  L  A  P  N  C  A  Y  T  C
                                                     (_)=acrB2
TCTTTTGTTAAATGCGATACGGATGAGGAAATCTGCGCCATGAGTGTCAAGACCCCTGCGGCCAATGATGCAGAACAAGgtaagata    1740
552   S  F  V  K  C  D  T  D  E  E  I  C  A  M  S  V  K  T  P  A  A  N  D  A  E  Q  A
                                                           (__)=acrB15
ttcgttctatggtttccgattcgtctcgatgtggtctaatttgtgccttccatagCCAATTCGGTGCCGGCCCCTCCGCAACCCTCA    1827
578                                                        N  S  V  P  P  P  Q  P  S
TATCGACCATCCTCCCCAACAACCACGCTGAAGAACTCGATCATCAATGCTGAGGCGAAACTGAACGAAAAGCGTGCTCGACTCCGA    1914
589   Y  R  P  S  S  P  T  T  T  L  K  N  S  I  N  A  E  A  K  L  N  E  K  R  A  R  L  R
AAGGCCAAAAATGACCACAAGCTTGCTATTTCTAAGATCAAGAAGGAGCTGACAATTACACCAATCGTCTTCAGACGGCACGGAT    2001
618   K  A  K  N  D  H  K  L  A  I  S  K  I  K  K  E  L  D  N  Y  T  N  R  L  Q  S  G  T  D
GAAAACAGGCAGAAGCAACGCTCTCTTCAATTGGAAAGGAACATTCGACAAACTGAAGAGGCTACCGCCGCTCTGGACAACCAGATC    2088
647   E  N  R  Q  K  Q  R  S  L  Q  L  E  R  N  I  R  Q  T  E  E  A  T  A  A  L  D  N  Q  I
GATAACTTGGGTAATGTTCCTGACGATGAGTATCAGGAGTGGGTTGAACAGAAGGCAAAGTACGAACGTGAATTGGAGCTCCTCAAA    2175
676   D  N  L  G  N  V  P  D  D  E  Y  Q  E  W  V  E  Q  K  A  K  Y  E  R  E  L  E  L  L  K
TCCGCCAAGGCAGAGATTGCTGCCACGCGTACCGCCAATGCTCGCGAGTTATCTTCATTGGAATCCGAGTTGAACTCTACCACGCAA    2262
705   S  A  K  A  E  I  A  A  T  R  T  A  N  A  R  E  L  S  S  L  E  S  E  L  N  S  T  T  Q
CGGCGCGAACGTCTGCAGGGTCGCCGAACCAGAGTGAATGAGCAGTACGAACGGATCATCTCGGCCAACGCACAGGGTCTCAATGAG    2349
734   R  R  E  R  L  Q  G  R  R  T  R  V  N  E  Q  Y  E  R  I  I  S  A  N  A  Q  G  L  N  E
CGAGAGCGCCGCGCTGCAGAGCAGTTTGCCCGGGAACAAGATCAGTCGAAGTTGGAGCAAAGTTTCAACGAACAATTCGCGAGCATC    2436
763   R  E  R  R  A  A  E  Q  F  A  R  E  Q  D  Q  S  K  L  E  Q  S  F  N  E  Q  F  A  S  I
AGTCAATCAGTGCAGGATTATCAGCTGCGCACCAGTCAATTGTGGCAACAGTGTACCGCCGTCGAACAAGCCCTCCAGCAGCAGTTG    2523
792   S  Q  S  V  Q  D  Y  Q  L  R  T  S  Q  L  W  Q  Q  C  T  A  V  E  Q  A  L  Q  Q  Q  L
CTCATGGAGCCCGCTCCGCTAACACCCGAAGGCGAGCTGCCCGGTACTAGTACGTTTGCCGACGCGCCCAGCGTGCCCTTGGGCACA    2610
821   L  M  E  P  A  P  L  T  P  E  G  E  L  P  G  T  S  T  F  A  D  A  P  S  V  P  L  G  T
TTGGCGTCAAATATGCCAAGCCACCGCTCGCTACTAGGACAGAGCTTTCCGCCGCTCAAGTCTAGTCCTCTGCAGCACTATGCTTCG    2697
850   L  A  S  N  M  P  S  H  R  S  L  L  G  Q  S  F  P  P  L  K  S  S  P  L  Q  H  Y  A  S
CCAATTGGAACTGCTCCGTCTCATCCGACTAGTCCAATCGCCGTCTCCATCCTACCAGCCTTTCTCCAGCTCGCCATTGGTAACGCG    2784
879   P  I  G  T  A  P  S  H  P  T  S  P  I  A  A  P  S  Y  Q  P  F  S  S  S  P  F  G  N  A
GCATCCTTCCTTGACCCGGACTTTGTCTACCGCGACCGTTCGTTCTCCAACCGCTCCGCACGCAGCAGCCTCTATGGCTCTGAGTTC    2871
908   A  S  F  L  D  P  D  F  V  Y  R  D  R  S  F  S  N  R  S  A  R  S  S  L  Y  G  S  E  F
CCCGGACGCGGATAACGGCCGTGTCCCCTTTGGCGTTGATCCTTTCGAGCTCGGTAACGAGAAACGACGCGGTTCGGGGATCTGAC    2958
937   P  D  A  I  T  A  R  R  V  P  F  G  V  D  P  F  E  L  G  N  E  K  R  R  G  S  G  D
AGCACCCCGCTCAACGGTCCATCTGGCCTACGTCCTATCTCCAGTCCTTTCCAGCGAGCCGCCAGTCGCGCAAGTGGAACCGGCAGT    3045
966   S  T  P  L  N  G  P  S  G  L  R  P  I  S  S  P  F  Q  R  A  A  S  R  A  S  G  T  G  S
GGCGGAAGCGGAGGAAGTGGTAGTGGTAGTGGCAGTCCTAGCTCTGCCGGCGTGGAAAGGGAAACTAAACGGAACTGCTTGCCAGCCAG    3132
995   G  G  S  G  G  S  G  S  G  G  S  P  S  S  A  R  G  K  G  N  *
CCAACTCAGCCGTCTGCAGTGACGACAAAAGCATTGGAGATTGATCCTTACTCGCCTGCCCATATGTCCATATTTGGTCCTCGGACA    3219
TGGCTCGGACTGTCCGTGTAGTGACTGGTTATATAGTTTGCCGTCGCCAACGGTCTATAGTATAGATCCTTGTGCTCGCCGCGTAGA    3306
GCCACATCTTATATTTTGGCGAAGAAAAAGAG                                                          3338
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tested in a transformation assay, but again neither complemented the *acrB2* mutation. Other cosmids within the region that also hybridized to BAC4B1, SW21D03, SW22F07, SW23H02, and SW23H06, were tested, and SW22F07, SW23H02, and SW23H06 were found to complement the *acrB2* mutation. Thus the order of genes in this region is, from centromeric to telomeric, *adCadD-acoB-atrC-acrB-creB* with *acrB* ~30 kb from *creB*.

Cosmid SW23H06 was selected for further analysis. An *Eco*RI cutdown that contained a 10-kb insert, c24F6-14, complemented *acrB2* in the transformation assay. c24F6-16 contained three *Cla*I fragments, which were cloned into pCB1004 (p4AcrB, p7AcrB, and p10AcrB), and p4AcrB, containing a 6-kb insert, was found to complement the *acrB2* mutation, while p7AcrB and p10AcrB did not. Plasmids containing ~2-kb *Sac*I or *Kpn*I deletions of each end of p4AcrB (p4AcrBSacIΔ and p4AcrB-KpnIΔ) failed to complement *acrB2* in the transformation assay. Thus *acrB2* was located in the middle of the 6-kb insert of p4AcrB.

Sequencing of the insert in p4AcrB revealed a 3111-bp open reading frame (GenBank accession no. AF485329; Figure 3). The *acrB* gene contained a single intron, which was determined by sequence analysis of PCR amplified cDNAs (see materials and methods), and the intronic sequence conforms to the consensus 5′ and 3′ junction and lariat sequences for fungal introns (Gurr *et al.* 1987). To determine the transcription start point (tsp) of the gene, 5′ RACE of RNA purified from D-glucose-grown mycelium was carried out using nested primers. Direct sequencing of the nested PCR product and of several subclones of this product mapped a single major tsp −221 bp from the translation start (Figure 3). A TATA-like sequence is located 33 bp upstream of the tsp, and a CCAAT box is located 155 bp upstream of the tsp. The *acrB* gene is not present in the *A. nidulans* expressed sequence tag (EST) database (containing EST information for cDNA clones from a mixed vegetative and 24-hr asexual development culture of *A. nidulans* strains FGSCA26 constructed in lambda Zap by R. Aramayo at Texas A&M University; http://www.genome.ou.edu/asperg.html), indicating a low level of expression under these conditions. The results of a Northern analysis using *acrB* as a probe were consistent with this, and *acrB* mRNA was below the level of detection in total RNA extracted from glucose-grown wild-type mycelia,

but was present in total RNA extracted from either maltose- or fructose-grown mycelia (data not shown). This expression pattern indicates transcriptional control in response to glucose, although only a single theoretical CreA binding site is present in the 312 bases of sequence determined 5′ to the major start point of transcription.

The *acrB* gene encodes a 1015-amino-acid polypeptide. AcrB is predicted to contain three transmembrane domains near the N terminus, at amino acid residues 158–180 (I), 214–236 (II), and 257–274 (III), and a coiled-coil region, at amino acid residues 596–753 (SMART protein motif analysis program; Schultz *et al.* 1998). The transmembrane domain prediction program, TMHMM2 (Krogh *et al.* 2001), predicts the most probable location and orientation of transmembrane helices from sequence data, and this program indicated that the N terminus of the AcrB protein and the region between the second and third transmembrane domains are most likely cytoplasmic, with the C-terminal region of the protein predicted to be external.

A hypothetical protein is in contig 1168 of the TIGR *Aspergillus fumigatus* Genome Database, which shows 76% identity with the *A. nidulans* sequence, but these are very closely related ascomycete species. Database searches (SwissProt/SpTrEMBL/PDB) using the BlastP program (Altschul *et al.* 1990) revealed that the AcrB protein was not highly similar to any protein in these databases. AcrB shows most sequence similarity with hypothetical proteins, one from *Neurospora crassa*, Q9C2R3 (31% identity over 1015 amino acids), and one from *Saccharomyces cerevisiae*, YG2K (24% identity over 861 amino acids). The hypothetical *N. crassa* protein contains three putative transmembrane domains and a coiled-coil domain, and although it shows low similarity along the length of the protein with AcrB, specific regions such as the transmembrane domains share higher sequence identity (55% identity over 24 amino acids). The *S. cerevisiae* hypothetical protein has a protein structure similar to that of AcrB, with three transmembrane domains and a coiled-coil region, but the sequence similarity is uniformly low throughout the protein.

Molecular analysis of the *acrB* mutant alleles: Three mutant alleles of *acrB* were sequenced to identify functional regions of the AcrB protein and to show that *acrB* rather than a suppressor of *acrB2* had been isolated. We used a PCR approach using primers spanning the *acrB*

Figure 3.—Location of the *acrB* mutations within the *acrB* gene. The complete sequence of the *acrB* genomic region is shown (GenBank accession no. AF485329). The single intron of 63 bp is depicted in lowercase letters and the 5′ and 3′ splice junction and lariat sequences are underlined. A putative TATA box and a putative CCAAT box are underlined and in boldface letters. The major start point of transcription is indicated above the DNA by the # symbol. A single theoretical CreA binding site in the 5′ untranslated region is double underlined, and a single theoretical AreA binding site in the 5′ untranslated region is underlined with a dashed line. The amino acid sequence is shown in single letter code beneath the DNA sequence, with numbered amino residues indicated on the left-hand side and numbered nucleotide positions indicated on the right-hand side, with the first nucleotide of the start codon as +1. The position and nature of the DNA sequence changes in the *acrB2*, *acrB14*, and *acrB15* alleles are shown, with (_) indicating a deletion of the base directly below and (+) indicating an addition of a base at the position below. The stop codon is indicated by an asterisk.

gene followed by direct sequencing of the PCR products (see materials and methods). The *acrB2* mutation is a single base pair deletion at nucleotide (nt) +1734 at the 5′ splice site of the only intron (Figure 3). This would be predicted to disrupt the splicing of the intron, resulting in a frameshift after amino acid 577 and the additional residues, GRYSFYGFRFVSMWSNLCLP, before terminating. The *acrB15* mutant allele contains a 2-bp deletion at nt +1815, which results in a frameshift after amino acid 584, with an addition amino acid sequence of ATLISTILPNNHAEELDHQC before truncating. Both *acrB2* and *acrB15* gene products contain all three transmembrane domains but lack the coiled-coil region. The *acrB14* allele contains a C-to-T transition plus a 1-bp insertion (T) at nt +627 that results in a frameshift after amino acid 209 and truncates 84 amino acids later. The additional amino acids are FARYHD CNGWFLPACMGPLYVDVGPEFCSRFSPCPGCHHSGR WRCREKWWCQCALRRYCSDSASHTQQRNTGFCRRP SCFSKNH. The *acrB14* mutant gene product lacks two of the three transmembrane domains and the coiled-coil region and, since it is also recessive to the wild-type allele, probably represents a null allele.

DISCUSSION

In the multicellular eukaryote, *A. nidulans*, CreA, encoded by the *creA* gene, is the master DNA-binding repressor required for carbon catabolite repression of a wide range of genes (Arst and Cove 1973; Bailey and Arst 1975; Arst and Bailey 1977; Hynes and Kelly 1977; Lockington *et al.* 1985; Dowzer and Kelly 1989, 1991; Kulmberg *et al.* 1993; Cubero and Scazzocchio 1994; Mathieu and Felenbok 1994; Scazzocchio *et al.* 1995; Shroff *et al.* 1996, 1997; Panozzo *et al.* 1998; Mathieu *et al.* 2000). The *creB* and *creC* genes were identified in some of the same genetic screens that identified mutations in *creA*, and it is clear from the phenotypes of *creB* and *creC* mutant strains that CreB and CreC play roles under both carbon catabolite repressing and carbon catabolite derepressing conditions. Compared with *creA* mutations, the *creB* and *creC* mutations lead to derepression of a restricted range of genes in the presence of a source of carbon catabolite repression such as glucose. However, compared with *creA* mutations, the *creB* and *creC* mutations have more marked effects on the expression of genes for the utilization of alternative sole carbon sources in the absence of a source of carbon catabolite repression (Hynes and Kelly 1977; Kelly and Hynes 1977; Kelly 1980; Arst 1981; Todd *et al.* 2000; Lockington and Kelly 2001). The *creB* gene encodes a deubiquitinating enzyme (Lockington and Kelly 2001) and the *creC* gene encodes a protein that contains five WD40-repeat motifs and a proline-rich region (Todd *et al.* 2000), and CreB and CreC are present in a complex *in vivo* under both carbon catabolite repressing and derepressing conditions

(Lockington and Kelly 2002). What remains unclear is whether the CreB/CreC regulatory deubiquitination network acts directly on CreA, affecting its stability, or whether it exerts its effects on carbon metabolism independently of CreA. A model has been put forward that is compatible with the existing data in *A. nidulans* in which a regulatory deubiquitination network involving the CreB/CreC complex acts directly on CreA, along with other target proteins, such that the effect of CreB and CreC on carbon catabolite repression is exerted directly via an effect on the stability of CreA (Lockington and Kelly 2002). This model is supported by experiments showing that protein modification and/or stability of CreA could be an important component of the carbon catabolite repression mechanism (Strauss *et al.* 1999). Alternatively, a model can be put forward in which the direct action of the CreB/CreC regulatory deubiquitination complex is on permeases, transporters, or other membrane proteins, with the effects on carbon catabolite repression being the consequence of altered concentrations or cellular localizations of signaling molecules or stabilization of membrane proteins involved in glucose sensing. There are parallels with this in yeast. Some yeast permeases, including Gap1p (general amino acid permease), are subject to ubiquitin-dependent regulation in response to nitrogen sources (Galan *et al.* 1996; Springael and Andre 1998; Rotin *et al.* 2000). The addition of ammonium to the medium leads to ubiquitination of Gap1, which is required for degradation of the permease. Proteins required for this process include the ubiquitin ligase, Npi1p (Springael and Andre 1998), and Bul1p and Bul2p, which form a complex with Npi1p (Yashiroda *et al.* 1996, 1998). Bro1p, which is also involved in the glucose-induced degradation of hexose transporters Hxt6/7 (Springael *et al.* 2002), and Npr1p, a protein kinase that prevents Gap1 from internalization and proteolysis (De Craene *et al.* 2001; Soetens *et al.* 2001), are also involved in this regulatory process. The ubiquitin hydrolase, Npi2p, is required to maintain the intracellular pool of ubiquitin (Swaminathan *et al.* 1999). It is possible that the CreB/CreC complex is required to stabilize permeases and transporters by the removal of ubiquitin and in this way affect intracellular concentrations or locations of glucose or other effector molecules, or it may stabilize membrane proteins involved in glucose sensing. Although a direct or indirect effect of CreB/CreC on CreA is discussed above as alternative models, in fact, both models could apply as they do not conflict with each other. To further characterize the roles of CreB and CreC in the cell, we have taken the approach of analyzing phenotypic suppressors of some or all of the phenotypes of *creB* and *creC* mutant alleles, to identify proteins involved in the ubiquitination of substrates that are deubiquinated by the CreB/CreC complex.

Phenotypic analysis of the effects of three *acrB* alleles has shown that mutations in *acrB* have a broader range

of phenotypes than the resistance to toxic compounds such as acriflavine, crystal violet, and malachite green previously described (ROPER and KAFER 1957), as they grow poorly on a range of sole carbon sources including sugars, starch, and ethanol, and they also grow poorly on some ω-amino acids such as β-alanine, GABA, and pyrrolidinone either as sole carbon and nitrogen sources or as nitrogen sources in the presence of D-glucose as a carbon source. Genetic analysis has shown that the *acrB2* mutation is a suppressor of the phenotypes conferred by mutations in *creB* and *creC*. Strains containing the *creB1937* and *creC27* mutations show derepressed expression of the *alcA* gene, in that there is inappropriate expression in the presence of D-glucose, and the presence of the *acrB2* mutation completely reverses the effects of these mutations as judged by plate testing. The reduced growth of the *acrB2*-containing strain on medium containing ethanol as a sole carbon source may indicate that this is due to a failure to induce *alcA* under either repressing or derepressing conditions due to the loss of *acrB* and that this failure in induction overrides the derepression caused by *creB* and *creC* mutations. The *creB1937* and *creC27* mutations lead to poor growth on D-quinate and D-glucuronate as sole carbon sources, and the *acrB2* mutation partially repairs this defect. These phenotypes are consistent with a regulatory defect associated with the membrane and signaling, but not with a simple interpretation that only permeases and transporters are affected, as in the case of ethanol, a fat soluble compound, no permease is required or exists.

The amino acid sequence of AcrB indicates that the protein contains membrane-spanning domains, indicating the probability of its residing in the membrane. There is also a coiled-coil region that would allow the prediction that the protein forms either a homodimer or a heterodimer. The striking aspect of the sequence is the lack of sequence similarity with proteins in databases from other eukaryotes, including those for which the entire genome has been sequenced, and it is clearly not a direct homolog of any gene in a characterized signaling pathway. If functional homologs exist they are very diverged in sequence from AcrB and thus unrecognizable. The CreB and CreC proteins are involved in a deubiquitination network that removes ubiquitin from target proteins, and although these target proteins are not yet directly identified, the mutant phenotypes indicate that they are proteins involved in carbon metabolism and its regulation. Unlike AcrB, CreB and CreC are well conserved among most eukaryotes other than yeast. The *acrB2* mutation suppresses phenotypes of the *creB* and *creC* mutations and this suggests that AcrB is involved in a process opposite to deubiquitination, such as a ubiquitin ligase pathway, since a failure to add ubiquitin to substrates could suppress the phenotypic effects of mutations that affect the removal of ubiquitin moieties. Thus the ubiquitination/deubiquitination net-

work in *A. nidulans* that involves CreB, CreC, and AcrB provides an ideal genetic and molecular genetic system in which to unravel regulation of protein stability.

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