Amino Acid Catabolism by an *areA*-Regulated Gene Encoding an L-Amino Acid Oxidase with Broad Substrate Specificity in *Aspergillus nidulans*

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The filamentous fungus *Aspergillus nidulans* can use a wide range of compounds as nitrogen sources. The synthesis of the various catabolic enzymes needed to breakdown these nitrogen sources is regulated by the *areA* gene, which encodes a GATA transcription factor required to activate gene expression under nitrogen-limiting conditions. The *areA102* mutation results in pleiotropic effects on nitrogen source utilization, including better growth on certain amino acids as nitrogen sources. Mutations in the *sarA* gene were previously isolated as suppressors of the strong growth of an *areA102* strain on L-histidine as a sole nitrogen source. We cloned the *sarA* gene by complementation of a *sarA* mutant and showed that it encodes an L-amino acid oxidase enzyme with broad substrate specificity. Elevated expression of this enzyme activity in an *areA102* background accounts for the strong growth of these strains on amino acids that are substrates for this enzyme. Loss of function *sarA* mutations, which abolish the L-amino acid oxidase activity, reverse the *areA102* phenotype. Growth tests with *areA102* and *sarA* mutants show that this enzyme is the primary route of catabolism for some amino acids, while other amino acids are metabolized through alternative pathways that yield either ammonium or glutamate for growth.

Aspergillus nidulans can use most amino acids as nitrogen sources, although different amino acids support growth to a greater or lesser extent (2, 3, 15). The synthesis of many nitrogen catabolic enzymes is regulated by nitrogen metabolite repression mediated by the product of the *areA* gene (2, 13). The AreA transcription factor contains a single C-terminal GATA-type zinc finger DNA binding domain and activates the expression of genes involved in nitrogen acquisition when nitrogen availability is limiting for growth (17). The *areA102* mutation results in a single amino acid substitution (Leu 683 to Val) in the loop of the GATA zinc finger. This leucine residue is universally conserved in GATA factors, and the conservative L683V change alters the binding affinity of the protein (17, 26).

The *areA102* mutation has pleiotropic effects on the utilization of a variety of amino acid and non-amino acid nitrogen sources and results in better growth on L-amino acids such as histidine, leucine, and lysine that are normally very poor nitrogen sources for *A. nidulans* (2, 12, 25). The *sarA* gene was initially identified in a collection of mutants isolated as suppressors of the *areA102* phenotype on histidine as a nitrogen source (24). As the utilization of several amino acids was altered in these mutants, the *sarA* gene was proposed to have a regulatory function.

Our objective in this study was to isolate and characterize the *sarA* gene and to determine its role in amino acid catabolism. This study has highlighted an enzyme activity that enables filamentous fungi to scavenge nitrogen from a wide range of amino acid substrates and has further defined the pathways of amino acid catabolism in *A. nidulans*.

MATERIALS AND METHODS

A. nidulans strains, media, and transformation. *Aspergillus* media and growth conditions were as described by Cove (7). Nitrogen sources were added at a final concentration of 10 mM and glucose at 1% (wt/vol). Genetic manipulations were carried out using techniques described by Clutterbuck (6). The *Aspergillus* strains used in this study were MH1 (*biA1*), MH8 (*biA1 areA102 niiA4*), MH50 (*yA1 areA102 pyroA4 riboB2*), MH9924 (*biA1 amdS::lacZ pyroA4 riboB2*), MH10065 (*biA1 areA102 sarA31 niiA4*), MH10066 (*biA1 areA102 sarA31 pyrG*[−]), MH10067 (*yA1 areA102 pyroA4 riboB2 sarA31*), MH10571 (*yA1 pyroA4 sarA::riboB riboB2*), MH10742 (*biA1 gdhB101 niiA4*), MH10743 (*biA1 areA102 gdhA10 gdhB101*), MH10744 (*biA1 pyroA4 gdhA10*), MH10745 (*biA1 areA102 gdhA10 pyroA4 niiA4*), MH10746 (*biA1 gdhA10 gdhB101 riboB2*), and MH10747 (*biA1 areA102 gdhB101 niiA4*). All strains used in this study are available on request. Gene symbols are those of Clutterbuck (6). *A. nidulans* strains were transformed according to the method of Andrianopoulos and Hynes (1). Transformants were selected on medium lacking riboflavin using the *riboB*⁺ gene from pPL3 (21) or on medium lacking pyridoxine using the *pyroA*⁺ gene from pI4 (23) as the selectable marker.

Molecular methods. Molecular methods were essentially as described by Sambrook et al. (27). *Escherichia coli* strain NM522, *supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM) 5(r_K[−] m_K⁺)* [F' *proAB lacZΔM15*], was used for all bacterial work. DNA was prepared using the High Pure Plasmid Isolation kit (Boehringer-Mannheim, Germany) and sequenced by the Australian Genome Research Facility (Brisbane, Australia). *A. nidulans* genomic DNA was isolated by the method of Lee and Taylor (18).

Complementation of the *sarA31* mutation. The *sarA* gene was cloned by functional complementation of the *sarA31* mutant with an *A. nidulans* genomic library in the autonomously replicating vector pRG3AMA1 (22). Transformants of MH10066 (*areA102 sarA31 pyrG*) were selected for uracil/uridine prototrophy on protoplast medium (1) containing biotin and with 10 mM ammonium tartrate as the sole nitrogen source. PyrG⁺ transformants were screened on glucose minimal medium containing 10 mM histidine as the sole nitrogen source. Genomic DNA from rapidly growing transformants was prepared and transformed into *E. coli*, and rescued plasmids were isolated from ampicillin-resistant colonies. Plasmids were screened by size, and restriction enzyme digests and representative plasmids were transformed back into MH10066 to confirm rescue of sequences that complement *sarA31*. A 2.1-kb NotI/KpnI fragment of a complementing plasmid pMD5302 was subcloned into pBlueScript SK(+) to create pMD5422 and sequenced.

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FIG. 1. Phenotype of *sarA* mutants on histidine, structure of the *sarA* gene, and *sarA* inactivation. (A) Wild-type (WT; MH1), *areA102* (MH8), *sarA31 areA102* (MH10065), and *sarAΔ areA102* (MH) strains were grown on glucose-minimal medium with 10 mM histidine as the sole nitrogen source for 2 days at 37°C. *sarA31/sarAΔ areA102* is a diploid strain formed between MH and MH10065. (B) The black boxes represent the coding region interrupted by a single intron. The direction of transcription is indicated by an arrow. Symbols in the promoter region represent potential AreA binding sites, where the open circles are TGATAA (−711, −391, −318, −304, and −191), the closed circle is AGATAA (−425), and the box is AGATAG (−668).

Nucleotide sequence accession number. This sequence has been deposited in GenBank (accession number AY688952).

Inactivation of the *sarA* gene. The disruption construct was created by removing 1 kb of the coding region of *sarA* by ligating a 3.7-kb *SmaI*-*XhoI* fragment of *riboB* from pPL1 (21) into the *StuI* and *XhoI* sites of pMD5422 to create pMA5655. This plasmid was linearized with *NotI* and transformed into MH50 (*areA102 riboB2⁺*). *Ribo⁺* transformants were screened on glucose-minimal medium containing 10 mM histidine as the sole nitrogen source. One transformant, MH10571, had a *sarA* mutant phenotype, and inactivation of the *sarA* gene was confirmed by Southern blot analysis with a 4.3-kb *NotI*-*KpnI* insert of pMD5422 as the probe. To confirm that the phenotype of MH10571 was the result of inactivation of the *sarA* gene, this strain was cotransformed with pMD5422 with the *pyrA* gene on pI4 (23) as the selectable marker. *Pyro⁺* transformants were selected on media lacking pyridoxine, and the cotransformants regained the *areA102* phenotype of good growth on histidine as a sole nitrogen source.

Protein comparison. The amino acid similarity of the *A. nidulans* predicted SarA protein and the *Neurospora crassa* Lao protein (GenBank accession number J05621) and predicted proteins from the *Magnaporthe grisea* genome (www.broad.mit.edu/annotation/fungi/magnaporthe/) and the *Fusarium graminearum* genome (www.broad.mit.edu/annotation/fungi/fusarium/) was determined by Gap analysis (11).

RESULTS

Isolation of the *sarA* gene. An *areA102 sarA31* double mutant grew more poorly on histidine than an *areA102* strain (Fig. 1) (24), and the *sarA* gene was cloned by complementation of this mutant phenotype. pMD5302 complemented the *sarA31* mutation in MH10067, and cotransformants carrying this plasmid grew as strongly as an *areA102* strain on L-histidine, L-leucine, and L-lysine as sole nitrogen sources. The insert in this plasmid, a 4-kb *KpnI*-*NotI* fragment, contained a 2.1-kb open reading frame interrupted by a single intron of 58 bp encoding a 685-amino-acid protein.

***sarA* encoded an L-amino acid oxidase enzyme.** The predicted product of the *sarA* gene had 52% amino acid similarity to the L-amino acid oxidase of *Neurospora crassa* encoded by the *lao* gene. There was general sequence conservation

throughout the predicted proteins and, in particular, conservation of the FAD binding site in the *N. crassa* protein. Similar proteins were predicted from the genome sequences of the plant pathogenic fungi *Magnaporthe grisea* and *Fusarium graminearum* (56% and 54% amino acid similarity, respectively).

Overexpression of an L-amino acid oxidase activity could result in phenotypic suppression, rather than genetic complementation, of the *sarA31* mutant. We tested this hypothesis by disrupting the wild-type *sarA* sequence with the *riboB*-selectable marker. One transformant, MH10571, grew poorly on histidine; the disruption of *sarA* was confirmed by Southern blot analysis (data not shown). The phenotype of the disruptant was the same as that of the *sarA31* mutant (Fig. 1A). We also made a diploid between the *sarA* disruptant and an *areA102 sarA31* strain. The diploid had a *sarA* mutant phenotype, indicating that the original *sarA* mutant could not be complemented by the mutant carrying the gene disruptant. Therefore, the cloned sequences represent the genomic *sarA* gene, and the *sarA* mutant phenotype is due to loss of L-amino acid oxidase activity.

L-Amino acid oxidase activity. To confirm that the level of L-amino acid oxidase activity determines the extent of growth on histidine as a nitrogen source, multiple copies of *sarA* were introduced into the *areA⁺* strain MH9924 by cotransformation with pPL3 (21). Overexpression of *sarA* in an *areA⁺* background would be expected to result in an *areA102*-like phenotype on histidine as the sole nitrogen source. The *sarA* copy numbers were determined in the transformants and found to correlate with their growth phenotypes. *Ribo⁺* transformants that lacked additional copies of the *sarA* gene had a wild-type phenotype, and the growth of transformants carrying one to four additional copies of *sarA* was intermediate between growth of the wild type and an *areA102* strain, while the growth of transformants carrying four to six additional copies of *sarA* was indistinguishable from that of an *areA102* strain on histidine as a sole nitrogen source (data not shown).

L-Amino acid oxidase activity also can produce hydrogen peroxide. Colony growth, morphology, or conidiation of the *areA102* strain was not detectably inhibited on histidine. To further elevate *sarA* expression, multicopy *sarA* strains were generated in an *areA102* background (MH50) by cotransformation with pPL3 and pMD5422. None showed evidence of growth inhibition or reduced conidiation when grown on histidine or other nitrogen sources (data not shown). Therefore, overexpression of *sarA* even in an *areA102* background did not result in detectable oxidative stress under conditions where hydrogen peroxide generation was elevated.

L-Amino acid oxidase activity and nitrogen source utilization. The *sarA* mutation also has a substantial effect on the utilization of L-leucine, L-lysine, L-methionine, and L-cysteine in an *areA102* background (24). Multicopy *sarA* strains generated in an *areA⁺* background grew better than the wild-type strain on these amino acids (data not shown). Therefore, these amino acids are substrates for the *sarA*-encoded enzyme. The utilization of amino acids metabolized exclusively via L-amino acid oxidase activity to ammonium will be *sarA* dependent and elevated in *areA102* mutants. L-Cysteine, L-histidine, L-leucine, L-lysine, L-methionine, L- α -amino butyrate, and L-citrulline are all substrates for the L-amino acid oxidase and are not detectably metabolized by any other enzyme activities. As this reac-

FIG. 2. The effect of *sarA*, *gdhA*, and *gdhB* mutations on amino acid utilization. Strains were grown on glucose-minimal medium containing amino acids as indicated at a concentration of 10 mM for 2 days at 37°C. The relevant genotypes of the strains are indicated. The strains (from left to right) are MH1, MH8, MH10745, MH10747, MH10743, MH10744, MH10742, MH10746, and MH10065.

tion released ammonium as the nitrogen source, growth on these amino acids also required *gdhA* function (Fig. 2). The *gdhA* gene encodes NADP-linked glutamate dehydrogenase, the major enzyme for the assimilation of ammonium into glutamate (3, 16).

Some amino acids are substrates for L-amino acid oxidase but also are substrates for other catabolic activities (Fig. 2). L-Tryptophan is metabolized by both *sarA*-dependent and *sarA*-independent catabolic activities that yield ammonium, as growth on this amino acid was *gdhA* dependent. Certain other amino acids that were substrates for L-amino oxidase were also metabolized by alternative catabolic activities that yielded glutamate. Growth on substrates metabolized via glutamate is dependent on the function of the *gdhB* gene encoding catabolic NAD-linked glutamate dehydrogenase to release ammonium from glutamate for glutamine biosynthesis (4, 15). These amino acids included L-alanine, L-isoleucine, L-ornithine, L-tyrosine, and L-valine. L-Phenylalanine metabolism requires *sarA* function, as well as a *sarA*-independent reaction(s) that produces both ammonium and glutamate. Growth of *areA102* strains on L-arginine, L-γ-amino butyrate, L-glycine, L-proline, L-serine, or L-threonine was not affected by the *sarA* mutation,

suggesting that these amino acids are not substrates for the *A. nidulans* L-amino acid oxidase.

DISCUSSION

The *sarA* gene was initially identified as an extragenic suppressor of the *areA102* phenotype on histidine and several other amino acids as sole nitrogen sources (24). We found that the *sarA* gene encodes an L-amino acid oxidase with a substrate specificity that is sufficiently broad to explain the pleiotropic phenotype of *sarA* mutants. A single *sarA* mutation that abolishes L-amino acid oxidase activity results in the simultaneous loss of the enhanced growth of *areA102* strains on these substrates.

The homologous *lao* gene of *N. crassa* encodes a precursor protein from which the mature enzyme is produced, following proteolytic cleavage at Ala129 (19). The cleavage site is preceded by basic residues at amino acid positions 1, 3, 6, 7, and 9 prior to Ala129 in *N. crassa*. There is some conservation of amino acids preceding the equivalent residue Ala138 in the predicted *A. nidulans* polypeptide, but only the residues at leading positions 6 and 7, which are identical to those found in the *N. crassa* protein, are basic.

The substrate specificity of the *N. crassa* L-amino acid oxidase enzyme is broad, with the best utilized including L-histidine, L- α -amino butyrate, L-canavanine, and L-tyrosine (29). In *N. crassa*, no *lao* structural gene mutants have been isolated, although a mutant postulated to have an altered *lao* promoter that increases *lao* expression has been described (5). The *A. nidulans* enzyme appears to have a similar substrate range, and there is a good correlation between the known substrates of the *N. crassa* enzyme and the effects of the *sarA* mutations on amino acid utilization. The substrate range of the *N. crassa* enzyme does not include L-glycine, serine, threonine, proline, or aspartate, none of which is *sarA* dependent for utilization by *A. nidulans*.

N. crassa L-amino acid oxidase is induced in the presence of amino acids, is not synthesized in the absence of carbon, and is regulated by *nit-2*, the functional homolog of *areA* (10, 20, 28, 29). *sarA* expression requires nitrogen limitation or starvation, but the addition of L-amino acids does not further increase L-amino acid oxidase activity (25). The *sarA* gene is regulated by the *areA* gene, and the *areA*-dependent response to nitrogen starvation is abolished by carbon starvation (25). This pattern of regulation mirrors that of the *A. nidulans* *fmdS*, which encodes formamidase (9, 12). Formamide, like histidine, is a nitrogen source but not a carbon source for *A. nidulans*.

The effect of the *areA102* mutation on *sarA* expression and hence growth on histidine is striking. The *areA102* mutation results in a single amino acid substitution (Leu 683 to Val) in the loop of the GATA zinc finger, which alters the binding affinity of the protein (17, 26). The wild-type protein recognizes H(A/C/T)GATA sequences with similar affinities. The mutant form of the protein binds more strongly to TGATA and less strongly to CGATA sites than does the wild-type protein. The *sarA* gene promoter contains seven potential AreA binding sites, five of which are TGATAA (Fig. 1B). Two NIT2 binding sites are known in the promoter of the *N. crassa* *lao* gene (8, 30). These sites both contain paired GATA sites separated by 3 and 10 bp, respectively. Mutations within the DNA binding domain of NIT2 can affect the ability of the protein to bind to these sites (30).

A. nidulans can use the *sarA* L-amino acid oxidase to catabolize many amino acids, but this enzyme is not highly expressed in a wild-type genetic background. One reason for the low activity in the wild type might be that the L-amino acid oxidase activity releases hydrogen peroxide in addition to ammonium and the corresponding keto acid. Hydrogen peroxide can cause oxidative damage to cells although *A. nidulans*, like other organisms, synthesizes a variety of catalases to help protect the cells (14). We did not detect an obvious viability cost associated with elevated *sarA* expression, but under natural conditions high levels of expression might be selected against. A number of forms of the *N. crassa* enzyme have been detected, and both intracellular and extracellular activities are produced, all of which are the product of a single gene (20, 28, 29). Both intracellular and extracellular activities in *A. nidulans* have also been detected (25). Secretion of a broad-specificity L-amino oxidase may provide a means to release ammonium from scarce and diverse sources while helping to protect cells from its toxic by-products. The combination of these factors suggest that, in the wild-type strain, *sarA* is expressed at a low level to

balance the generation of toxic products and the ability to scavenge nitrogen from the environment.

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