Characterization of the *amdR*-Controlled *lamA* and *lamB* Genes of *Aspergillus nidulans*

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ABSTRACT

Four *Aspergillus nidulans* genes are known to be under the control of the *trans*-acting regulatory gene *amdR*. We describe the isolation and initial characterization of one of these *amdR*-regulated genes, *lamA*. The *lam* locus, however, was found to consist of two divergently transcribed genes, the *lamA* gene, and a new gene, also under *amdR* control, which we have designated *lamB*. Using recombinant DNA techniques we have constructed a strain of *A. nidulans* lacking a functional *lamB* gene. Experiments conducted with this strain demonstrate that *lamB*, like *lamA*, is involved in utilization of 2-pyrrolidinone in *A. nidulans*. Metabolism of a related compound, γ-amino butyric acid (GABA) is not affected. We also provide evidence that the conversion of exogenous 2-pyrrolidinone to endogenous GABA requires a functional *lamB* gene. The expression of both *lamA* and *lamB* is subject to carbon and nitrogen metabolite repression in addition to *amdR*-mediated induction by ω-amino acids.

THE mechanism by which multiple environmental signals control the expression of individual genes is the subject of much investigation. The regulation of genes required for the utilization of substrates which can serve as carbon and/or nitrogen sources in eukaryotic microorganisms provides a model for the study of more complex regulatory systems.

The *Aspergillus nidulans lamA* gene is required for utilization of 2-pyrrolidinone, the lactam of γ-amino butyric acid (GABA) (ARST, PENFOLD and BAILEY 1978). Both 2-pyrrolidinone and the ω-amino acid, GABA, can serve as sole nitrogen and/or carbon sources for *A. nidulans*. The proposed pathway for the metabolism of 2-pyrrolidinone and GABA is shown in Figure 1 (from ARST, PENFOLD and BAILEY 1978). The genes participating in 2-pyrrolidinone (*lamA*, *gatA*) and GABA (*gabA*, *gatA*) utilization are under the control of a positively acting regulatory gene, *amdR*, also known as *intA* (ARST 1976). Induction by *amdR* is dependent on the presence of ω-amino acid inducers. The acetamidase structural gene (*amdS*) constitutes a fourth gene regulated by *amdR* (HYNES and PATEMAN 1970a,b). The *amdS*, *gabA* and *lamA* genes are also subject to nitrogen metabolite repression mediated by the *areA* gene (ARST and COVE 1973; ARST 1976; ARST, PENFOLD and BAILEY 1978), while the *gatA* gene is at most only weakly affected by this control mechanism (I. B. RICHARDSON, S. HURLEY and M. J. HYNES, unpublished data). The *amdS* and *gatA* genes are also known to be subject to carbon catabolite repression (ARST and COVE 1973; HYNES and KELLY 1977; BAILEY and ARST 1975).

An understanding of the mechanism by which the expression of the *lamA*, *gatA*, *gabA* and *amdS* genes is regulated can only be achieved with detailed molecular analysis. The *amdS* and *gatA* genes as well as the *amdR* regulatory gene have been isolated (HYNES, CORRICK and KING 1983; ANDRIANOPOULOS and HYNES 1988; I. B. RICHARDSON, S. HURLEY and M. J. HYNES, unpublished data).

We report here the isolation of the *lamA* gene and the fortuitously discovered *lamB* gene. Using a combination of recombinant DNA and classical genetic techniques we demonstrated that *lamB* is a new member of the *amdR* regulon and is required for conversion of exogenous 2-pyrrolidinone to endogenous GABA. We show that both *lamA* and *lamB* are subject to carbon and nitrogen metabolite control.

MATERIALS AND METHODS

Strains: The strains used in this study are listed in Table 1. All the markers have been described (CLUTTERBUCK 1984).

Media, genetic techniques and *Aspergillus* transformation: The growth media used have been described (COVE 1966). 2-Pyrrolidinone medium refers to medium containing 1% glucose as a carbon source and 10 mM 2-pyrrolidinone as a nitrogen source, unless otherwise noted. Where 2-pyrrolidinone provided a sole carbon source the concentration of 2-pyrrolidinone was raised to 50 mM.

Standard techniques for genetic analysis were used (CLUTTERBUCK 1974). The preparation and transformation of *Aspergillus nidulans* protoplasts has been described (TILBURN *et al.* 1983). In most experiments cotransformation,

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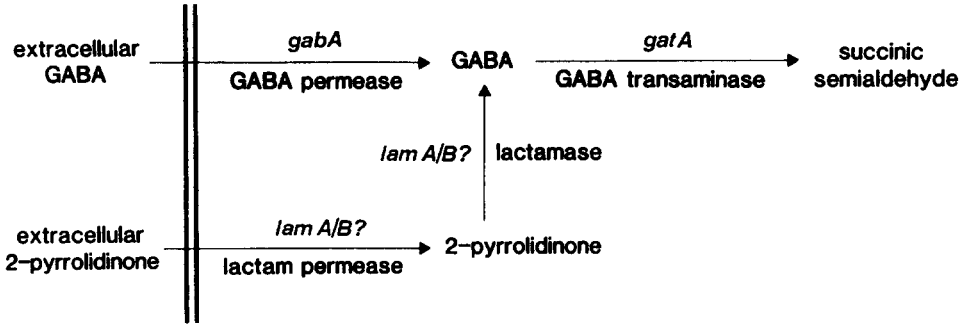


FIGURE 1.—Proposed pathway of GABA and 2-pyrrolidinone catabolism (from ARST, PENFOLD and BAILEY 1978).

TABLE 1

Genotypes of strains used

Strain	Genotype
MH54 (WT)	*biA1; niiA4*
MH3018	*pabaA1, yA2; argB1*
MH3075	*niiA4, lamA5, riboB2, trpC801*
MH3032	*yA1; amdA7; lamA5; riboB2*
MH3005	*pabaA1; lamA5*
MH3610	*pabaA1; lamA1*
MH3019	*biA1; argB1; methG1*
MH2671	*pabaA1; prn-309; cnxJ1*
MH6	*biA1; amdR6^c*
MH8	*biA1; areA102; niiA4*
MH341	*su-adE20, yA1, adE20; areA217; riboB2*
MH345	*biA1; amdR44; pyroA4; niiA4*
MH1055	*biA1; wA3; gatA312; niiA4*
LARG-RS1	*pabaA1, yA1; gatA312*
LARG-RS3	*pabaA1, yA1; gatA312; lamB*::pLARG

Strains and transformants not listed in the table are described in the text. The meaning of gene symbols have been described (CLUTTERBUCK 1984). Strains containing *lamA*, *trpC* and *argB* alleles were originally kindly provided by H. N. ARST, W. E. TIMBERLAKE and A. UPSHALL, respectively.

using a second selectable plasmid, was employed. Cotransformation frequencies varied with 10–90% of transformants carrying the selected plasmid also receiving a functional copy of the unselected plasmid.

pAmPh, a plasmid which contains the *Escherichia coli* bleomycin resistance gene under the control of the *Neurospora crassa am* promoter (B. AUSTIN and B. TYLER, personal communication) was used in cotransformation experiments with LARG1. Bleomycin resistant transformants were selected on protoplast regeneration medium (TILBURN *et al.* 1983) containing 1 μg/ml Blenoxane (Bristol Laboratories).

DNA and RNA preparation: Genomic DNA was prepared from Aspergillus by the method of YELTON, HAMER and TIMBERLAKE (1984) with the modifications described by ANDRIANOPOULOS and HYNES (1988). RNA was prepared by a procedure developed for the isolation of *N. crassa* RNA (REINERT, PATEL and GILES 1981).

Isolation of the *lamA* gene: The *trpC801 lamA5* strain, MH3075, was transformed with a cosmid gene bank constructed by YELTON, TIMBERLAKE and VAN DEN HONDEL (1985). Trp$^+$ transformants were selected on protoplast regeneration media (TILBURN *et al.* 1983) without tryptophan supplementation. Transformants were replicated directly from regeneration media to 2-pyrrolidinone medium using sterile velvet cloths.

Cosmid sequences were recovered from the Lam$^+$ Aspergillus transformant by the following procedure. For each treatment approximately 400 ng of transformant genomic DNA was partially digested with 0.1 unit of *Bgl*II for 1 hr at 37°C. Following heat inactivation of the restriction endonuclease, the digested DNA was diluted to a final volume of 100 μl and incubated in the presence of 1 unit of T4 ligase for 16 hr at 14°C. The ligation reaction mixture was extracted with phenol/chloroform and precipitated. The ligated DNA was resuspended in TCM (100 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 10 mM $CaCl_2$) and introduced into *E. coli* by transformation selecting for resistance to 25 μg/ml ampicillin.

Southern and DNA dot blots: For Southern blotting, 1–2 μg of Aspergillus genomic DNA were fractionated, following restriction endonuclease digestion, by electrophoresis through a 0.6% agarose gel. ^{32}P-Labeled molecular weight standards were prepared by end-labeling *Hin*dIII-digested lambda DNA using T4 DNA polymerase. The DNA was transferred to a nitrocellulose membrane (BA-85, Schleicher & Schuell Co.) by the method described by HYNES, CORRICK and KING (1983).

DNA samples were prepared for dot blots by a 15-min RNase treatment (1 μg/ml). The samples were denatured by heating in boiling water for 2–3 min, cooled on ice and an equal volume of denaturation solution (4.8 M formaldehyde, 1.8 M NaCl, 0.18 M trisodium citrate) was added. DNA was applied to a nitrocellulose membrane with the help of a dot-blot apparatus (Schleicher & Schuell Co.) and baked for 16 hr at 65°C.

The hybridization and stringent washing procedures used have been described (DAVIS and HYNES 1987; HYNES, CORRICK and KING 1983).

Northern and RNA dot blots: Northern blots were prepared by the electrophoresis of RNA in a 1% agarose gel (2.2 M formaldehyde) and transfer of the RNA to a nylon membrane (Zetaprobe, Bio-Rad) in 50 mM NaOH. RNA samples were prepared for dot blots by the addition of an equal volume of denaturation solution (see DNA dots). A procedure identical to that used for DNA dot blots was used to apply the RNA samples to a nitrocellulose membrane. Hybridization and washing procedures were as for Southern and DNA dot blots.

RESULTS

Isolation of the *A. nidulans lamA* gene: The *lamA* gene was isolated from a cosmid gene bank (YELTON, TIMBERLAKE and VAN DEN HONDEL 1985) by complementation of the *lamA5* mutation. The library consists of wild-type Aspergillus genomic DNA inserted in a cosmid vector containing the *A. nidulans trpC* gene and bacterial genes specifying ampicillin and chloramphenicol resistance in *E. coli*. The cosmid library was introduced into a *trpC801 lamA5* strain of *A.*

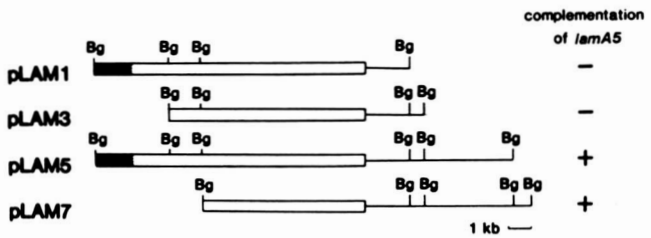


FIGURE 2.—Plasmids containing cosmid sequences recovered from the Trp$^+$ Lam$^+$ transformant, MLM-28C. *Bgl*II (Bg) sites only are shown. Open boxes represent cosmid sequences; shaded boxes represent *A. nidulans* sequences adjacent to the other side of the cosmid cloning site and lines represent *A. nidulans* insert sequences. Complementation of the *lamA5* mutation was tested by cotransformation and plating on medium containing 2-pyrrolidinone as the sole nitrogen source.

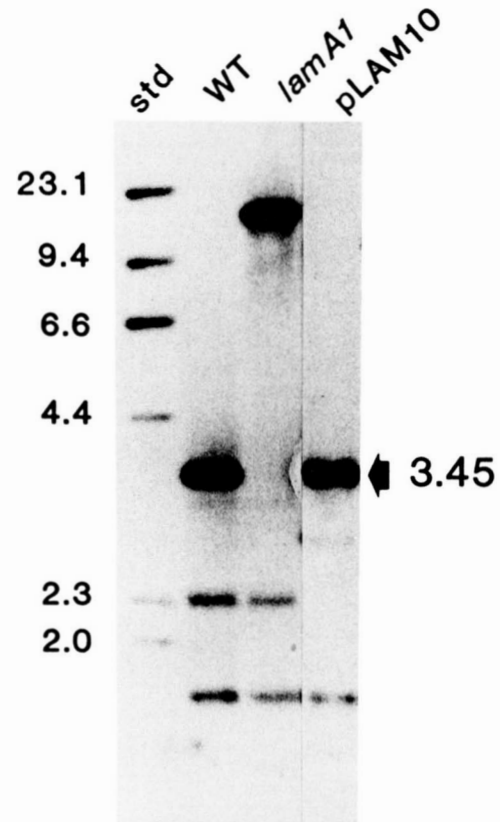
nidulans (MH3075) by transformation. Three thousand trp$^+$ transformants were tested for the ability to utilize 2-pyrrolidinone as a sole nitrogen source. A single Lam$^+$ transformant (MLM-28C) was obtained.

Cosmid sequences were recovered from the *Aspergillus* transformant by transformation of *E. coli*. MLM-28C genomic DNA was partially digested with the restriction endonuclease *Bgl*II. Partial digestion conditions were chosen such that the bulk of genomic DNA was of a size greater than 10 kb in order to facilitate the rescue of substantial portions of sequence adjacent to the cosmid vector drug resistance genes. The digested DNA was ligated and introduced into *E. coli* by transformation. Eight ampicillin-resistant transformants were obtained. The transformants were subsequently shown to also be resistant to 15 μg/ml chloramphenicol. The frequency with which bacterial transformants were produced was extremely low (<1 per 400 ng Aspergillus genomic DNA).

Plasmid DNA was prepared from the transformants. Analysis of the *Bgl*II restriction fragments of overlapping plasmid clones indicated that sequences adjacent to both sides of the cosmid vector cloning site had been recovered. In a preliminary experiment, transformation of a *lamA5* strain of *A. nidulans* with plasmid mini-preps, showed that pLAM7 carried an intact *lamA* gene. On that basis pLAM7 and 3 other rescued plasmids (pLAM1, 3 and 5) were chosen for further study (Figure 2).

Plasmid DNA preparations of pLAM1, 3, 5 and 7 were purified on CsCl gradients and tested by cotransformation of the *trpC801 lamA5* strain. The *trpC* plasmid, pHY201 (YELTON, HAMER and TIMBERLAKE 1984), was used for primary selection of Trp$^+$ transformants. The results are shown in Figure 2. Both pLAM5 and pLAM7 gave a high frequency of cotransformants able to utilize 2-pyrrolidinone. No Lam$^+$ cotransformations were observed among 50 pLAM1 and 50 pLAM3 cotransformants tested.

Based on the information obtained from the overlapping plasmids pLAM1, 3, 5 and 7, two subclones



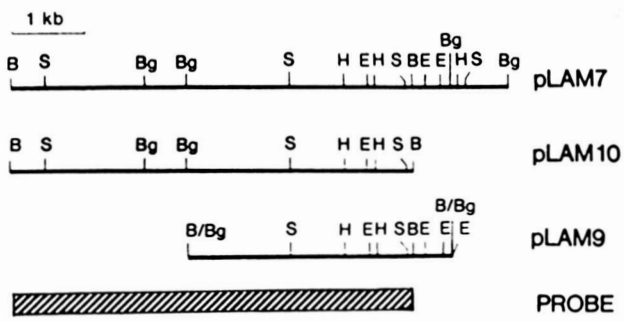


FIGURE 3.—Southern blot analysis of the translocation strain *lamA1*, MH3610 (upper figure) and restriction maps of inserts in the rescued plasmid pLAM7 and the derivative plasmids pLAM9 and pLAM10 (lower figure). DNA from the *lam*$^+$ strain, MH2671 (WT), the *lamA1* mutant strain MH3610 and pLAM10 were digested with *Sac*I, run on a 0.6% agarose gel and blotted to nitrocellulose. The filter was hybridized to the *Bam*HI fragment shown as a shaded box in the lower figure. This fragment was gel purified and ^{32}P-labeled by nick translation. The probe hydridized to three *Sac*I fragments in wild-type DNA. The 3.45-kb fragment only was altered in the *lamA1* mutant. Lambda DNA digested with *Hin*dIII and end labeled with T4 DNA polymerase was run for size standards (std). pLAM10 and pLAM9 were, respectively, *Bam*HI and *Bgl*II subclones of pLAM7 cloned into the *Bam*HI site of pUC18. BamHI, B; *Sac*I, S; *Bgl*II, Bg; *Hin*dIII, H and *Eco*RI, E.

of pLAM7 were constructed in pUC18. Restriction maps of pLAM9 and pLAM10 are shown in Figure 3. pLAM10 was shown to transform a *lamA5* strain of *A. nidulans* to Lam$^+$ both in cotransformation exper-

iments (12 Lam$^+$ cotransformants of 25 tested) and by direct selection on regeneration medium containing 2-pyrrolidinone as a sole nitrogen source. Lam$^+$ transformants were obtained with pLAM9 only at a low frequency (2 of the 75 cotransformants tested) and none were obtained by direct selection. Southern blot analysis of the two pLAM9 transformants demonstrated that one was the result of a gene conversion event. In the second transformant a homologous integration event at the *lamA* locus had occurred (results not shown). As Lam$^+$ transformants were generated only by rare homologous recombination events at the *lamA* locus it was considered unlikely that pLAM9 contained the entire *lamA* gene.

Southern blot analysis confirmed the integrity of the pLAM10 insert. No differences in restriction sites between the plasmid insert and Aspergillus genomic DNA were detected with one exception (six restriction endonucleases tested). A *Bam*HI site was created at the junction with the cosmid vector that was not present in Aspergillus genomic DNA.

Location of the translocation breakpoint in the *lamA1* strain: The *lamA1* mutation is associated with a translocation involving linkage groups VII and VIII (Arst, Penford and Bailey 1978). Southern blot analysis of DNA from the strain carrying the translocation showed that the translocation breakpoint is located in a 3.45-kb *Sac*I restriction fragment of pLAM10 (Figure 3).

Transcriptional analysis of pLAM10: We have shown that pLAM10 hybridizes to two divergently transcribed mRNAs of 4.0 and 1.0 kb (M. E. Katz and M. J. Hynes, unpublished data). Both transcripts are more abundant in mycelia grown in the presence of the inducer β-alanine (Figure 4). Various subclones of pLAM10 with deletions extending into the region coding for the 4-kb transcript were constructed. These deleted plasmids failed to complement the *lamA5* mutation (results not shown). These results were consistent with the 4-kb transcript being the *lamA* message. The gene coding for the 1-kb mRNA has been designated *lamB*. The size of the RNA species detected were consistent with previous results showing that two polypeptides of 39 kDa and greater than 100 kDa were inducible by β-alanine (M. J. Hynes and J. A. King, unpublished data).

Inactivation of *lamB*: We have explored the role of the 1.0-kb transcript in 2-pyrrolidinone metabolism in *A. nidulans* by creating an insertion mutation in the sequences coding for the transcript. A plasmid (pLARG) was designed that could, by a single crossover event between the *lam* sequences in pLARG and homologous genomic sequences, interrupt the transcribed region. pLARG was constructed by the addition of a selectable marker to a pUC18 derived plasmid containing an internal fragment of the region

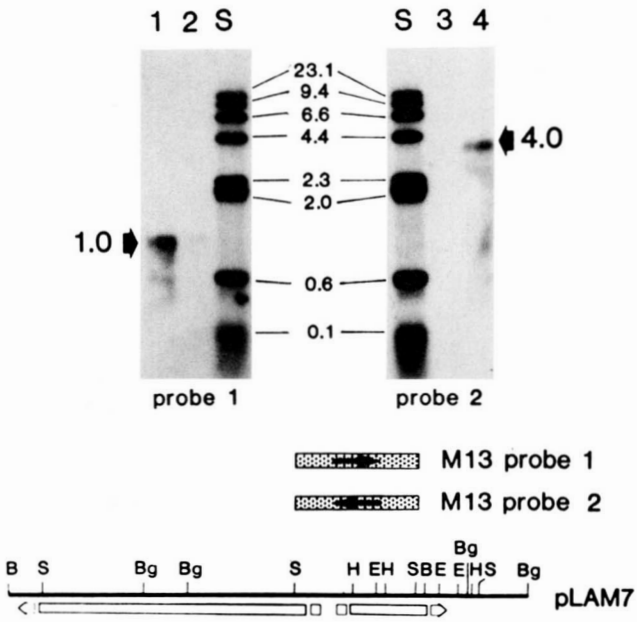


FIGURE 4.—β-Alanine induction of two divergently transcribed RNA species from the *lam* region. Total RNA was prepared from mycelium grown in 50 mM alanine (lanes 2 and 3) or 50 mM β-alanine (lanes 1 and 4) as the sole nitrogen source and subject to Northern blot analysis. Single stranded probes used were the indicated *Sac*I subclone of pLAM7 inserted in both orientations in M13mp18. ^{32}P-Labeling of the probes used standard hybridization probe primer and the Klenow fragment of DNA polymerase I. Standard (S) size markers were end labeled lambda *Hin*dIII fragments. Restriction enzyme abbreviations are as for Figure 3. The approximate extents of the two transcripts are shown below the restriction map of pLAM7. The 5′ and 3′ termini of the transcripts have not been determined precisely.

transcribed into the 1.0-kb mRNA. A 3.4-kb *Xba*I fragment containing the *argB* gene (Upshall 1986; Miller, Miller and Timberlake 1985) was inserted into the *Xba*I site of the pUC18 polylinker (Figure 5). The resulting construct was introduced into an *argB*$^-$ strain of *A. nidulans*, MH3018. 150 arg$^+$ transformants were tested for altered growth characteristics on 2-pyrrolidinone media. The growth of one transformant was greatly reduced on 2-pyrrolidinone as a sole nitrogen source (Figure 5). Utilization of GABA was not affected (results not shown).

Southern analysis of the transformant (LARG1) confirmed that the transforming plasmid, pLARG, had inserted by homology into the *A. nidulans* genome (Figure 5). No additional copies of the plasmid were detected. We have therefore demonstrated that the disruption of the *lamB* gene results in reduced 2-pyrrolidinone utilization in *A. nidulans*.

Two crosses were performed to confirm that the Lam$^-$ phenotype of the transformant was a result of the plasmid integration event in sequences adjacent to *lamA*. The LARG1 transformant was crossed to a *lamA*$^-$ strain. No Lam$^+$ segregants were recovered (out of 230 tested) indicating that the *lam*$^-$ mutation in the transformant was closely linked to *lamA*. A

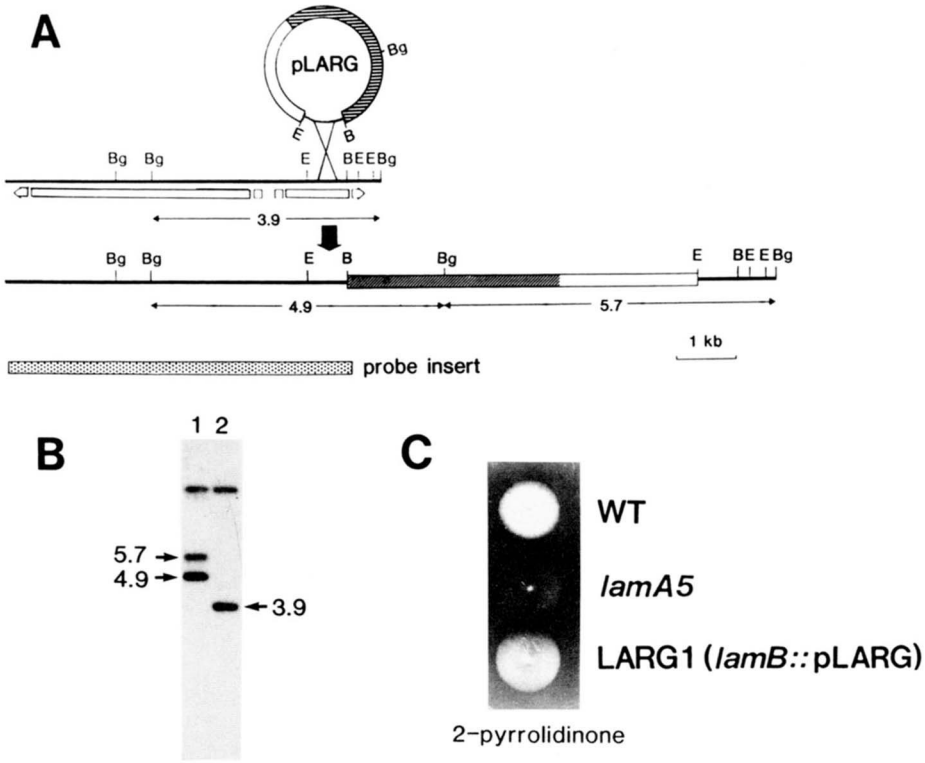


FIGURE 5.—Inactivation of the *lamA* gene. (A) Integration of pLARG by a single homologous crossover event in the transformant LARG1. pLARG contains the *A. nidulans argB* gene (shaded region) inserted into pUC18 (open box) and the *Bam*HI, *Eco*RI fragment of *lamB* (line). The size of predicted *Bgl*III restriction fragments in LARG1 are shown below the restriction map. Restriction enzyme abbreviations are as for Figure 3. In *Bgl*II digested wildtype genomic DNA the probe hydridizes to a 3.9-kb fragment, a 0.6-kb fragment not visible on the blot and a large fragment extending beyond the 3′ end of the *lamA* gene. In LARG1 the 3.9 kb fragment is replaced by two new fragments of 4.9 kb and 5.7 kb due to the insertion of pLARG which contains a *Bgl*II site. (B) Southern blot analysis of DNA from the LARG1 transformant (lane 1) and the wild type strain, MH2671 (lane 2) digested with *Bgl*II. The blot was probed with ^{32}P-labeled pUC18 plasmid containing the insert indicated in (A). (C) Utilization of 2-pyrrolidinone (10 mM) as the sole nitrogen source by a wild-type strain (WT), a *lamA5* strain (MH3005) and the transformant LARG1 containing the *lamB*::pLARG insertion mutation. The medium contained 1% glucose as a carbon source and incubation was at 37° for 3 days.

second cross to the *argB*$^-$ strain MH3019, demonstrated that the *argB* gene of pLARG is linked to the Lam$^-$ phenotype. 192 Arg$^+$ segregants of the cross all grew poorly on 2-pyrrolidinone medium. Arg$^-$ segregants were not tested since supplementation with arginine prevented scoring of growth on 2-pyrrolidinone as a nitrogen source.

It was necessary to address the possibility that the integration of pLARG had caused or was associated with a mutation in the nearby *lamA* gene. Complementation tests in heterozygous diploids were not possible as neither the *lamA5* mutation (ARST, PENFOLD and BAILEY 1978) nor the *lamB*::pLARG gene disruption were completely recessive. In an alternative approach, the *lamB*::pLARG transformant strain LARG1, was retransformed with plasmids carrying the *lamA* gene (pLAM10 and pLAM15) or the *lamB* gene (pLAM9) and a selectable plasmid, pAmPh (see MATERIALS AND METHODS). Bleomycin-resistant cotransformants were tested for their ability to utilize 2-pyrrolidinone as a sole source of nitrogen. Lam$^+$ transformants were generated with the *lamB* clone (pLAM9—9 of 41 cotransformants tested) but not the *lamA* gene (pLAM10—none observed in 59 cotransformants). These results confirm that the insertion of pLARG has created a mutation in a second gene involved in 2-pyrrolidinone utilization in *A. nidulans*.

Partial suppression of the effects of the *gatA312* mutation by the *lamB*::pLARG mutations: Mutations in the GABA aminotransferase structural gene (*gatA*) result in inhibited growth on GABA and 2-pyrrolidinone media, presumably due to the toxic effect of high levels of accumulated endogenous GABA (ARST 1976; ARST, PENFOLD and BAILEY 1978). The inhibited growth of *gatA*$^-$ strains on 2-pyrrolidinone is suppressed by mutations which block the conversion of 2-pyrrolidinone to GABA. Mutations in both *lamA* and *amdR* genes prevent accumulation of endogenous GABA derived from 2-pyrrolidinone. If the *lamB*::pLARG mutation affects a step in the conversion of exogenous 2-pyrrolidinone to GABA, the *lamB*::pLARG mutation should protect a *gatA*$^-$ strain from 2-pyrrolidinone toxicity. The *lamB*::pLARG strain, LARG1, was crossed to a *gatA*$^-$

strain, MH1055. Approximately 25% of the segregants of the cross were strongly inhibited by the presence of GABA in the medium but only partially inhibited by 2-pyrrolidinone. To confirm that partial inhibition by 2-pyrrolidinone was a consequence of the *lamB*::pLARG mutation, the presence of *lamB*::pLARG allele in 2 segregants of this class was verified by an outcross. The absence of the *lamB*::pLARG allele was also confirmed in 2 segregants inhibited by both GABA and 2-pyrrolidinone.

Multiple copies of *lamA* lead to altered growth on 2-pyrrolidinone: It was noted that when pLAM10 was introduced into a *lamA5* strain of *A. nidulans*, the growth of some of the transformants on 2-pyrrolidinone medium was stronger than that of a wild-type strain. To determine whether multiple copies of the *lamA* gene can cause increased growth on 2-pyrrolidinone medium, a strain of *A. nidulans* that already possesses a functional copy of the *lamA* gene was transformed. The *lamA*$^+$ *prn-309* containing strain, MH2671, is unable to utilize proline as a nitrogen source due to the *prn-309* mutation. Proline-utilizing transformants were obtained by cotransformation with pLAM15 and pAN222, a plasmid carrying the *prn* genes (DURRENS *et al.* 1986). pLAM15 is a derivative of pLAM10 lacking the 0.55 kb *Bam*HI/*Hin*dIII fragment upstream of the 5′ end of *lamA* (Figure 3). pLAM15 contains the entire *lamA* gene as shown by transformation of a *lamA5* strain of *A. nidulans* to Lam$^+$ (M. E. KATZ and M. J. HYNES, unpublished data). Of 50 transformants tested, 11 exhibited stronger growth on 2-pyrrolidinone medium than the parent strain. The colony morphology of an additional 15 cotransformants was significantly altered. The growth of these 15 transformants was inhibited by the presence of 2-pyrrolidinone in the media.

It has been shown that, in crosses with transformants carrying multiple copies of a plasmid, the progeny receive a variable, usually lower, number of plasmid copies due to unequal crossover events among tandemly integrated copies (TILBURN *et al.* 1983; UPSHALL 1986; KELLY and HYNES 1987). If the inhibited phenotype was due to the presence of more copies of the *lamA* gene than are present in transformants that grow strongly on 2-pyrrolidinone, we expected segregants from outcrosses of the inhibited transformants to have variable plasmid copy numbers and display all 3 phenotypes (wild-type, stronger and inhibited). This was the case. A cross with a transformant that had increased growth on 2-pyrrolidinone yielded no strongly inhibited progeny.

DNA dot blot hybridizations confirmed that a few copies of *lamA* lead to enhanced growth on 2-pyrrolidinone but a large number of copies caused 2-pyrrolidinone inhibition (Figure 6). These results indicated that the *lamA* function is the rate limiting step in 2-pyrrolidinone utilization. Multiple copies of pLAM9 carrying the *lamB* gene do not increase growth on 2-pyrrolidinone media. In fact, growth on 2-pyrrolidinone media, is decreased in many pLAM9 transformants, presumably due to titration of the *amdR* regulatory gene product (M. E. KATZ and M. J. HYNES, unpublished data).

Regulation of *lamA* and *lamB* expression: In an effort to quantitate *amdR* mediated induction of *lamA* and *lamB* and to investigate the role of carbon and nitrogen metabolite control, RNA was prepared from a wildtype strain and a number of mutant strains exposed to a variety of growth conditions. Triplicate RNA dot blots were probes with *lamA* or *lamB* specific probes. As a control, a third identical set of dots was probed for RNA from the constitutively expressed *argB* gene (UPSHALL 1986).

The data indicated that the two transcripts were regulated similarly (Figure 7). In the presence of glucose the levels of both messengers was increased 2–4 fold in the presence of inducer. Carbon limitation resulted in an increase in RNA levels in the absence of inducer and these levels were greatly increased by the addition of inducer. Induction by β-alanine was abolished in the *amdR44* strain and, in fact, both messengers were barely detectable. The *amdR6*^{c} mutation leads to increased utilization of 2-pyrrolidinone. The effect of this mutation was to cause low constitutive *lamA* expression but hyperinducible expression of the *lamB* transcript (Figure 7). The addition of ammonium eliminated β-alanine induction, a result consistent with *areA*-mediated nitrogen metabolite control of both genes. In an *areA*$^-$ strain, in the presence of inducer, message levels were below those of the wild type strain under uninduced conditions.

DISCUSSION

The *lamA* gene of *A. nidulans* has previously been shown to be involved in the conversion of exogenous 2-pyrrolidinone to GABA and probably to be under the control of the *amdR* (*intA*) positively acting regulatory gene (ARTS, PENFOLD and BAILEY 1978). We have isolated the *lamA* gene from a cosmid gene bank by complementation of the *lamA5* mutation.

Two divergently transcribed RNA species of 1 kb and 4 kb have been detected by probing Northern blots with cloned DNA. The 4 kb species has been found to be encoded by sequences necessary for *lamA* complementation and is likely to be the *lamA* message. The adjacent sequence, designated *lamB*, encoding the 1-kb RNA, has been shown to be involved in 2-pyrrolidinone utilization by the creation of an insertion mutation. This has been confirmed in experiments to be reported elsewhere in which it has been shown that both *lamA* and *lamB* sequences are necessary (and sufficient) to allow the 2-pyrrolidinone non-

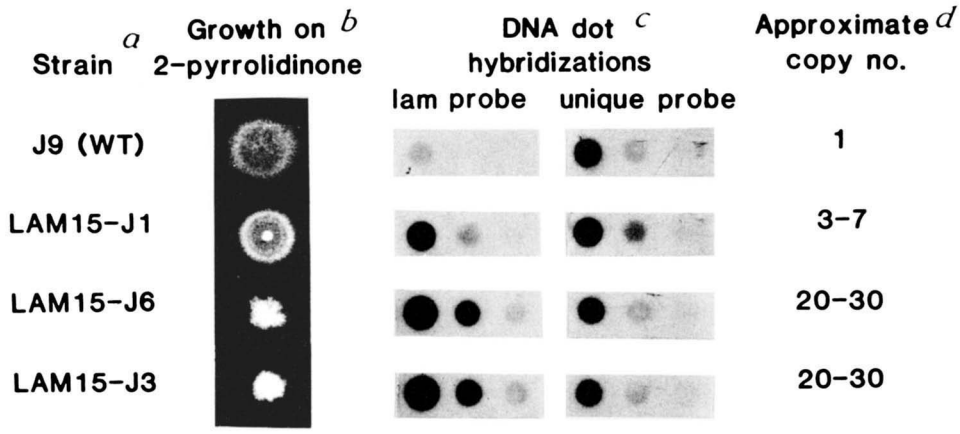
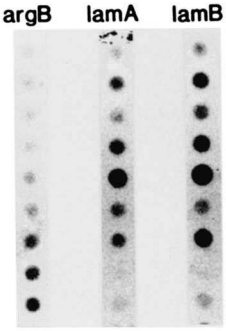


FIGURE 6.—Growth properties and DNA dot hybridizations of *A. nidulans* transformants carrying multiple copies of the *lamA* plasmid, pLAM15. (a) The genotype of the parent strain MH2671 is listed in Table 1. The three cotransformants listed have received copies of pAN222 and pLAM15 as described in RESULTS. (b) Growth on 2-pyrrolidinone medium. Medium and incubation conditions were as for Figure 5. (c) Serial 5-fold dilutions of genomic DNA from the parent and transformant strains were applied to a nitrocellulose membrane as described in MATERIALS AND METHODS. Duplicate DNA dot blots were hybridized with a nick-translated gel-purified *Bam*HI fragment, the pLAM10 insert (Figure 3) and a unique probe, a nick-translated gel-purified fragment containing part of the single copy gene, *facB* (M. E. KATZ and M. J. HYNES, unpublished data). (d) Approximate copy number was determined by a comparison of hybridization intensities of the serially diluted DNA.



Relevant genotype [a]	RNA dot hybridizations [b] argB	lamA	lamB	C source [c]	N source [d]	Approx. mRNA levels [e] lamA	lamB
WT				gluc	ala	1	1
WT				gluc	β-ala	3	3
WT				gluc	NH_4^+ / β-ala	1	1
WT				-	ala	3	3
WT				-	β-ala	24	24
amdR6[c]				gluc	ala	2	1
amdR6[c]				gluc	β-ala	2	6
amdR44				gluc	β-ala	0.1	<0.1
areA217				gluc	β-ala	0.4	0.2

FIGURE 7.—Dot blot analysis of RNA from wild-type and mutant strains. (a) Total RNA was prepared from 4 strains: MH54 (WT), MH6 (*amdR6*[c]), MH345 (*amdR44*) and MH341 (*areA217*). The complete genotypes are listed in Table 1. (b) Twofold serial dilutions of each RNA sample were applied to a nylon membrane as described in MATERIALS AND METHODS. Three identical blots each carrying four dilutions of each RNA sample were prepared. A single set of dilutions from each dot blot hybridization is shown. (c, d) RNA was prepared from mycelium grown for 18 hr in minimal medium containing 1% glucose and 10 mM ammonium, then transferred for 4.5 hr to medium containing the following carbon (C) and nitrogen (N) sources: 1% glucose (gluc), 10 mM alanine (ala), 10 mM β-alanine (β-ala) and 10 mM ammonium (NH_4^+). Alanine and β-alanine are weak carbon sources as well as nitrogen sources. (e) Approximate mRNA levels were determined by hybridization intensities of the serially diluted RNA. The levels expressed are relative to WT uninduced levels in the presence of a source of carbon catabolite repression (1% glucose).

utilizing species *A. terreus* to grow on this nitrogen source (M. E. KATZ and M. J. HYNES, unpublished data).

The leaky phenotype resulting from the *lamB* insertion mutation probably explains the failure to isolate *lamB* mutations in a previous study (ARST, PENFOLD and BAILEY 1978). In addition, the fact that both *lamA* and *lamB* mutations are incompletely recessive to wild type prevents the identification of two genes in this region by the use of complementation tests. Only the use of molecular techniques has allowed the discovery of the *lamB* gene.

Levels of both the *lamA* and *lamB* transcripts are regulated by induction by ω-amino acids mediated by the *amdR* gene, directly confirming the conclusion of ARST, PENFOLD and BAILEY (1978) with respect to *lamA*. There is now evidence for control of expression of five genes by the *amdR* regulatory gene. Specific sequences in the 5′ region of the *amdS* and *gatA* genes have been implicated in *amdR* binding (I. B. RICHARDSON, S. HURLEY and M. J. HYNES, unpublished data). Related sequences have been found in the intergenic region between the *lamA* and *lamB* genes and there is evidence that at least one of these sequences is functional in *amdR* product binding (M. E. KATZ, I. B. RICHARDSON and M. J. HYNES, unpublished data). It will be of considerable interest to determine whether bidirectional transcription is regulated by common sites of action for *amdR*.

The *areA* gene is the major regulatory gene mediating nitrogen metabolite repression in *A. nidulans* (ARST and COVE 1973). Previous evidence suggested that the *lamA* gene is subject to *areA* control (ARST, PENFOLD and BAILEY 1978). Both *lamA* and *lamB* RNA levels are reduced by growth in the presence of ammonium and in a strain containing the *areA217* loss of function mutation. The low level of *lam* RNA in an *areA217* strain cannot be attributed solely to a lack of β-alanine uptake as the levels are below that of the wild-type strain in the absence of inducer. Further support for this is provided by the observation that strains containing the altered function *areA102* allele (ARST and COVE 1973) utilize 2-pyrrolidinone poorly but are not defective in the utilization of either β-alanine or GABA as sole nitrogen sources (our unpublished data). This indicates that the *areA* gene probably affects the *lam* gene directly rather than via the uptake of exogenous inducer. Again it will be of importance to determine if *areA* mediated regulation of both *lam* genes is via a common site (or sites) of action.

Evidence is presented for expression of both the *lamA* and *lamB* genes being strongly regulated by carbon limiation. Little is known at present about carbon catabolite repression in *A. nidulans* and it cannot be excluded that some of the effects of carbon limitation are via inducer uptake. However even under noninduced conditions elevated messenger levels were observed. Therefore it is likely that the *lam* genes, like many other catabolic genes involved in both carbon and nitrogen metabolism, are subject to both nitrogen and carbon metabolite control.

The *lamA* and *lamB* gene products appear to be very limiting for 2-pyrrolidinone utilization with even a slight increase or decrease in levels affecting growth. This is shown by the lack of complete recessiveness of *lamA* and *lamB* mutations in heterozygous diploids and the increased 2-pyrrolidinone utilization shown by transformants containing increased copy numbers of the *lamA* gene. In addition some $amdR^c$ mutations result in significantly better growth than wildtype strains on 2-pyrrolidinone medium. Transformants containing very high copy numbers of the *lamA* gene, however, are inhibited on 2-pyrrolidinone medium. This is most likely to be due to an accumulation of GABA which is known to be toxic (ARST 1976). Greatly increased uptake of 2-pyrrolidinone and conversion to GABA probably makes transamination of GABA (the *gatA* function) the rate limiting step.

ARST, PENFOLD and BAILEY (1978) were unable to determine whether the *lamA* product is involved in lactam uptake or in lactamase activity. Attempts to detect lactamase activity *in vitro* were unsuccessful. The discovery of the *lamB* gene reported here makes it most likely that one of the genes encodes a lactamase and the other a permease. However other possibilities cannot be excluded, *e.g.*, a complex between the gene products could function as a lactamase or permease or both; or, less likely, one gene could regulate the expression of the other. The effects of the *lamB* insertional inactivation are less extreme than those of *lamA* mutations. In addition, the *lamB*::pLARG mutation only weakly protects a *gatA312* strain from 2-pyrrolidinone toxicity indicating only a partial prevention of GABA formation. As pointed out previously (ARST, PENFOLD and BAILEY 1978) permease mutants are often leaky due to alternative uptake systems or to passive diffusion. We therefore favour the view that the *lamB* gene encodes a permease and the *lamA* gene encodes a lactamase. The situation, however, remains unresolved.

The *lamA* and *lamB* genes constitute a gene cluster and add to the considerable number of such clusters detected in *A. nidulans* [see ARST and SCAZZOCCHIO (1985) for review]. In many cases a permease encoding gene forms part of the cluster. The functional significance of clusters is not clear. Many examples show that genes subject to control by a particular regulatory gene do not have to be linked. There may however be genomic domains affecting general levels of gene activity as suggested by studies with the *SpoC1* gene cluster (MILLER *et al.* 1987). Apparently gene clusters can be disrupted both experimentally or during evolution without detectably affecting function. Thus the *lamA* and *lamB* genes are functional when integrated via separate plasmids into the genome of *A. terreus* (M. E. KATZ and M. J. HYNES, unpublished data). Although the nitrate reductase and nitrite reductase genes of *A. nidulans* form a cluster, this is not the case in *N. crassa* (MARZLUF 1981). In addition the order of genes does not appear to be of functional importance since the gene order in the quinate utilization gene cluster in *A. nidulans* and *N. crassa* are different (GRANT *et al.* 1988).

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