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A *lacZ* Reporter Fusion Method for the Genetic Analysis of Regulatory Mutations in Pathways of Fungal Secondary Metabolism and Its Application to the *Aspergillus nidulans* Penicillin Pathway

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Secondary metabolism, usually superfluous under laboratory conditions, is intrinsically elusive to genetic analysis of its regulation. We describe here a method of analyzing regulatory mutations affecting expression of secondary metabolic genes, with an *Aspergillus nidulans* penicillin structural gene (*ipnA* [encoding isopenicillin *N*-synthase]) as a model. The method is based on a targeted double integration of a *lacZ* fusion reporter gene in a chromosome different from that containing the penicillin gene cluster. The *trans*-acting regulatory mutations simultaneously affect *lacZ* expression and penicillin biosynthesis. One of these mutations (*npeE1*) has been analyzed in detail. This mutation is recessive, prevents penicillin production and *ipnA′::′lacZ* expression, and results in very low levels of the *ipnA* message at certain times of growth. This indicates that *npeE* positively controls *ipnA* transcription. We also show that this tandem reporter fusion allows genetic analysis of *npeE1* by using the sexual and parasexual cycles and that *lacZ* expression is an easily scorable phenotype. Haploidization analysis established that *npeE* is located in chromosome IV, but *npeE1* does not show meiotic linkage to a number of known chromosome IV markers. This method might be of general applicability to genetic analysis of regulation of other fungal secondary metabolic pathways.

Secondary metabolism in microbes is often elusive to genetic analysis, because most, if not all, pathways classified in this category are dispensable under laboratory conditions. The penicillin biosynthetic pathway (22) is a prototype of such pathways in filamentous ascomycetes and has been extensively used as a model for at least four reasons. (i) It is a rather simple pathway, and only three enzymes are required to convert primary metabolites (three amino acids) into penicillin; (ii) the corresponding structural genes, which are clustered, have been cloned and characterized from several species; (iii) the end product can be sensitively detected with a bioassay; and (iv) it is of obvious biotechnological interest. As a consequence, a wealth of information about this pathway has been accumulated. In contrast, regulation of penicillin biosynthesis is largely unelucidated, possibly because the absence of a sexual cycle in *Penicillium chrysogenum* has hindered formal genetic analysis of the putative regulatory mechanisms. This problem has been circumvented by using *Aspergillus nidulans* (26), a closely related plectomycete amenable to formal genetic studies (9) and for which sophisticated molecular biology techniques are available (31).

By using molecular techniques to analyze transcription of the *A. nidulans ipnA* gene (encoding isopenicillin *N*-synthase, a key enzyme catalyzing the central step in the pathway), we have described two modes of transcriptional regulation of a penicillin structural gene. Carbon regulation in response to the availability of a preferred carbon source modulates *ipnA* expression through the action of a yet undefined negative-acting regulatory gene (5, 16, 17, 27). pH regulation positively regulates *ipnA* transcription in response to alkaline ambient pH (18) through the action of the zinc finger protein PacC (29).

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We and others have previously used *lacZ* fusions for functional analysis of the *ipnA* promoter (5, 27). As in bacteria, expression of these fusions can be scored in plate assays with the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We therefore designed a method to score for *trans*-acting regulatory mutations impairing expression of an *ipnA′::′lacZ* gene, which is described here. The method has been validated by the genetic characterization of a previously unidentified gene regulating *ipnA* transcription and penicillin biosynthesis. The method can, in principle, be applied to study the regulation of expression of any other gene of fungal secondary metabolism, provided that two additional copies of its promoter can be tolerated. While this work was under review, a similar, independent analysis of *A. nidulans* mutations involved in the regulation of the penicillin pathway with a *lacZ* reporter gene was published (6).

MATERIALS AND METHODS

Strains, media, and genetic techniques. The *A. nidulans* strains used in this work are listed in Table 1. With the exception of *npeE1*, all strains carried previously described markers (10). Haploidization analysis was modified according to the method of McCully and Forbes (25) with Benlate as the haploidization agent (32). All other genetic techniques were performed according to the methods described by Clutterbuck (9). *ipnA′::′lacZ* expression was scored in glucose-minimal medium (12) containing X-Gal (32 μg/ml) and 0.08% sodium desoxycholate. The *bgA0* mutation (19), which leads to loss of endogenous β-galactosidase (β-Gal), was scored in 1% lactose-minimal medium with the X-Gal and desoxycholate concentrations given above. This was only possible in those strains which were not simultaneously carrying the reporter fusions. The heterozygous *npeE*$^+$*/npeE1* diploid BPE-D1 (used to test the dominance relationship of *npeE1* in a *bgA0* homozygous background), in which only one of the chromosome III homologs carried a double copy of the reporter fusion, was constructed from strains BPE-6 and BPE-3. The *bgA0* strain BPE-3 was constructed by crossing WG355 and G719. The second parent of the diploid described above (BPE-6) was constructed from BPE-2 (the original *npeE1* mutant strain isolated after mutagenesis) in two steps as follows. First, BPE-2 was marked with a *niaD* loss-of-function mutation (obtained after selection of spontaneous mutations conferring resistance to 10 mM chlorate [13]) to yield strain BPE-4. Second, BPE-4 was crossed with BPE-5 (also a *bgA0* strain obtained by crossing WG355 and A237), and BPE-6 was selected from the progeny. The

TABLE 1. Characteristics of strains used in this study

Strain	Genotype	Source
WG355	*biA1 argB2 bgA0*	P. Punt (Rijswijk, Holland).
BPE-1	*biA1* [*ipnA′::′lacZ argB*$^+$*]*$_2$ *bgA0*	This work
BPE-2	*biA1* [*ipnA′::′lacZ argB*$^+$]$_2$ *bgA0 npeE1*	This work
G719	*pabaA1 wA3 pyroA4 sF211*	J. Clutterbuck (Glasgow, Scotland)
BPE-3	*biA1 wA3 argB2 bgA0 sF211*	This work
BPE-4	*biA1* [*ipnA′::′lacZ argB*$^+$]$_2$ *bgA0 niaD npeE1*	This work
A237	*pabaA1 yA2 trpC801*	Fungal Genetics Stock Center, Kansas City, Kans.
BPE-5	*pabaA1 yA2 argB2 bgA0 trpC801*	This work
BPE-6	*pabaA1 yA2* [*ipnA′::′lacZ argB*$^+$]$_2$ *bgA0 trpC801 npeE1*	This work
BPE-D1	Diploid strain between BPE-3 and BPE-6	This work
A615	*sulA1 adE20 acrA1 wA3 actA1 methG1 nicA2 lacA1 sB3 choA1 riboB2 chaA1*	Master strain, Fungal Genetics Stock Center, Kansas City, Kans.
BPE-DM	Diploid strain between BPE-6 and A615	This work
HNA-1	*yA2 acuK248 meaA8 pabaB22 pyroA4*	H. Arst (London, United Kingdom)
HNA-2	*yA2 inoB2*	H. Arst (London, United Kingdom)
HNA-3	*biA1 inoB2*	H. Arst (London, United Kingdom)
HNA-4	*pabaA1 yA2 biA1 palC4 bzuA1*	H. Arst (London, United Kingdom)
CS-87	*biA1 argB2 methG1*	C. Scazzocchio (Paris, France)

presence of the double copy of the reporter fusion in BPE-6 was verified by Southern analysis with the appropriate probes. BPE-6 was also used as a parent of diploid strain BPE-DM, used in haploidization analysis. Although the master strain A615 did not carry a *bgA0* marker, *bgA* is located in chromosome III (10), and therefore segregation of *bgA0* from the double copy of the reporter fusions (integrated at *argB*, which is also in chromosome III) upon haploidization would have required mitotic recombination and would have been very infrequent. In addition, *npeE* alleles were scored in glucose-minimal medium with X-Gal (repressing conditions which prevent β-Gal activity encoded by *bgA*).

For penicillin production, Northern (RNA) analysis, and β-Gal assays in mycelial protein extracts, the different strains were cultured at 37°C with vigorous shaking in penicillin-production broth (PPB) with 3% lactose, a derepressing carbon source (17). Penicillin bioassays were also performed as described in reference 17.

Molecular techniques. Plasmid pCiAnlacZ, carrying a translational fusion between the first 12 codons of the *ipnA* gene and *lacZ*, whose expression is driven by the *ipnA* promoter, has been described previously (21, 27). Transformation was done according to the method of Tilburn et al. (30), with 5 of transforming DNA. DNA was isolated from purified *argB*$^+$ transformants and analyzed by Southern blot hybridization as described previously (27), with either a 3.2-kb fragment containing the *argB* gene or a 2-kb *Bam*HI *ipnA* promoter fragment (isolated from pCiAnlacZ) as the probe. RNA isolation from lyophilized mycelial samples taken at three different times of growth and Northern analysis with *ipnA*- or actin-specific probes were performed according to the method of Espeso and Peñalva (17). For β-Gal assays, mycelia were grown for the indicated times in lactose-PPB and frozen in liquid nitrogen. Protein extraction (from lyophilized mycelia) and quantitative β-Gal assays were performed as detailed by Pérez-Esteban et al. (27).

RESULTS

An overview of the selection procedure. Our method (Fig. 1) is based on targeted tandem integration of two copies of a *lacZ* fusion in a chromosomal location different from that of the gene whose regulation is being analyzed (*ipnA* in this example). Expression of these fusion genes results in blue staining of the colonies in X-Gal-containing plates. Mutations increasing or decreasing the activity of the reporter gene (i.e., increasing or decreasing the blue staining of mutant colonies) would enable identification of *trans*-acting regulatory genes. The presence of two identical copies of the reporter fusion eliminates the possibility of selecting a *lacZ* structural gene or down-promoter mutations, which would result in a white colony phenotype and which would be scored as affecting expression in a negative way. Other more extensive *cis*-acting mutations (such as deletions) simultaneously preventing expression of both reporter genes are discarded in a later step (vide infra). Plasmid integration is mitotically stable and precludes undesirable effects of variations in plasmid copy number on gene expression. A copy number higher than the one used here would possibly result in meiotic instability and was therefore not considered.

We have used integration at the *argB* locus (chromosome III) for the *ipnA′::′lacZ* reporter fusions. The penicillin gene cluster is located in chromosome VI. A bona fide *trans*-acting mutation would affect expression of *ipnA′::′lacZ* fusions (in chromosome III) as well as expression of the recipient's *ipnA* gene (chromosome VI). Eventually, such a mutation may simultaneously affect expression of other penicillin structural genes, and either isopenicillin *N*-synthase or the products of these other structural genes may be limiting for penicillin bio-

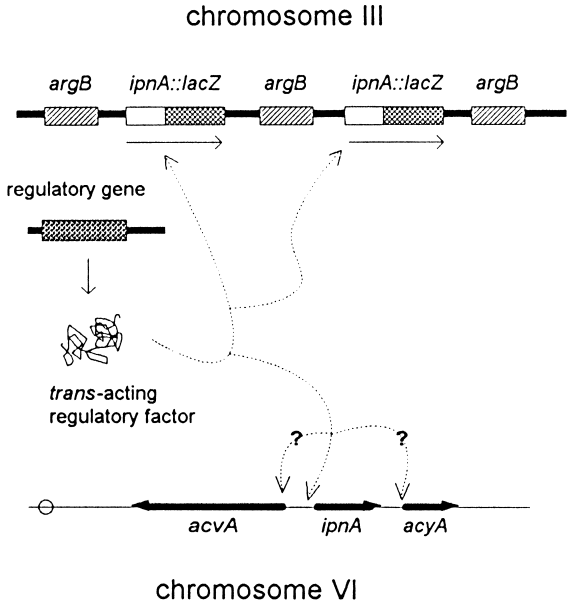

FIG. 1. Schematic representation of the selection procedure. Mutations in genes encoding *trans*-acting regulatory proteins controlling *ipnA* transcription will simultaneously affect expression of twin reporter *ipnA′::′lacZ* genes (integrated in chromosome III) as well as that of the resident *ipnA* gene (in the penicillin gene cluster, located on chromosome VI). These mutations might also affect expression (as indicated by question marks) of other penicillin structural genes, such as *acvA* and/or *acyA* (see reference 22 for a review) and/or other yet unidentified structural genes. This could potentially affect levels of exported penicillins.

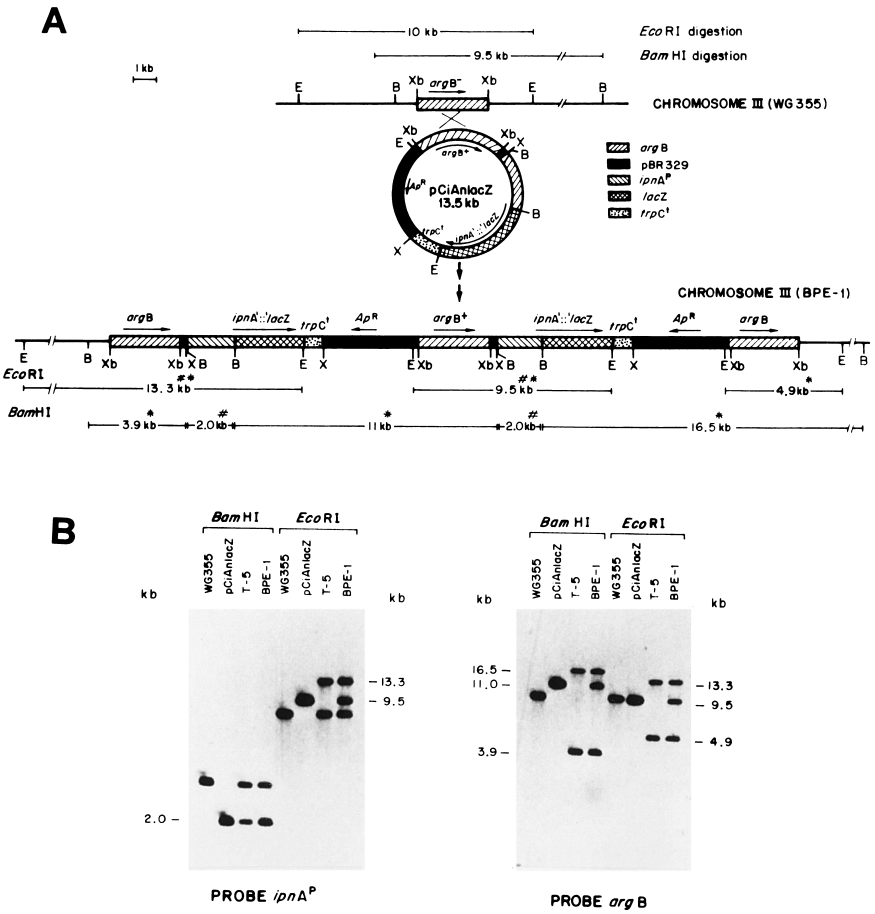

FIG. 2. Construction of strain BPE-1, used for mutagenesis, by double integration of the reporter fusion at the *argB* locus. (A) Scheme of the double integration event of pCiAnlacZ at *argB*. Symbols are as indicated. Restriction sites are as follows: E, *Eco*RI; B, *Bam*HI; X, *Xho*I; and Xb, *Xba*I. The *ipnA* promoter ($IPNA^P$) and a transcriptional terminator from the *trpC* gene ($trpC^t$) are indicated. Construction of this plasmid is described in reference 21. Restriction fragments from the *argB* region resulting from *Eco*RI or *Bam*HI digestion of genomic DNA from a transformant (BPE-1) carrying such a double integration event are shown, with the predicted sizes given in kilobases. Fragments hybridizing to an *argB* probe are indicated by ∗, while those hybridizing to an $ipnA^p$ probe are indicated by #. (B) Southern analysis of DNA from strain BPE-1, showing that its hybridization pattern corresponds to a double integration event at *argB*. T-5 indicates DNA from a strain with a single integration of pCiAnlacZ at the same chromosomal location; WG355 indicates DNA from the recipient strain; lanes labelled pCiAnlacZ contain DNA from the transforming plasmid. The *argB*-hybridizing band corresponding to the resident *argB* locus is split either into two different bands after the single-copy integration event (strain T-5) or into three different bands after the double integration (BPE-1). Integration of pCiAnlacZ creates a single (T-5) or a double (BPE-1) 2-kb *Bam*HI fragment hybridizing with the $ipnA^p$ (note that the intensity of this band in the BPE-1 lane is twice that corresponding to T-5). Because integration events in both T-5 and BPE-1 are at *argB*, the mobility of *Bam*HI of *Eco*RI bands corresponding to the resident *ipnA* gene of WG355 is not modified.

synthesis. Therefore, mutations simultaneously affecting *lacZ* expression and penicillin biosynthesis should represent *trans*-acting regulatory mutations.

Construction of the parental strain BPE-1 and selection of mutations impairing β-Gal expression. Plasmid pCiAnlacZ (21) contains an *ipnA′*::*′lacZ* fusion in an $argB^+$ background. This plasmid was transformed into *A. nidulans* WG355 (an *argB* strain carrying a *bgA0* mutation, resulting in the absence of endogenous β-Gal. Southern analysis allowed us to identify several transformants carrying a double integration of the plasmid at *argB*. This tandem integration event carried by one of these transformants was definitively characterized with *argB*- and *ipnA*-specific probes (Fig. 2). This transformant, designated BPE-1, was chosen for mutant selection. Colonies of BPE-1 stain blue in X-Gal-containing glucose-minimal medium (Fig. 3A). Quantitative in vitro β-Gal assays indicated that BPE-1 has approximately twice the activity detected in a transformant (T-5 [Fig. 2]) carrying a single-copy integration of pCiAnlacZ at *argB* (data not shown), confirming that both fusion genes are being expressed. A spore suspension of strain BPE-1 was UV light mutagenized and spread on X-Gal–minimal medium plates. Because colonies with increased β-Gal staining were more difficult to detect than those showing reduced staining (because the parental strain already shows significant blue staining [Fig. 3A]), we concentrated on mutants with reduced staining.

A screen of approximately 9,000 colonies yielded seven clones which reproducibly showed either a white (four mutants) or a convincing faint blue (three mutants) phenotype. Quantitative β-Gal assays with mycelial extracts (described below) confirmed that white and faint blue mutant strains, respectively, showed undetectable or substantially reduced levels of this activity. Penicillin levels produced by the four white strains were similar to those of the parental strain, and Southern analysis revealed that these four mutants had lost both *ipnA′*::*′lacZ* genes (data not shown), possibly by excision resulting from UV irradiation. These four strains were therefore discarded. The three other mutants, showing substantially reduced (but detectable) β-Gal activity, produced less penicillin than the parental strain when cultured in liquid PPB (data not

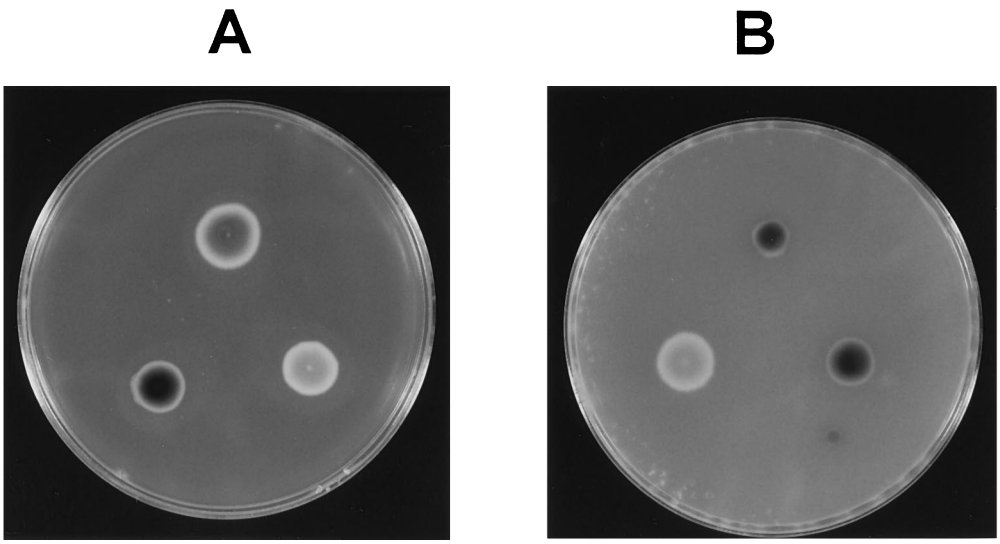

FIG. 3. β-Gal activity on X-Gal-containing plates in colonies of the indicated strains. (A) Top, BPE-2 (*npeE1*); left, BPE-1 (*npeE*$^+$); right, WG355. (B) Top, BPE-D1 diploid strain (*npeE1*/*npeE*$^+$); right, BPE-1; left, BPE-2. Dark blue, indicating strong (wild-type) staining of the colonies, appears black in the figure, while pale blue (mutant) staining appears grey.

shown), indicating that they were carrying mutations in *trans*-acting regulatory genes affecting *ipnA* transcription. Southern blot analysis (not shown) demonstrated that these three mutant strains conserved the two copies of the reporter fusion and that no detectable rearrangements in these genes had been induced by the mutagenic treatment. Although this shows the validity of our method for isolating putative regulatory mutations of *ipnA* transcription, we could not predict, in light of the results described above, that our reporter system would be suitable for their genetic analysis, because the reporter fusions, essential for scoring the presence of these mutations, could be excised after mitotic or meiotic intrachromosomal recombination events. Therefore, we characterized in detail the most promising of the three mutant strains described above. This mutant, designated BPE-2, showed markedly reduced levels of β-Gal in plates (Fig. 3A) and had wild-type morphology and conidiation. The mutation carried by this strain (described below) was designated *npeE1* (*npe*, impaired in penicillin biosynthesis [14]; see Discussion). *npeE1* is a temperature-sensitive mutation, the mutant phenotype being evident at 37°C but not at 25°C.

***npeE1* prevents penicillin biosynthesis and transcription of *ipnA*.** Quantitative β-Gal assays with mycelial extracts from strains BPE-1 and BPE-2 corresponding to three different times of growth (12, 24, and 36 h after inoculation) confirmed that the *npeE1* mutation nearly abolished expression of the *ipnA′*::*′lacZ* fusions (Fig. 4A). In addition, the BPE-2 mutant strain produced approximately 10 times less penicillin than its parent, BPE-1 (Fig. 4B), despite the fact that BPE-1 and BPE-2 show very similar growth patterns (Fig. 4D). This strongly suggested that this mutation affects a *trans*-acting factor simultaneously regulating the twin reporter fusions in chromosome III and (at least) the *ipnA* gene (in chromosome VI). To confirm this, RNA was isolated from mycelial samples of these cultures (corresponding to 12, 24, and 36 h of growth) and analyzed by Northern hybridization (Fig. 4C). Espeso and Peñalva showed that steady state levels of *ipnA* mRNA under the conditions used here (carbon-derepressed conditions) are constant and elevated throughout the growth of the culture (17). This was shown to be the case for the BPE-1 (*npeE*$^+$) strain at the three times sampled (Fig. 4C). In contrast, *ipnA* transcript levels in mycelia of the *npeE1* strain were slightly reduced at 12 h and were nearly undetectable at both 24 and 36 h. Because *npeE1* affects *ipnA* transcript steady state levels as well as *lacZ* expression driven by the *ipnA* promoter (also described below), we conclude that *npeE* encodes a protein regulating (either directly or indirectly) transcription of (at least) the *ipnA* gene.

***npeE1* is recessive in diploids.** A heterozygous (*npeE1*/*npeE*$^+$) diploid was constructed in which one of the two chromosome III homologs carried the twin reporter gene fusion in a *bgA0*/*bgA0* background. For this and other crosses (described below), it was necessary to introduce into the *npeE1* strain markers not present in the BPE-1 parental strain. Because the presence of the *npeE1* mutation cannot be readily scored in the absence of the reporter fusions, these markers were introduced by crossing with *argB* mutant strains. Because the reporter fusions in the *npeE1* parental strain are closely linked to *argB*$^+$ (the marker carried by the transforming plasmid [Fig. 2B]), *argB*$^+$ progeny from these crosses were likely to carry the double reporter fusion. This (as well as the segregation of the wild-type and mutant *npeE* alleles) was then verified on X-Gal plates (note that *npeE1* does not completely prevent *ipnA′*::*′lacZ* expression [Fig. 3A]). In addition, all parental strains contained the *bgA0* mutation, which eliminates endogenous β-Gal. This ensured that all of the progeny carried *bgA0*, which cannot be scored in the presence of the activity encoded by the fusion genes.

Southern analysis of the heterozygous diploid strain described above (designated BPE-D1) confirmed that, compared with BPE-1, this strain carried an additional *argB*-hybridizing band corresponding to the resident *argB*$^+$ gene in that chromosome III which does not contain the two copies of pCiAnlacZ (Fig. 5). The diploid *npeE1*/*npeE*$^+$ strain had β-Gal levels similar to those of the BPE-1 *npeE*$^+$ parental strain, as measured in qualitative plate assays (Fig. 3B), indicating that *npeE1* is a recessive mutation. This was further confirmed by quantitative in vitro assays (Fig. 4A). (In fact the diploid strain had approximately twice the β-Gal levels of the control, haploid *npeE*$^+$ strain at the three times sampled.) The diploid

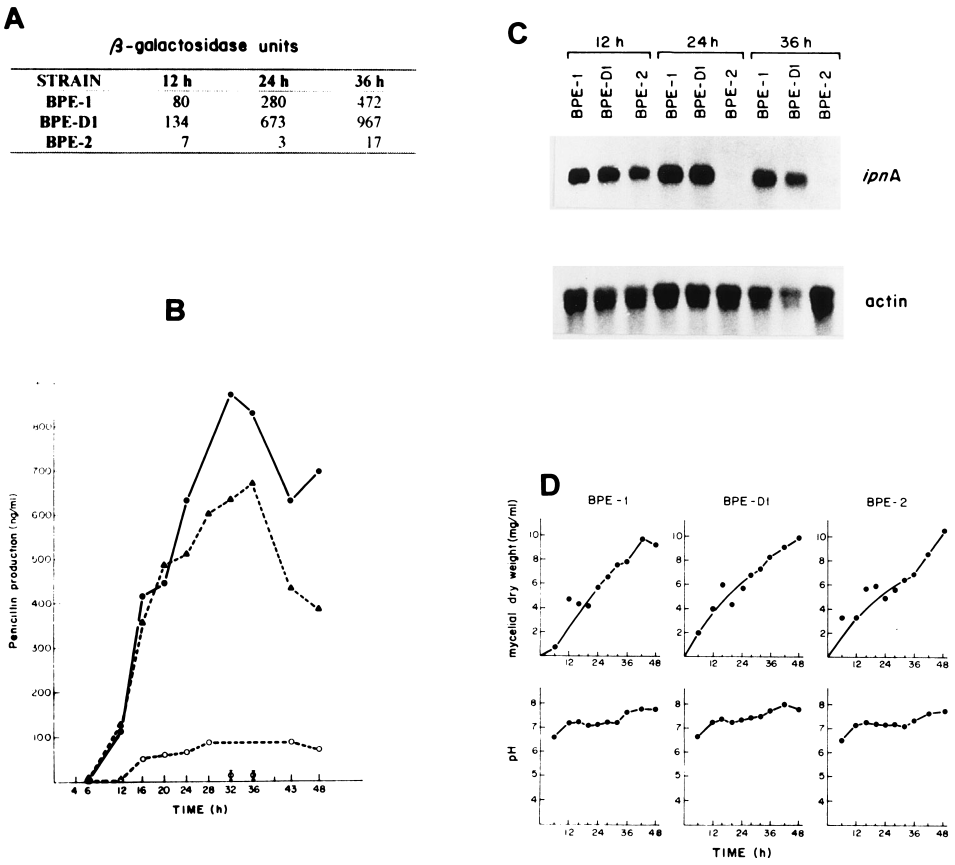

FIG. 4. Molecular phenotype and recessiveness of *npeE1*. (A) Quantitative β-Gal assays of mycelial extracts (at three different growth times) of the indicated strains. (B) Time courses of extracellular penicillin accumulation in the cultures shown above. ●, BPE-1 (*npeE*$^+$); ▲, BPE-D1 (*npeE*$^+$/*npeE1*); ○, BPE-2 (*npeE1*). (C) Northern analysis of *ipnA* transcript levels in these mycelia. Actin message levels are shown as loading controls. (D) Mycelial growth and external pH changes.

strain also had wild-type penicillin production levels (Fig. 4B) and the normal pattern of *ipnA* transcription (as measured by Northern analysis [Fig. 4C]). Therefore, *npeE1* is recessive for all phenotypic criteria, suggesting, together with the results presented above, that *npeE1* is a loss-of-function mutation in a gene encoding a positive-acting factor regulating *ipnA* transcription.

***npeE1* is located in linkage group IV.** We next tested whether the twin reporter technique could be used in assigning the *npeE1* mutation to a linkage group by parasexual genetics. Therefore, a diploid (strain BPE-D2) between an *npeE1* strain and a master strain was constructed and haploidized in the presence of Benlate. The master strain carried an *actA* (actidione resistance) marker in chromosome III. Therefore, haploid segregants showing actidione resistance (thereby having inherited chromosome III from the master strain) were most likely to lack the twin reporter fusion, whereas actidione-sensitive (i.e., *actA*$^+$) segregants most likely have received the chromosome carrying the *ipnA*′::′*lacZ* genes, whose presence was essential to score for the *npeE1* mutation. This presence could also be confirmed by the blue staining of the colonies (in either the *npeE*$^+$ or *npeE1* background [Fig. 3]). The genotype of 620 purified sectors all staining pale (*npeE1*) or deep (*npeE*$^+$) blue on X-Gal plates was analyzed in detail. As expected, only a minor proportion of these sectors (~5%, possibly representing mitotic recombinants or disomics for chromosome III) were actidione resistant (Table 2). Notably, *npeE1* segregated independently of all markers of the master strain with the exception of *methG1*, located on chromosome IV (Table 2). All clones carrying *npeE1* simultaneously were *methG*$^+$, and 93% of the *npeE*$^+$ clones were *methG1*. Therefore, we conclude that *npeE* is located in chromosome IV. Twenty-one segregants which were *methG*$^+$ *npeE*$^+$ possibly represent chromosome IV disomics, mitotic recombinants, or strains in which reversion to *methG*$^+$, which takes place spontaneously with great frequency (20), has occurred.

In addition, it was formally possible (although rather unlikely) that the original mutant strain BPE-2 could have acquired two independent (and recessive [described above]) mutations, one (*npeE1*) affecting expression of *ipnA* (and *ipnA*′::′*lacZ* fusions) and the other affecting penicillin production. If so (and provided that these mutations were located in different chromosomes), they could have segregated during haploidization analysis. To test this possibility (data not shown), we used four pairs of haploid strains derived from the haploidization described above. Each pair was isogenic with respect to several markers representing a set of different chromosomes, with the exception of chromosome IV. For this, one strain in each pair was *npeE*$^+$ while the other was *npeE*. The four pairs were chosen so as to reveal segregation independent of *npeE* of the penicillinless phenotype. Although penicillin production varied with the genetic background (for unknown reasons, different auxotrophic requirements reduce penicillin biosynthesis), in all cases penicillin levels produced by the *npeE*$^+$ strains were at least seven times higher than those produced by their corresponding *npeE1* partners. This, to-

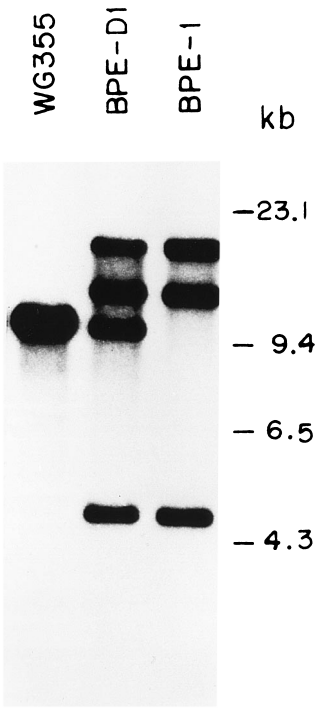

FIG. 5. Southern blot analysis of DNA from strain BPE-D1 with an *argB* probe. The hybridization pattern corresponding to this diploid strain results from superimposing the patterns of strains WG355 and BPE-1, the two haploid strains used to construct BPE-D1.

gether with the results presented above, strongly indicates that a single mutation (*npeE1*) simultaneously reduces *ipnA* transcription and penicillin production.

The double reporter technique allows scoring of *npeE1* in sexual cross progeny. We next tested whether this method could also be used in sexual crosses, in which the presence of two copies of the *ipnA'*::*'lacZ* gene could result in unequal crossing over or intrachromosomal recombination leading to their loss. To test this and with the aim of establishing linkage to previously mapped loci, we carried out sexual crosses between *npeE1* strains and strains (see Materials and Methods and Table 1) carrying a variety of chromosome IV markers: *acuK248*, *meaA8*, *pyroA4*, *methG1*, *inoB2*, *palC4*, and *bzuA1*. Progeny containing the double *ipnA'*::*'lacZ* integration from the *npeE1* parental strain were selected by their deep (*npeE*$^+$) or pale (*npeE1*) staining in X-Gal plates (Fig. 3A). In all cases, *npeE1* segregated independently of the other chromosome IV markers (data not shown). We conclude that *npeE* is probably located in a region of chromosome IV which does not yet contain known genetic markers. This is not unusual in *A. nidulans*. For example, *pacA* (encoding an acid phosphatase), also located in chromosome IV according to haploidization analysis, has resisted attempts to detect meiotic linkage(s) (7). In chromosome I, *uapA* and *camA* segregate in sexual crosses independently of located markers and each other (2). In any case, the experiments described above demonstrate that the technique described here can be used to monitor penicillin regulatory mutations in meiotic crosses.

DISCUSSION

We describe here a twin reporter gene method for the genetic analysis of regulatory mutations in pathways of secondary metabolism in *A. nidulans*. We have used this technique to isolate regulatory mutations in the penicillin biosynthetic pathway. One of these mutations (*npeE1*) has been analyzed in detail to show that this technique also allows scoring of these mutations in haploidization and meiotic analysis as well as allowing study of dominance relationships in diploids.

Although not typical of secondary metabolic pathways, in the case of the penicillin pathway, detection of the end product (usually benzylpenicillin) by bioassay is highly sensitive. Therefore, the bioassay allows isolation and genetic analysis of mutations affecting this pathway. However, the model organism *A. nidulans* produces only modest amounts of penicillin. This causes significant difficulty in detecting and scoring mutations, particularly those affecting regulation, which might not necessarily result in complete loss of penicillin biosynthesis. Many factors (for example colony size) influence antibiotic production by colonies growing on a solid substrate, thereby limiting the value of in situ bioassays in culture plates. More reliable scoring methods, such as those based on bioassays of liquid culture supernatants, are limited by the number of samples which can be analyzed. Moreover, the final output of the pathway (rather than changes in the expression of a single gene) is measured and auxotrophic mutations often affect final penicillin (and perhaps other secondary metabolite) yields in an unpredictable manner. These variations hinder scoring in crosses

TABLE 2. Chromosomal location of *npeE* by haploidization analysis

Chromosomal marker[a]	Haploid segregants			
	npeE$^+$		*npeE1*	
	No.	%	No.	%
sulA1 (I)	160	54	175	54
sulA$^+$	135	46	150	46
adE20 (I)	141	48	167	51
adE$^+$	154	52	158	49
pabaA1 (I)	132	45	150	46
pabaA$^+$	163	55	175	54
acrA1 (II)	137	46	132	41
acrA$^+$	158	54	193	59
actA1 (III)	24	8	10	3
actA$^+$	271	92	315	97
methG1 (IV)	274	93	0	0
methG$^+$	21	7	325	100
nicA2 (V)	137	46	155	48
nicA$^+$	158	54	170	52
lacA1 (VI)	140	47	168	52
lacA$^+$	155	53	157	48
choA1 (VII)	113	38	122	38
choA$^+$	182	62	203	62
riboB2 (VIII)	143	48	203	62
riboB$^+$	152	52	122	38
trpC801 (VIII)	135	46	113	35
trpC$^+$	160	54	212	65

[a] Chromosomes containing genetic markers in the master strain are indicated by Roman numerals in parentheses.

involving a number of auxotrophies. An example of the difficulty in genetically analyzing regulatory mutations in this pathway is provided by the penicillin-nonproducer mutations *npeC007* and *npeD0045* (11), which were incorrectly assigned to chromosomes IV and II, respectively, by haploidization and meiotic linkage analysis (24). However, using more sensitive scoring techniques available for *pal* mutations, Shah et al. (28) showed them to be *palA* mutant (*palA* is located in chromosome III) and *palF* mutant (*palF* is in chromosome VII) alleles, respectively. Characterization of the *npeE1* mutation shows that our method is useful not only for identification but also for genetic analysis of regulatory mutations affecting penicillin biosynthesis. *npeE1* defines a positive-acting regulatory gene of *ipnA* transcription essential for penicillin production. Mutations impairing penicillin biosynthesis have been already described for *A. nidulans* (14) and belong to four different complementation groups, denoted *npeA*, *npeB*, *npeC*, and *npeD* (11), none of which is located in chromosome IV (24), to which the *npeE1* mutation has been assigned by haploidization analysis. Therefore, *npeE1* identifies a new *npe* gene. Another previously identified gene affecting penicillin production located in linkage group IV is *palC*, encoding a product involved in ambient pH signal transduction to the PacC zinc finger transcription factor (29), a major, positive-acting regulator of the penicillin pathway and of *ipnA* gene expression (18, 28, 29). *npeE1* is not a *palC* allele. First, *palC4* recombines freely with *npeE1* (48 parental versus 66 recombinant progeny in a cross with the markers in repulsion [data not shown]). Second, extant *palC* mutations (mimicking the effects of growth at acidic ambient pH) nearly abolish penicillin biosynthesis but, in contrast to *npeE1* (Fig. 4E), do not prevent *ipnA* transcription in the presence of a derepressing carbon source (18). Third, *npeE1* does not prevent growth at pH 8, in contrast to extant *palC* mutations.

We have not yet tested whether the two other mutations isolated in this screen, resulting in a less marked reduction in β-Gal levels, are allelic to *npeE1*. Neither of them is a loss-of-function mutation in *pacC*, a transcriptional activator of *ipnA* (15, 18, 29), as indicated by their lack of effect on secretion of extracellular acid and alkaline phosphatases, whose expression is also under *pacC* control. In addition, as does the *npeE1* strain (described above), the two other mutant strains grow normally at pH 8, indicating that they are not carrying a loss-of-function mutation in any of the six identified *pal* genes (3), which would reduce *ipnA* transcription in a repressing carbon source and prevent penicillin production (15, 18). These data indicate that the screen was not saturating.

Finally, the allocation of *npeE* to a region of chromosome IV (the smallest in *A. nidulans*) which probably does not contain identified markers (as determined by meiotic recombination) has to be considered not only in the context of previously reported cases of lack of meiotic linkage (for example, references 2 and 7 and described above) but also in the context of the existence of several regions of the *A. nidulans* genome which have been shown to be dispensable under laboratory conditions, such as the penicillin cluster itself (23), the Spo1 gene cluster (1) or, most notably, a region in chromosome III centromere-distal to the *areA* gene which contains at least 0.5% of the *A. nidulans* genome (8). This possibly indicates that the *A. nidulans* genetic map is far from being saturated. Most *A. nidulans* mutations have been isolated on the basis of growth tests made under laboratory conditions. *A. nidulans* is a saprophyte fungus showing remarkable physiological versatility (4), which must be advantageous in natural environments. A significant amount of its genetic information may therefore encode functions which are only beneficial in its natural habitats and would therefore elude genetic analysis performed under laboratory conditions. The technique used here for the well-known penicillin pathway as well as "trapping" reporter gene techniques will help in identifying some of these functions.

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