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Use of Reporter Genes To Identify Recessive *trans*-Acting Mutations Specifically Involved in the Regulation of *Aspergillus nidulans* Penicillin Biosynthesis Genes

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Starting from three amino acid precursors, penicillin biosynthesis is catalyzed by three enzymes which are encoded by the following three genes: *acvA* (*pcbAB*), *ipnA* (*pcbC*), and *aat* (*penDE*). To identify *trans*-acting mutations which are specifically involved in the regulation of these secondary metabolism genes, a molecular approach was employed by using an *Aspergillus nidulans* strain (AXTII9) carrying *acvA-uidA* and *ipnA-lacZ* gene fusions integrated in double copies at the chromosomal *argB* gene. On minimal agar plates supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), colonies of such a strain stained blue, which is indicative of *ipnA-lacZ* expression. After mutagenesis with UV light, colonies were isolated on agar plates with lactose as the carbon source, which produced only a faint blue color or no color at all. Such mutants (named Prg for penicillin regulation) most likely were defective in *trans*-acting genes. Control experiments revealed that the mutants studied still carried the correct number of gene fusions. In a fermentation run, mutants Prg-1 and Prg-6 exhibited only 20 to 50% of the *ipnA-lacZ* expression of the wild-type strain and produced only 20 to 30% of the penicillin produced by the wild-type strain. Western blot (immunoblot) analysis showed that these mutants contained reduced amounts of *ipnA* gene product, i.e., isopenicillin N synthase. Both mutant Prg-1 and mutant Prg-6 also differed in *acvA-uidA* expression levels from the wild type. Segregation analysis indicated that for both mutants the Prg phenotype resulted from mutation of a single gene. Two different complementation groups, which were designated *prgA1* and *prgB1*, were identified. However, the specific activity of the *aat* (*penDE*) gene product, i.e., acyl coenzyme A:6-aminopenicillanic acid acyltransferase, was essentially the same for the mutants as for the wild-type strain, implying that the last step of the penicillin biosynthetic pathway is not affected by the *trans*-acting mutations identified.

Penicillin is produced as an end product by filamentous fungi exclusively, notably by *Aspergillus* (*Emericella*) *nidulans* and *Penicillium chrysogenum* (reviewed in references 1, 9, 13, and 30). Penicillin is synthesized as a classical secondary metabolite from three amino acid precursors: L-α-aminoadipic acid, L-cysteine, and L-valine. In *P. chrysogenum*, L-α-aminoadipic acid is an intermediate of the L-lysine biosynthetic pathway (reviewed in references 1, 9, 13, 30, and 36). Recently, it was shown that L-α-aminoadipic acid can also be obtained by the reversal of the last two steps of the L-lysine biosynthetic pathway (15). The importance of this pathway for penicillin biosynthesis, however, has not been clarified yet. For *A. nidulans*, the analysis of lysine-auxotrophic mutants strongly implied that, for penicillin biosynthesis, L-α-aminoadipic acid is also provided by catabolic conversion of L-lysine by a yet-unidentified pathway (41).

Starting from these amino acids, penicillin biosynthesis in both *A. nidulans* and *P. chrysogenum* is catalyzed by three enzymes which are encoded by three genes. The genes are organized into a single cluster. *acvA* (*pcbAB*) and *ipnA* (*pcbC*), encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase and isopenicillin N synthase (IPNS), respectively, are divergently transcribed. The last step of penicillin biosynthesis is catalyzed by acyl coenzyme A:6-aminopenicillanic acid acyltransferase (AAT), encoded by the *aat* (*penDE*) gene, which is located downstream of the *ipnA* gene (reviewed in references 1, 9, 28, 30, and 36).

The biochemistry of penicillin biosynthesis is rather well understood. Since in recent years recombinant techniques have been developed for some filamentous fungi, several groups have begun to investigate the molecular regulation of penicillin biosynthesis. So far, the greatest progress has been made with *A. nidulans*, because in contrast to *Penicillium chrysogenum* which is used for penicillin production industrially, this fungus has a sexual cycle. Hence, classical genetic techniques which together with molecular techniques facilitate a thorough analysis of the genetic regulation of metabolic pathways, including penicillin biosynthesis, can be applied to *A. nidulans* (2, 11, 12, 26, 39).

By using *lacZ* and *uidA* reporter genes, it was shown that the expression of penicillin biosynthesis genes *acvA* and *ipnA* of *A. nidulans* was repressed by L-lysine (8). In addition, it was demonstrated that expression of *ipnA*, but not of *acvA* gene fusions, was repressed when, instead of lactose, glucose was used as the carbon source (5). The repression of expression of *ipnA* gene fusions by carbon sources is mediated, at least in part, at the transcriptional level, because the steady-state level of *ipnA* mRNA decreased when mycelia were fermented with certain carbon sources (16).

So far, only the wide-domain regulatory gene *pacC* has been reported to positively affect *ipnA* transcription (17, 35). However, pathway-specific *trans*-acting regulatory genes which specifically activate the expression of the penicillin biosynthesis genes have not been identified yet. This is of considerable interest because the genetic regulation of penicillin biosynthe-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype and/or selectable markers in *A. nidulans*	Source or reference(s)
Strains		
E. coli		
DH5α	F$^-$ ϕ80d/*lacZ* M15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ$^-$ *thi-1 gyrA96 relA1*	BRL
A. nidulans		
R21	*yA2 pabaA1*	18
WG355	*biA1 bgaO argB2*	18, 42
WG357	*yA2 pabaA1 bgaO*	This study
AXB4A	*biA1 bgaO argB2*::pAXB4A ArgB$^+$ WG355 derivative carrying pAXB4A	5
AXTII9	*biA1 bgaO argB2*::(pAXB4A)$_2$ ArgB$^+$ WG355 derivative carrying pAXB4A	5
NI · 4	*pabaA1 pyrG89 npeA0049/1 niaD*	25
Prg-1	*prgA1 biA1 bgaO argB2*::(pAXB4A)$_2$ ArgB$^+$ AXTII9 derivative	This study
Prg-6	*prgB1 biA1 bgaO argB2*::(pAXB4A)$_2$ ArgB$^+$ AXTII9 derivative	This study
Prg-6.1	*prgB1 yA2 pabaA1 bgaO argB2*::(pAXB4A)$_2$ ArgB$^+$	This study
Prg-41	*prg-41 biA1 bgaO argB2*::(pAXB4A)$_2$ ArgB$^+$ AXTII9 derivative	This study
Plasmids		
pILJ16	*argB*$^+$	20
pAXB4A	pTRAN2 derivative; *acvA-uidA ipnA-lacZ argB**[a]	5

[a] *argB**, mutant *argB* allele which can complement chromosomal *argB* mutations.

sis presently represents the most advanced model system for studying the regulation of the biosynthesis of a secondary metabolite in fungi. Hence, the exciting question of whether specific regulators for secondary metabolism genes evolved or whether regulators of the primary metabolism took over this task can be tackled. This investigation might also give hints concerning the evolution of secondary metabolism, which is currently intensely discussed (43). Furthermore, penicillin is still a valuable therapeutic antibiotic and the overexpression of regulatory genes could lead to higher yields of penicillin in production strains of *P. chrysogenum.*

Until now, the isolation of mutants involved in biosyntheses of important secondary metabolites has been extremely laborious. For example, mutants of *P. chrysogenum* producing more or less penicillin than the precursor strain have been isolated classically, i.e., after mutagenization of a production strain, mutants have been fermented and scored for higher or lower levels of penicillin production (37). Here, by the combination of classical and molecular techniques, mutants of *A. nidulans* carrying mutations which are specifically involved in *trans* in the regulation of the expression of the penicillin biosynthesis genes *acvA* and *ipnA* were isolated. Mutants were first identified on agar plates by using a translational fusion of *ipnA* with the *lacZ* reporter gene. The phenotype of mutants identified on agar plates was stable during cultivation in fermentation medium, indicating that this approach is feasible for the isolation of mutants which are deregulated under production conditions. Some mutants were further characterized both physiologically and genetically. The results obtained indicated that the mutants isolated most likely carry mutations in positively acting regulatory genes which specifically affect both *acvA* and *ipnA* gene expression.

MATERIALS AND METHODS

Strains and plasmids. Bacterial and fungal strains used in this study are listed in Table 1. Vectors and plasmids were propagated in *Escherichia coli* DH5α. For construction of *A. nidulans* WG357, strains WG355 and R21 (Table 1) were crossed. Ascospores were plated on *Aspergillus* minimal medium (AMM) agar plates with lactose as the carbon source and supplemented with *p*-aminobenzoic acid (PABA) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 50 μg/ml). One of the resulting progeny that had yellow spores, was auxotrophic for PABA, and did not produce blue color indicative of the *bgaO* mutation (18) was named WG357 (Table 1).

Media. Seed culture medium and fermentation medium were prepared as previously described (5). Experimental cultures contained 4% (wt/vol) lactose as the carbon source. As a minimal medium, the AMM of Pontecorvo et al. (29) was used essentially according to the following recipe: $NaNO_3$, 6.0 g/liter; KCl, 0.52 g/liter; KH_2PO_4, 1.52 g/liter; $MgSO_4 \cdot 7H_2O$, 0.52 g/liter; and 1 ml of trace element solution [$FeSO_4 \cdot 7H_2O$, 1 g/liter; $ZnSO_4 \cdot 7H_2O$, 8.8 g/liter; $CuSO_4 \cdot 5H_2O$, 0.4 g/liter; $MnSO_4 \cdot 4H_2O$, 0.15 g/liter; $Na_2B_4O_7 \cdot 10H_2O$, 0.1 g/liter; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05 g/liter] per liter. The pH of the medium was adjusted to 6.5 with 10 M KOH. As the carbon source, either 1% (wt/vol) lactose or 1% (wt/vol) glucose was used. To obtain distinct colonies of *A. nidulans*, deoxycholate (0.06%, wt/vol) was added to agar plates. If required, biotin (300 μl; 2 mg/100 ml), PABA (300 μl; 0.1 g/100 ml), or L-arginine (500 μl; 2 g/100 ml) was added to the flasks. *Aspergillus* complete medium agar plates were prepared as previously described (33).

Fermentation conditions. Seed and experimental cultures with fermentation medium or AMM (20 ml in 250-ml unbaffled flasks) were incubated at 26°C and 250 rpm on a flatbed rotary shaker (B. Braun Biotech, Melsungen, Federal Republic of Germany). Seed cultures were inoculated with 1 ml of a conidial suspension and incubated for 24 h. Mycelia were washed twice with 0.9% (wt/vol) sterile NaCl solution by centrifugation and finally resuspended in 14 ml of sterile NaCl solution. Fermentation medium (20 ml) supplemented with appropriate vitamins (biotin or PABA) in 250-ml flasks was inoculated with 1 ml of mycelial suspension. Cultures were further incubated up to 96 h. At different time points several flasks were harvested.

Penicillin bioassay and determination of dry weight. Penicillin bioassays using *Bacillus calidolactis* C953 as an indicator organism and determination of the dry weights of cultures were carried out essentially as previously described (5).

Mutagenesis. Conidiospores of strain AXTII9 were exposed to UV irradiation at 254 nm (UV_{254} irradiation) in 0.1 M $MgSO_4$ solution until the viable count was decreased to 2 to 5%. The spore suspension was diluted and plated on AMM agar plates with lactose as the carbon source, supplemented with biotin and X-Gal (50 μg/ml) as an indicator for *ipnA-lacZ* expression.

Genetic techniques. Complementation tests, sexual crosses, and characterization of the resulting progeny were performed according to the method of Pontecorvo et al. (29).

Standard DNA techniques. For small-scale preparation of *A. nidulans* chromosomal DNA, the technique of Raeder and Broder (32) was used. Standard techniques in the manipulation of DNA were as detailed by Sambrook et al. (34). For Southern blot analysis, transfer of DNA from agarose gels onto nylon membrane (Hybond N$^+$; Amersham, Little Chalfont, United Kingdom) was performed as previously described (7). The labelling of DNA probes with fluorescein-11-dUTP was carried out essentially according to the method of Feinberg and Vogelstein (19) by using an ECL random prime kit (Amersham). Detection of hybridized fragments by Southern blot analysis was performed with an ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions.

β-Gal and β-Glu activity assays. β-Galactosidase (β-Gal) and β-glucuronidase (β-Glu) activities were determined in crude extracts from mycelia grown in

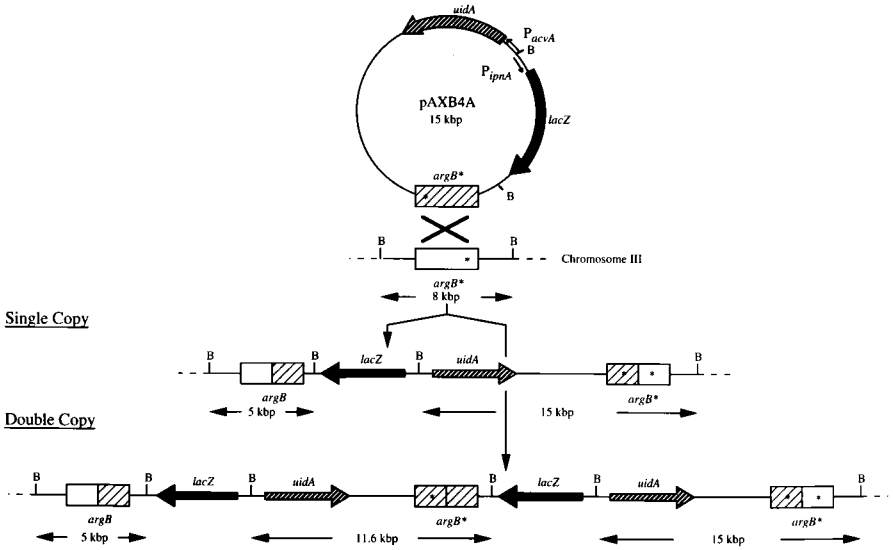

FIG. 1. Diagrammatic representation of possible homologous recombination events for plasmid pAXB4A integrated in a single copy or in double copies at the chromosomal *argB* gene locus of *A. nidulans* (5). The restriction map around the *argB* locus with and without integrated pAXB4A plasmid is shown. The *argB* gene on the plasmid is labelled by stripes, and the chromosomal *argB* is labelled by an open box. Mutations in *argB* are indicated by asterisks. Sizes of *Bam*HI fragments which were detected by use of the specific *argB* probe described in the legend to Fig. 3 are given. Abbreviations: *argB**, mutated *argB* allele which can complement the chromosomal *argB2* mutation by homologous recombination; B, *Bam*HI restriction site; P_{acvA}, promoter region of the *acvA* gene; P_{ipnA}, promoter region of the *ipnA* gene. *lacZ* is the *E. coli* β-galactosidase gene, and *uidA* is the *E. coli* β-glucuronidase gene.

fermentation medium as previously described (5). β-Gal and β-Glu activities at each time point were measured in cell extracts from three simultaneously incubated cultures. Differences in absolute activities between experiments were observed, although activities at time points within each fermentation experiment were consistent, as indicated by the standard deviations.

Protein gel electrophoresis and immunoblotting. Electrophoretic analysis of proteins was performed on a 10% (wt/vol) polyacrylamide slab gel according to the method of Laemmli (23). Each slot was loaded with either 72 or 14 μg of crude cellular protein extract of different *A. nidulans* strains. Proteins were detected by Coomassie blue staining. For immunoblotting, unstained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels were electroblotted onto nitrocellulose (40). Filters were blocked with 5% (wt/vol) milk powder in TTBS (20 mM Tris · Cl [pH 7.6], 137 mM NaCl, 0.1% [vol/vol] Tween 20) for 90 min at room temperature with gentle agitation. For detection, filters were extensively washed with TTBS (once for 15 min and four times for 5 min) and then incubated with primary antibodies (anti-IPNS antiserum; dilution, 1:3,000) with 0.5% (wt/vol) bovine serum albumin (BSA) in TTBS for 90 min. Filters were extensively washed as described above and subsequently incubated with protein A-horseradish peroxidase conjugate (dilution, 1:3,000; Bio-Rad, Richmond, Calif.) for 90 min with 0.5% (wt/vol) BSA in TTBS. After a last washing as described above, detection was carried out with an ECL kit (Amersham) according to the manufacturer's instructions.

Antiserum. Rabbit antiserum against the *P. chrysogenum* IPNS was kindly provided by SmithKline Beecham Pharmaceuticals (Worthing, United Kingdom) and was described previously (6). Since the specificity of this antiserum against *A. nidulans* IPNS was low, the antibodies were first saturated with crude extracts of non-penicillin-producing *A. nidulans* NI · 4 (*npe0049*) carrying a deletion of the whole penicillin biosynthesis gene cluster, including the *ipnA* gene (25). Precipitates were centrifuged for 30 min at 6°C, and the supernatant was used for the immune reaction.

AAT activity assay. AAT specific activities were determined as previously described (6).

Determination of protein concentrations. Protein concentrations were determined according to the method of Bradford (4).

RESULTS

Isolation of mutants carrying *trans*-acting mutations specifically involved in the expression of *A. nidulans* penicillin biosynthesis genes. Previously, the construction of *A. nidulans* transformants containing integrated copies of plasmid pAXB4A encoding *ipnA-lacZ* and *acvA-uidA* gene fusions (Fig. 1) was described (5). By using such strains, the expression of penicillin biosynthesis genes *acvA* and *ipnA*, encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase and IPNS, respectively, can be monitored simultaneously simply by measuring β-Gal and β-Glu activities. As also reported previously, some of the transformants carried plasmid pAXB4A integrated in double or multiple copies at the chromosomal *argB* gene (6).

Here, one of the strains, named AXTII9, contained plasmid pAXB4A integrated in double copies precisely at the chromosomal *argB* gene locus. This was demonstrated by Southern blot analysis, which, for strain AXTII9, showed the characteristic band pattern, i.e., three bands of 15, 11.6, and 5 kbp and of equal intensity detected by the use of an *argB* probe (Fig. 1; see Fig. 3, lane 3). Additional evidence for double-copy integrated plasmid pAXB4A resulted from the measurement of *acvA-uidA* and *ipnA-lacZ* expression during a fermentation run. Strain AXTII9 gave values for β-Glu and β-Gal activities which were exactly twice as high as those produced by a previously characterized strain carrying plasmid pAXB4A integrated in a single copy at the chromosomal *argB* gene locus (Fig. 1; see Fig. 3, lane 2) (5).

In addition, strain AXTII9 carries the *bgaO* mutation (5, 18), i.e., the strain has no detectable endogenous β-Gal activity. However, *bgaO* mutants grow as well as wild-type strains with lactose as a sole carbon source (reference 18 and our own observations). *A. nidulans* appears to possess an alternative and so far unknown mode of lactose utilization which is not dependent on the presence of β-Gal activity (18).

On AMM agar plates supplemented with either X-Gal (AMM–X-Gal agar plates) or 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Glu) as the substrate for β-Gal or β-Glu, respectively, colonies of strain AXTII9 stained blue (Fig. 2). Because of the *bgaO* mutation, all of the β-Gal activity of strain AXTII9 derived from *ipnA-lacZ* expression. Lactose was used as the carbon source because penicillin titers and levels of expression of *ipnA* gene fusions were highest with this carbon source, whereas the use of glucose led to an about two- to threefold repression of *ipnA* expression and to reduced penicillin titers in the fermentation broth (5). Because of the sensitivity of X-Gal, *ipnA-lacZ* expression on AMM agar plates

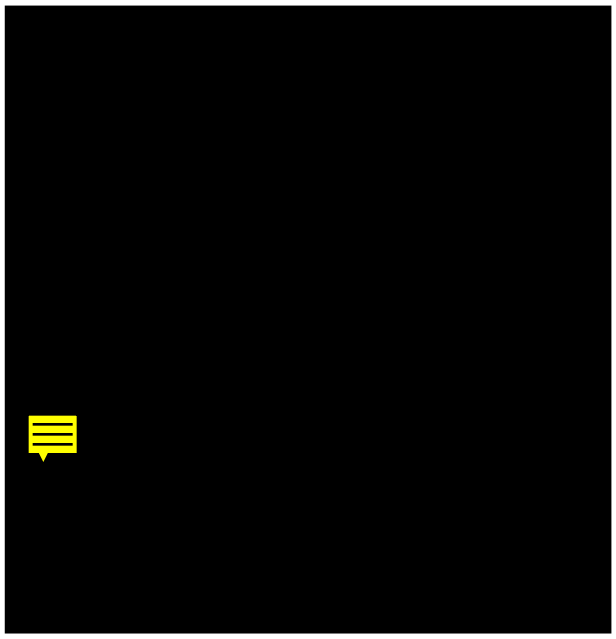

FIG. 2. *A. nidulans* AXTII9 (wild type) and mutant strains Prg-1, Prg-6, and Prg-41 grown on AMM agar plates with 1% (wt/vol) lactose supplemented with biotin and X-Gal (35 μg/ml) to detect expression of *ipnA-lacZ* gene fusions. The wild-type strain WG355 without gene fusions does not produce blue color around colonies under these conditions because of the *bgaO* mutation, which is also present in all mutant strains (data not shown).

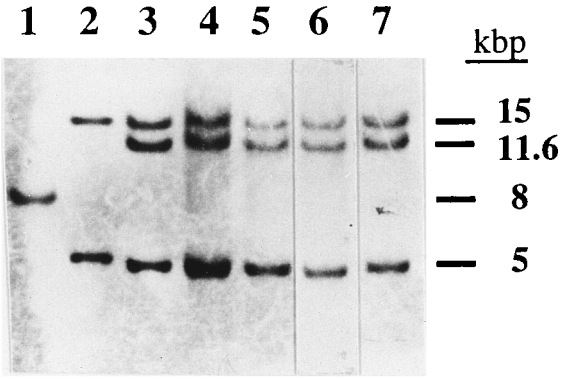

FIG. 3. Southern blot analysis of chromosomal DNA from wild-type and mutant *A. nidulans* strains. Chromosomal DNA of the different strains was digested with *Bam*HI. Southern blot analysis was carried out essentially as previously described (7). The blot was hybridized under stringent conditions (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]; 60°C) with an *argB* probe consisting of a fluorescein-11-dUTP-labelled *Hin*dIII fragment (1.7 kbp) of plasmid pILJ16 encoding a part of the *A. nidulans argB* gene (20). Single-copy and double-copy or multicopy integration of plasmid pAXB4A at the chromosomal *argB* gene locus are indicated by two (5 and 15 kbp) and three bands (15, 11.6, and 5 kbp), respectively, as illustrated in Fig. 1. The untransformed wild-type strain WG355 shows a single band of 8 kbp hybridizing with the *argB* probe (5). Sizes of hybridized fragments are indicated on the right. Lanes: 1, untransformed wild-type strain WG355; 2, strain AXB4A carrying plasmid pAXB4A integrated in a single copy at the chromosomal *argB* gene locus; 3, AXTII9 carrying plasmid pAXB4A in double copies; 4, mutant strain Prg-1; 5, Prg-6; 6, Prg-41; 7, Prg-6.1.

with glucose can still be detected by the appearance of blue-stained colonies.

The rationale for the specific identification of *trans*-acting mutations involved in the expression of penicillin biosynthesis genes can be summarized as follows. After mutagenesis of strain AXTII9, colonies which did not produce blue color were isolated on AMM agar plates with lactose supplemented with X-Gal. Cells of such colonies most likely carried *trans*-acting mutations affecting the expression of *ipnA-lacZ* gene fusions in *trans*. The appearance of mutants carrying *cis*-acting mutations, i.e., mutations in the *ipnA* promoter or the *lacZ* gene, seemed to be unlikely, since such mutations would probably not be detected because of the second gene fusion located on the chromosome (Fig. 1).

Approximately 10^8 conidiospores of strain AXTII9 were exposed to UV_{254} irradiation. The spore suspension was spread on AMM agar plates with lactose as the carbon source, supplemented with biotin and X-Gal (50 μg/ml) as an indicator for *ipnA-lacZ* expression. Approximately 15,000 colonies were examined. Most of these showed the wild-type phenotype, i.e., production of blue color on AMM–X-Gal agar plates with lactose. About 100 colonies with a white or faint blue phenotype were purified. The most promising mutants with a clear and reproducible phenotype, i.e., white colonies, were named Prg-1 (for penicillin regulation), Prg-6, and Prg-41 (Fig. 2).

White colonies could have lost the gene fusions by DNA rearrangements or excision of the gene fusions after exposure of conidiospores to UV irradiation. To exclude this possibility, Southern blot analysis was performed (Fig. 3). In the mutant strains Prg-1, Prg-6, and Prg-41, the gene fusions were still present and integrated in double copies at the *argB* gene locus (Fig. 3, lanes 4 to 6, and Fig. 1).

Furthermore, the mutant strains Prg-1, Prg-6, and Prg-41 still showed blue-stained colonies on AMM–X-Gal agar plates with glucose as the carbon source, indicating that the gene fusions were still functional.

The mutants have been grown on *Aspergillus* complete medium and AMM agar plates for many generations. Revertants have never been observed. All conidiospores of mutant strains spread on AMM agar plates with lactose and X-Gal (35 μg/ml) formed white colonies, indicating that the mutants were stable.

During a fermentation run Prg mutants produced less penicillin, had lower levels of expression of *ipnA-lacZ* than the wild-type strain and had *acvA-uidA* expression levels different from those of the wild-type strain. For further characterization, mutants and the wild-type strain were grown in fermentation medium. This would enable us to analyze whether the mutant phenotype was also prominent during cultivation in fermentation medium, i.e., whether the isolation of mutants on agar plates is feasible for the identification of mutations which affect the penicillin biosynthesis genes under production conditions. If the mutations acted in *trans*, they should have an impact on the production of penicillin. Finally, it was important to determine the growth rates of the mutants in order to analyze whether the mutations exhibited pleiotropic effects.

The results of a fermentation run with lactose as the carbon source are shown in Fig. 4. Compared with the wild type, all mutants tested had lower levels of expression of the *ipnA-lacZ* fusions, as indicated by β-Gal specific activity (Fig. 4C). The apparent lack of *ipnA-lacZ* expression on AMM agar plates was thus reflected by reduced expression of the gene fusions in a fermentation run. Furthermore, the mutations also affected the expression of the *acvA-uidA* gene fusions, which was reduced in mutant Prg-1 (Fig. 4D). In mutant Prg-6, *acvA-uidA* expression differed among fermentation runs. As shown in Fig. 4D, Prg-6 generally showed reduced values compared with Prg-1 and the wild type. In some fermentation runs, however, these values rose above the level, at least, for mutant strain Prg-1 (data not shown).

Despite this possible difference, all mutants showed a re-

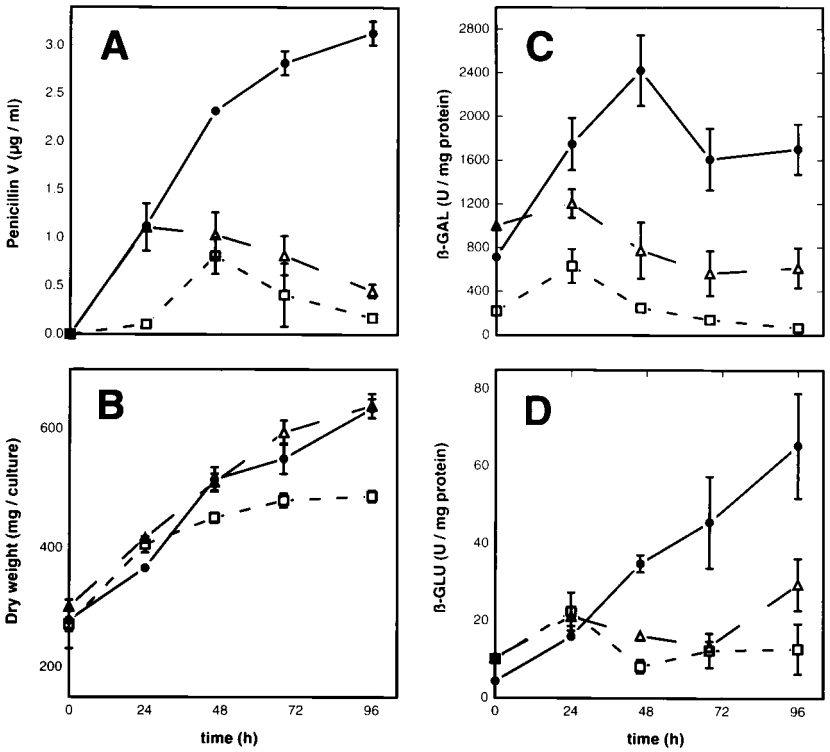

FIG. 4. Fermentation run with 4% (wt/vol) lactose as the carbon source of the *A. nidulans* wild-type strain AXTII9 (●) and mutant strains Prg-1 (△) and Prg-6 (□) carrying plasmid pAXB4A (*acvA-uidA* and *ipnA-lacZ* fusions) integrated in double copies at the chromosomal *argB* gene locus. Data at each time point represent the mean and standard deviation of values for three simultaneously harvested flasks. The values at 0 h represent data for seed cultures. The promoterless vector pTRAN2 integrated at the *argB* gene locus produced only 10 to 20 U of β-Gal specific activity and no β-Glu background activity (5). (A) Penicillin V titer. (B) Dry weight. (C) Expression of *ipnA-lacZ* gene fusions determined as β-Gal specific activity. (D) Expression of *acvA-uidA* gene fusions determined as β-Glu specific activity. Differences in *acvA-uidA* expression of Prg-6 were observed among fermentation runs. Generally, Prg-6 showed reduced levels. In some fermentation runs, however, *acvA-uidA* expression of Prg-6 was increased, at least to levels above those observed for Prg-1 (data not shown).

duced penicillin titer in the fermentation broth (Fig. 4A). In addition, the growth rate of mutant strains was measured by determining the mycelial dry weight. The mutant strain Prg-1 grew as well as the wild-type strain and reached the same mycelial mass (Fig. 4B). Hence, the reduced penicillin titer in the medium of Prg-1 cultures was caused by drastically reduced penicillin production. This strongly supports the notion that the mutant harbors a *trans*-acting mutation, because the effect on penicillin production was independent of the expression of gene fusions. The mutant strain Prg-6, however, grew more slowly and reached a lower mycelial mass than Prg-1 or the wild-type strain (Fig. 4B).

The mutants Prg-1 and Prg-6 contained smaller cellular amounts of IPNS. To independently confirm that the mutants were in fact defective in *trans*-acting factors, the cellular amounts of IPNS were determined. Since the expression of *ipnA-lacZ* gene fusions was reduced in the mutants, this should be reflected by a lower cellular content of IPNS. Hence, Western blot (immunoblot) analysis using anti-IPNS antiserum was carried out. Mycelia were grown in fermentation medium for 46 h at 26°C with lactose as the carbon source. In order to quantify precisely the cellular concentration of IPNS, two different amounts of crude extracts (72 and 14 μg) were subjected to Western blot analysis. By using antiserum against the *P. chrysogenum* IPNS, the corresponding *A. nidulans* enzyme was detected, as shown in Fig. 5. As expected, no band was visible with crude extracts of the negative control strain NI · 4 carrying a deletion of the whole penicillin biosynthesis gene cluster, including the *ipnA* gene (25) (Fig. 5, lanes 1 and 5). Crude extract of mutant Prg-1 was loaded in lanes 3 and 7, and crude extract of Prg-6 was loaded in lane 8. The intensity of the IPNS bands in these lanes was clearly reduced compared with that of the IPNS bands in lanes loaded with crude extracts of wild-type strains (Fig. 5, lanes 2, 4, 6, and 9). The difference in band intensity was particularly noticeable in lanes which were loaded with 14 μg of crude extract only (Fig. 5, lanes 5 to 9). Hence, the intracellular amount of IPNS was reduced in both mutants. The smallest cellular amount of IPNS was detected in mutant Prg-6, which showed a hardly visible IPNS band (Fig. 5, lane 8). This finding agrees well with results obtained for the expression of the *ipnA-lacZ* gene fusions, which was reduced in both mutants and at the lowest level in Prg-6 (Fig. 4C).

Compared with the wild-type strain, the mutants showed essentially the same level of AAT specific activity. So far, the mutants were characterized with respect to the genes and proteins involved in the first and second reaction of the penicillin biosynthetic pathway. The third and final step, however, is catalyzed by AAT encoded by *aat* (*penDE*) (reviewed in references 1, 9, and 13). To determine whether the *prg* mutations identified also affected the last step, AAT specific activity was measured during a fermentation run. The results are shown in Fig. 6.

In both mutants, AAT activity was clearly detectable and in about the same amount as in the wild-type strain. Hence, the *trans*-acting mutations of mutant strains Prg-1 and Prg-6 do not seem to be involved in the regulation of the last step of penicillin biosynthesis.

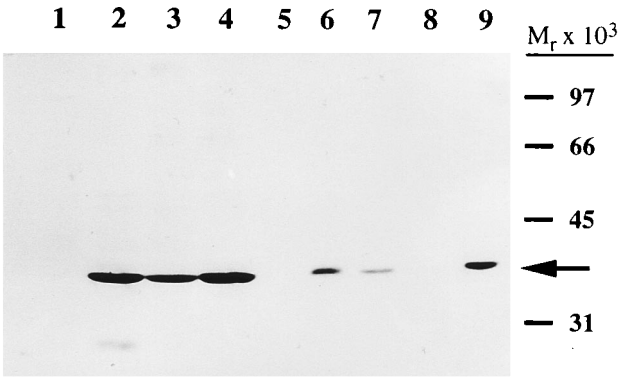

FIG. 5. Detection of IPNS in mutants Prg-1 and Prg-6 by Western blot analysis. Experimental cultures (20 ml) were grown in fermentation medium with 4% (wt/vol) lactose and harvested after 46 h. Immunoblotting was carried out as stated in Materials and Methods. To show the specificity of antibodies, the non-penicillin-producing strain NI · 4 (*npe0049*), carrying a deletion of the whole penicillin biosynthesis gene cluster, including the *ipnA* gene (25), was used as a negative control (lanes 1 and 5). Low-molecular-weight standards from Bio-Rad were used as markers. The IPNS band is indicated by the arrow. Lanes 1 to 4 contained 72 μg of crude extract each; lanes 5 to 9 contained only 14 μg of crude extract. Lanes: 1 and 5, *A. nidulans* NI · 4 (*npe0049*); 2 and 6, wild-type strain AXTII9; 3 and 7, mutant strain Prg-1; 4 and 9, wild-type strain WG355; 8, mutant strain Prg-6.

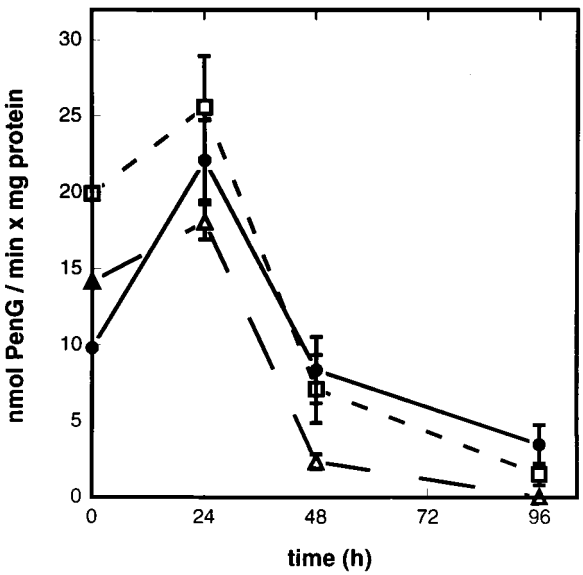

FIG. 6. Time course of AAT specific activity of the *A. nidulans* wild-type strain AXTII9 and mutant strains Prg-1 and Prg-6. Mycelia were grown in fermentation medium with 4% (wt/vol) lactose as the carbon source for 96 h. At different time points two flasks of each strain were harvested. Measurements were made in triplicate for each culture. Standard deviations are shown. The values at 0 h represent data of seed cultures. Symbols: ●, wild-type strain AXTII9; △, mutant strain Prg-1; □, mutant strain Prg-6.

The mutations belong to different complementation groups. To test whether the mutations can be complemented in *trans*, wild-type strain WG357 (*pabaA1 yA2 bgaO*) (Table 1) was coinoculated onto *Aspergillus* complete medium agar plates with mutant strain Prg-1 or Prg-6. After 36 h of growth, vegetative hyphae were transferred onto AMM agar plates with 2% (wt/vol) glucose but without further supplements to force the generation of heterokaryons. These were purified and transferred onto AMM–X-Gal (35 μg/ml) agar plates with lactose. Heterokaryons of the wild-type strain with both Prg-1 and Prg-6 stained the agar blue, providing evidence that the mutations can be complemented in *trans* and thus are recessive.

To check whether these mutations belong to different complementation groups, *A. nidulans* Prg-6.1, carrying the *prg-6* mutation, gene fusions integrated in double copies at the chromosomal *argB* gene locus, and, in addition, an auxotrophic marker (*pabaA1*; auxotrophy for PABA) and having a spore color (*yA2*; yellow conidiospores) different from that of Prg-1, was constructed. For construction of the mutant strain Prg-6.1, ascospores of a sexual cross of strains WG357 and Prg-6 leading to yellow conidiospores (*yA2*) were tested first on AMM–X-Gal (50 μg/ml) agar plates with glucose. With glucose as the carbon source, the mutants as well as the wild-type strain still produced blue color. Therefore, blue color showed the presence of *ipnA-lacZ* fusions. Blue colonies were further tested for production of blue color on AMM–X-Gal (35 μg/ml) agar plates with lactose as the sole carbon source. In contrast to wild-type strains carrying the gene fusions, Prg mutants did not produce blue color on the latter plates. By determining these two phenotypes, mutant strain Prg-6.1 was identified. Southern blot analysis confirmed that strain Prg-6.1 carried the gene fusions integrated in double copies at the chromosomal *argB* gene (Fig. 3, lane 7). This analysis was essential because some progeny of the cross had lost a copy of the gene fusions during meiosis. In addition, the Prg phenotype of strain Prg-6.1 was independently confirmed by cultivation of mycelia in fermentation medium. Strain Prg-6.1 reached about 30% of the penicillin titer of a wild-type strain (data not shown).

For complementation analysis, heterokaryons of mutant strains Prg-1 and Prg-6.1 were isolated. Several heterokaryons were tested. All of them showed a wild-type phenotype on AMM agar plates with lactose and X-Gal (35 μg/ml), indicating that strains Prg-1 and Prg-6.1 could complement each other. The mutations *prg-1* and *prg-6* thus belonged to different complementation groups and were designated *prgA1* and *prgB1*, respectively.

Segregation analysis. Prg mutant strains belonging to either the *prgA1* or the *prgB1* complementation group were sexually crossed with a strain phenotypically wild type with respect to Prg phenotype. Progeny producing blue-stained colonies on AMM–X-Gal (50 μg/ml) agar plates with glucose were checked for their Prg phenotype on AMM–X-Gal (35 and 28 μg/ml) agar plates with lactose. For *prgA1*, 45.2% of the progeny showed the mutant phenotype and 54.8% showed the wild-type phenotype. Analysis of data by the χ^2 test (data not shown) indicated that this proportion represented a 1:1 mutant/wild-type phenotypic segregation. For *prgB1*, 51% of the progeny showed the mutant phenotype, also indicating a 1:1 mutant/wild-type segregation. Therefore, in both mutants Prg-1 and Prg-6, the Prg phenotype resulted from mutation of a single gene, *prgA1* and *prgB1*, respectively.

DISCUSSION

In this study, by using *ipnA-lacZ* gene fusions, mutants which are defective in *trans*-acting genes involved in the regulation of penicillin biosynthesis were specifically isolated. Two mutants (Prg-1 and Prg-6) with a reproducible phenotype, i.e., white colonies on AMM–X-Gal agar plates with lactose as the carbon source (Fig. 2), were characterized in detail. In both mutants, the expression of *ipnA-lacZ* gene fusions was still detectable on AMM agar plates with glucose, indicating that the mutants carry mutations in genes which are particularly important for the expression of penicillin biosynthesis genes with

lactose as the carbon source. At least for Prg-1, the possibility that the mutant is defective in lactose utilization can be excluded because the growth rates of Prg-1 with lactose as the carbon source both on AMM agar plates (data not shown) and in fermentation medium were indistinguishable from those of the wild-type strain (Fig. 4B).

The mutations of mutants Prg-1 and Prg-6 were complemented in *trans* and assigned to different complementation groups designated *prgA1* and *prgB1*, respectively. Therefore, the mutations are recessive. Segregation analyses showed that in both mutants Prg-1 and Prg-6, a single mutation (*prgA1* or *prgB1*) caused the Prg phenotype.

Consistent with their phenotypes on AMM–X-Gal agar plates with lactose, i.e., white colonies, in fermentation medium the mutants Prg-1 (*prgA1*) and Prg-6 (*prgB1*) showed reduced expression of *ipnA-lacZ*, which was independently reflected by reduced intracellular amounts of IPNS, the gene product of the *ipnA* gene.

Although *ipnA-lacZ* expression was used as the sole criterion to identify mutations, in both mutants *acvA-uidA* expression was also affected. Mutant Prg-1 had lower levels of *acvA-uidA* expression than the wild-type strain. For Prg-6, this could not be precisely determined because of variations during fermentation runs. Prg-6 generally showed reduced levels of *acvA-uidA* expression (Fig. 4D). However, in some fermentation runs, Prg-6 had levels of *acvA-uidA* expression that were at least higher than those observed for Prg-1 (data not shown). The basis of this variation has not been clarified yet. Despite this possible difference, the mutations exhibited a strong impact on the penicillin titer, which in the fermentation broth of both mutants reached about 20% of that of the wild-type strain. These results provide evidence that the recessive mutations identified affect the regulation of penicillin biosynthesis genes *ipnA* and *acvA* in *trans*. This finding is in agreement with observations that in the intergenic region of *acvA* and *ipnA* there are *cis*-acting elements which affect the expression of both genes (38). Furthermore, both *acvA* expression and *ipnA* expression were repressed by L-lysine (8), again indicating the presence of common mechanisms regulating both genes. However, in addition there are regulatory circuits which are specific for *ipnA*, e.g., in contrast to *acvA* expression, *ipnA* expression was repressed by glucose (5). Since in the mutants the expression of both *acvA* and *ipnA* was affected, the mutations *prgA1* and *prgB1* most likely represent *trans*-acting genes which are involved in the regulation of expression of both genes.

However, the mutations do not seem to affect the regulation of *aat*, because in the mutant strains, AAT specific activity was essentially the same as in the wild type. By using gene fusions, analysis of *aat* regulation revealed that its expression pattern differed in some aspects from those of *acvA* and *ipnA* (24). Thus, it seems plausible to expect additional factors which specifically regulate *aat*. So far, the identification of three regulatory genes of biosyntheses of secondary metabolites in fungi has been reported. First, the *aflR* gene of both *Aspergillus flavus* and *Aspergillus parasiticus* seems to regulate aflatoxin biosynthesis in a positive way (44). Second, in a previous study, a mutation in *A. nidulans* called *creA* which is involved in carbon catabolite repression of many genes was identified (3). Espeso and Penalva (16) reported that *creA* mutations had an impact on carbon catabolite repression of *ipnA* transcription, although penicillin production was still reduced by glucose in certain *creA* mutants (5). Further studies revealed, however, that the deletion of the putative CREA binding site upstream of the *ipnA* gene had no effect on carbon catabolite repression of *ipnA* transcription (17). Hence, it is unlikely that CREA plays a major role in carbon catabolite repression of penicillin biosynthesis.

Third, in contrast to *creA*, the *pacC* mutation clearly affects the regulation of penicillin biosynthesis (17, 35). *PacC* mutants were originally isolated by Dorn (14) in a screening program to identify mutants which had lost the ability to produce phosphatase activity. *PacC* mutants were able to produce phosphatase activity under alkaline conditions but not under acidic conditions. Subsequent studies by Arst, Jr., and colleagues revealed that the *pacC* sequence contains three putative C_2H_2 zinc fingers (10, 17). PACC is proposed to act as a negative regulator of alkaline pH-specific genes at neutral or acidic pHs and as an activator of such genes at alkaline pHs. In addition, it was found that *pacC* mutants produced about two times more penicillin than wild-type strains (9a, 35). The *pacC* gene product seems to act on the penicillin biosynthesis gene *ipnA* (17). However, because PACC is a wide-domain regulator originally identified by using deregulated phosphatase activity as the phenotype, in this study we aimed to isolate mutants defective in positive *trans*-acting elements which are specifically involved in the regulation of penicillin biosynthesis genes. As shown here, the putative regulatory genes *prgA1* and *prgB1* seem to represent such elements and to indicate that in addition to being regulated by PACC and a yet-unidentified regulator mediating carbon catabolite repression, penicillin biosynthesis is also regulated by positively acting pathway-specific factors.

The use of gene fusions to identify *trans*-acting regulators in bacteria has been reported previously (21, 31). Here, we demonstrate that a similar molecular approach is also feasible for the identification of *trans*-acting mutations specifically involved in the biosynthesis of a secondary metabolite in a fungus. Since gene fusions can be used with most, if not all, fungi, e.g., with *P. chrysogenum* and *Acremonium chrysogenum* (22, 27), the method described here is applicable to other fungi as well. Furthermore, because the phenotype of mutants isolated on agar plates was stable during a fermentation run under production conditions, the strategy presented here might have implications for industrial screening programs. In addition, it provides an excellent tool for the cloning by complementation of *trans*-acting genes. This is in particular valuable for metabolic pathways which do not allow identification of deregulated mutants on agar plates.

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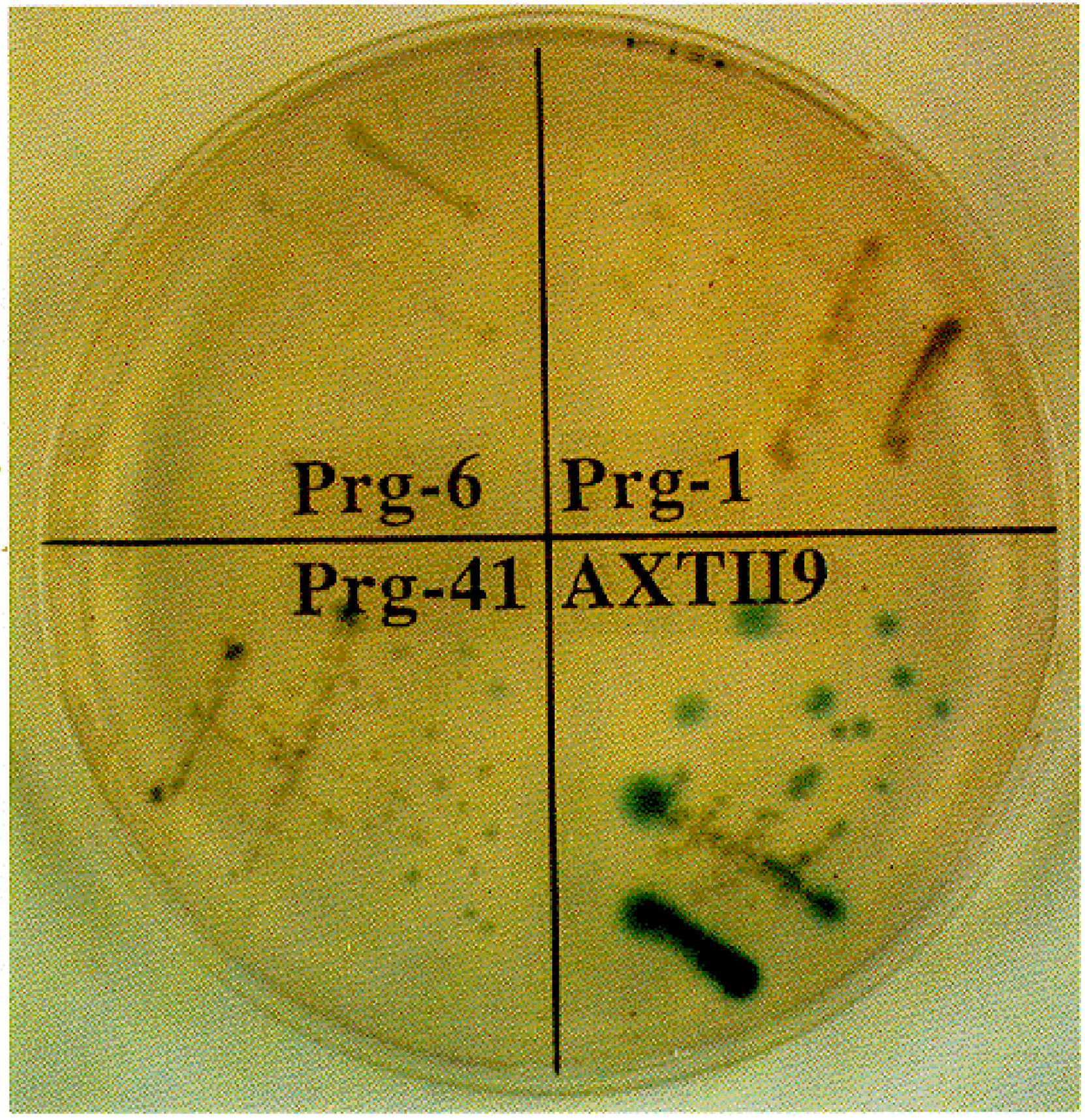
Prg-6
Prg-1
Prg-41
AXTII9