Penicillin, a classical secondary metabolite found in some filamentous fungi, and cephalosporin, another widely used antibiotic, belong to the group of β-lactam antibiotics, which are still of great importance for the treatment of infectious diseases (37). The hydrophobic cephalosporins are produced by some gram-positive and gram-negative bacteria as well as by filamentous fungi, e.g., Streptomyces coelicolor. The biosynthesis of penicillin and cephalosporin starts from the amino acid precursors L-α-amino adipic acid, L-cysteine, and L-valine. Previous data suggested that certain amino acids play a role in the regulation of its biosynthesis. Therefore, in this study the effects of externally added amino acids on both Aspergillus (Emericella) nidulans penicillin production and expression of the bidirectionally oriented biosynthesis genes acvA (pcbAB) and ipnA (pcbC) were comprehensively investigated. Different effects caused by amino acids on the expression of penicillin biosynthesis genes and penicillin production were observed. Amino acids with a major negative effect on the expression of acvA-uidA and ipnA-lacZ gene fusions, i.e., histidine, valine, lysine, and methionine, led to a decreased ambient pH during cultivation of the fungus. An analysis of deletion clones lacking binding sites for the pH-dependent transcriptional factor PACC in the intergenic regions between acvA-uidA and ipnA-lacZ gene fusions and in a pacC5 mutant (PacC5-5) suggested that the negative effects of histidine and valine on acvA-uidA expression were due to reduced activation by PACC under acidic conditions. These data also implied that PACC regulates the expression of acvA, predominantly through PACC binding site ipnA3. The repressing effect caused by lysine and methionine on acvA expression, however, was even enhanced in one of the deletion clones and the pacC5 mutant strain, suggesting that regulators other than PACC are also involved.

Since penicillin is synthesized from amino acid precursors, we believed it conceivable that amino acids play an important role in the regulation of its biosynthesis. Evidence for this hypothesis came from the observation that in both A. nidulans and P. chrysogenum, the addition of L-lysine to fermentation medium led to reduced penicillin titers (8, 12). Since AA is a branch-point metabolite between the lysine and penicillin biosynthetic pathways, lysine inhibition was suggested to operate at one or more steps of the lysine pathway (28). Consistent with this assumption was the notion that in P. chrysogenum, one target for lysine regulation is homocitrulline synthase, which catalyzes the initial reaction of lysine biosynthesis. Lysine was found to cause both feedback inhibition of homocitrulline synthase activity and repression of its synthesis (14, 26). In agreement with these findings, it was shown that the AA pool available for penicillin production was reduced in lysine-grown mycelia (23, 38).

In A. nidulans, lysine led to reduced expression of both acvA and ipnA gene fusions (8), indicating an additional and more direct effect on the expression of penicillin biosynthesis genes. Furthermore, in P. chrysogenum, the addition of L-methionine to the medium (final concentration, 20 nM) led to a three- to fourfold increase in the production of cephalosporin C (16, 44). The increased production of this β-lactam compound was paralleled by increased steady-state levels of mRNAs of cephalosporin biosynthesis genes pcbAB (acvA), pcbC (ipnA), cefEF, and, to a slight extent, cefG (44). This finding suggests that the methionine effect was mediated via the expression of biosynthesis genes. In addition, Lara et al. (25) reported an inductive effect of L-glutamate on penicillin biosynthesis in P. chrysogenum.

Because of these limited data, we wished to study comprehensively whether the presence of different amino acids in the medium affected A. nidulans penicillin biosynthesis and, in particular, the expression of penicillin biosynthesis genes acvA.
and ipnA. Furthermore, a molecular mechanism which accounts for the repressing effects of some amino acids is elucidated.

MATERIALS AND METHODS

**A. nidulans** strains. The fungal strains used in this study are listed in Table 1.

**Media and fermentation conditions.** Fermentations were carried out with *Aspergillus* minimal medium (AMM) or fermentation medium (FM) essentially as described previously (7, 39). Seed cultures (20 ml of AMM or FM in 250-ml flasks) contained 4% (wt/vol) lactose as the carbon source and were supplemented with or without one Experimental cultures (20 ml of AMM or FM in 250-ml flasks) contained 4% (wt/vol) glucose as the carbon source. These cultures were incubated with or without L-amino acids (final concentration, 50 mM) or biotin (0.3 μg/ml) or L-aminobenzoic acid (15 μg/ml) added to the flasks.

**β-GLU and β-GAL activity assays.** The expression of *acvA-uidA* and *ipnA-lacZ* gene fusions was monitored by β-glucuronidase (β-GLU) and β-galactosidase (β-GAL) activities, respectively. They were measured in mycelial extracts from cells grown in AMM with or without L-amino acids. The final amino acid concentrations in AMM and FM cultures were 10 and 50 mM, respectively. Experimental cultures were inoculated with 1 ml of the seed culture suspension. They were incubated for 48 h at 26°C because the measured effects on gene expression were most pronounced after this incubation time. The effects of exogenously added L-amino acids on the expression of *acvA-uidA* and *ipnA-lacZ* gene fusions were measured with the well-defined AMM. For this purpose, mycelia were harvested by filtration through Mira cloth (Calbiochem). Crude protein extracts were obtained by grinding the mycelia to a fine powder in liquid nitrogen. For preparation of cell extracts, a method previously described in detail was followed (7). In AMM, fungi hardly produce any penicillin, so penicillin production was determined with FM (see below). When required, biotin (0.3 μg/ml) or L-aminobenzoic acid (15 μg/ml) was added to the flasks.

**Determination of protein concentrations.** Protein concentrations were determined according to the method of Bradford (5).

**Activity assays for acidic and alkaline phosphatases.** For determination of the activities of acidic and alkaline phosphatases, a plate assay was applied essentially according to the method of Dorn (15). The expression of *acvA-uidA* and *ipnA-lacZ* gene fusions was measured with mycelia grown for 48 h in AMM with lactose as the carbon source. Penicillin titers were determined from cultures grown in FM for 48 h (see Materials and Methods). In total, three progeny carrying both the *pacC5* mutation and the gene fusions were analyzed. Data for one of these progeny (PacC5-5) are shown. The other progeny gave essentially the same results (data not shown).

**Genetic techniques.** Sexual crosses and characterization of the resulting progeny were performed according to the methods of Pontecorvo et al. (33). For construction of *A. nidulans* FLIRT-2-3, strains G191 and FLIRT (Table 1) were crossed. Ascospores were plated on AMM agar plates with glucose as the carbon source and supplemented with p-aminobenzoic acid and X-Gal. One of the progeny was designated FLIRT-2-3. It was auxotrophic for p-aminobenzoic acid (pabaA1) and stained the agar blue on AMM-X-Gal agar plates with glucose as the carbon source, indicating an ipnA-lacZ gene fusion.

To construct a strain carrying the *acvA-uidA* and *ipnA-lacZ* gene fusions (Fig. 1B) and the *pacC5* mutation, strain FLIRT-2-3 was crossed with strain G0156 (pacC5). Several progeny lacking acidic phosphatase activity, indicative of the presence of the *pacC5* mutation, auxotrophic for p-aminobenzoic acid, and producing blue color on AMM-glucose-X-Gal agar plates, were isolated. The presence of the *acvA-uidA* and *ipnA-lacZ* gene fusions integrated in a single copy at the chromosomal *argB* gene locus was checked by Southern blot analysis of chromosomal DNA of the progeny as described previously (7). These progeny gave essentially identical results. Therefore, data for only one of these progeny, PacC5-5 (Table 1), are shown in Table 2.

**RESULTS**

**Effects of adding various amino acids to the medium on the expression of penicillin biosynthesis genes acvA and ipnA and on penicillin production.** In order to comprehensively test the effects of most of the proteinogenic amino acids on the expres-
sion of penicillin biosynthesis genes. *A. nidulans* FLIRT was used. This strain contains the full-length intergenic region of 872 bp between *A. nidulans* genes *acvA* and *ipnA* fused in frame to *Escherichia coli* reporter genes *uidA* and *lacZ*, resulting in the generation of *acvA-uidA* and *ipnA-lacZ* gene fusions (Fig. 1B). These gene fusions were integrated in a single copy at the chromosomal *argB* gene locus, allowing the precise determination of the expression of both genes simultaneously within one strain (7, 39) (Fig. 1B). Because strain FLIRT carries the complete intergenic region fused to reporter genes, this strain was designated wild type with respect to the expression of the *acvA* and *ipnA* gene fusions. Strain FLIRT was grown in AMM with lactose as the carbon source (see Materials and Methods). The expression of the *acvA-uidA* and *ipnA-lacZ* gene fusions was monitored by determination of β-GLU and β-GAL specific activities, respectively. L-Amino acids were added to the medium at a final concentration of 10 mM. The results are shown in Fig. 2.

Different effects caused by the addition of amino acids were observed. On the basis of these effects, the amino acids were grouped into four classes. (i) Some amino acids led to increased *acvA-uidA* expression but without a major effect on *ipnA-lacZ* expression (Fig. 2, group I). One of these amino acids, glutamate, was previously found to increase the amount of penicillin in *P. chrysogenum* (25). (ii) The amino acids methionine, leucine, isoleucine, phenylalanine, valine, histidine, and lysine led to the repression of both *acvA-uidA* expression and *ipnA-lacZ* expression (Fig. 2, group II). (iii) The amino acids tyrosine, tryptophan, proline, and AA had no major effect on *acvA-uidA* expression but led to the repression of *ipnA-lacZ* expression (Fig. 2, group III). (iv) The amino acids serine and arginine did not affect the expression of either gene fusion (Fig. 2, group IV).

To exclude possible artifacts due to the reporter genes used, the experiments were repeated with different gene fusion constructs, i.e., *acvA-lacZ* and *ipnA-uidA*. With these reporter gene constructs, basically the same results were obtained (data not shown). In general, and with either reporter gene, *acvA* expression was more sensitive to exogenously added amino acids than *ipnA* expression (Fig. 2).

In the following experiments, we mainly concentrated on the analysis of some amino acids which showed repressing effects on the expression of both gene fusions. To further support the results obtained for the effects of these amino acids on gene expression, penicillin titers were measured with and without amino acids. Therefore, *A. nidulans* FLIRT was grown in FM with 4% lactose as the carbon source for 48 h, and supernatant fluid was analyzed for penicillin production. Because of the greater mycelial mass reached in FM than in AMM, the concentration of added amino acids was 50 mM (see Materials and Methods). The results are shown in Fig. 3. The repressing effects of histidine, valine, lysine, and methionine on the expression of gene fusions (Fig. 2) were reflected in decreased penicillin titers (Fig. 3). Determining the expression of the gene fusions in FM with L-amino acids (data not shown) gave essentially the same results as those observed in minimal medium (AMM) with L-amino acids (Fig. 2); i.e., the expression of the gene fusions was repressed. These results suggested that the effects of amino acids on penicillin titers were mediated, at least in part, by modulation of the expression of the penicillin biosynthesis genes *acvA* and *ipnA*.

**Deletion of specific DNA fragments of the intergenic region between *acvA-uidA* and *ipnA-lacZ* reduced the repressing effect of histidine and valine but not of lysine and methionine on the expression of both gene fusions.** To analyze the effects of amino acids at the molecular level, we used *A. nidulans* strains which carried deletions in the intergenic region between the *acvA-uidA* and *ipnA-lacZ* gene fusions (Fig. 4). Like the wild-type strain FLIRT, these strains contained various deletion constructs integrated in a single copy at the chromosomal *argB* gene locus (39). The strains were cultivated in AMM with lactose as the carbon source. L-Amino acids were added to the medium at a final concentration of 10 mM. The results are shown in Fig. 4.

Compared to that in the wild type (strain FLIRT), *acvA-uidA* expression in deletion strain Δ183–312 was significantly less repressed by histidine and valine, suggesting that an important cis-acting element involved in repression by these...
Amino acids are located within nucleotides (nt) 183 to 312 of the intergenic region. Interestingly, the repression caused by the addition of lysine and methionine was even stronger in this deletion strain. In strain Δ1–493 and Δ43–182 showed hardly any repression of ipnA-lacZ expression by histidine and valine, whereas repression by lysine and methionine was unchanged. This result suggested that cis-acting elements involved in the effects of histidine and valine are located between nt 43 and 182. Strain Δ183–312 showed only a slight relief of repression by these amino acids, but as was observed for the expression of acvA-uidA, the repressing effect of lysine and methionine on ipnA-lacZ expression was enhanced. It is thus conceivable that cis-acting elements located between nt 183 and 312 and involved in the relief of repression by histidine and valine act mainly on acvA-uidA expression and less on ipnA-lacZ expression.

In summary, these results indicated that, at least for repression by histidine and valine, cis-acting DNA elements mediating these effects are located in the promoter regions which were deleted in strains Δ183–312, Δ43–182, and Δ1–493. The repressing effect of methionine on ipnA-lacZ seems to be due to one or more elements located between nt 433 and 569.

It was intriguing to note that in strain Δ43–182, two of the four PACC binding sites identified in vitro (42) and designated ipnA1 and ipnA2 (17) were missing (Fig. 4). In strain Δ183–312, another of these sites, ipnA3, was lacking (Fig. 4). PACC is a transcriptional factor which mediates the pH-dependent regulation of several A. nidulans genes, including the ipn4A gene (19, 42). The intergenic region between acvA and ipnA of A. nidulans was found to contain four functional PACC binding sites bound by a glutathione S-transferase–PACC fusion protein in vitro (42) (Fig. 4). At an alkaline ambient pH, PACC activates the transcription of alkaline-expressed genes and prevents the transcription of acid-expressed genes (2, 42). The full-length form of PACC, which predominates at an acidic ambient pH, is not functional and must be specifically proteolyzed to yield the functional (for both positive and negative roles) version containing the N-terminal 40% of the protein (2, 30).

It is therefore conceivable that at least some of the effects of amino acids could be due to the action of PACC and thus to an ambient pH. To test this hypothesis, pH values of supernatant fluid from cultures incubated with and without amino acids were determined. As shown in Fig. 5 for wild-type strain FLIRT, all tested amino acids which resulted in reduced gene expression led to a decreased ambient pH, suggesting a role for PACC in at least some of the effects of exogenously added amino acids. This assumption was further supported by the observation that threonine led to an increased ambient pH of 7.5 and simultaneously to slightly increased acvA-uidA expression. This observation that threonine led to an increased ambient pH of 7.5 and simultaneously to slightly increased acvA-uidA expression led to a decreased ambient pH, suggesting a role for PACC in at least some of the effects of exogenously added amino acids. This assumption was further supported by the observation that threonine led to an increased ambient pH of 7.5 and simultaneously to slightly increased acvA-uidA expression.
and valine on acvA-uidA expression are due to a decrease in the ambient pH as the result of the metabolism of these amino acids.

The stronger repression caused by lysine and methionine in both the pacC5 mutant strain (PacC5-5) and deletion clone Δ183–312 (lacking PACC binding site ipnA3) is difficult to reconcile with an effect exclusively due to the action of PACC. It thus seems likely that regulators other than PACC are also involved (see Discussion).

In contrast to acvA-uidA expression, ipnA-lacZ expression was still repressed by exogenously added valine and histidine in mutant PacC5-5. As was observed for acvA-uidA expression, the repression of ipnA-lacZ expression by lysine and methionine was still detectable in PacC5-5. Formally, these data imply that PACC is less involved in the regulation of ipnA-lacZ expression than of acvA-uidA expression by these amino acids (see Discussion).

**DISCUSSION**

Our data suggested that the acvA gene of *A. nidulans*, like ipnA (19, 30, 42), is regulated by the pH-dependent transcription factor PACC. In addition, we demonstrated that most amino acids, when added to the medium, affect the expression of gene fusions consisting of the penicillin biosynthesis genes acvA and/or ipnA fused to reporter genes. The repressing effects of histidine and valine on acvA-uidA expression are most likely due to the action of PACC and therefore to the pH of the culture broth.

Four lines of evidence support these conclusions. (i) The expression of acvA-uidA gene fusions was significantly increased in pacC5 mutant strains. This finding is consistent with the observation that the steady-state levels of acvA mRNA were increased at an alkaline ambient pH, a characteristic of genes regulated by PACC (18). (ii) The growth of *A. nidulans* with histidine and valine led to a lower ambient pH. At this pH, most or at least some of the PACC molecules are inactive (30, 42). At an alkaline ambient pH, however, PACC acts as an activator for the expression of both ipnA (19) and acvA (18; this study). Therefore, histidine and valine led to the acidification of the medium, caused the accumulation of the inactive form of PACC, and resulted in reduced acvA-uidA expression. (iii) In the deletion strain Δ183–312, the addition of histidine and valine caused a lower pH but did not lead to a reduction in acvA-uidA expression. In this deletion strain, one of the four PACC binding sites identified in vitro, ipnA3, spanning nt 265 to 270 (17, 42) (Fig. 4), was missing. Taken together, these findings suggest that in this deletion strain, the expression of the acvA-uidA gene fusion could not be further repressed by acidification because the absence of PACC binding site ipnA3 made it almost PACC independent. These results also imply that PACC binding site ipnA3 is the major site for PACC regulatory activity of acvA-uidA expression. PACC binding sites at nt 149 to 154 (ipnA1) and 174 to 179 (ipnA2) contributed less, and those at nt 509 to 514 (ipnA4A) and 524 to 529 (ipnA4B) apparently did not contribute to the activation of acvA-uidA expression by PACC (Fig. 4). These findings agreed well with those of Espeso and Penálva (17), who had precisely analyzed the functionality of the PACC binding sites identified in vitro for ipnA-lacZ expression in vivo. The authors found that site ipnA3 was most important for PACC-dependent ipnA expression, whereas sites ipnA2 and ipnA4 were less important, although site ipnA2 was bound with the highest affinity by a glutathione S-transferase–PACC fusion protein in vitro. Binding site ipnA1 was apparently not required for PACC-dependent ipnA expression (17) (Fig. 4). Hence, binding site
ipnA3 seems to be of major importance for PACC-dependent expression of both gene acvA and gene ipnA. When this site was lacking (strain Δ183–312), the repressing effects of histidine and valine were no longer detectable. (iv) In a pacC5 mutant strain (PacC5-5) with a constitutively active PACC protein, no repression by histidine or valine could be detected. This result demonstrates the importance of PACC for repression by these two amino acids.

The pH of the culture supernatant of the pacC5 mutant strain (PacC5-5) was higher than that of the wild-type strain. Therefore, it was conceivable that due to the increased ambient pH, the mutant had a reduced uptake of some amino acids, thus avoiding repressing intracellular concentrations of histidine, valine, or derivatives. However, this situation was unlikely, because in strains with the wild-type pacC gene and with gene fusions having deletions of certain PACC binding sites, there was hardly any detectable repression either. The only difference between these deletion strains and wild-type strain FLIRT was the deletion of nucleotides in the intergenic region between the acvA-uidA and ipnA-lacZ gene fusions. This fact supports the assumption that the repressing effects of histidine and valine on acvA-uidA expression were mediated, at least in part, by an ambient pH via the transcriptional factor PACC. This fact is also consistent with the result that exogenously added threonine led to a slightly increased ambient pH and to increased acvA-uidA expression in the wild type, suggesting that PACC activity was increased under these conditions.

The addition of lysine and methionine also resulted in a decrease in both the ambient pH and the expression of the acvA-uidA and ipnA-lacZ gene fusions. Although this pattern was similar to that observed for histidine and valine, the effects of methionine and lysine seemed to be mediated via different mechanisms, although some contribution of PACC could not be entirely excluded. This conclusion was drawn from the observation that, in contrast to those of histidine and valine, the repressing effects of lysine and methionine on acvA-uidA expression were even increased in deletion clone Δ183–312, lacking PACC binding site ipnA3 (Fig. 4). Furthermore, in strain PacC5-5, the constitutively active PACC5 protein could not overcome the repressing effects of lysine and methionine (Fig. 5), making the involvement of PACC unlikely. So far, the regulatory mechanisms that mediate the repressing effects of these amino acids have not been elucidated. For methionine, the analysis of deletion clones indicated that a major cis-acting DNA element responsible for its effect on acvA-uidA expression is located between nt 433 and 569. For lysine, no DNA region which could be involved has yet been identified. The effects of lysine and methionine in the medium are mediated by regulatory mechanisms which appear to differ even between these two amino acids. It is interesting to note that lysine and methionine are closely related to the precursor amino acids by regulatory mechanisms which appear to differ even between the region spanned by nt 433 to 569 of the intergenic region between acvA and ipnA, which was found to be important for the methionine effect (Fig. 4). Therefore, an understanding of the effects of exogenously added methionine at the molecular level awaits further studies.

The analysis of deletion clones implied the involvement of PACC in the regulation of ipnA-lacZ expression by amino acids. This implication was contradicted, however, by results obtained with strain PacC5-5, in which, in contrast to acvA-uidA expression, ipnA-lacZ expression was still repressed by histidine and valine. It is conceivable that the different effects on the two gene fusions in strain PacC5-5 were due to the pacC5 mutation. Small changes in the ambient pH enable the mutant as well as the wild-type PACC protein to regulate the expression of acvA. This finding may be due to the organization of the bidirectionally oriented promoter region between acvA and ipnA. For the expression of ipnA, the pH signal may need to be more pronounced. It is tempting to speculate that acvA expression is subject to more sensitive regulation than ipnA expression. This hypothesis is supported by the observation in this study that acvA was regulated to a greater extent by amino acids than was ipnA. This idea is also consistent with the finding that the expression of acvA is rate limiting for penicillin production (24) and thus represents a critical step in penicillin biosynthesis.

It was also conceivable that a GCN4-like factor (reviewed in reference 21) was involved in the effects of exogenously added amino acids. In A. nidulans, the expression of the argB gene, encoding ornithine transcarbamoylase, was increased when cells were starved for certain amino acids (20, 32), indicating that general amino acid control is present in this fungus. This result was further supported by the findings of Wanke et al. (45), who recently cloned a GCN4 homolog of A. niger. Some of the amino acid effects reported here could thus be due to the action of a GCN4 analog in A. nidulans. Thus, the addition of excess leucine could lead to reduced amounts of isoleucine and valine by feedback inhibition, causing activation of the GCN4 system (reviewed in reference 21). Furthermore, exogenously added aminotriazole, which causes histidine starvation and thus presumably induces GCN4-analogous regulators, was found to increase penicillin production in certain P. chrysogenum strains (22). It was suggested that this increase was due to the induction of lysine biosynthesis by histidine starvation, leading to increased AA levels and thus to increased penicillin titers. However, no correlation between the increased expression of penicillin biosynthesis genes and ornithine transcarbamoylase specific activity, which was measured as an indicator of the activity of a GCN4-analogous regulator, was detected for any of the amino acids analyzed here (data not shown). This finding suggests that for the effects described here, the participation of a GCN4-like factor is unlikely.

In summary, some of the effects of exogenously added amino acids on the biosynthesis of penicillin are mediated via the pH-dependent transcriptional regulator PACC. This finding may have implications for other biosynthetic pathways which are regulated by exogenously added amino acids.

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