

A Homologue of the *Aspergillus velvet* Gene Regulates both Cephalosporin C Biosynthesis and Hyphal Fragmentation in *Acremonium chrysogenum*[∇]

Jacqueline Dreyer,¹ Heiko Eichhorn,² Ernst Friedlin,² Hubert Kürnsteiner,² and Ulrich Kück^{1*}

Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität, Universitätsstr. 150, D-44780 Bochum, Germany,¹ and Sandoz GmbH, A-6250 Kundl, Austria²

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The *Aspergillus nidulans velvet* (*veA*) gene encodes a global regulator of gene expression controlling sexual development as well as secondary metabolism. We have identified the *veA* homologue *AcveA* from *Acremonium chrysogenum*, the major producer of the β -lactam antibiotic cephalosporin C. Two different disruption strains as well as the corresponding complements were generated as a prelude to detailed functional analysis. Northern hybridization and quantitative real-time PCR clearly indicate that the nucleus-localized AcVEA polypeptide controls the transcriptional expression of six cephalosporin C biosynthesis genes. The most drastic reduction in expression is seen for *cefEF*, encoding the deacetoxycephalosporine/deacetylcephalosporine synthetase. After 120 h of growth, the *cefEF* transcript level is below 15% in both disruption strains compared to the wild type. These transcriptional expression data are consistent with results from a comparative and time-dependent high-performance liquid chromatography analysis of cephalosporin C production. Compared to the recipient, both disruption strains have a cephalosporin C titer that is reduced by 80%. In addition to its role in cephalosporin C biosynthesis, *AcveA* is involved in the developmentally dependent hyphal fragmentation. In both disruption strains, hyphal fragmentation is already observed after 48 h of growth, whereas in the recipient strain, arthrospores are not even detected before 96 h of growth. Finally, the two mutant strains show hyperbranching of hyphal tips on osmotically nonstabilized media. Our findings will be significant for biotechnical processes that require a defined stage of cellular differentiation for optimal production of secondary metabolites.

The filamentous fungus *Acremonium chrysogenum* is the exclusive producer of the pharmaceutically relevant β -lactam antibiotic cephalosporin C, which is structurally related to penicillins. The latter is synthesized by two other filamentous fungi, namely *Penicillium chrysogenum* or *Aspergillus nidulans* (11). While *P. chrysogenum* is used mainly for the industrial production of penicillin, work with *A. nidulans* has focused on the understanding of the complex regulation of β -lactam biosynthesis in filamentous fungi. The first two steps of the biosynthesis of penicillin and cephalosporin C are identical in all fungal producers and involve the activity of *N*-(5-amino-5-carboxypentanoyl)-L-cysteiny-D-valine synthase (ACV) synthetase (ACVS) and isopenicillin *N*-synthetase (IPNS). Interestingly, the genes for the two enzymes *pcbAB* and *pcbC* are not only highly homologous in the three fungal species but also have a very similar gene organization. They are located adjacently on the chromosome in a divergent orientation and share a common promoter sequence (5, 32, 40). This clustered gene organization has been found for many fungal genes encoding enzymes for specific metabolic pathways. In addition to β -lactam antibiotics, this includes secondary metabolites such as aflatoxin, gibberellins, or lovastatin (27, 48). Mainly molecular genetic investigations have shown that the coordinated regulation of gene clusters is controlled by narrow or broad domain regulators (20).

In *A. chrysogenum*, global regulators such as CPC1, CRE1, and PACC control expression of the *pcbAB* and *pcbC* genes (16, 39, 41), whereas in *A. nidulans*, another set of factors has been discovered to be involved in the coregulation of the homologous genes (5, 20). One such global regulator is *velvet* (*veA*), a developmental gene first isolated and described in *A. nidulans* as a regulator of fungal morphogenesis (18, 29). While wild-type strains conidiate in the presence of light and are aconidial in the dark, mutation of the *veA* gene eliminates this light dependency, thus allowing conidiation to occur also in the dark (29). In addition, *veA* deletion strains are unable to develop sexual structures (cleistothecia), even under inducing conditions, and the overexpression of the *veA* gene leads to the formation of cleistothecia also under unfavorable conditions, where the wild type produces few or no sexual structures (21). These findings led to the conclusion that *veA* negatively regulates asexual development, while positively regulating sexual development. Recent studies have demonstrated the involvement of *veA* also in the secondary metabolism of different aspergilli. Examples include versicolorin synthesis in *Aspergillus parasiticus*, cyclopiazonic acid, aflatrem, and aflatoxin synthesis in *Aspergillus flavus* (7, 10) or sterigmatocystin and β -lactam biosynthesis in *Aspergillus nidulans* (19). In the latter, *veA* regulates transcriptional expression of the two penicillin biosynthesis genes. After deleting the *veA* gene, an increase in the *ipnA* (*pcbC*) gene expression was observed, while *acvA* (*pcbAB*) gene expression was no longer detectable (19). This led to an overall decrease in penicillin production in the deletion strain.

Until now, it has been unknown whether *velvet*, a highly

* Corresponding author. Mailing address: Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität, Universitätsstr. 150, D-44780 Bochum, Germany. Phone: 49-234-3226212. Fax: 49-234-3214184. E-mail: ulrich.kueck@ruhr-uni-bochum.de.

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TABLE 1. *A. chrysogenum* strains used in this study

| Strain | Characteristic(s) | Reference |
|----------------------------|--|-----------|
| A3/2 | Producer strain (recipient) | 36 |
| ΔveA | $\Delta veA::hygB$ | This work |
| $\Delta veA::veA$ | $\Delta veA::hygB; veA(p)::nat$ | This work |
| KiveA | $veA::hygB$ | This work |
| KiveA::veA | $veA::hygB; veA(p)::nat$ | This work |
| $\Delta veA::veA_egfp$ | $\Delta veA::hygB; gpd(p)::veA::egfp::trpC(t)$ | This work |
| $\Delta veA::veA_t_egfp$ | $\Delta veA::hygB; gpd(p)::veA::egfp::trpC(t)$ | This work |

conserved gene in the aspergilli, is also involved in the control of cephalosporin C biosynthesis. Here, we present the molecular characterization and functional analysis of the *veA* homologue from *A. chrysogenum*. This is the first report showing that a *velvet* homologue controls biosynthesis of the β -lactam antibiotic cephalosporin C. In addition, we demonstrate that the time-dependent onset of hyphal fragmentation and hyphal branching in *A. chrysogenum* requires the presence of a full-size *A. chrysogenum* VEA (AcVEA) polypeptide. Our findings will have a significant impact on the implementation of further strain improvement programs.

MATERIALS AND METHODS

Strains, DNA extraction, culture conditions, and transformation of *A. chrysogenum*. Competent *Escherichia coli* cells of strain K-12 XL1-Blue (Stratagene) were used for routine DNA manipulations (6). Construction, maintenance, and isolation of recombinant plasmids were performed using standard techniques (37). The cephalosporin C producer strain A3/2 (36) from *A. chrysogenum* was

used as a recipient, and all transformants (Table 1) were obtained by a procedure described previously (24, 47). The transgenic strains were selected on media containing either 10 U/ml of hygromycin B or 25 μ g/ml nourseothricin. Cultures were grown in liquid complex culture medium (CCM) (28) or production medium (38) with 55 g/liter dextrin instead of saccharose at 27°C and 180 rpm in Erlenmeyer flasks and in the dark. Fungal DNA extraction was done as described previously (14). Cultures for microscopic studies were started with a 5% inoculum from a 2.5-day preculture as described previously (14).

Isolation and sequencing of genomic DNA. For the isolation of a *veA* gene fragment of *A. chrysogenum*, heterologous primers 8/10 (see Table 2) were designed on the basis of the *N. crassa* genome sequence (available at <http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>) and used for PCR amplification with genomic DNA from A3/2 as template. The resulting amplicon was cloned into vector pDrive (QIAGEN, Hilden, Germany) and used for subsequent DNA sequencing by a custom sequencing service (MWG Biotech, Ebersberg, Germany). The amplicon obtained by this PCR approach was further employed to screen an *A. chrysogenum* cosmid library (custom-made cosmid library using vector P1920; MWG Biotech, Ebersberg, Germany). The screening resulted in the isolation of cosmid E11 containing the entire *AcveA* open reading frame (ORF), together with flanking regions, and FASTA (31) was used for comparisons of nucleotide and amino acid sequences. Alignments were made using the CLUSTAL W program (44) provided by the European Bioinformatics Institute and formatted using the multiple sequence alignment editor GeneDoc (<http://www.psc.edu/biomed/genedoc>).

Generation of an insertional mutant. The screening of an *A. chrysogenum* cosmid library using the *AcveA* gene as a probe led to the isolation of cosmid E11, carrying the entire *AcveA* open reading frame together with flanking sequences. For in vitro transposition, we used a novel transposition vector, pGPS2.1-hph, that was constructed as follows. The *Swa*I-restricted plasmid pGPS2.1 (New England Biolabs) was ligated with an *Hpa*I fragment from vector pZHK2 (23) carrying the *hph* gene. The recombinant vector contains the *hph* gene under the control of the *trpC* promoter together with the chloramphenicol acetyltransferase resistance (Cm^R) gene. Both genes are flanked by the right- and left-hand borders from transposon Tn7 (New England Biolabs), designated

TABLE 2. Oligonucleotides used in this study

| Oligonucleotide | Specific sequence (5' to 3') ^a | Specificity (position) ^b |
|-----------------|---|-------------------------------------|
| 8 | CAATCCTGAAACTTTTGGCGCT | <i>AcveA</i> (2021–2044) |
| 10 | ACTTTTCAGTACAACGCCAACTTTTTCCTC | <i>AcveA</i> (1681–1710) |
| 30 | <u>GAGCICCTTGTCTGTAGCGCTTGCC</u> | <i>AcveA</i> (91–110) |
| 31 | <u>GCGGCCGCACGATATGGCTTCGGATG</u> | <i>AcveA</i> (1364–1381) |
| 32 | <u>GCGGCCGCCATCTACTTATTCCTTTGC</u> | <i>hph</i> from pZHK2 |
| 33 | <u>TCTAGATGCCAACACATTTGCGTGCC</u> | <i>AcveA</i> (3001–3020) |
| 34 | <u>ACTAGTGGTATGGCGAGGTTACGCGT</u> | <i>AcveA</i> (5069–5088) |
| 35 | <u>TCTAGAAACGACGGCCAGTGCCAAGC</u> | <i>trpC(p)</i> from pZHK2 |
| 42 | AGATCAAGACTTCGCCCTTC | <i>AcveA</i> (1991–2010) |
| 49 | GGCCGGCTACATGGTGAATA | <i>AcveA</i> (2981–3000) |
| 52 | GATCCTTGCTAGCGCCGGAC | <i>AcveA</i> (26–45) |
| 57 | TCTCGTTTTACCTCCACCCA | <i>AcveA</i> (1301–1320) |
| 59 | CAGGTATCAAACGCAACAG | <i>AcveA</i> (3061–3080) |
| Ac_IPNS_U1 | ACCAGTCCGACGTGACGGAAT | <i>pcbC</i> gene |
| Ac_IPNS_L1 | TGGGTGATATGGGCCATGTAG | <i>pcbC</i> gene |
| Ac_cefEF_U1 | CCGTAACCACCAAGGTTATCT | <i>cefEF</i> genes |
| Ac_cefEF_L1 | CTCCTCGCTTCCGTTCTTGA | <i>cefEF</i> genes |
| Ac_ACVS_U1 | CGTTGCGCACCGTAACC | <i>pcbAB</i> genes |
| Ac_ACVS_L1 | CACTGCCTGGATAAAGTCCATGA | <i>pcbAB</i> genes |
| Ac_cefG_U1 | AAGAGCAAACCTGCGATGGA | <i>cefG</i> gene |
| Ac_cefG_L1 | TCTGTGCCGTTGATTTCTTCT | <i>cefG</i> gene |
| Ac_cefD1_U1 | GAACGACTTGATGACGGAATA | <i>cefD1</i> gene |
| Ac_cefD1_L1 | TCCCCGACATAGACAAATATTGTTG | <i>cefD1</i> gene |
| Ac_cefD2_U1 | CTAGCCCCAGGCCATCT | <i>cefD2</i> gene |
| Ac_cefD2_L1 | CGATGCAGATGGACGACTTGT | <i>cefD2</i> gene |
| Ac_act_U3 | GCGACGTCGATGTCCGTAA | Actin gene |
| Ac_act_L3 | AGAAGGAGCAAGAGCAGTGATCTC | Actin gene |
| 66 | <u>CCATGGCCACCCCTTCGCTCAT</u> | <i>AcveA</i> (1390–1405) |
| 74 | <u>CCATGGCGAGCGCGGGCGGCGG</u> | <i>AcveA</i> (2219–2235) |
| 75 | <u>CCATGGTGAATACGCTGAACTT</u> | <i>AcveA</i> (2971–2986) |

^a Recognition sequences for endonucleases that were used for the construction of knockout cassettes are underlined. For further details, see the text.

^b Nucleotide positions for *AcveA* gene are from accession no. AM410093 in the EMBL data library.

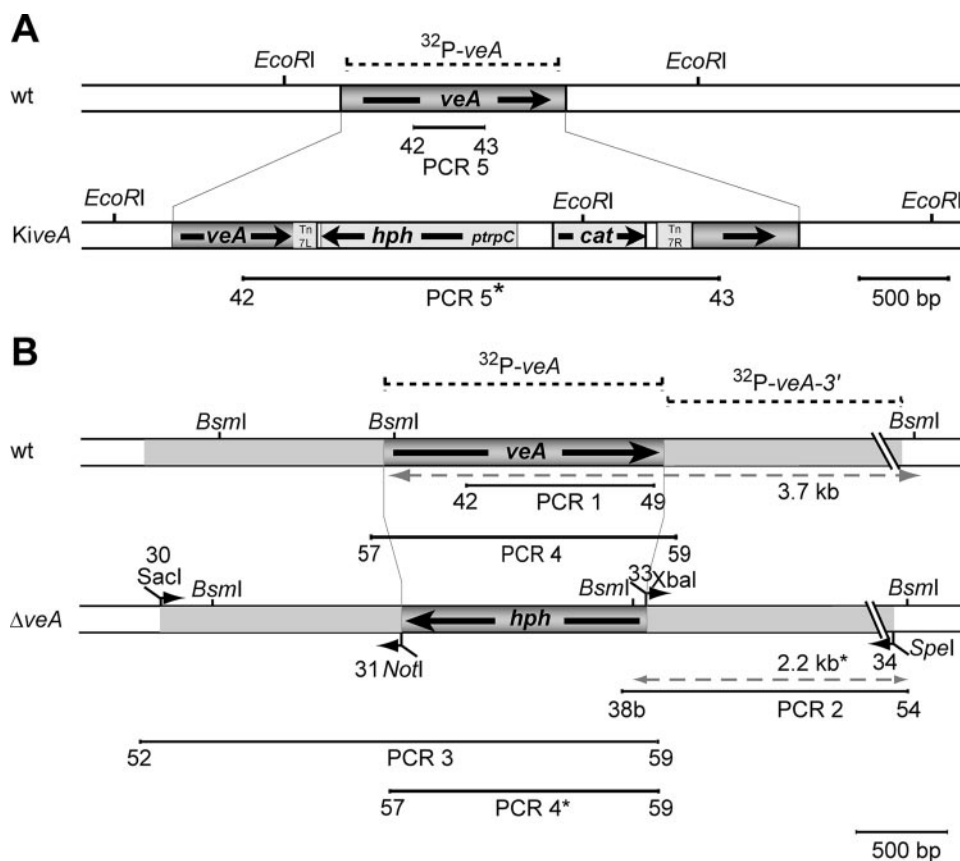


FIG. 1. Disruption of the *AcveA* gene in *A. chrysogenum*. The *AcveA* ORF is represented by the dark gray bar. (A) Generation of an insertional mutant. Schematic representation of the *AcveA* open reading frame before and after insertion of the transposon. (B) Generation of a ΔveA strain: genomic organization of the *A. chrysogenum veA* locus from wild-type (wt) and knockout strains. The disruption cassette was constructed by amplifying the *AcveA* flanking regions by PCR. The restriction sites necessary for further cloning were inserted through the primers. The 5' and 3' flanking regions present on the disruption cassette are depicted by the light gray regions. Fragments expected in Southern blot analysis with the 3' *AcveA*-flanking probe against *BsmI*-restricted DNA from the wild-type or the knockout strain are indicated by light-gray dashed double-headed arrows. The locations of the oligonucleotide primers used for PCR amplifications and the sizes of the expected amplicons are indicated by the numbers at the respective lines. The DNA fragment used for Southern blot analysis is represented by the dashed line over the map of the wild-type locus.

Tn7R and Tn7L, respectively. pGPS2.1-*hph* was used in the genome priming system for in vitro transposition of the *hph* gene into cosmid E11, according to the manufacturer's recommendations. The recombinant transposon inserts randomly into the target DNA (in vitro), and selection of clones with the mutagenized DNA was performed on chloramphenicol-containing media. The resulting recombinant cosmid, E11-*hph*, carries an insertion at nucleotide position 850 of the *AcveA* ORF and received the designation *AcveAt*. *A. chrysogenum* insertional mutants, designated as knock-in (*KiveA*), carrying E11-*hph* can be selected on hygromycin B-containing media.

Deletion of the *AcveA* gene. To replace the *AcveA* open reading frame with the hygromycin phosphotransferase gene (*hph*), deletion vector pVEAKO was constructed as follows. Approximately 1.3 kb and 2.0 kb of the upstream and downstream regions of the *AcveA* gene together with primer pairs 30/31 and 33/34 (see Table 2), respectively, were amplified by PCR using genomic DNA from A3/2 as template. Both amplicons are terminated by *SacI*/*NotI* (upstream region) or *XbaI*/*SpeI* (downstream region) restriction sites. Together with the *hph* cassette derived from plasmid pZHK2 and terminated by *NotI*/*XbaI* restriction sites, these fragments were inserted into vector pBluescript KS+. The resulting vector, pVEAKO, contains a 4.8-kb insert with the *hph* gene flanked by genomic sequences from *A. chrysogenum*. This insert is terminated by *SacI* and *SpeI* restriction sites, as shown in Fig. 1B. Transformation of recipient strain A3/2 was done with the 4.8-kb *SacI*/*SpeI* fragment by standard protocols (46). DNA extraction and Southern blot analysis were performed as described previously (14) using standard procedures (37). Screening for deletion mutants was performed by a PCR-based approach using primer pair 57/59 (see Table 2),

leading to amplicons of 1,780 bp in the wild-type gene and 1,594 bp for the knockout cassette. Transformants lacking the 1,780-bp PCR product were further tested by PCR using primer pairs 42/49 and 52/59 (see Table 2) in order to amplify fragments representing the *AcveA* ORF or upstream region of the *AcveA* gene.

Complementation of *AcveA* disruption strains. For complementation of the *AcveA* gene in both disruption strains, cosmid E11 was modified by insertion of the nourseothricin resistance cassette. For this purpose, we used pG-Nat1 (22), which carries a modified Transprimer element, derived from pGPS2.1 (New England Biolabs) that includes the *nat1* gene for selection of transformants. Screening of recombinant cosmids was done by Southern blot analysis using the *AcveA* ORF as probe. A single clone (E11-*nat1*), with a transposon insertion outside of the *AcveA* ORF was used for further experiments, and transformation into ΔveA and *KiveA* strains generated the $\Delta veA::veA$ and *KiveA::veA* complements, respectively.

Construction of plasmids for fluorescence microscopy. pEHN1-nat is a fungal expression vector carrying the enhanced green fluorescent protein (*egfp*) gene under the control of the *Aspergillus nidulans gpd* promoter and *trpC* terminator; it also contains the *nat1* gene for selection of transgenic strains (22). Using the unique *NcoI* restriction site, this vector was used to generate fusions of the *egfp* gene with the entire or truncated *AcveA* ORF. For amplification of the *AcveA* derivatives, primer pairs 66/75 and 66/74 were used (see Table 2). The length of the ORF in *AcveAt* corresponds to the truncated open reading frame that is contained on cosmid E11-*hph* (see above).

Microscopy and image analysis. Hyphal morphology at different cultivation times (48 to 120 h) was analyzed using a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany). Images were captured with an Axiovision digital imaging system and processed with Adobe Photoshop 6.0 software. Fluorescence microscopy was carried out with an AxioImager microscope (Zeiss, Jena, Germany) using an XBO 75 xenon lamp for fluorescence excitation. Fluorescence was studied using Chroma filter sets 41017 (exciter, HQ470/40; emitter, HQ525/50; beam splitter, Q560lp) (Chroma Technology Corp., Rockingham, VT) for EGFP detection. Images were captured with a Photometrix Cool Snap_{HQ} camera (Roper Scientific) and MetaMorph (version 6.3.1; Universal Imaging). Recorded images were edited with MetaMorph and Adobe Photoshop 6.0 software.

RNA isolation. RNA extraction from mycelia, grown in production media as mentioned above, was done using the TRIzol solution (Invitrogen) and RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Total RNA from mycelia grown in CCM was isolated as previously described (15), and the integrity of RNAs was verified by agarose gel and Northern blot analysis according to standard procedures (37).

qRT-PCR. Quantification of mRNA levels derived from the *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF*, and *cefG* genes was performed in two independent experiments. Total RNA was reverse transcribed using the high-capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's protocol. cDNA was analyzed in quantitative real-time PCR (qRT-PCR) assays in triplicates on an ABI 7900HT Fast real-time PCR machine (Applied Biosystems) using Power SYBR green master mix (Applied Biosystems) according to the manufacturer's recommendation. As a reference, the mean cycle threshold values from an amplicon derived from the actin mRNA were used for normalization. All primers used are given in Table 2.

Titer analysis of cephalosporin C. Culture broth from 100-ml liquid shake flask cultures, grown in production medium, was used for high-performance liquid chromatography (HPLC). Quantification of cephalosporin C was performed as previously described (39).

Nucleotide sequence accession number. The sequence of the *AcveA* gene has been deposited in the EMBL data library under accession no. AM410093.

RESULTS

Isolation and characterization of the *veA* homologue gene from *Acremonium chrysogenum*. In a pilot study, we have compared five protein-coding genes (coding for diphthamine biosynthesis methyltransferase, eIF-2 α , eIF5, ATP synthase subunit beta, and DNA-directed RNA polymerase III second largest chain) from *A. chrysogenum* with the corresponding sequences from filamentous fungi that were used in genome sequencing projects. We found that *A. chrysogenum* shares a sequence similarity of 80% with *N. crassa* at the amino acid sequence level (J. Kamerewerd and U. Kück, unpublished data). Therefore, heterologous primers 8/10 (see Table 2) based on conserved domains of the *N. crassa velvet* sequence were used to obtain a 360-bp amplicon using *A. chrysogenum* genomic DNA as template. This product was further used to screen an *A. chrysogenum* cosmid library leading to the isolation of cosmid E11. Using a primer-walking strategy, the complete nucleotide sequence of the *A. chrysogenum* homologue of *NcveA*, including the 1.3-kb upstream and 2.0-kb downstream region, was determined. Annotation of the sequence identified an ORF of 514 amino acids that is interrupted by a single intron. The predicted gene was named *AcveA*, and an alignment of the derived amino acid sequence with those from velvet proteins of other ascomycetes shows high conservation in the N-terminal regions (data not shown). Overall, the amino acid sequence has 54%, 37%, 33% and 28% identity with FvVEA from *Fusarium verticillioides*, NcVEA from *Neurospora crassa*, the hypothetical MgVEA of *Magnaporthe grisea*, and the VEA from *Aspergillus nidulans*. We also compared the sequence with a putative classical bipartite nuclear localization signal (NLS) motif, NLSI, which recently was predicted for the *A. nidulans* VEA polypep-

tide (43). Comparing this N-terminal sequence with the *A. chrysogenum* AcVEA sequence, we found considerable conservation between both sequences, although two highly conserved Lys residues are missing in the *A. chrysogenum* sequence, as well as in the velvet sequences from *Fusarium verticillioides* and *Magnaporthe grisea*. Using the computer program PSORTII (<http://psort.nibb.ac.jp>), another nuclear localization signal, PIGSKRK (NLSII), was predicted close to the C-terminal end of the putative polypeptide. This motif is less conserved in the compared sequences from other filamentous fungi. As demonstrated below, the function of the predicted NLSII is further supported by localization studies of AcVEA using fluorescent microscopy. To determine the gene copy number of *AcveA* in the *A. chrysogenum* genome, Southern blot analyses were performed with the complete *AcveA* gene as a probe. Using BsmI-, EcoRI-, or PstI- restricted genomic DNA from *A. chrysogenum* as a template, single hybridizing restriction fragments were obtained, indicating that *AcveA* is a single-copy gene (data not shown).

Generation of strains with a deleted or disrupted *AcveA* gene. To study the function of *velvet* in *A. chrysogenum*, two different strains carrying a disrupted copy of *AcveA* were constructed. In addition, the two strains were complemented with a wild-type copy of *AcveA*, and the resulting strains were used for further analysis.

In the first attempt, we used recombinant cosmid E11-*hph* for generation of a fungal insertional mutant (*KiveA*). As described in Materials and Methods, E11-*hph* carries an *AcveA* ORF that is disrupted at nucleotide position 850 by the modified transposon Tn7 (Fig. 1A). Transformation of the wild-type strain of *A. chrysogenum* gave transformants that were characterized by a PCR-based strategy. Using DNA from all transformants as a template, primer pair 42/43 (see Table 2) generates two different PCR products (PCR 5 and 5*). While the 350-bp fragment from PCR 5 is derived from the wild-type *AcveA* gene, the 3.0-kb fragment of PCR 5* is obtained from the disrupted *AcveA* gene. As expected, most fungal transformants produced the PCR 5 as well as the PCR 5* product. However, we identified 2 transformants out of 90 that showed only the larger 3.0-kb fragment of PCR 5* (Fig. 2A). Both are therefore likely to contain a disrupted *AcveA* gene that was received by homologous recombination with cosmid E11-*hph*.

To confirm further the disruption event, both transformants were analyzed by Southern hybridization. When probed with the *AcveA* gene, EcoRI-restricted genomic DNA from both strains showed two fragments of 2.5 kb and 3.3 kb after hybridization. In contrast, the wild-type strain gave only a single band of 3.0 kb (Fig. 2C). Both insertional mutants displayed identical phenotypes on all media tested (data not shown), and thus a single clone, designated *KiveA*, was used for further analysis.

In the second attempt, a knockout strain was constructed by a strategy previously described for *A. chrysogenum* (14). Plasmid pVEAKO contains a genomic sequence of *A. chrysogenum* of about 5.0 kb, in which the *AcveA* ORF was substituted for by the 1.4-kb *hph* resistance gene. The SacI/SpeI fragment from pVEAKO (Fig. 1B) was used to generate hygromycin-resistant *A. chrysogenum* transformants. Using a PCR-based screening procedure, fungal clones were identified in which the *AcveA* locus was substituted for by the *hph* gene via homologous

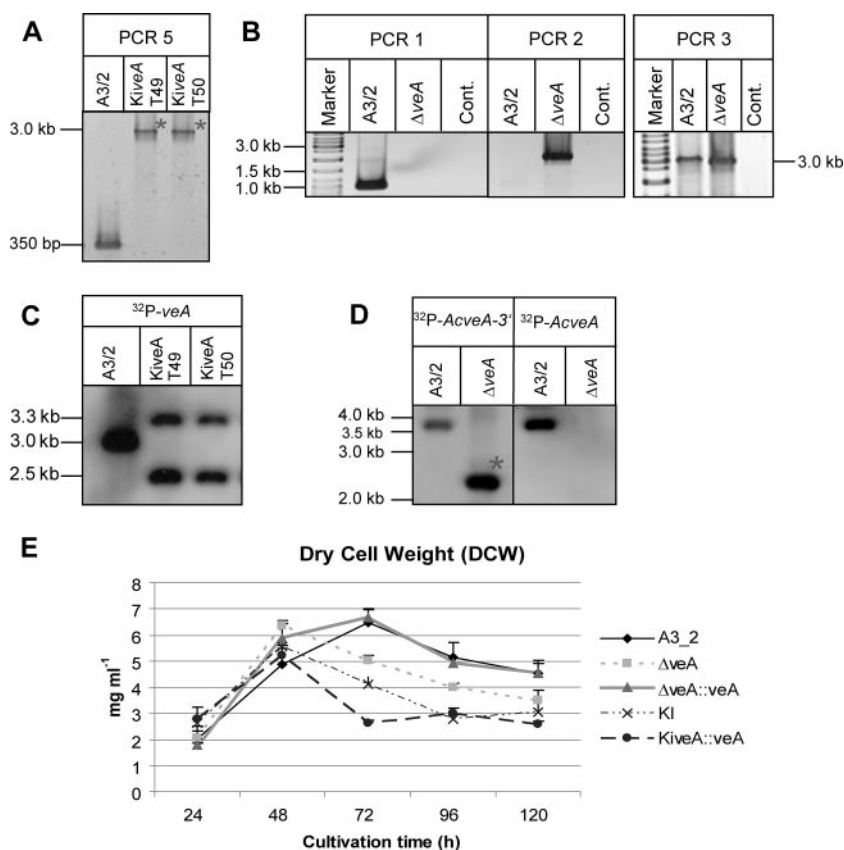


FIG. 2. Characterization of *AcveA* disruption strains. Shown are the results of PCR analysis of the recipient strain A3/2 and the disruption strains *KiveA* (A) and ΔveA (B) using genomic DNA as template. The 2.2-kb amplicon generated by PCR 2 can only be obtained after insertion of the *veA* knockout cassette by homologous recombination into genomic DNA. Cont., control. (C and D) Southern analysis of strains A3/2, *KiveA* (C), and ΔveA (D). (C) The genomic DNA of the recipient and two insertional mutants (*KiveA* T49 and *KiveA* T50) was digested with *EcoRI* and hybridized with the ^{32}P -*AcveA* probe. The hybridization bands of 3.3 kb and 2.5 kb in the transformants indicate the occurrence of the homologous recombination event depicted in Fig. 1A. (D) Genomic DNA was digested with *BsmI*. Expected band sizes after hybridization with the ^{32}P -*AcveA*-3' probe are as follows: A3/2, 3.7 kb; ΔveA , 2.2 kb (marked with an asterisk). As expected, hybridization with the ^{32}P -*AcveA* probe gained a single band of 3.7 kb for the A3/2 strain. (E) Growth curves of the A3/2, ΔveA , $\Delta veA::veA$, *KiveA* (KI), and *KiveA::veA* strains determined as mg DCW per ml liquid CCM. The mean values of three independent experiments with respective standard deviations are shown.

recombination. As seen in Fig. 1B, the use of primer pair 57/59 (see Table 2) leads to an amplicon of 1.7 kb (PCR 4) in the wild-type gene, while the 1.5-kb band of PCR 4* originates from the knockout cassette. Transformants lacking PCR 4 were further tested with primer pairs 42/49 (PCR 1) and 38b/54 (PCR 2) for verification of the *AcveA* deletion by homologous integration (Fig. 2B). PCR 1 is only feasible in the recipient strain A3/2, whereas genomic DNA from a ΔveA strain lacks any binding sites for primers 42/49. Further verification of the homologous integration event came from PCR 2. While primer 38b binds to the knockout cassette, primer 54 binds specifically to a sequence outside of the homologous targeting region. The 2.3-kb fragment of PCR 2 is indicative for the disruption event that occurred through homologous recombination in the ΔveA strain. As expected, A3/2 lacks any amplification product.

To analyze the deletion by Southern blotting, genomic DNA was digested with *BsmI* and hybridized with probes corresponding either to the 3' region of the *veA* gene or the *veA* ORF. The 3' probe detected a 3.7-kb fragment in the wild-type strain, which was absent in the knockout strain, in which a 2.2-kb band resulted from an additional *BsmI* site of the *hph*

cassette. Rehybridization of the membrane with the *veA* ORF probe resulted in the expected 3.7-kb band for the recipient strain and complete absence of a signal in ΔveA , thereby confirming the construction of a knockout strain (Fig. 2D).

In summary, using A3/2 as a recipient, we constructed two different strains as a prelude to functional analysis of the homologue of the *Aspergillus velvet* gene in *A. chrysogenum*. *KiveA* carries a truncated version of *AcveA* that codes for a predicted polypeptide of 264 amino acids, while ΔveA lacks any codon of the *AcveA* ORF. Our strain construction was completed by generating two strains with the mutants described above as recipients. Using cosmid E11-*natI*, both strains were complemented with the full-size *AcveA* wild-type gene that is contained in the transforming DNA. These strains were further characterized and carry ectopically integrated copies of the *AcveA* gene (data not shown). As summarized in Table 1, five different strains served as a basis for the detailed functional analysis. It is important to note that the dry cell weight (DCW) of all strains was measured at different time points (24 to 120 h) (Fig. 2E). No significant differences in DCW were observed, although the strains are distinguishable by their morphological and physiological features.

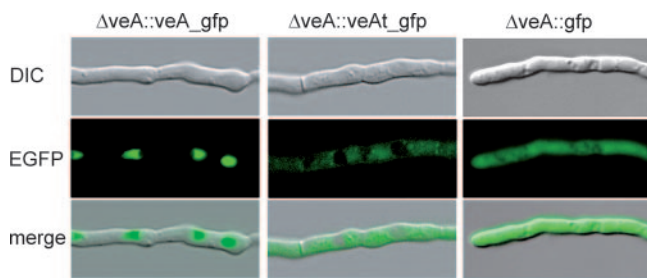


FIG. 3. Fluorescence microscopy of two strains carrying *gfp* fusions with the full-length ($\Delta veA::veA_gfp$) or truncated ($\Delta veA::veAt_gfp$) *AcveA* gene as indicated. Transformants carrying solely the *egfp* gene ($\Delta veA::gfp$) served as a control. DIC, differential interference contrast.

Subcellular localization of AcVEA. To determine the subcellular localization of the AcVEA protein and its truncated version, we generated *egfp*-tagged fusion constructs. While plasmid *pveA_egfp* contains the full-size *AcveA* gene fused to the *egfp* reporter gene, *pveAt_egfp* contains a truncated version of *AcveA* coding for the 264 N-terminal amino acids of the wild-type gene. Transformation of both plasmids into recipient ΔveA resulted in the $\Delta veA::veA_egfp$ and $\Delta veA::veAt_egfp$ strains. Microscopic investigation of both strains gave clearly different fluorescence patterns (Fig. 3). The $\Delta veA::veA_egfp$ strain shows that the complete AcVEA protein is targeted into the nucleus, producing a fluorescence pattern that coincides with the staining pattern of nucleic acid dyes (data not shown). The $\Delta veA::veAt_egfp$ strain provides a fluorescence pattern that is distributed throughout the cytoplasm and seems to be excluded from nuclei and vacuoles of the fungal cell. The fluorescence of the $\Delta veA::veAt_egfp$ strain can clearly be distinguished from the bright uniformly distributed fluorescence of strains synthesizing solely the EGFP. These results indicate that the predicted NLSII of *AcveA* is most probably functional. The truncated mRNA of *AcveAt* lacking the NLSII sequence is translated into a shortened version of the *AcveA* polypeptide that is unable to enter the nucleus.

Transcriptional expression of *AcveA*. Next, we investigated the transcriptional expression of *AcveA* in the five different strains described above. For the hybridization analysis, RNA from all strains grown for 48, 72, 96, and 120 h on rich medium was used in a comparative investigation. As an example, Fig. 4A shows a hybridization pattern which was obtained with RNA from the 48- and 120-h-grown cultures. Northern blot analysis shows that the wild-type *veA* transcript is ~ 2.5 kb in length, while the insertional mutant *KiveA* produces a transcript of only ~ 1.5 kb. The generation of a stable 1.5-kb transcript can be explained by the presence of eukaryotic polyadenylation signals that are preceded by stop codons in all reading frames in the bacterial transposon (26). This leads to the premature polyadenylation of the mRNA, and thus most probably to a truncated *AcveA* polypeptide (AcVEAT). Interestingly, the 1.5-kb transcript seems to accumulate already after 48 h, while for the larger transcript of 2.5 kb, the highest transcript level was detectable after 120 h of growth (Fig. 4A).

Altered expression of cephalosporin biosynthesis genes in strains with a disturbed *AcveA* gene. For *Aspergillus nidulans*, *velvet* is known to be a global regulator of genes involved in

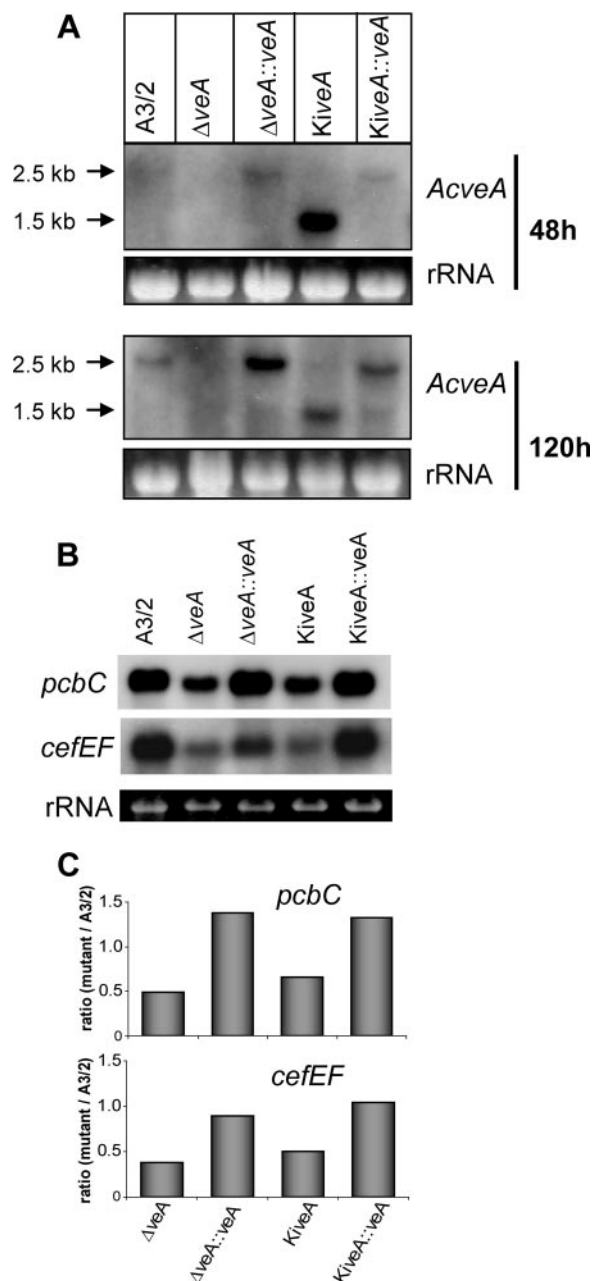


FIG. 4. Transcript analysis of *AcveA*, *pcbC*, and *cefEF* expression in five strains as indicated. Total RNA was isolated after cultivation on CCM. (A) Northern hybridization of RNA isolated at two different time points with the *AcveA* gene as a probe. (B) Northern analysis with RNA of cultures grown for 120 h. Probes were used as indicated. (C) Quantification of the autoradiogram shown in panel B by densitometry using Scion Image software (Scion Corporation, Frederick, MD) and normalized with rRNA. Gene products: *cefEF*, deacetoxycephalosporine synthase; *pcbC*, IPN synthase.

secondary metabolism of this fungus. This includes the three penicillin N biosynthesis genes, two of which are homologous to the *pcbAB* and *pcbC* from *A. chrysogenum*. Both encoded enzymes are involved in the first two steps of cephalosporin C biosynthesis in *A. chrysogenum*. We investigated the transcriptional expression of *pcbAB* and *pcbC* together with the *cefDI*,

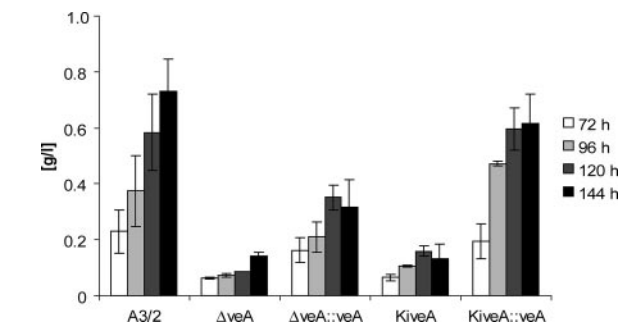
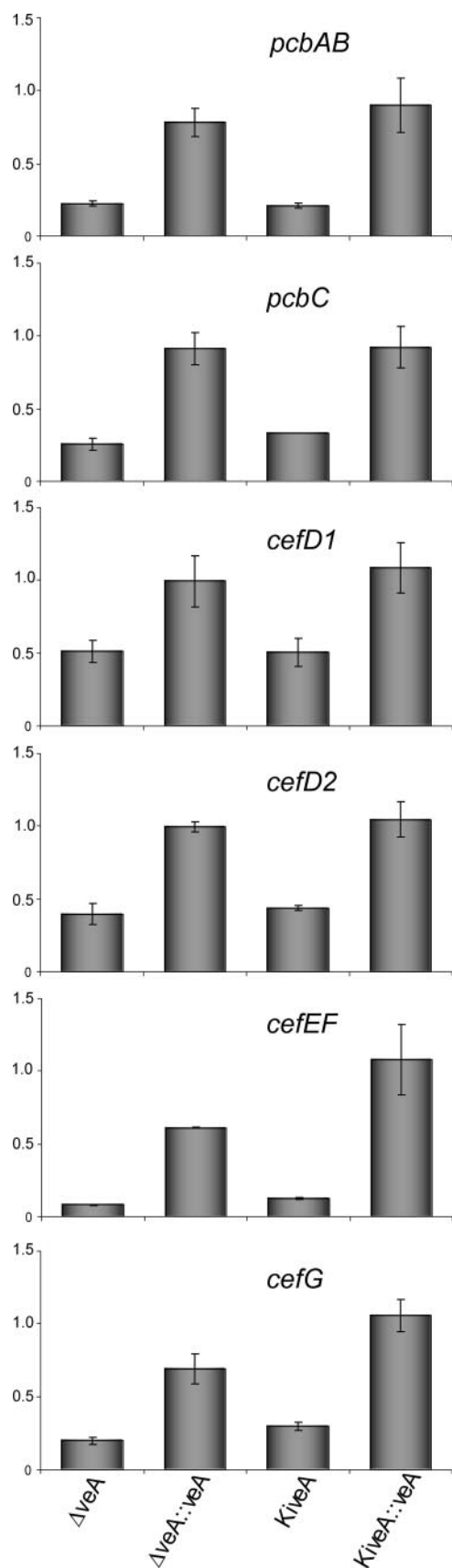


FIG. 6. HPLC quantification of cephalosporin C. All strains were grown in production media and harvested after the indicated time. The supernatant of the cultures was analyzed by HPLC as described in Materials and Methods.

cefD2, *cefEF*, and *cefG* genes that are specific for cephalosporin C biosynthesis. RNA derived from all five strains was isolated at different time points of growth in batch culture with production media. In Fig. 4B, Northern blot analysis for the *pcbC* and *cefEF* genes at 120 h is shown, and for standardization, the amount of loaded RNA is shown. *pcbC* and *cefEF* show the highest transcriptional expression of all cephalosporin C biosynthesis genes and were therefore chosen for a quantitative analysis. As can be seen in Fig. 4B and C, the level of transcripts of both genes is highly abundant in A3/2, but clearly reduced in both disruption strains. The wild-type level was partially restored when the full-length *AcveA* gene was reinserted into the knockout strains. Both complements show an increase of the *pcbC* and *cefEF* transcript level. A more quantitative analysis of the transcripts was done by qRT-PCR using actin mRNA as internal standard. As can be seen in Fig. 5, the transcript levels of all genes were clearly decreased in both disruption strains. Quantification by qRT-PCR of the *pcbC* and *cefEF* transcripts thereby confirms data obtained by Northern blot analysis (Fig. 4C). The most drastic reduction is seen in the case of the *cefEF* transcript. While *pcbAB*, *pcbC*, *cefD1*, *cefD2*, and *cefG* transcript levels are reduced by about 20 to 50%, the *cefEF* transcript level decreases to less than 13% in both disruption strains. In both complements, the wild-type phenotype is almost fully restored, and these data were further confirmed by cephalosporin C titer quantification.

Disruption of the *AcveA* gene results in reduction of cephalosporin C. In the next set of experiments, cephalosporin C was quantified by HPLC. For this analysis, culture broths of those mycelia were used, which were employed for the RNA extractions as described above. For this comparative analysis, five different strains were investigated in parallel and HPLC values for all strains were taken at four different time points (Fig. 6). The amounts produced by the ΔveA and KiveA strains

FIG. 5. Quantitative real-time PCR analysis of the *pcbAB*, *pcbC*, *cefD1*, *cefD2*, *cefEF*, and *cefG* transcripts in the indicated strains. Data are given as ratio of mutant to A3/2 with the corresponding standard deviations. Results were tested for the significance of differential expression at $P = 0.001$ using REST (33). Gene products: *pcbAB*, ACV synthetase; *cefG*, deacetylcephalosporine acetyltransferase. All other products are given in the legend to Fig. 4.

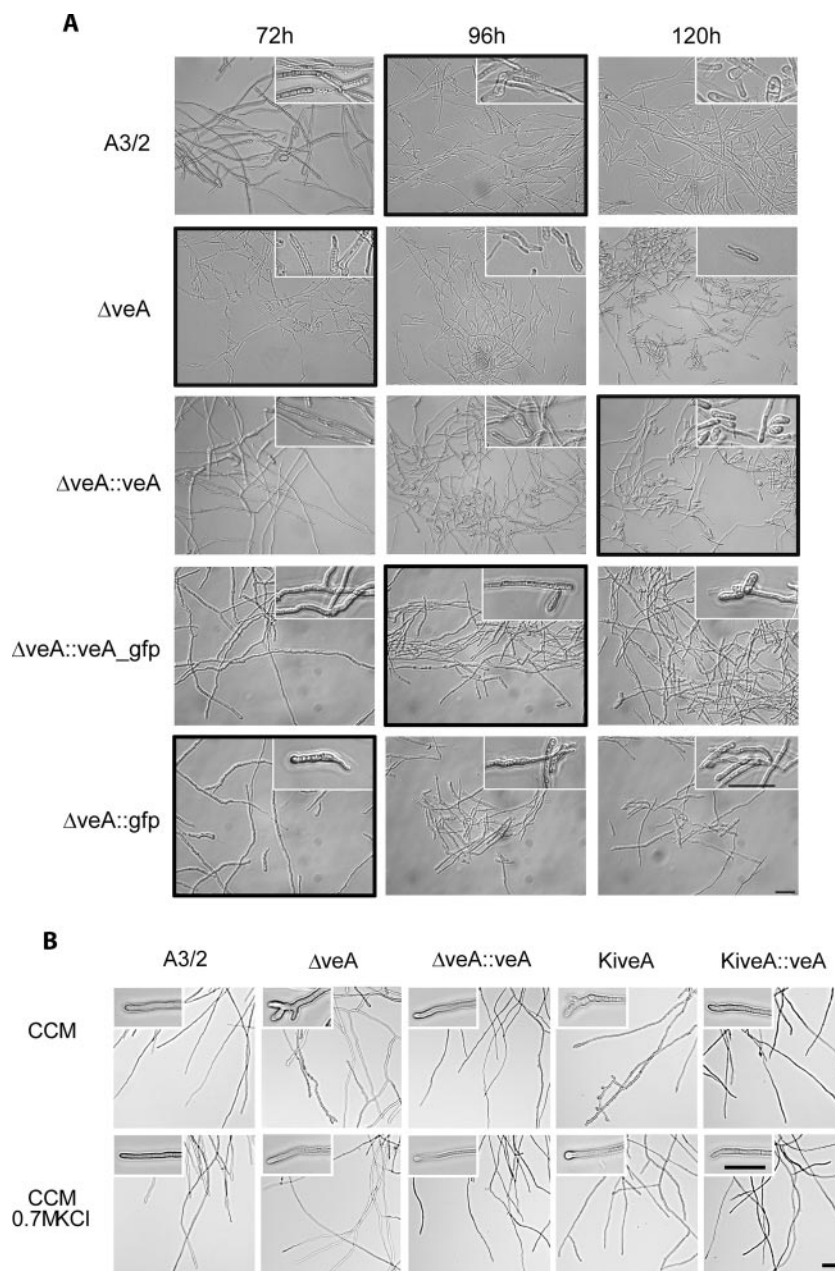


FIG. 7. Microscopic investigations of *A. chrysogenum* strains as indicated. (A) For arthrospore formation, all strains were grown at 27°C and 180 rpm in liquid CCM. At the assigned time points, mycelial morphology was analyzed by differential interference contrast microscopy, and representative microscopic fields are depicted. Insets show an enlargement of characteristic mycelial structures, and black frames indicate the beginning of arthrospore formation. (B) Growth of the recipient strain and transformants in complete medium with (CCM plus 0.7 M KCl) and without (CCM) the addition of the osmotic stabilizer KCl. Insets show an enlargement of characteristic mycelial tips. The scale bar represents 20 μ m in all panels.

are similar and account for only approximately 20% of the amount produced by A3/2. Compared to the disruption strains, the $\Delta veA::veA$ and $KiveA::veA$ complemented strains show an increase in cephalosporin C titer reaching amounts similar to that of the wild type. These results correlate well with the overall decreased expression of the cephalosporin C biosynthesis genes in disruption strains observed by Northern blot and qRT-PCR analyses.

Disturbance of *AcveA* leads to an altered hyphal morphology. We have previously shown that ACFKH1 and CPC1, two global regulators of secondary metabolism in *A. chrysogenum*, have a strong impact on fungal arthrospore morphology (14). The fragmentation of long hyphae is an active developmental process in cellular differentiation of *A. chrysogenum* and results in the formation of spherical cells called arthrospores (9, 30, 35). It was therefore logical to investigate also the

impact of *AcveA* on fungal morphology, since *AcveA* homologues in aspergilli have a significant effect on fungal development. For our microscopic investigations, fungal morphology was monitored after 48, 72, 96, and 120 h of growth in liquid medium, using the five different strains described above. Arthrospore formation in the A3/2 strain starts at 96 h and is correlated with an increase in the yield of cephalosporin C biosynthesis (1, 30). As shown in Fig. 7A, microscopic examinations revealed, however, that the observed hyphal fragmentation in the disruption strain is different from arthrospore formation. While A3/2 arthrospores can be described as rounded short hyphal fragments with one or two compartments, the hyphal fragments of the ΔveA strain are on average larger and often have one tapered end. Measurements of the length of the hyphal fragments smaller than 20 μm showed that in the recipient strain about 57% of the cells are larger than 10 μm and the remaining cells are smaller than 10 μm . In contrast, the ΔveA strain showed a distribution of 84% of the hyphal fragments being larger than 10 μm and only 16% being smaller. This hyphal fragmentation can already be seen after 72 h in ΔveA . Most importantly, the A3/2 phenotype is restored when ΔveA is complemented with *AcveA* ($\Delta veA::veA$) and arthrospores are not formed before 96 h. After reintroduction of the *AcveA* gene, arthrospores have an even more spherical appearance than in the recipient strain, with 53.7% of the fragments being larger than 10 μm and the rest (46.3%) being smaller. These data were confirmed when the ΔveA strain was complemented with the *veA_gfp* fusion gene or with the *gfp* gene. While the fusion protein obviously restored the A3/2 phenotype, GFP itself is not able to prevent hyphal fragmentation at 72 h.

The two strains with a disturbed *veA* (ΔveA and *KiveA*) gene did not show any obvious phenotype when grown on solid CCM (data not shown). In static liquid culture, the mycelial growth of recipient A3/2 typically consists of slender hyphal filaments that almost exclusively display lateral branching. In contrast, both disruption strains show altered hyphal tip morphology with frequent dichotomous branching or hyperbranching (Fig. 7B). However, this phenotype could be restored by the addition of osmotic stabilizers, indicating that the change in morphology could be due to an alteration in cell wall composition.

DISCUSSION

The homologue of the *Aspergillus velvet* gene from *A. chrysogenum* shows a high degree of sequence similarity and carries a putative nuclear localization signal at the C-terminal end. During her pioneering work with *Aspergillus nidulans*, E. Käfer (18) generated the velvet mutant, showing delayed and reduced sexual reproduction together with enhanced conidiation in the dark. Molecular investigations later identified the *velvet* gene encoding a 537-amino-acid-long polypeptide that is conserved in all filamentous ascomycetes investigated so far (21, 25). It was further shown that in the original velvet mutant, a VEA polypeptide shorter by 36 amino acids than the wild-type protein is most probably produced. This truncation is located at the N terminus and affects a functional NLS that could be specifically recognized by the general and nuclear importin α (43). Yeast two-hybrid analysis has shown that the *A. nidulans*

NLS interacts with importin α and deletion of the VEA-NLS motif weakens this interaction, indicating that additional VEA motifs interact with importin α (34). An appropriate candidate sequence might be NLSII, predicted by us to be between amino acid positions 454 and 460. This prediction is consistent with our observation that only the full-length AcVEA polypeptide, and not the truncated version, shows nuclear localization by fluorescent microscopy. Two nuclear localization signals have frequently been reported for proteins from higher as well as lower eukaryotes (2, 34). The transcriptional activator Gcn4p from yeast, for example, has been shown to carry two nuclear localization signals, NLS1 and NLS2. While the nuclear localization function of NLS1 is relatively unspecific, NLS2 mediates specific translocation by a heterodimeric importin α/β complex (34). Another case was described for the *Aspergillus oryzae* HapB subunit of the CCAAT-binding complex. This polypeptide carries two C-terminal NLS motifs. Only when both were mutated was a complete loss of the nuclear localization ability observed (12).

Expression of the late cluster gene *cefEF* is severely affected by disruption of *AcveA*. The involvement of *veA* in the regulation of secondary metabolism has been shown for different species of aspergilli, where *veA* is necessary for the synthesis of different metabolites (7, 10, 19). In *A. nidulans*, the first two genes of penicillin biosynthesis were analyzed and *veA* was shown to repress the transcription of the isopenicillin synthase gene *ipnA* and to be necessary for the expression of *acvA*. Since the expression of *acvA* is the rate-limiting step in penicillin biosynthesis, this led to a reduction in penicillin production (19). However, recently, the opposite was observed using two different *veA* disruption strains (42). These authors used reporter genes to demonstrate that VeA acts as a repressor mainly on *pcbAB* (*acvA*) expression. In addition, penicillin V synthesis was increased under *veA*-inducing conditions.

While in *A. nidulans* and *P. chrysogenum* the penicillin biosynthesis genes are found in a single cluster, in *A. chrysogenum* the genes involved in cephalosporin biosynthesis are organized in at least two clusters located on different chromosomes. The *pcbAB*, *pcbC*, *cefD1*, and *cefD2* genes are linked in the so-called "early" cephalosporin cluster, while the "late" cluster contains the *cefEF* and *cefG* genes. These genes are involved in the last two steps, which are specific for cephalosporin C biosynthesis (4, 40). Knowledge about the molecular regulation of β -lactam biosynthesis in *A. chrysogenum* is, however, still very limited. Here, we analyzed the effect of *AcveA* on the cephalosporin C titer and transcriptional expression of the "early" genes as well as the "late" genes, *cefEF* and *cefG*. The quantification of Northern hybridizations as well as qRT-PCR experiments showed that disruption of *AcveA* represses transcription of all cephalosporin C biosynthesis genes. Similarly, all downstream-acting enzymes are downregulated in the disruption strains. The strongest reduction was observed for *cefEF* transcription, which decreased to less than 15% in both disruption strains. We also determined the cephalosporin C content by HPLC and saw that it correlates well with the reduction in transcription levels. The amount of penicillin N produced, however, was less drastically altered (data not shown), thus indicating that the effect of *AcveA* is more pronounced on the late cluster of cephalosporin biosynthesis genes. The strong reduction of *cefEF* gene expression together

with a reduction to less than 50% for the *cefG* gene can account for the strong decline in cephalosporin C biosynthesis, since deacetylcephalosporin C acetyltransferase, encoded by *cefG*, is a very labile enzyme requiring continuous resynthesis throughout the fermentation process (8). Moreover, *cefG* is very poorly expressed in *A. chrysogenum* compared to other genes of the cephalosporin pathway (*pcbAB*, *pcbC*, and *cefEF*) (45), and the overexpression of this gene was shown to result in increased cephalosporin C production (13), indicating that it is a limiting factor.

AcveA seems to be involved in the control of hyphal fragmentation and cell wall biosynthesis. In filamentous fungi, secondary metabolism and morphological alterations are often linked phenomena, suggesting that their onset has a common regulation. The molecular determinants connecting both processes, however, remain largely undefined. It was therefore the aim of this study to see whether global regulators that have been reported to connect secondary metabolism and morphogenesis in *A. nidulans* (19) play a similar role in another β -lactam producer. In *A. chrysogenum*, the formation of uni- or bicellular swollen hyphal fragments called arthrospores seems to be correlated with high-yield cephalosporin C production in the corresponding production strains (14). This is consistent with observations that the phase of hyphal differentiation coincides with the maximum rate of β -lactam biosynthesis (30). We recently have shown that the RFX1 transcription factor CPR1, but not the interacting forkhead transcription factor AcFKH1, controls arthrospore formation in *A. chrysogenum* (14). The fragmentation of hyphae observed in this contribution is different from typical round-ended short hyphal arthrospores. All hyphal fragments observed in the *AcveA* disruption strains are longer than typical arthrospores, indicating that cell wall integrity in these strains is disturbed. The fungal cell wall is critical for cell viability and pathogenicity, serving as a protective shell and providing cell morphology. The main macromolecular components in the cell walls of higher fungi (*Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes*) are mannoproteins, 1,3- β -glucan, and chitin, although wall composition frequently varies markedly between species of fungi. In yeast, it is known that the major mannoproteins are present both at the surface and in deeper layers of the cell wall, shielding the cell wall glucan. Removal of most of the mannoproteins results in an increased porosity of the cell wall (49). Deletion of the *veA* gene in *A. nidulans* resulted in reduced transcript levels of the mannoprotein-encoding gene *mnpA*, without altering cell wall integrity (17). In *Fusarium verticillioides*, however, the *veA* homologue *Fvve1* was shown to be necessary for normal cell wall composition and integrity (25). Disruption of the *AcveA* gene led to changes in hyphal tip morphology and hyphal fragmentation, indicating that similar to *FvVE1* it might have an influence on cell wall composition. The hyperbranching and dichotomous branching observed in the disruption strains were restored by the addition of osmotic stabilizers, a further indication of an altered cell wall composition. A disturbance in cell wall composition, and thus rigidity, could also be the cause of the premature fragmentation observed under liquid shaking culture conditions. This could indicate that the ΔveA and *KiveA* strains do not produce regular arthrospores. This effect is more prominent in the ΔveA strain, where the hyphal fragments often displayed an altered morphology and the culture

was also depleted of the typical spherical arthrospores that are present in large amounts in the $\Delta veA::veA$ strain.

Are velvet homologues differently regulated in different fungi? The cellular function of velvet in *A. nidulans* is so far poorly understood. It was recently demonstrated that *velvet* most probably acts downstream of a phytochrome that represses sexual development under red-light conditions (3). It was also suggested that the phytochrome acts in the cytoplasm on red-light photoperception since it is excluded from the nuclei. Fungal phytochromes show a close relationship to bacterial phytochromes carrying an additional response regulator domain. These modules function in bacterial two-component signal transduction systems, and it can therefore be assumed that AcVEA, as a component of a signal transduction pathway, shuffles between the cytoplasm and the nucleus and thus controls directly or indirectly differential gene expression. This assumption is supported by the recent findings of a light-dependent subcellular mobility of the *A. nidulans* VEA polypeptide (43). In the dark, VEA is located mainly in the nuclei, while in the presence of light, VEA associates with filamentous bodies in the cytoplasm. As preliminary data, we found a similar light-dependent subcellular localization in *A. chrysogenum* cells grown in liquid CCM (J. Dreyer and U. Kück, unpublished observation). Stinnett and coworkers (43) discuss how phytochromes, similar to data obtained with velvet, are associated with electrodense particles and possibly are part of a single cytoplasmic complex. Whether this complex controls identical cellular functions in different filamentous fungi remains to be determined. However, Li et al. (25) have pointed out that the mode of control by *velvet* homologues in different fungi may be different, since *velvet* deletion phenotypes observed in *Fusarium* and *Aspergillus* species suggest different regulation of cellular processes. This would also explain the different regulation of early β -lactam biosynthesis genes in *A. nidulans* and *A. chrysogenum*, as demonstrated by us and others (19, 42).

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