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Protein Kinase A Regulates Growth, Sporulation, and Pigment Formation in *Aspergillus fumigatus*▽

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***Aspergillus fumigatus* is an opportunistic human pathogenic fungus causing severe infections in immunocompromised patients. Cyclic AMP (cAMP) signal transduction plays an important role in virulence. A central component of this signaling cascade is protein kinase A (PKA), which regulates cellular processes by phosphorylation of specific target proteins. Here we describe the generation and analysis of *A. fumigatus* mutants expressing the gene encoding the catalytic subunit of PKA, *pkaC1*, under control of an inducible promoter. Strains overexpressing *pkaC1* showed high PKA activity, reduced growth, sporulation deficiency, and formation of a dark pigment in the mycelium. These data indicate that cAMP-PKA signaling is involved in the regulation of important processes, such as growth, asexual reproduction, and biosynthesis of secondary metabolites. Furthermore, elevated PKA activity led to increased expression of the *pksP* gene. The polyketide synthase PksP is an essential enzyme for production of dihydroxynaphthalene-melanin in *A. fumigatus* and contributes to virulence. Our results suggest that increased *pksP* expression is responsible for pigment formation in the mycelium. Comparative proteome analysis of the *pkaC1*-overexpressing strain and the wild-type strain led to the identification of proteins regulated by the cAMP-PKA signal transduction pathway. We showed that elevated PKA activity resulted in activation of stress-associated proteins and of enzymes involved in protein biosynthesis and glucose catabolism. In contrast, proteins which were involved in nucleotide and amino acid biosynthesis were downregulated, as were enzymes involved in catabolism of carbon sources other than glucose.**

The importance of *Aspergillus fumigatus* as a human pathogenic fungus has increased notably over the last years. Today, *A. fumigatus* is the most important airborne fungal pathogen causing invasive mycoses (for an overview, see references 4 and 28). Sensing of environmental stimuli and transduction of the corresponding signal via the cyclic AMP (cAMP) signaling cascade play an essential role in the virulence of a variety of human and plant pathogenic fungi, including *Cryptococcus neoformans*, *Magnaporthe grisea*, and *A. fumigatus* (1, 9, 19). They enable the fungus to adapt to changing environmental conditions, e.g., after invasion of the host tissue, by activation of factors which protect the pathogen against defense mechanisms of the host immune system. In eukaryotes, exogenous signals are sensed by defined transmembrane receptors on the surface of the cell, resulting in activation of receptor-bound heterotrimeric G proteins. In their inactive state, these G proteins consist of three subunits, designated Gα, Gβ, and Gγ. The Gα subunit binds GDP. After binding of a signal molecule to the receptor, GDP is exchanged with GTP. Subsequently, the G protein dissociates from the receptor and the Gα subunit is released from the βγ heterodimer. The Gα-GTP monomer formed by GpaB activates the adenylate cyclase (ACYA) that generates cAMP from ATP.

A central component of the cAMP signaling cascade is protein kinase A (PKA). PKA is a serine/threonine kinase which is conserved in eukaryotes. In the inactive state, PKA forms a heterotetrameric complex, consisting of two PKA catalytic (PKAC) subunits that are bound by two regulatory (PKAR) subunits. Each PKAR subunit has an autophosphorylation site for the PKAC subunit as well as two tandem copies of a cAMP binding site. After binding of two molecules of cAMP to these binding motifs, the catalytic and regulatory subunits dissociate as a result of a conformational change of the heterotetramer. The activated catalytic subunits are now able to phosphorylate target proteins, such as transcription factors.

As a counterpart of ACYA, phosphodiesterases hydrolyze intracellular cAMP to AMP to prevent constitutive activation of PKA and to reset the signaling cascade for the response to new environmental signals. For *A. fumigatus*, this model of cAMP signaling is based on results obtained by analysis of mutants of the cAMP cascade. Genes encoding the Gα subunit GpaB, the adenylate cyclase ACYA, the PKA catalytic subunit PKAC1, and PKAR were deleted, and the corresponding mutants were analyzed (18, 19, 34). The Δ*pkaC1*, Δ*pkaR*, and Δ*acyA* mutants were severely delayed in growth and sporulation, whereas the Δ*gpaB* mutant showed only a slight decrease in growth rate and spore formation. In contrast to its nearly unaffected growth, the Δ*gpaB* mutant showed a significant attenuation in virulence (19), underlining the importance of the cAMP-PKA signaling cascade for the virulence of this fungus.

In the *A. fumigatus* genome, two different genes for PKA catalytic subunits were identified, namely, *pkaC1* and *pkaC2* (19). Somehow PKAC2 is not active, because its nucleotide binding site does not contain the consensus sequence necessary for binding of ATP. Furthermore, deletion of *pkaC1* resulted in a complete loss of PKA activity (19). This led to the assump-

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)
PKAC1-F-Xba	TCTAGAAATGCCGACTTTAGGAGGTCTC
PKAC1-R-Xba	TCTAGAGTATCTCTCAGATGCGTC
PKAC1-int-F	AGA CGC CGT CGA CTT GCT C
acuD-F	CGGATCCGAAGGACAGGAAC
PKAR-for	CTTGTCATCACGTCTGTCCTC
PKAR-rev	GACGATGACTCGAATGTGGTTG

tion that PKAC1 is the single active PKAC subunit in *A. fumigatus*.

The aim of this study was to identify proteins of *A. fumigatus* that were regulated by PKA. Ectopic integration of *pkaC1* under control of the inducible promoter of the isocitrate lyase gene (*acuDp*) (2) resulted in transgenic mutants whose level of active catalytic subunits could be drastically increased. The mutants were analyzed phenotypically and by comparative two-dimensional (2D) gel electrophoresis to identify putative target proteins/genes of PKA.

MATERIALS AND METHODS

Fungal and bacterial strains, media, and growth conditions. The *A. fumigatus* ATCC 46645 wild-type strain was used for DNA isolation and to generate *pkaC1*Oex mutants. An *A. fumigatus pksP* strain was used for generation of *pksP-pkaC1*Oex mutants. Due to a mutation in the *pksP* gene, this strain is impaired in dihydroxynaphthalene (DHN)-melanin biosynthesis, producing white conidia and showing strong attenuation in virulence (15). A *pksPp-lacZ* strain (18) was used for the generation of *pksPp-lacZ-ΔpkaR* and *pksPp-lacZ-pkaC1*Oex mutants. The *pksPp-lacZ* strain contains the *pksP* promoter fused with the *lacZ* reporter gene for the quantification of *pksP* expression. *A. fumigatus* was cultivated at 37°C in *Aspergillus* minimal medium (AMM) as described previously (32). For solid medium, AMM containing 1.5% (wt/vol) agar was used. For transformation of *Escherichia coli*, strain TOP10F′ (Invitrogen, Germany) was used. *E. coli* strains were grown at 37°C in LB medium supplemented with 100 μg ml^{-1} of ampicillin.

Standard DNA techniques. Standard techniques for manipulation of DNA were carried out as described previously (22). Chromosomal DNA of *A. fumigatus* was prepared using a Master Pure yeast DNA purification kit (Epicentre). For Southern blot analysis, chromosomal DNA of *A. fumigatus* was digested with SacI. DNA fragments were separated in an agarose gel and blotted onto Hybond N+ nylon membranes (GE Healthcare Bio-Sciences, Germany). Labeling of DNA probes, hybridization, and detection of DNA-DNA hybrids were performed using DIG labeling mix, DIG Easy Hyb, and a CDP-Star ready-to-use kit (Roche Applied Science, Germany), respectively, according to the manufacturer's recommendations.

Generation of recombinant plasmids and transformation of *A. fumigatus*. Plasmid p*acuDpkaC1* was used for inducible overexpression of the *pkaC1* gene in *A. fumigatus*. For construction of p*acuDpkaC1*, a 1-kb fragment of the *acuD* promoter was isolated via BamHI restriction from plasmid pDsRed_*icl*p (2) and inserted into the BamHI-digested plasmid pUC18 (Fermentas, Germany), resulting in plasmid pUC*acuD*p. Using primers PKAC1-F-Xba and PKAC1-R-Xba (Table 1) and *A. fumigatus* wild-type chromosomal DNA as a template, the *pkaC1* gene was amplified by PCR, employing Bio-X-Act Short DNA polymerase (Bioline, Germany). The resulting 2-kb PCR product was inserted into plasmid pCR2.1TOPO (Invitrogen, Germany) by TOPO-TA cloning and verified by sequencing. After digestion with XbaI, the *pkaC1*-carrying DNA fragment was inserted into the XbaI restriction site of pUC*acuD*p, resulting in plasmid pUC*acuDpkaC1*. This plasmid was cut with SfoI and SmaI, and the 3.2-kb blunt-ended fragment comprising the *acuD* promoter in frame with the *pkaC1* sequence was inserted into the SfoI site of plasmid pAN8-1 (GenBank accession number Z32751), containing the phleomycin resistance cassette. The resulting plasmid was designated p*acuDpkaC1*.

For deletion of the *pkaR* gene in *A. fumigatus*, using pyrithiamine resistance as a selection marker, plasmid pCR2.1Δ*pkaR-ptrA* was generated as follows. A PCR product carrying the *pkaR* gene, including 1-kb flanking regions, was obtained by using primers PKAR-for and PKAR-rev and genomic *A. fumigatus* DNA as a template. The DNA fragment was ligated into vector pCR2.1TOPO (Invitrogen, Germany), resulting in plasmid pCR2.1*pkaR*. This plasmid was digested with NheI. Blunt ends were then generated by applying the DNA polymerase I large (Klenow) fragment (New England Biolabs, Germany). The product was digested with XmaI. The pyrithiamine resistance gene was inserted as a 2-kb DraI/XmaI fragment obtained from plasmid pCR2.1*mpkA-ptrA* (a gift from V. Valiante). For transformation, the *pkaR* deletion construct was amplified by PCR, using primers PKAR-for and PKAR-rev and plasmid pCR2.1Δ*pkaR-ptrA* as a template. Transformation of *A. fumigatus* was carried out using protoplasts as described previously (32). When selection for phleomycin resistance was used, phleomycin (Invivogen) was added to the medium to a final concentration of 80 μg ml^{-1}. When selection for pyrithiamine resistance was used, pyrithiamine (Sigma-Aldrich, Germany) was added to the medium to a final concentration of 0.1 μg ml^{-1}.

Quantification of conidial production. To investigate the effect of *pkaC1* overexpression on production of conidia in *A. fumigatus*, 10^5 conidia were plated on AMM agar plates containing glucose (50 mM) and glucose (50 mM)-acetate (100 mM) as carbon sources. After 3 days, conidia were harvested in 10 ml 0.9% (wt/vol) NaCl–0.1% (vol/vol) Tween 80, filtered using a cell strainer (Becton Dickinson, Germany), and counted using a Thoma chamber.

Protein extraction from *A. fumigatus*. For protein extraction, 10^8 conidia of the *A. fumigatus* wild type and the *pkaC1*Oex strain were inoculated in 100 ml AMM containing 50 mM glucose and cultivated for 16 h on a rotary shaker. After this precultivation step, the mycelium was harvested by filtration using Miracloth, washed, transferred to AMM containing 100 mM acetate as the sole carbon source (inducing conditions), and incubated for a further 8 hours. Mycelium was harvested and immediately frozen in liquid nitrogen. After grinding of the mycelium to a fine powder, using a mortar and pestle, proteins were extracted for proteome analysis as described previously (17). For determination of PKA activity, 100 mg of the ground mycelium was resuspended in 500 μl ice-cold extraction buffer (25 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA), and a PKA assay (Promega, Germany) was applied. Protein extracts were centrifuged for 10 min at 13,000 rpm at 4°C, and the supernatant was used for further experiments.

Proteome analysis. Proteome analysis of *A. fumigatus* was carried out essentially as described previously (14, 17). In brief, protein extracts for 2D gel electrophoresis were purified by phenol extraction (13). The dried protein pellets were resuspended in lysis buffer. The absolute amount of 300 μg protein was applied via anodic cup loading to rehydrated IPG strips with a nonlinear pH gradient from 3 to 11 (GE Healthcare Bio-Sciences, Germany). The second dimension of electrophoresis was performed on an Ettan DALTsix system (GE Healthcare Bio-Sciences, Germany). Gels were stained with colloidal Coomassie blue (20). For each strain analyzed, three replicas of three gels were performed. Images were analyzed with Delta 2D software (version 3.4; Decodon, Germany). After background subtraction and normalization, spots were quantified using % spot volumes. Only spots with a ratio of >2 and a P value of <0.05 using Student's t test were regarded as significantly regulated. Protein spots were excised manually and digested with trypsin according to the protocol of Bruker Daltonics (adapted from the method described in reference 24). The samples were analyzed by matrix-assisted laser desorption ionization–tandem time of flight (Ultraflex 1; Bruker Daltonics, Germany) and subsequently identified by searching the NCBI database, using the MASCOT interface (MASCOT 2.1.03; Matrix Science, United Kingdom) with the following parameters: Cys as an *S*-carbamidomethyl derivative, Met in oxidized form (variable), one missed cleavage site, and a peptide mass tolerance of 200 ppm. Hits were considered significant according to the MASCOT score ($P < 0.05$). Database analysis was refined by using Protein Scape 1.3 software (Protagen, Germany).

β-Gal activity assays. β-Galactosidase (β-Gal) activity was determined with *o*-nitrophenyl-β-D-galactoside (ONPG) as the substrate. Extinction of the sample was measured at 420 nm. Specific activities were calculated as previously described for *Aspergillus nidulans* (5).

Determination of cAMP-dependent PKA activity. For determination of PKA activity, the PepTag assay for nonradioactive detection of cAMP-dependent PKA (Promega, Germany) was applied. This assay uses a positively charged kemptide coupled to a fluorescent dye as a substrate specific for PKA. Visualization of the kemptide was done by using UV light. The PKA activity assay was applied as described previously (19). Protein extracts were adjusted to identical concentrations. PKA holoenzymes in the sample were activated by incubation with 1 μM cAMP. The quantity of free catalytic PKA subunits was determined by incubation of the samples without the addition of cAMP.

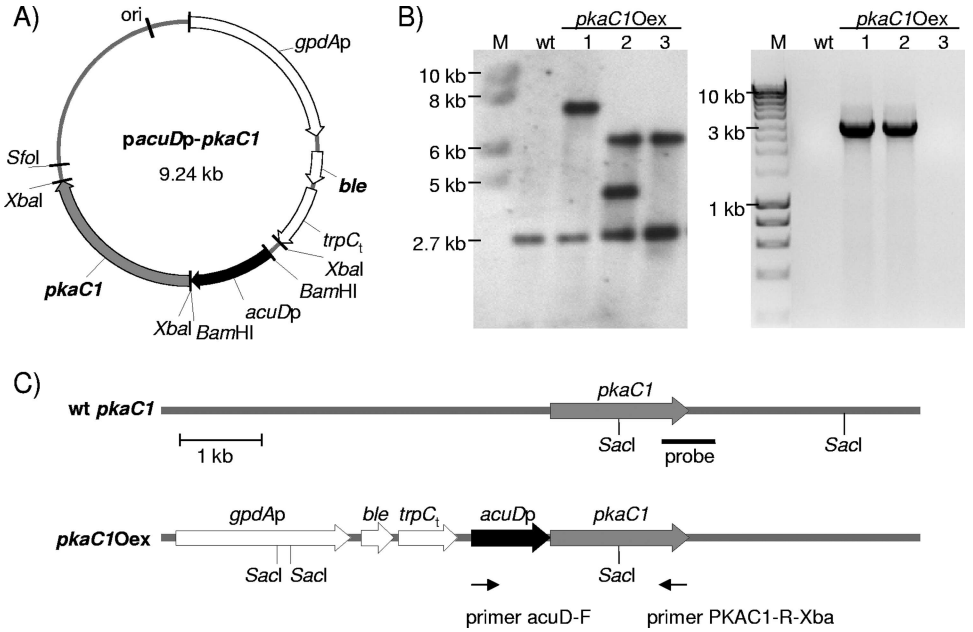

FIG. 1. Generation of inducible *pkaC1*-overexpressing *A. fumigatus* strains. (A) Map of plasmid p*acuD*p-*pkaC1* used for transformation of *A. fumigatus* wild type. *pkaC1*, *pkaC1* gene amplified from *A. fumigatus* genomic DNA, including 3′-untranslated region; *acuDp*, promoter of the *A. fumigatus acuD* gene; *gpdAp*, promoter of the *gpdA* gene from *A. nidulans*; *ble*, phleomycin resistance gene; $trpC_t$, terminator sequence. (B) Southern blot analysis (left) and PCR analysis (right) of the wild type (wt) and *pkaC1*Oex transformant strains. Lanes M, DNA marker. (C) Schematic drawings of the *A. fumigatus* wild-type *pkaC1* genomic locus and the *pkaC1*Oex construct.

RESULTS

Generation of *pkaC1*-overexpressing mutants. To identify target proteins and metabolic pathways regulated by cAMP-PKA signal transduction, an *A. fumigatus* strain with enhanced PKA activity was generated. This enhanced activity was achieved by overexpressing the *pkaC1* gene, encoding the catalytic subunit of PKA, which leads to an excess of free PKAC subunits. To exclude the possibility that constitutive overexpression results in a drastic growth defect, sporulation deficiency, or even lethality, the expression of *pkaC1* was placed under the control of the *A. fumigatus acuD* promoter, which is activated by C_2 carbon sources (2). Therefore, overexpression of *pkaC1* was controllable by using different carbon sources, e.g., acetate for induction and glucose for repression. A schematic map of plasmid p*acuDp-pkaC1*, used for transformation of the *A. fumigatus* wild type, is shown in Fig. 1A. As a resistance marker, the phleomycin resistance gene *ble* under control of the *gpdA* promoter from *A. nidulans* was employed.

Southern blot analysis was performed to identify *pkaC1*-overexpressing mutants. Genomic DNAs of selected transformants and the wild-type strain were digested with SacI. For hybridization, a 640-bp probe specific for detection of the 3′ region of the *pkaC1* gene was used, as presented schematically in Fig. 1C. As shown in Fig. 1B (left), a band with a size of 2.7 kb was obtained for all tested strains, indicating the presence of the endogenous *pkaC1* gene. Additional bands with different sizes were obtained for transformants 1, 2, and 3, indicating successful integration of an additional *pkaC1* sequence. To verify the integration of the complete *acuDp-pkaC1* sequence, PCR analysis of the transformants was carried out. By using the specific primers acuD-F and PKAC1-R-Xba, a DNA fragment with a size of 2.8 kb was obtained only when the complete sequence, starting from the *acuD* promoter and ending at the 3′-untranslated region of the *pkaC1* gene, was present in the genomes of the transformants. As shown in Fig. 1B (right panel), transformants 1 and 2, but not 3, gave the expected signal. Transformant strain 1, designated *pkaC1*Oex, was used for further studies, including proteome analysis.

Phenotypic analysis of the *pkaC1*-overexpressing mutant. To compare the phenotypes of the *pkaC1*Oex strain and the wild type, conidia of these strains were point inoculated on agar plates containing different carbon sources. After incubation for 72 h at 37°C on AMM agar plates with glucose as the sole carbon source, the wild-type and *pkaC1*Oex strains showed no differences (Fig. 2A and B). To induce *pkaC1* expression via the *acuD* promoter, conidia were inoculated on AMM agar plates containing 100 mM acetate as the sole carbon source. In contrast to growth on glucose, the *pkaC1*Oex mutant was not able to germinate (Fig. 2C). Microscopic analysis showed that *pkaC1*Oex conidia were swollen but were not able to germinate (data not shown). To test whether the conidia were still viable at this stage (at 4 days postinoculation), a drop of glucose solution was added to the conidia on the agar plate. The conidia started to germinate (data not shown). In contrast, conidia of the wild type were able to germinate and to grow normally on acetate as the sole carbon source. To enable the *pkaC1*Oex strain to germinate under inducing conditions, conidia were inoculated on AMM agar plates containing 100 mM acetate and 50 mM glucose. As shown in Fig. 2C, the addition of glucose to acetate-containing medium enabled the conidia to germinate. However, in comparison to the wild-type strain, which grew normally on

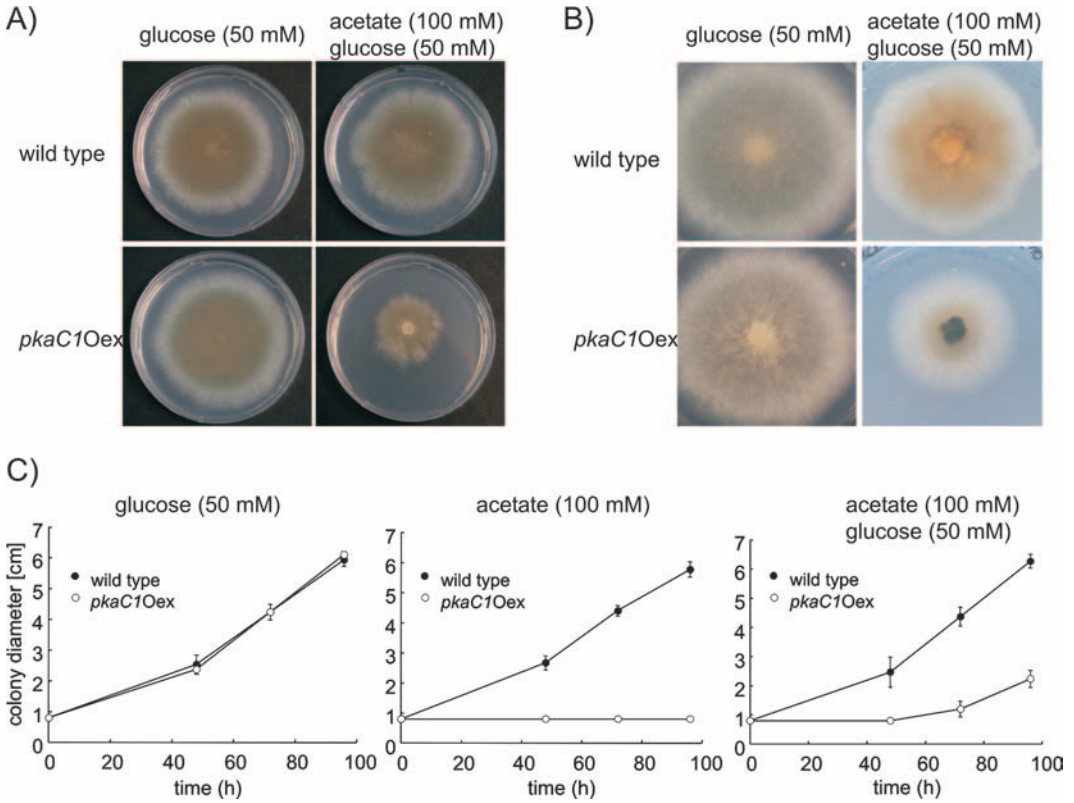

FIG. 2. Phenotypic analysis of the *pkaC1*Oex mutant under noninducing (glucose) and inducing (acetate-glucose) conditions. Front views (A) and back views (B) of 5-day-old colonies grown on AMM agar plates with glucose and acetate-glucose are shown. (C) Determination of radial growth of wild-type and *pkaC1*Oex strains cultivated on medium containing different concentrations of glucose and/or acetate as the carbon source. Spores (10^3) were point inoculated, and the plates were incubated at 37°C for the indicated times.

glucose-acetate medium, the *pkaC1*Oex strain exhibited a drastically altered phenotype. Radial growth of the mutant was strongly reduced (Fig. 2A and C), and the mycelium produced a dark pigment visible on the back side of the agar plates (Fig. 2B).

PKA enzyme activity was increased in the *pkaC1*-overexpressing strain. To determine whether induced overexpression of *pkaC1* resulted in active PKAC subunits, PKA activity was measured in protein extracts of the *pkaC1*Oex mutant and compared to that of the wild-type strain. For this purpose, PKA activity was assayed using a colored peptide (kemptide) specifically recognized and phosphorylated by PKA. For generation of protein extracts, the *A. fumigatus* strains were preincubated in AMM-glucose and then shifted to AMM-acetate for induction of *pkaC1* overexpression. As shown in Fig. 3, the nonphosphorylated kemptide migrated to the cathode, whereas the phosphorylated and therefore negatively charged kemptide migrated to the anode. The stronger the signal, the larger was the amount of phosphorylated kemptide in the protein extract, indicating increased PKA activity. This assay clearly showed that in the wild type incubated without cAMP, hardly any PKA activity was detectable. This was due to the regulatory subunits, which bind most of the catalytic subunits and keep them inactive. In contrast, a strong signal due to phosphorylated kemptide was detectable after addition of cAMP to the wild-type extract. This obviously was the result of cAMP molecules binding to PKAR subunits, followed by the release of active PKAC1 subunits.

The PKA activity pattern of the *pkaC1*Oex mutant was different from that of the wild type, as strong PKA activity was detectable even without the addition of cAMP (Fig. 3). This result indicated that PKA activity in the *pkaC1*Oex mutant was

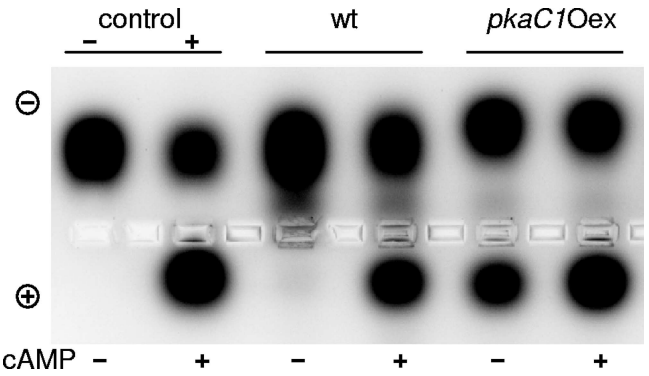

FIG. 3. Analysis of PKA activity of wild-type (wt) and *pkaC1*Oex transformant strains. Depending on the PKA activity, the net charge of the substrate kemptide was altered with respect to its phosphorylation status. As a positive control (+), the purified PKAC subunit was used, and as a negative control (−), protein extraction buffer without enzyme was used.

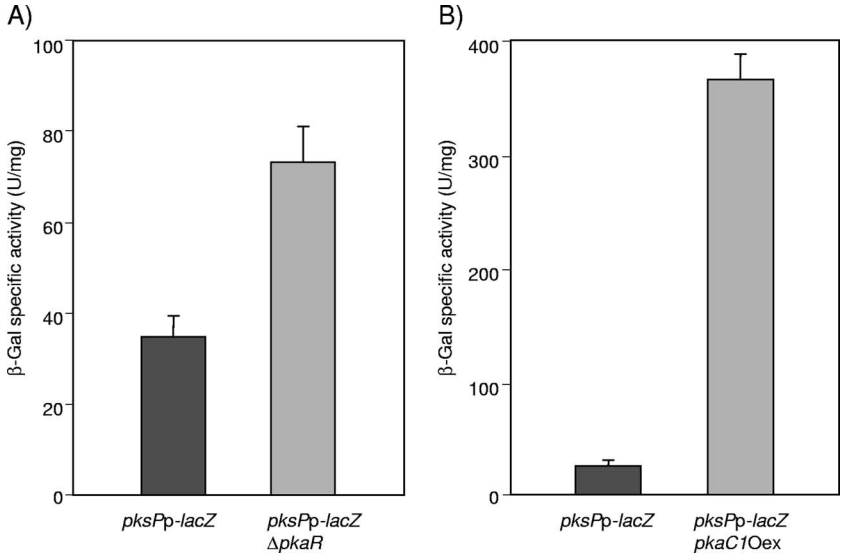

FIG. 4. Determination of specific β-Gal activity for quantification of *pksPp-lacZ* expression. (A) The *pksPp-lacZ* strain, bearing a *pksP* promoter-*lacZ* gene fusion, and the *pksPp-lacZ* Δ*pkaR* mutant, with a deletion of the *pkaR* gene, were cultivated for 28 h in AMM. Protein extracts were analyzed for β-Gal activity. (B) The *pksPp-lacZ* and *pksPp-lacZ-pkaC1*Oex strains were precultivated for 16 h in AMM. The mycelia were then shifted to acetate-containing medium and further incubated for 8 h for induction of *pkaC1* overexpression. Protein extracts were analyzed for β-Gal activity.

significantly increased after induction of *pkaC1* expression. The presence of free catalytic subunits in the cytoplasm thus resulted in high PKA activity, irrespective of the availability of cAMP.

Overexpression of *pkaC1* and deletion of *pkaR* resulted in increased β-Gal activity in a *pksPp-lacZ* reporter strain. *A. fumigatus* mutants with enhanced PKA activity, either due to overexpression of *pkaC1* or as a result of deletion of *pkaR* (34), develop a darkly pigmented mycelium. Previously, the *pksP* gene, encoding a polyketide synthase, was identified to be essential for DHN-melanin biosynthesis in *A. fumigatus* (15, 30). To investigate a possible influence of PKA activity on expression of the *pksP* gene, two different mutants, the Δ*pkaR* and *pkaC1*Oex strains, were generated using the *pksPp-lacZ* strain, containing the *pksP* promoter fused with the *lacZ* reporter gene. Expression of the *pksPp-lacZ* gene fusion in *A. fumigatus* was determined by measurement of the β-Gal activity in protein extracts of the *pksPp-lacZ* Δ*pkaR* mutant, the *pksPp-lacZ-pkaC1*Oex mutant, and the parental strain. Figure 4 shows that specific β-Gal activity was increased 2-fold in the *pksPp-lacZ* Δ*pkaR* strain (Fig. 4A) and 10-fold in the *pksPp-lacZ-pkaC1*Oex strain (Fig. 4B), indicating that in *A. fumigatus* expression of the *pksP* gene is positively regulated by enhanced PKA activity.

Overexpression of *pkaC1* led to reduced conidiation. In previous experiments, it was shown that cAMP signal transduction regulates conidiation (19). To investigate the effect of *pkaC1* overexpression on production of conidia, wild-type and *pkaC1*Oex strains were grown on AMM agar plates containing glucose and glucose-acetate as carbon sources. After 3 days, the conidia were harvested and counted (Fig. 5). On glucose (repressing conditions), the number of conidia derived from the *pkaC1*Oex mutant was similar to that calculated for the wild type. In contrast, under *pkaC1* overexpression conditions (acetate-containing medium; inducing conditions), the number of conidia produced by the *pkaC1*Oex mutant reached only 18% of the number of conidia produced by the wild type on the same medium. This indicates that *pkaC1* overexpression severely reduced the production of conidia.

Identification of putative PKA target proteins by proteome analysis. A comparative proteome analysis was performed to identify putative target proteins of PKA. For this purpose, the *pkaC1*Oex strain was preincubated in glucose-containing medium to allow germination of the conidia. *pkaC1* overexpression was then induced by shifting the mycelia to acetate-containing medium. This experiment led to the identification of proteins that were differentially synthesized compared with the

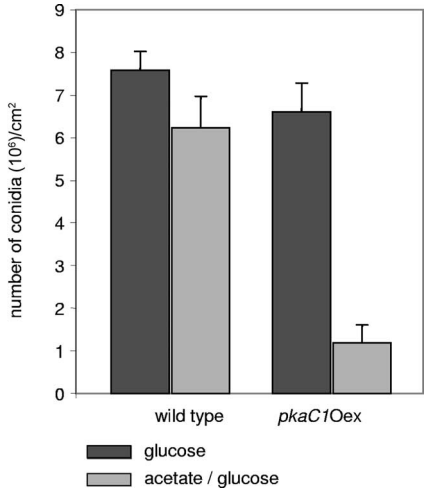

FIG. 5. Quantification of sporulation of wild-type and *pkaC1*Oex strains. AMM agar plates containing glucose or acetate-glucose were inoculated with 10^5 spores. After incubation for 3 days at 37°C, the number of conidia was determined.

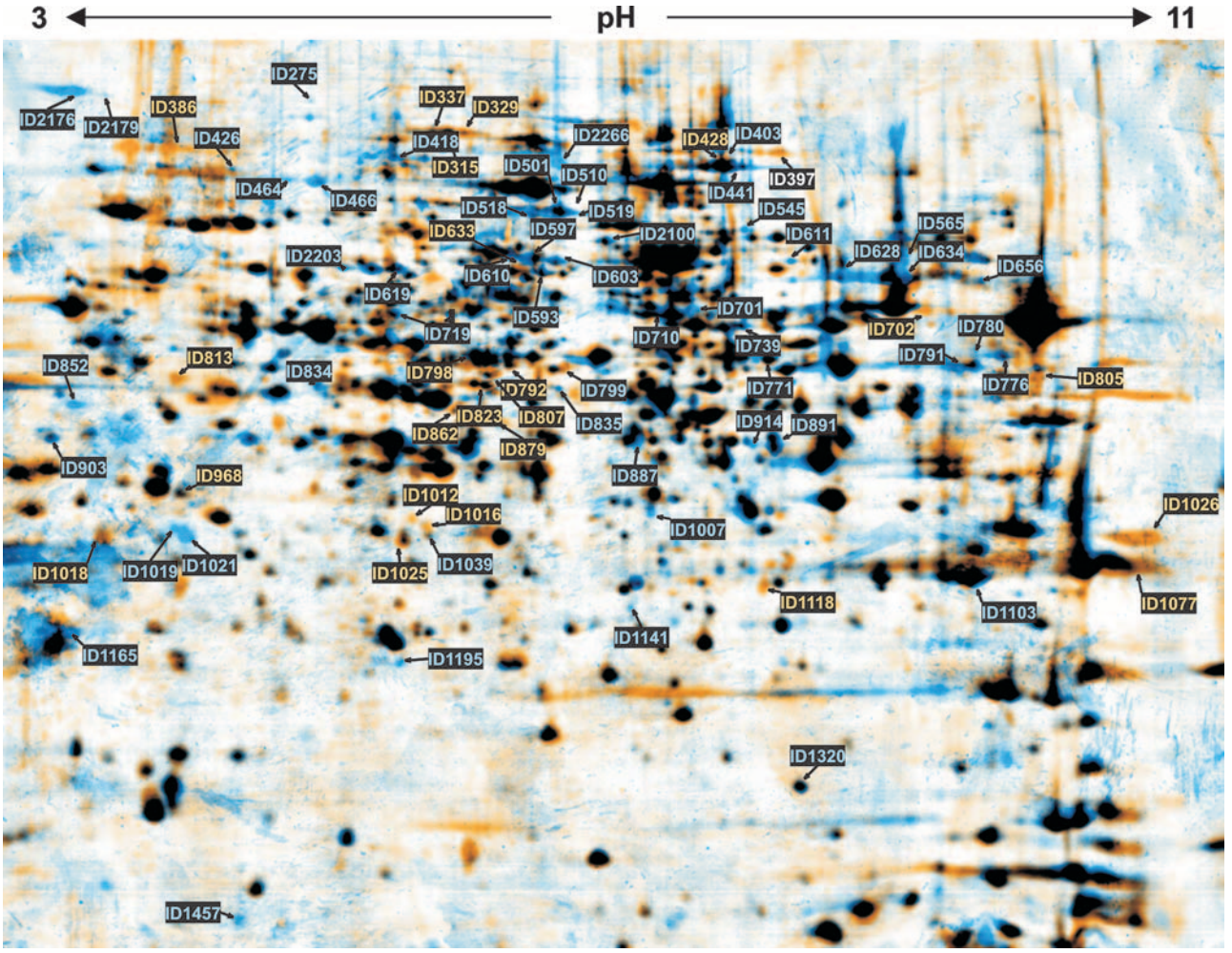

FIG. 6. 2D gel electrophoresis of protein extracts of the wild type and the *pkaC1*Oex strain. To identify proteins that were synthesized due to elevated PKA activity, conidia of both strains were preincubated in AMM containing glucose (noninducing conditions). The mycelia were then transferred to AMM containing 100 mM acetate as the sole carbon source and further incubated for 8 hours. Under these conditions, *pkaC1* expression was strongly induced in the *pkaC1*Oex strain, in contrast to the wild-type strain. The figure shows a dual-channel image produced by Delta2D 3.5 software after alignment of all images, using an implemented warping strategy. The blue areas represent the control gel for the wild type, and the orange areas represent the gel for the *pkaC1*Oex strain (black areas show overlap of both strains). The orientation of isoelectric focusing is indicated. The numbers (spot identities) refer to proteins whose levels changed significantly in the *pkaC1*Oex strain in comparison to those in the wild-type strain (Tables 2 and 3).

wild-type strain (Fig. 6). Overall, 98 protein spots on the 2D gel containing the wild-type proteins showed an increase of the spot volume of larger than twofold (P value of <0.05). In contrast, the 2D gel for the *pkaC1*Oex strain showed only 44 upregulated protein spots. Hence, 69% of the differentially synthesized proteins were downregulated in the *pkaC1*Oex strain, and 31% were upregulated compared with the wild type. By mass spectrometry, 53 of the 98 downregulated and 28 of the 44 upregulated protein spots were identified. The results are summarized in detail in Tables 2 and 3. Among the upregulated spots, many proteins were identified that are involved in protein biosynthesis, e.g., ribosomal proteins and translation elongation factors. Some of the downregulated proteins identified are involved in the biosynthesis of nucleotide bases and amino acids. For example, the carbamoyl phosphate synthetase large subunit is a key enzyme in pyrimidine and arginine biosynthesis (12). Ade1p is involved in purine biosynthesis (11).

Some important metabolic enzymes were also found to be regulated differentially. Among others, malate dehydrogenase, involved in the citric acid cycle, the phosphoenolpyruvate carboxy kinase of gluconeogenesis, and ribose 5-phosphate isomerase A of the pentose phosphate pathway were downregulated in the *pkaC1*Oex mutant. Furthermore, enzymes were identified that are involved in degradation or activation of acetate (acetyl-coenzyme A [acetyl-CoA] synthase), ethanol (alcohol dehydrogenase), propionate (methylcitrate synthase), and fatty acids (3-ketoacyl-CoA ketothiolase). Interestingly, two enzymes of the glyoxylate cycle were identified, namely, isocitrate lyase and malate synthase, whose synthesis was downregulated in the *pkaC1*Oex strain. The glyoxylate cycle allows the utilization of C_2 carbon sources, such as ethanol or acetate, and is present only in bacteria, plants, and fungi.

Proteins involved in cytoskeleton assembly and cell cycle regulation were identified, i.e., β-tubulin and a septin family

TABLE 2. Downregulated proteins of *A. fumigatus pkaC1*Oex strain in comparison to the wild type after induction of *pkaC1* expression

Putative function and spot no.[a]	Protein name	Locus tag	% Sequence coverage	Regulation (fold)
Protein biosynthesis				
403	Translation elongation factor EF-2 subunit, putative	AFUA_2G13530	13.5	3.7
441	Translation elongation factor EF-2 subunit, putative	AFUA_2G13530	19.4	5.6
418	Polyadenylate-binding protein	AFUA_1G04190	50.5	2.4
852	40S ribosomal protein S0	AFUA_3G13320	50.5	8.1
Purine biosynthesis				
426	Ade1p protein	AFUA_6G04730	27.5	2.3
Pyrimidine/arginine biosynthesis				
275	Carbamoyl-phosphate synthetase, large subunit	AFUA_2G10070	18.2	3.2
Tricarboxylic acid cycle				
1007	Malate dehydrogenase, NAD dependent	AFUA_6G05210	71.5	3.0
Glyoxylate cycle				
565	Malate synthase	AFUA_6G03540	32.4	2.4
628	Malate synthase	AFUA_6G03540	25.4	2.7
634	Malate synthase	AFUA_6G03540	37.9	2.7
2100	Isocitrate lyase (AcuD)	AFUA_6G02860	17.4	2.4
Gluconeogenesis				
619	Phosphoenolpyruvate carboxykinase (AcuF)	AFUA_6G07720	28.3	2.8
2203	Phosphoenolpyruvate carboxykinase (AcuF)	AFUA_6G07720	34.6	2.5
Pentose phosphate pathway				
1039	Ribose 5-phosphate isomerase A	AFUA_6G10610	18.4	2.1
Acetate degradation				
501	Acetyl-CoA synthetase (FacA)	AFUA_4G11080	16.0	3.5
510	Acetyl-CoA synthetase (FacA)	AFUA_4G11080	18.2	11.0
518	Acetyl-CoA synthetase (FacA)	AFUA_4G11080	26.7	2.8
519	Acetyl-CoA synthetase (FacA)	AFUA_4G11080	29.7	4.8
2266	Acetyl-CoA synthetase (FacA)	AFUA_4G11080	30.4	7.6
Alcohol degradation				
891	Alcohol dehydrogenase (ADH1)	AFUA_7G01010	37.7	2.8
914	Alcohol dehydrogenase (ADH1)	AFUA_7G01010	33.7	2.6
Fatty acid degradation				
791	3-Ketoacyl-CoA ketothiolase (Kat1), putative	AFUA_1G12650	45.5	3.0
Propionate degradation				
771	Methylcitrate synthase	AFUA_6G03590	40.0	2.4
Electron transport and reduction				
835	Flavohemoprotein	AFUA_8G06080	22.9	2.2
2179	Assimilatory sulfite reductase	AFUA_6G08920	17.0	2.2
Cytoskeleton assembly				
701	Tubulin beta-2 subunit	AFUA_7G00250	22.6	3.2
710	Tubulin beta-2 subunit	AFUA_7G00250	23.3	2.0
780	Septin	AFUA_5G03080	21.6	2.7
Protection against oxidative stress				
464	Mycelial catalase Cat1	AFUA_3G02270	34.6	2.1
466	Mycelial catalase Cat1	AFUA_3G02270	37.8	5.8
Siderophore biosynthesis				
656	L-Ornithine N-5-oxygenase	AFUA_2G07680	45.5	2.7

Continued on following page

TABLE 2—*Continued*

Putative function and spot no.[a]	Protein name	Locus tag	% Sequence coverage	Regulation (fold)
Hemolysis				
1457	Asp hemolysin	AFUA_3G00590	50.4	2.4
Others and proteins of unknown function				
597	GMC oxidoreductase	AFUA_3G01580	49.1	2.4
603	GMC oxidoreductase	AFUA_3G01580	25.9	8.0
610	GMC oxidoreductase	AFUA_3G01580	41.3	2.8
545	AMP-binding domain protein, putative	AFUA_1G05980	38.3	3.6
593	Pyruvate decarboxylase PdcA, putative	AFUA_3G11070	37.1	2.1
611	Pyridine nucleotide-disulfide oxidoreductase (Nfrl), putative	AFUA_7G02070	37.1	3.2
719	Homocitrate synthase	AFUA_4G10460	22.2	2.1
739	Gamma-butyrobetaine hydroxylase subfamily, putative	AFUA_2G14970	43.3	2.6
776	Acyl-CoA dehydrogenase family protein	AFUA_5G06500	24.6	2.2
834	2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase, putative	AFUA_5G12460	27.9	2.0
903	Ran/Spi1 binding protein	AFUA_5G12180	45.7	2.5
887	Unknown function	AFUA_6G11850	57.4	2.4
1019	Short chain dehydrogenase, putative	AFUA_4G08710	59.6	3.6
1021	Unknown function	AFUA_8G00550	63.1	7.9
1103	Outer mitochondrial membrane protein porin	AFUA_4G06910	33.0	2.4
1141	DlpA domain protein	AFUA_4G10940	29.4	2.1
1165	Unknown function	AFUA_5G14680	63.0	2.1
1195	Rho-GDP dissociation inhibitor	AFUA_5G11380	29.4	2.2
1320	HEX1	AFUA_5G08830	30.3	2.3
2176	PH domain protein	AFUA_4G12450	20.9	8.3

[a] Some spots represent different isoforms of the same protein. Proteins were identified by peptide mass fingerprinting. Spot numbers are shown in Fig. 6.

protein were downregulated and Cdc48 was found to be upregulated in the *pkaC1*Oex mutant. Two isoforms of the catalase Cat1 were significantly downregulated, as well as the L-ornithine-N-5-oxygenase, an essential enzyme of siderophore biosynthesis. For some proteins, no function was assigned in the database. These spots were designated proteins of unknown function.

DISCUSSION

Signal transduction via cAMP is a central signaling pathway in all eukaryotic cells to mediate cellular responses to environmental stimuli. In a large number of studies, elements of this cascade were identified, and their roles in different cellular processes, such as growth, development, and reproduction, and in the infection process were analyzed (for an overview, see references 18, 23, and 33). In this study, we further characterized the role of cAMP in cellular processes of the opportunistic human pathogenic fungus *A. fumigatus*. A central enzyme of cAMP signaling is PKA. Here we investigated the influence of enhanced PKA activity on *A. fumigatus*, and additionally, we aimed at the identification of PKA target proteins by a proteomic approach.

The generation of *A. fumigatus* strains with altered PKA activity was achieved by ectopic integration of the *pkaC1* sequence under control of the *acuD* promoter. In such a strain, overexpression of *pkaC1* was inducible, leading to an excess of free catalytic subunits. Consequently, the increase of PKA activity was independent of the intracellular cAMP level. This finding implies that in the *pkaC1* overexpression strain, the intracellular level of PKAR subunits is not sufficient to bind and inactivate the excess of additionally generated PKAC1 subunits. A similar increase in PKA activity was observed by Zhao and coworkers (34) by deletion of the gene encoding the regulatory subunit of PKA, *pkaR*. In contrast to the case for the Δ*pkaR* mutant, the PKA activity in the *pkaC1*Oex strain could be increased further by the addition of cAMP. This is due to the cAMP-induced release of PKAC1 subunits bound by endogenous PKAR subunits.

Increased PKA activity in the *pkaC1*Oex mutant led to phenotypic changes similar to those of other fungal mutants with elevated PKA activity. For example, in *Neurospora crassa*, *Aspergillus niger*, and *Colletotrichum lagenarium*, deletion of *pkaR* resulted in reduced growth and conidiation (6, 26, 27). This agrees well with the observation that sporulation capacity was significantly reduced in the *A. fumigatus pkaC1*Oex mutant. Similar results were obtained for other *A. fumigatus* mutants affected in cAMP signaling, i.e., the Δ*gpaB*, Δ*acyA*, and Δ*pkaC1* mutant strains (18, 19). In *A. nidulans*, overexpression of the *pkaA* gene, encoding one of the two PKAC subunits in this fungus, resulted in reduced sporulation but had no influence on growth (25), whereas deletion of *pkaA* resulted in increased sporulation and a severe growth defect. Furthermore, *pkaR* mutants of *N. crassa* and *A. niger* displayed apolar growth (6, 26). By deletion of *ubc1*, the PKAR subunit-encoding gene in *Ustilago maydis*, cytokinesis and budding were affected (10). In conclusion, in different fungi PKA regulates growth, cell proliferation, and sporulation.

Interestingly, the *pkaC1*Oex mutant was unable to germinate on AMM-acetate agar plates. This is in contrast to the case for the Δ*pkaR* mutant, which is able to germinate when acetate is the sole carbon source (T. Heinekamp, unpublished data). The polarization defect of the *pkaC1*Oex strain was not due to a delayed death of the conidia. This was demonstrated

TABLE 3. Upregulated proteins of *A. fumigatus pkaC1*Oex strain in comparison to the wild type after induction of *pkaC1* expression

Putative function and spot no.[a]	Protein name	Locus tag	% Sequence coverage	Regulation (fold)
Protein biosynthesis				
805	Translation elongation factor EF-1 alpha subunit, putative	AFUA_1G06390	33.0	3.0
428	Translation elongation factor EF-2 subunit, putative	AFUA_2G13530	31.3	2.1
315	Elongation factor EF-3, putative	AFUA_7G05660	53.8	2.5
329	Elongation factor EF-3, putative	AFUA_7G05660	39.1	5.6
337	Elongation factor EF-3, putative	AFUA_7G05660	20.1	2.1
823	Translation elongation factor EF-Tu, putative	AFUA_1G12170	68.0	2.1
968	40S ribosomal protein S0	AFUA_3G13320	57.2	2.3
1026	Cytosolic large ribosomal subunit protein L7A	AFUA_6G12990	53.1	3.3
1077	Cytosolic small ribosomal subunit S4, putative	AFUA_3G06840	62.8	2.2
1118	60S ribosomal protein P0	AFUA_1G05080	51.1	3.3
C1 metabolism				
397	C1-THFS protein	AFUA_3G08650	29.0	3.4
879	Methylenetetrahydrofolate dehydrogenase	AFUA_8G05330	34.9	2.6
Pyridoxine biosynthesis				
1012	Pyridoxine biosynthesis protein	AFUA_5G08090	31.8	2.3
Pyruvate metabolism				
792	Pyruvate dehydrogenase complex alpha subunit, putative	AFUA_1G06960	58.6	2.4
798	Phosphatidyl synthase	AFUA_4G11720	24.3	4.6
799	Phosphatidyl synthase	AFUA_4G11720	25.9	3.1
813	Phosphatidyl synthase	AFUA_4G11720	24.1	2.9
ATP synthesis				
702	Mitochondrial F1 ATPase subunit alpha, putative	AFUA_8G05320	50.5	2.7
Pentose phosphate pathway				
1016	Ribose 5-phosphate isomerase A	AFUA_6G10610	48.9	2.4
Purine degradation				
807	Allantoicase	AFUA_3G12560	44.9	4.9
Protein-folding				
862	Hsp70 chaperone, putative	AFUA_1G07440	42.4	2.4
Cell cycle and protein degradation				
386	Cell division control protein Cdc48	AFUA_2G17110	47.5	4.3
Others				
633	Choline oxidase (CodA), putative	AFUA_8G04090	54.6	2.1
1018	HAD superfamily hydrolase, putative	AFUA_5G08270	52.0	2.8
1025	Carbonyl reductase, putative	AFUA_5G09400	55.1	2.0

[a] Some spots represent different isoforms of the same protein. Proteins were identified by peptide mass fingerprinting. Spot numbers are shown in Fig. 6.

by inoculating conidia on agar plates supplemented with acetate as the sole carbon source. After incubation for 4 days, glucose was added to the medium and the conidia started to germinate, indicating that the conidia were viable. A role of PKA in germination could also be shown for other aspergilli. In a *pkaC*-overexpressing *A. niger* mutant, germination was delayed (26). The same was observed for an *A. nidulans* PKA-deficient mutant. In contrast, conidia of a mutant overexpressing *pkaB*, encoding a putative second catalytic subunit of PKA of *A. nidulans* with minor importance, were not able to germinate (21). For a *pkaR* deletion mutant of *A. fumigatus*, retarded spore germination and a severe reduction of spore viability were reported (34). This indicates the importance of cAMP signaling for conidial germination. After induction of PKA activity, the mycelium of the *pkaC1*Oex strain changed its color due to the formation of a dark pigment. The same was observed in the *pkaR* mutant (34), implying that high PKA activity regulates the biosynthesis of this pigment. The polyketide synthase PksP is a key enzyme in the biosynthesis of the pigment DHN-melanin in *A. fumigatus* (15, 29). Mutants deficient for PksP produce white conidia instead of the gray-green conidia of the wild type and are strongly attenuated in virulence. Expression of the gene was found to be predominantly in the conidia and phialides but was also detected in hyphae during the infection process (16). Liebmann and coworkers suggested that regulation of *pksP* depends on cAMP signaling, based on the observation that in both Δ*gpaB* and Δ*pkaC1* deletion mutants of *A. fumigatus*, *pksP* expression was drastically reduced (18, 19). These data make it very likely that DHN-melanin is the pigment produced due to high PKA ac-

tivity in both the *pkaC1*Oex strain and the Δ*pkaR* mutant. Because overexpression of *pkaC1* in a *pksP* mutant did not result in pigment formation (Heinekamp, unpublished data), these data indicate that the pigment produced in the *pkaC1*-overexpressing strain is in fact DHN-melanin. Consistently, as shown here, β-Gal activity was strongly increased in both a Δ*pkaR* strain and a *pkaC1*-overexpressing strain with an integrated reporter construct consisting of a fusion of the *pksP* promoter and the *lacZ* reporter gene. Previously, Zhao et al. (34) reported for a Δ*pkaR* mutant that the hyphal cell wall appeared thicker and more pigmented than that of the wild type. Therefore, increased *pksP* expression in *A. fumigatus* hyphae could result in DHN-melanin formation and pigmentation in hyphae of *pkaC1*Oex and Δ*pkaR* mutant strains.

Comparative proteomics of the wild type and a mutant strain with enhanced PKA activity was used for the identification of PKA target proteins and of the metabolism or biosynthesis pathways that are regulated by cAMP-PKA. In general, there are two straightforward approaches to generate mutants with enhanced PKA activity. On the one hand, deletion of the regulatory subunit results in increased PKA activity (34). On the other hand, as shown here, overexpression of the catalytic subunit also results in a strong increase of PKA activity. We could clearly show that both strategies led to mutants exhibiting similar phenotypes with regard to growth, pigmentation, and conidiation. However, for proteome analyses, it is important to compare strains with similar growth rates. Considering the severe germination and growth defects of the *pkaR* mutant, we chose the induced *pkaC1* overexpression approach. By preincubating the *pkaC1*Oex mutant and the wild-type strain in glucose-containing medium (repressing conditions), normal germination and growth were achieved. The mycelia were then shifted to acetate-containing medium (inducing conditions). This experimental setup excluded possible side effects from germination defects and differences in growth rates.

The proteomic data imply that PKA regulates the utilization of C sources. This was inferred from the observation that a high PKA level induced the biosynthesis of enzymes necessary for growth on glucose. In contrast, low PKA activity increased the levels of enzymes for catabolism of C sources others than glucose, e.g., ethanol or acetate. Accordingly, enzymes essential for utilization of C_2 carbon sources are less synthesized in the *pkaC1*Oex strain. This is consistent with the reduced growth rate of the *pkaC1*Oex strain on AMM agar plates with acetate as the carbon source. However, the finding that isocitrate lyase, a central enzyme of the glyoxylate cycle, was synthesized less (2.4-fold) in the *pkaC1*Oex strain also depicts the inherent complexity of the experimental setup of this study. The transcriptional regulation of isocitrate lyase is mediated by the *acuD* promoter, the same promoter that was used to induce *pkaC1* overexpression. Therefore, interference of *pkaC1* induction may be caused by elevated PKA activity.

PKA was found to regulate several important biosynthesis pathways, i.e., enzymes involved in nucleotide and amino acid biosynthesis. Based on uracil auxotrophic mutants bearing a mutation of the *pyrG* gene, encoding orotidine-5′-phosphate decarboxylase, it was shown that de novo pyrimidine biosynthesis is essential for germination and virulence of *A. fumigatus* (7). Interestingly, two protein spots, representing septin and β-tubulin, which are involved in cytoskeleton assembly, were downregulated by induced *pkaC1* expression. Septins play an important role in morphogenesis and virulence in different fungi, e.g., *Candida albicans* (31) and *U. maydis* (3). In *U. maydis*, a septin essential for establishing normal cellular morphology for infection of plants was found to be regulated by the cAMP pathway (3). The proteome data also indicate that cell division and growth are reduced by high PKA activity. This correlates well with the growth defect observed for the Δ*pkaR* and *pkaC1*Oex strains. Therefore, PKA regulates fungal morphogenesis.

Some proteins, i.e., the translation elongation factor EF-2 (spots 403, 441, and 428) and the ribose 5-phosphate isomerase (spots 1039 and 1016), appeared as multiple spots (up- and downregulated) on the gels. This phenomenon might be caused by different posttranslational modifications in the wild-type and mutant strains resulting in altered electrophoretic properties of the respective proteins. For instance, the regulation of EF-2 by phosphorylation is well known from studies of *Cryptococcus neoformans* (8).

Taken together, the results of the proteome analysis demonstrate the diversity in cellular processes regulated by PKA in *A. fumigatus*. Signaling via cAMP-PKA plays an important role, especially in regulating C source sensing and metabolism. Further analysis of the PKA-mediated regulation of these cellular processes will help to determine the details of the multifactorial virulence process of *A. fumigatus*.

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