PcchiB1, encoding a class V chitinase, is affected by PcVelA and PcLaeA, and is responsible for cell wall integrity in *Penicillium chrysogenum*

Jens Kamerewerd,¹ Ivo Zadra,² Hubert Kürnsteiner² and Ulrich Kück¹

Correspondence

Ulrich Kück
ulrich.kueck@rub.de

¹Christian Doppler Laboratory for 'Fungal Biotechnology', Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität Bochum, Universitätsstraße 150, 44780 Bochum, Germany

²Anti Infectives Microbiology, Sandoz GmbH, Biochemiestraße 10, 6250 Kundl, Austria

Penicillin production in *Penicillium chrysogenum* is controlled by PcVelA and PcLaeA, two components of the regulatory velvet-like complex. Comparative microarray analysis with mutants lacking PcVelA or PcLaeA revealed a set of 62 common genes affected by the loss of both components. A downregulated gene in both knockout strains is *PcchiB1*, potentially encoding a class V chitinase. Under nutrient-depleted conditions, transcript levels of *PcchiB1* are strongly upregulated, and the gene product contributes to more than 50 % of extracellular chitinase activity. Functional characterization by generating *PcchiB1*-disruption strains revealed that PcChiB1 is responsible for cell wall integrity and pellet formation in *P. chrysogenum*. Further, fluorescence microscopy with a DsRed-labelled chitinase suggests a cell wall association of the protein. An unexpected phenotype occurred when knockout strains were grown on media containing *N*-acetylglucosamine as the sole C and N source, where, in contrast to the recipient, a penicillin producer strain, the mutants and an ancestral strain show distinct mycelial growth. We discuss the relevance of this class V chitinase for morphology in an industrially important fungus.

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INTRODUCTION

Penicillium chrysogenum is the main industrial producer of the β -lactam antibiotic penicillin. In this filamentous ascomycete, developmental processes and secondary metabolism are tightly linked and controlled by diverse conserved regulators. Among these are PcVelA and PcLaeA, two global regulators, which have recently been shown to control penicillin biosynthesis as well as hyphal and conidiophore morphogenesis (Hoff *et al.*, 2010a). Homologues of both proteins have been intensively studied in the model fungus *Aspergillus nidulans* (Calvo, 2008; Bok & Keller, 2004; Sarikaya Bayram *et al.*, 2010). For example, it has been shown that both proteins, together with VelB, form the core of a regulatory heterotrimeric complex, which we refer to as the velvet complex (Bayram *et al.*, 2008a). The general regulatory function of these proteins is highly conserved in diverse filamentous fungi (Calvo, 2008), and it has been shown that velvet-like complexes exist in other ascomycetes (Hoff *et al.*, 2010a; Wiemann *et al.*, 2010).

Despite its importance for development and secondary metabolism, so far only a few high-throughput transcriptional analyses have been conducted with strains lacking components of the velvet complex. Cary *et al.* (2007) performed a microarray analysis with a ΔveA mutant of *Aspergillus flavus* to analyse the expression levels of 5002 genes. That study identified 136 differentially regulated genes in the disruption strain compared with the control strain, some of these representing known aflatoxin biosynthetic genes. In *Aspergillus fumigatus*, about 20–40 % of all secondary metabolite biosynthesis genes are negatively affected by the loss of *laeA*, as detected by whole-genome microarray analysis (Perrin *et al.*, 2007).

Using a *PcvelA* knockout strain, our previous microarray study to understand the expression profile of various genes involved in secondary metabolism and morphogenesis identified 1771 genes up- or downregulated in *P. chrysogenum* (Hoff *et al.*, 2010a).

In this study, we present a follow-on microarray analysis with a knockout *PclaeA* mutant to identify genes collectively affected by the loss of both regulator proteins, PcVelA and PcLaeA. By this approach, we identified *Pc22g01100*, which encodes a putative class V chitinase, as being strongly downregulated in $\Delta PcvelA$ and $\Delta PclaeA$ mutant strains.

Chitinases (EC 3.2.1.14) represent an important subgroup of cell wall-modifying enzymes and hydrolyse chitin

Abbreviation: qRT-PCR, quantitative RT-PCR.

Seven supplementary figures and four supplementary tables are available with the online version of this paper.

The microarray data discussed in this paper are available from the NCBI Gene Expression Omnibus (GEO) under accession number GSE18585.

randomly at internal sites to generate soluble, low-molecular-mass chito oligomers. Further morphogenetic, autolytic and nutritional roles have been found for fungal chitinases (Adams, 2004; Gooday *et al.*, 1992; Sahai & Manocha, 1993). In a widely accepted model, chitinases are necessary for the plasticity of the fungal cell wall in a concerted action with chitin synthase and β -1,4-*N*-acetylglucosaminidase (Bartnicki-Garcia, 1973; Rast *et al.*, 1991).

All fungal chitinases belong to the glycosyl hydrolase family 18 (GH18) (Seidl, 2008; Henrissat & Bairoch, 1993), and some chitinases seem to have different biological functions, while others are redundant. The genomes of filamentous ascomycetes and basidiomycetes so far analysed contain multiple genes encoding predicted or characterized chitinases, which, according to their structural similarities and reaction mechanisms, can be divided into subclasses III (subclass B) and V (subclass A). Recently, based on phylogenetic analysis, a third subclass C, with similarity to killer toxins from *Kluyveromyces lactis*, has been described (Gruber *et al.*, 2011; Seidl, 2008; Seidl *et al.*, 2005).

Several groups have reported disruption strains that lack distinct genes for diverse class V chitinases. However, most of these reports showed that the corresponding mutants have no apparent morphological phenotype. Because of its strong downregulation in both knockout strains, we wanted to determine whether *Pc22g01100* is functionally responsible for some of the described phenotypes observed in $\Delta PcvclA$ and $\Delta PclaeA$ mutants.

Here, we provide a detailed functional analysis of *Pc22g01100*, which according to its homology to related fungal chitinase genes was named *PcchiB1*. Using *PcchiB1* knockout mutants and rescued strains for a functional analysis, we observed significant morphological and physiological phenotypes. So far, these phenotypes are distinct from comparable chitinase knockout strains of other filamentous fungi.

METHODS

Strains and culture conditions. *Escherichia coli* strain XL1-Blue MRF' was used for general plasmid construction and maintenance (Jerpseth *et al.*, 1992). Cloning and propagation of nucleic acids were

performed using standard protocols according to Sambrook & Russell (2001). The *P. chrysogenum* strains used in this study are listed in Table 1. All *P. chrysogenum* cultures were grown, unless otherwise stated, in baffled shake flasks with liquid minimal medium (MM), complete medium (CCM) and production medium at 27 °C and 130 r.p.m., or on MM and CCM solid medium containing 2% (w/v) agar-agar, as described elsewhere (Haas *et al.*, 1997; Minuth *et al.*, 1982). For fluorescence microscopic investigations, *P. chrysogenum* was grown in static liquid CCM cultures for 1–2 days at 27 °C. For quantitative RT-PCR (qRT-PCR) analysis, strains were grown as previously described (Hoff *et al.*, 2010b). All liquid media were inoculated with 10^8 conidiospores. For point inoculations, 10^5 spores were spotted on solid MM with or without 2% (w/v) *N*-acetyl-D-glucosamine (AppliChem).

Transformation of *P. chrysogenum*. Transformation of linear restriction fragments and circular plasmids into *P. chrysogenum* was performed according to Hoff *et al.* (2010b), with some modifications: solid MM containing 5% (w/v) KCl or 20% (w/v) sucrose was used to regenerate protoplasts transformed with the *ptrA* selection marker from *Aspergillus oryzae* (Kubodera *et al.*, 2000). At 24 h after transformation, the medium was overlaid with 11 ml top agar containing 0.7 μ g pyrithiamine ml⁻¹. Germinating protoplasts were transferred onto solid MM supplemented with pyrithiamine (0.7 μ g ml⁻¹) as a selectable substrate. To obtain homokaryotic deletion mutants, single spore isolates were selected and further analysed.

Preparation and analysis of nucleic acids. Preparation of nucleic acids, hybridization of membrane-transferred genomic DNA and total RNA, microarray analysis, PCR and qRT-PCR were carried out as previously described (Hoff *et al.*, 2010b).

In silico sequence analysis. The sequence of *Pc22g01100* published by van den Berg *et al.* (2008) served as template for further analysis (accession no. AM920437). *In silico* prediction of the translation initiation site of *PcchiB1* was performed with the deduced cDNA sequence using the web interface of the NetStart 1.0 server (<http://www.cbs.dtu.dk/services/NetStart/>; Pedersen & Nielsen, 1997). The SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>; Bendtsen *et al.*, 2004) was used to predict signal peptides, and functional domains of the PcChiB1 protein were determined by Interproscan (Zdobnov & Apweiler, 2001). Multiple protein sequences were aligned with CLUSTAL_X (Thompson *et al.*, 1997) by changing the gap opening penalty to the value of 15.00 and the gap extension penalty to 0.3 combined with 25% in the 'delay divergent sequences' option. Alignments were edited and analysed with GeneDoc (Nicholas *et al.*, 1997). Phylogenetic analysis was performed with 1000 bootstraps using the maximum-parsimony method implemented in PHYLIP (Felsenstein, 2005). qRT-PCR analysis was conducted with Opticon Monitor 3.1 software as provided by the manufacturer (MJ Research) and LinRegPCR (Ruijter *et al.*, 2009). Microarray data

Table 1. Fungal strains used in this study

Strain	Genotype	Reference or source
P2niaD18	<i>niaD</i> ⁻	Hoff <i>et al.</i> (2008)
$\Delta Pcku70$	$\Delta Pcku70::nat1; niaD$ ⁻	Hoff <i>et al.</i> (2010b)
$\Delta PclaeA$	$\Delta PclaeA::ble; \Delta Pcku70::nat1; niaD$ ⁻	Hoff <i>et al.</i> (2010a)
Q176	Mutagenized derivative of strain NRRL1951	Backus <i>et al.</i> (1946)
Wisconsin 54-1255	Mutagenized derivative of strain Q176	Elander (1983)
$\Delta PcchiB1m9$	$\Delta PcchiB1::ble; \Delta Pcku70::nat1; niaD$ ⁻	This study
$\Delta PcchiB1m22$	$\Delta PcchiB1::ble; \Delta Pcku70::nat1; niaD$ ⁻	This study
$\Delta PcchiB1m22-re$	$\Delta PcchiB1::ble; \Delta Pcku70::nat1; niaD$ ⁻ ; <i>PgpdA::PcchiB1-DsRed::TtrpC, ptrA</i>	This study

were analysed as previously described by Hoff *et al.* (2010b). Heat maps of expression data and cladograms were generated with TreeView (Page, 1996).

Chitinase assay. To detect extracellular chitinase activity, six biological replicates were grown for 240 and 312 h in MM at 130 r.p.m. and 27 °C. After filtering cultures, three aliquots of each culture medium were taken to determine the amount of secreted proteins, quantified by the Bradford assay (Bradford, 1976). The chitinase assay was performed according to Lopes *et al.* (2008) with the dye-labelled chitin derivative CM-chitin-RBV (Loewe Biochemica; Wirth & Wolf, 1990). Extracellular chitinase activity was assayed photometrically at 550 nm with a DU 7400 spectrophotometer (Beckman). The specific chitinase activity was measured as A_{550} (μg secreted protein) $^{-1}$ according to Pócsi *et al.* (2009).

Microscopic analysis. Pellet formation was analysed in cultures grown in MM at 130 r.p.m. and 27 °C for 240 and 312 h. Bright-field images were taken with a Leica MZ16FA stereomicroscope using the Leica Application Suite 2.0 software (Leica Microsystems). Measurement of pellet size was carried out with an Olympus SZX10 stereomicroscope (Olympus Europa) and ImageJ (<http://rsbweb.nih.gov/ij/>; National Institutes of Health). Fluorescence microscopic investigation was performed with an AxioImager microscope (Carl Zeiss MicroImaging) using a XBO75 Xe or HBO100 Hg lamp for fluorescence excitation. Calcofluor white and filipin staining was observed using Chroma filter set 31000v2 (Chroma Technology). DsRed fluorescence was visualized with Chroma filter set 41035 (Chroma Technology). For cell wall staining of *P. chrysogenum*, samples were incubated for 5 min with 100 $\mu\text{g ml}^{-1}$ calcofluor white (Fluorescent Brightener 28, Sigma-Aldrich) prior to microscopic investigation. The cell membrane of *P. chrysogenum* was stained with filipin (filipin III, Sigma-Aldrich), applied to the sample at a final concentration of 1 $\mu\text{g ml}^{-1}$. Images were captured with a Cool SnapHQ camera (Photometrics) and MetaMorph software (version 6.3r6, Molecular Devices). Recorded images were processed with MetaMorph and Adobe Photoshop CS4.

Calcofluor assay. Solid (MM) was supplemented with calcofluor white (Fluorescent Brightener 28, Sigma-Aldrich) to a final concentration of 200 $\mu\text{g ml}^{-1}$. From a series of 10-fold dilutions derived from a starting suspension of 10^7 conidia ml^{-1} , aliquots of 10 μl were spotted on solid medium and incubated for 4 days.

Construction of *PcchiB1* disruption strains. The sequences and specificities of all oligonucleotides used in this study are given in Supplementary Table S1. To construct a *PcchiB1*-specific knockout cassette, the 5' and 3' flanking regions of *PcchiB1* were amplified by PCR using genomic DNA from P2niaD18 (Table 1) as a template. Primers 5'ChiB1_MluI and 5'ChiB1_BamHI were used for amplification of the 5' flanking region containing a linker for *MluI* and *BamHI* endonucleases, resulting in an amplicon of 1.9 kb. The 1.9 kb 3' flanking region of *PcchiB1* was amplified with the oligonucleotides 3'ChiB1_XbaI and 3'ChiB1_HindIII (Supplementary Table S1). Both PCR fragments were subcloned in pDrive (Supplementary Table S2; Qiagen). Subsequently, both fragments were cloned via *MluI* and *BamHI* (5' flanking region) or *XbaI* and *HindIII* (3' flanking regions) into plasmid pD-Phleo (Hoff *et al.*, 2010b). The resulting recombinant plasmid, pPcchiB1-KO (Supplementary Table S2), carrying the *ble* gene adjacent to the 5' and 3' flanks of the *PcchiB1* locus, was cleaved with *MluI* and *XbaI*, and the 4.6 kb fragment was transformed into ΔPcku70 (Table 1).

Rescue of *PcchiB1* disruption strains. For complementation analysis, $\Delta\text{PcchiB1m22}$ was transformed with plasmid pPcchiB1-DsRed encoding the PcChiB1 protein fused with DsRed at its C terminus (Supplementary Table S2). For efficient expression, the

strong constitutive *gpdA* promoter and the *trpC* terminator from *A. nidulans* (Punt *et al.*, 1990; Mullaney *et al.*, 1985) were used to express the recombinant construct.

RESULTS

Comparative array analysis

We recently reported a microarray analysis using ΔPcvelA and its recipient ΔPcku70 for comparison, which identified 1771 genes that were differentially regulated in ΔPcvelA (Hoff *et al.*, 2010a). Here, in a similar comparative experimental approach with ΔPcku70 as a reference strain, we used microarrays to identify sequences affected by the loss of *PcLaeA*. Isolation of mRNA was performed from a ΔPclaeA knockout mutant grown on production medium for 48, 60 and 96 h. Considering a twofold change in expression levels as a threshold, we found a total of 1282 down- and 789 upregulated genes (Supplementary Fig. S1). Comparable with our previous report for the ΔPcvelA mutant, penicillin biosynthetic genes were downregulated after 60 and 96 h of growth (Hoff *et al.*, 2010a), while other genes for secondary metabolism showed less consistent mRNA levels (Fig. 1a).

However, we found a number of genes involved in conidiophore development that showed significantly altered mRNA levels, which is in agreement with our identification of *PcLaeA* as a main regulator of conidiophore development in *P. chrysogenum* (Fig. 1b; Hoff *et al.*, 2010a). We further detected reduced expression levels of five transcripts encoding putative class III or V chitinases in the ΔPclaeA strain. While the transcripts of the class III chitinases *Pc22g13690* and *Pc21g15780* were reduced after 60 and 96 h of growth, the expression levels of genes encoding the class V chitinases *Pc22g01100*, *Pc13g09520* and *Pc20g02250* were downregulated at every time point analysed (Fig. 1c, Supplementary Table S3). For *Pc22g01100*, the decreased mRNA level was confirmed by qRT-PCR analysis (Fig. 1d).

To identify genes affected by the loss of both *PcvelA* and *PclaeA*, we compared array data obtained previously (Hoff *et al.*, 2010a) with that obtained in this study from the corresponding knockout mutants. The transcriptome analysis showed rather small overlaps of 46 down- and 16 upregulated genes (Fig. 2). Interestingly, BLAST analysis of these sequences revealed that a relative high number of genes presumed to encode C6 zinc cluster transcription factors are found in the group of downregulated sequences (Supplementary Table S4). Notably, the overlap also includes *Pc22g01100*, which shows 13- to 79-fold and 51- to 86-fold reduced transcript levels in ΔPclaeA and ΔPcvelA , respectively (Supplementary Table S3; Hoff *et al.*, 2010a).

Sequence analysis of gene *Pc22g01100*

In the published *P. chrysogenum* genome, *Pc22g01100* is annotated as a putative pseudogene with an undefined start codon (accession no. AM920437; van den Berg *et al.*,

Fig. 1. Expression levels of transcripts affected by the loss of *PclaeA*. The heat maps (a–c) illustrate data from microarray analysis of $\Delta PclaeA$. Transcripts encoding proteins involved in secondary metabolism (a), conidiophore development (b) and chitinases (c) were analysed. Red squares indicate upregulation of gene expression, green squares downregulation. The colour of each square represents the log₂ fold change in expression levels at a given time point. (d) The transcript level of gene *Pc22g01100* as determined by qRT-PCR analysis. At the given time points, log₂ ratios of expression levels compared with the recipient $\Delta Pcku70$ are shown.

2008). Thus, to identify a potential ATG start codon, we generated a cDNA clone for further sequencing of the ORF. *In silico* analysis with the NetStart 1.0 prediction server (Pedersen & Nielsen, 1997) identified two putative

ATG start codons within the ORF. The ATG triplet at nucleotide position 301 is the most probable start site of translation (data not shown), and the corresponding annotation of the *Pc22g01100* coding sequence predicts a

Fig. 2. Venn diagrams of differentially regulated genes in $\Delta PclaeA$ and $\Delta PcvelA$. Only genes which were differentially expressed at all time points (48, 60 and 96 h) were considered. The number of genes in common is given in bold type in the intersection.

protein of 427 aa with an estimated molecular mass of 47 kDa.

The identity of the predicted Pc22g01100 protein was further confirmed by alignment with putative homologous proteins (Supplementary Fig. S2). The protein most similar to Pc22g01100 is a putative class V chitinase from *P. chrysogenum*, Pc13g00130 (accession no. CAP91082.1), sharing 68 % amino acid identity with Pc22g01100. In addition, chitinases ChiB1 from *A. fumigatus* (XP_747065.1) and CTS1 from *Coccidioides immitis* (Q1E3R8.1), with 62 and 57 % identity with Pc22g01100, respectively, were considered orthologous sequences (Supplementary Fig. S2).

Comparison of these four chitinases with homologous chitinases from *Hypocrea jecorina* (chitinase 18-7, accession no. DAA05855.1), *A. nidulans* (ChiB, XP_662475.1), *P. chrysogenum* (Pc13g09520, CAP92021.1) and *A. fumigatus* (AFUB_037900, EDP52624.1) revealed a lack of N-terminal sequences in this second group (Supplementary Fig. S2).

As indicated by *in silico* analysis, the N-terminal sequence of Pc22g01100 most likely represents a putative signal peptide for secretion, which is in accordance to the extracellular localization of Pc22g01100 predicted by WoLF PSORT (Horton *et al.*, 2007). Considering the high similarity of Pc22g01100 to chitinase ChiB1 from *A. fumigatus* (Escott *et al.*, 1998), its gene was named *PcchiB1*.

Using the maximum-parsimony method implemented in PHYLIP (Felsenstein, 2005), we performed a phylogenetic analysis using the predicted protein sequence of PcChiB1 together with six other putative class V chitinases of *P. chrysogenum* listed in the CAZy database (Cantarel *et al.*, 2009). The phylogenetic tree in Fig. 3 confirms the close relationships with other chitinases, as was predicted from the alignment (Supplementary Fig. S2). Interestingly, ChiB, a well-characterized chitinase from *A. nidulans* (Yamazaki *et al.*, 2007) locates together with related sequences in a sister clade; the four other class V chitinases from *P. chrysogenum* (Pc14g01550, Pc16g15030, Pc20g02250 and Pc22g25160) are less closely related to PcChiB1.

Analysis of *PcchiB1* transcript levels and chitinase activity

We used Δ Pcku70 (Hoff *et al.*, 2010b) as a recipient for homologous recombination to construct a *PcchiB1* deletion strain. The screening of 18 primary bleomycin-resistant transformants by PCR analysis using primers ChiB1_for_N and ChiB1_rev_N (Supplementary Table S1) with specificity for the *PcchiB1* gene resulted in a specific amplicon of 402 bp for each transformant (data not shown). Two of these independently obtained transformants were selected to isolate homokaryotic strains from single spore isolates. The resulting mutant strains lacking the *PcchiB1*-specific amplicon were designated m9 and m22. Southern analysis confirmed that no additional recombinant DNA was present in the nuclear DNA of either mutant (Supplementary Fig. S3).

Fig. 3. Phylogenetic tree of class V chitinases from *P. chrysogenum* (P.c. or Pc+sequence identifier), *A. nidulans* (A.n.), *A. fumigatus* (A.f.), *C. immitis* (C.i.), *Trichoderma virens* (T.v.) and *H. jecorina* (H.j.). For *P. chrysogenum*, sequence identifiers were used according to van den Berg *et al.* (2008). *A. fumigatus* and *T. virens* chitinases are listed by protein models of the corresponding databases. Protein sequences of *A. nidulans*, *C. immitis* and *H. jecorina* are represented by GenBank accession numbers. The tree was calculated by the maximum-parsimony method with 1000 bootstrap replicates. Bootstrap values are given for each node.

For characterization of *PcchiB1* transcript levels and chitinase activity in the disruption mutants Δ PcchiB1m9 and Δ PcchiB1m22, recipient Δ Pcku70 with a deleted *Pcku70* gene for efficient gene replacement (Hoff *et al.*, 2010b) and its ancestor, the penicillin producer P2niaD18 (Hoff *et al.*, 2008), served as control strains (Table 1). Northern analysis revealed that the *PcchiB1*-specific transcript accumulated significantly after 312 h of growth in the reference strain P2niaD18 and the recipient Δ Pcku70, whereas no transcript was detected in either knockout strain (Fig. 4).

We further analysed chitinase activity in the supernatant of all strains investigated, and detected about 50 % less extracellular chitinase activity in the knockout mutants after 240 and 312 h of growth in MM (Fig. 5). Intracellular chitinase activity was generally lower for each strain and did not exceed 25 % of extracellular activity (data not shown). These results demonstrate that PcChiB1 contributes to most of the extracellular chitinase activity in *P. chrysogenum*.

Fig. 4. Northern analysis of *PcchiB1* transcript levels. At the indicated time points RNA was isolated from cultures grown in liquid MM. As a control, the *Pcgpd* gene with a constitutive expression level was analysed.

Phenotypic characterization of the disrupted *PcchiB1* strains

When the control and mutant strains were grown on solid CCM, we observed no aberrant mutant phenotype compared with the recipient Δ Pcku70 and P2niaD18. However, when the knockout strains were grown in nutrient-depleted MM for more than 240 h, we detected stable pellet formation in submerged cultures, as characterized by highly entangled, densely packed hyphae. The control strains P2niaD18 and Δ Pcku70 lost this compact mycelial morphology after growth for more than 240 h, resulting in highly fragmented and dispersed hyphae with the formation of only a few small fringed pellets (Fig. 6).

In contrast, Δ PcchiB1m9 and Δ PcchiB1m22 retained the pellet morphology for a growth period of at least 312 h (Fig. 6). The pellet size from at least two biological replicates with a Δ Pcku70 or Δ PcchiB1 background was quantified in submerged cultures with production medium. Both strains grew continuously as mycelial pellets with a pellet diameter of 190–220 μm over a period of 96–168 h (Supplementary Fig. S4). In Δ Pcku70, pellet size clearly decreased after prolonged growth for 240 h, resulting in a mean diameter of 40 μm per pellet. As expected from the microscopic analysis,

the pellet size in Δ PcchiB1m22 decreased only slightly after 240 h of growth, when the mean pellet size was about 175 μm .

Our microscopic analyses and quantification of pellet sizes at different time points indicate that the pellet morphology of Δ Pcku70 is lost during prolonged growth, regardless of the media composition. In contrast the mutant's morphology remains constant over a period of 312 h. Despite the obvious differences in morphology, the mutant and control strains show identical dry weight (data not shown).

Modified cell wall composition of the disrupted *PcchiB1* strains

To analyse whether cell wall integrity was affected by the lack of PcChiB1, both mutants and the control strains were grown on solid MM containing calcofluor white, and as a control, on media lacking calcofluor. All strains exhibited equal conidial germination rates (Supplementary Fig. S5); therefore, restricted growth on calcofluor-containing medium indicates an altered cell wall composition. Different dilutions of conidia were inoculated for 4 days, and the growth of mutant mycelium was compared with mycelium from the parental strains. As can be seen in Fig. 7(a),

Fig. 5. Chitinase activity in the supernatant of cultures after 240 and 312 h of growth in MM. The chitinase activity of each strain was analysed with six biological replicates by the measurement of the A_{550} per microgram of secreted protein.

Fig. 6. Pellet formation of different strains in MM at the indicated time points.

P2niaD18 and both mutants exhibited similar phenotypes on MM, while the fitness of ΔPcku70, recently shown to be more stress sensitive than P2niaD18 (Hoff *et al.*, 2010b), was slightly reduced. In contrast, growth of all strains was significantly reduced when cultivated on MM supplemented

with calcofluor white. However, the two mutant strains were less affected than the control strains (Fig. 7a).

To confirm that the observed phenotype is due to deletion of *PcchiB1*, ΔPcchiB1m22 was rescued by reintroducing a

Fig. 7. Growth assays. (a) Cell wall integrity assay on MM supplemented with 200 μg calcofluor white ml⁻¹ and MM without calcofluor as a control. The indicated number of conidia was allowed to germinate for 4 days. (b) Growth characteristics of mutant strains on solid MM after 6 days and on medium with 2% (w/v) GlcNAc as sole carbon and nitrogen source after 12 days.

plasmid encoding wild-type PcChiB1 fused to DsRed (pPcchiB1-DsRed, Supplementary Table S2). Successful reintegration of the recombinant *PcchiB1* gene was verified by PCR (data not shown) and Southern analysis (Supplementary Fig. S3). The phenotype of the rescued strain Δ PcchiB1m22-re resembled that of the recipient strain Δ Pcku70. From this analysis we surmise that deletion of *PcchiB1* results in a modified cell wall composition, thus generating mutants with increased resistance towards calcofluor. Remarkably, the microscopic analysis showed no obvious morphological differences when the mutant cell wall was stained with calcofluor white (Supplementary Fig. S6).

Since PcChiB1 represents the main extracellular chitinase activity in *P. chrysogenum*, we investigated fungal growth with colloidal chitin as sole carbon and nitrogen source. In addition, mutants were grown on solid or in liquid media containing the chitin monomer *N*-acetylglucosamine (GlcNAc) as the sole N and C source. While with colloidal chitin we observed no growth differences compared with the control strains P2niaD18 and Δ Pcku70 (data not shown), a significant difference was seen with GlcNAc. The disruption strains showed distinct mycelial growth, whereas both control strains were unable to propagate even after prolonged incubation for 12 days (Fig. 7b). This analysis was further extended by testing the producer strain Wisconsin 54-1255 (Elander, 1983) and its ancestor Q176 (Backus *et al.*, 1946). With both liquid and solid media, Q176 showed the same growth phenotype as the two mutant strains. In contrast, Wisconsin 54-1255, as well as the rescued strain Δ PcchiB1m22-re, were unable to use GlcNAc efficiently (Fig. 7b, Supplementary Fig. S7).

The ability of knockout strains to grow on GlcNAc was further analysed at the transcriptional level by Northern hybridization using two different probes with homology to either *NAG1* from the yeast *Candida albicans*, *Pc22g10040* (accession no. XM_002564967), or *CreA* from *A. nidulans*, *Pc20g13880* (XM_002563820). *NAG1* encodes a glucosamine-6-phosphate deaminase, and has previously been shown to be involved in GlcNAc catabolism (Natarajan & Datta, 1993), whereas *CreA* represents a putative regulator of carbon catabolism. While no strain-specific transcript levels were detected for the *NAG1* homologue (data not

shown), the *CreA* probe showed a distinct strain-specific expression pattern, with increased expression levels in Q176 and both chitinase mutants compared with the recipient strain Δ Pcku70 (Fig. 8a). No *CreA* transcript was detected in the rescued mutant Δ PcchiB1m22-re (Fig. 8b) and in the penicillin producer strains Wisconsin 54-1255 and P2niaD18 (data not shown).

Chitinase PcChiB1 is associated with the cell wall

Furthermore, we studied the *in vivo* localization of PcChiB1 in the rescued strain Δ PcchiB1m22-re. As can be seen in Fig. 9, the recombinant PcChiB1 protein fused to DsRed was visible at vacuole-like structures and at the periphery of conidiospores or vegetative fungal cells. To discriminate between the cell wall and the cell membrane, we used the cell wall-staining dye calcofluor white (Elorza *et al.*, 1983; Maeda & Ishida, 1967) and the cell membrane-specific dye filipin (Pearson *et al.*, 2004; Takeshita *et al.*, 2008). At septal structures, the two plasma membranes of the separated hyphal compartments were clearly distinguished by filipin staining, while the PcChiB1-DsRed-specific fluorescence was located between the membranes at the septum, indicating an association of PcChiB1 with the fungal cell wall (Fig. 9).

DISCUSSION

Identification of common genes affected by *PcLaeA* and *PcVelA*, two core components of the velvet-like complex

We recently described the velvet-like complex of the industrial β -lactam producer *P. chrysogenum*, which regulates asexual and hyphal development in *P. chrysogenum*. The disruption of genes for *PcVelA* and *PcLaeA*, two core elements of this complex, leads to significantly reduced penicillin titres caused by a strong transcriptional silencing of the penicillin biosynthetic gene cluster (Hoff *et al.*, 2010a).

The phenotypes of Δ PcvelA and Δ PclaeA, such as altered hyphal branching and positive regulation of the penicillin biosynthetic genes, as well as their physical interaction,

Fig. 8. Northern analysis of *PccreA* transcript levels in strains grown for 10 days in liquid MM with GlcNAc as sole carbon and nitrogen source. (a) Northern analysis of mutants Δ PcchiB1m9 and -m22 in comparison with recipient Δ Pcku70 and strain Q176. (b) Northern analysis of mutants Δ PcchiB1m9 and -m22 in comparison with the rescued strain Δ PcchiB1m22-re. As a control, the *Pcgpd* gene with a constitutive expression level was analysed.

Fig. 9. Fluorescence microscopy of conidiospores and vegetative cells of *P. chrysogenum*. (a) Overview of conidiospore and vegetative hypha. (b) Detail of cell wall and septum of a vegetative hypha. Samples were analysed by differential interference contrast (DIC) microscopy, stained with dyes (calcofluor, filipin) as indicated, or were investigated with a DsRed-specific filter set for detection of the PcChiB1–DsRed fusion protein (PcChiB1). The arrows point to a septum.

imply that both proteins directly or indirectly regulate a common subset of genes. We therefore compared previously reported microarray data for the $\Delta PcvlA$ mutant (Hoff *et al.*, 2010a) with data obtained here using the $\Delta PclaeA$ disruption strain.

To our knowledge, this is the first report from a comparative analysis of array data from two strains lacking main components of a velvet-like complex. This approach successfully identified genes concertedly influenced by the loss of both PcLaeA and PcVelA. One of the most downregulated genes in both knockout mutants was *Pc22g01100*, encoding a class V chitinase.

We recently reported a hyperbranching phenotype for $\Delta PcvlA$ and, to a lesser extent, for $\Delta PclaeA$ (Hoff *et al.*, 2010a). In addition, $\Delta PcvlA$ showed an enhanced pellet formation in submerged cultivation. This was retained for a prolonged growth period, resulting in a higher dry weight compared with the control strain, and suggests a delay in autolysis. Furthermore, calcofluor staining revealed an enhanced fluorescence in the hyphal tips of $\Delta PcvlA$, indicating increased chitin content in this region (Hoff *et al.*, 2010a). A *Neurospora crassa* strain with a deleted homologue of VeA displays a slightly disturbed hyphal branching pattern on solid medium (Bayram *et al.*, 2008b). Similarly, deletion of VeA homologues in *Acremonium chrysogenum* and *Fusarium verticilloides* causes altered hyphal branching, which is thought to be caused by cell wall defects (Dreyer *et al.*, 2007; Li *et al.*, 2006). Due to its strong downregulation in both knockout strains, we asked

whether *Pc22g01100* is functionally responsible for some of the described phenotypes observed in $\Delta PcvlA$ and $\Delta PclaeA$.

$\Delta Ppch1B1$ shows novel and distinct morphological and physiological phenotypes

The *Ppch1B1* transcript is strongly induced when *P. chrysogenum* is grown for a prolonged period. More than 10 days of growth in batch cultures depletes nutrients, which suggests that *Ppch1B1* expression is induced by a lack of carbon and/or nitrogen sources. Increased transcript levels of *Ppch1B1* indicate a functional role for its gene product in degrading chitin from the fungal cell wall in order to supply the fungus with new nutritional resources. Similarly, increased expression levels have been reported for *ech42*, encoding the class V chitinase CHIT42 of *Trichoderma atroviride* and *ChiB* from *A. nidulans*, when grown under various nutrient-depleted conditions (Donzelli & Harman, 2001; Mach *et al.*, 1999; Pócsi *et al.*, 2009; Pusztahelyi *et al.*, 2006; Seidl *et al.*, 2005). Therefore, nutrient-dependent regulation of *Ppch1B1* expression in *P. chrysogenum* is proposed to represent a conserved function common to various class V chitinases.

Consistent with *Ppch1B1* transcript levels, the overall extracellular chitinase activity detected in the supernatant of control and mutant strains increases during prolonged growth. However, after 10 days of growth, chitinase activity is reduced by about 50 % in the disruption mutant, suggesting that PcChiB1 provides the major extracellular chitinase activity in *P. chrysogenum* under these conditions.

Since *PcchiB1* disruption strains exhibit residual extracellular chitinase activity, the remaining extracellular chitinases have to compensate for the lack of PcChiB1 in *P. chrysogenum*. This is in contrast with results obtained from *A. fumigatus* with a deleted *ChiB1* gene. While after 6 days of growth the wild-type strain exhibits maximum extracellular chitinase activity, the disruption strain shows almost no residual chitinase activity (Jaques *et al.*, 2003).

In *P. chrysogenum*, the maximum level of extracellular chitinase activity is accompanied by the onset of pellet disintegration in the two control strains, P2niaD18 and Δ Pcku70. Conversely, the reduced chitinase activity in both knockout strains, Δ PcchiB1m9 and Δ PcchiB1m22, correlates with stable pellet formation after prolonged growth, albeit that dry cell weight was identical in all strains investigated. This suggests that PcChiB1 is required for cell wall integrity. PcChiB1 seems thus to be responsible for the initial breakdown of hyphal structures, leading to the disintegration of pellets, but is not exclusively responsible for the complete lysis of fungal cell walls. The remaining extracellular chitinase activity detected in the autolytic cultures of the knockout mutants, and the presence of six other class V chitinases encoded in the genome, mean that further chitinases are likely involved in the lysis of *P. chrysogenum* cultures under nutrient-depleted conditions.

Similar results have been reported for *A. nidulans*, whereby strong induction of chitinase ChiB is observed in cultures starved for carbon sources (Pócsi *et al.*, 2009; Pusztahelyi *et al.*, 2006). In this fungus, the induction of gene expression is consistent with the increase in ChiB protein levels and extracellular activity, and correlates well with the observed pellet disintegration discussed above (Pócsi *et al.*, 2009; Shin *et al.*, 2009; Yamazaki *et al.*, 2007). In contrast to the unaltered dry cell weight in *PcchiB1* mutants of *P. chrysogenum* and a *ChiB1* mutant of *A. fumigatus* (Jaques *et al.*, 2003), deletion of *ChiB* in *A. nidulans* results in a slower decrease in mycelial dry weight in carbon-starved cultures. This is accompanied by an almost complete loss of extracellular chitinase activity (Pócsi *et al.*, 2009; Shin *et al.*, 2009; Yamazaki *et al.*, 2007). However, since overexpression of *ChiB* does not drive the reduction of dry cell weight, Shin *et al.* (2009) suggest that ChiB is necessary, but not sufficient, for autolysis in *A. nidulans*. In conclusion, it can be suggested that additional chitinases yet to be identified contribute to the autolytic process in response to nutrient depletion in *P. chrysogenum*, *A. nidulans* and *A. fumigatus*. This hypothesis is also supported by the observation that staining of hyphae of Δ PcchiB1 mutants with the chitin-intercalating fluorescent dye calcofluor white revealed wild-type-like fluorescence in the mutant cell wall, thus indicating that the *PcchiB1* deletion does not cause a major change in the chitin content of the cell wall. However, when we used calcofluor white to analyse cell wall integrity, deletion of *PcchiB1* conferred enhanced resistance to calcofluor white. We propose that hydrolysis of chitin microfibrils is reduced in the Δ PcchiB1 mutant. According to the chitin turnover model of Bartnicki-Garcia (1973), which requires a delicate

balance of chitinases and chitin synthases to provide cell wall plasticity, a lower level of chitin hydrolysis results in a less severe interfering effect of calcofluor white due to lower chitin microfibril assembly rates. In agreement with the proposed role of PcchiB1 in the initial breakdown of hyphal structures, this would cause the higher resistance of the disruption mutants to calcofluor white, while the chitin content of their cell walls remains mainly unaltered.

Although the stable pellet formation observed for Δ PcchiB1 in submerged cultures is reminiscent of the pellet morphology of Δ PcvelA (Hoff *et al.*, 2010a), notable phenotypic differences concerning dry weight in submerged cultures and calcofluor staining of hyphae are obvious when the two strains are compared. The drastic decrease in transcript levels of *PcchiB1* is probably not exclusively responsible for the increase in cell dry weight and calcofluor fluorescence observed for Δ PcvelA. Other genes involved in chitin catabolism, such as *NagA* encoding β -1,4-*N*-acetylglucosaminidase (Hoff *et al.*, 2010a; Díez *et al.*, 2005), most probably are also affecting the phenotype of this deletion strain.

Fungal growth with colloidal chitin as the sole carbon and nitrogen source indicated that PcChiB1 activity is not a rate-limiting step in the catabolism of externally supplied chitin. Probably, the remaining activities provided by other chitinases are sufficient to degrade exogenous chitin. An unexpected, and to our knowledge novel, phenotype was detected when the Δ PcchiB1 mutant and control strains were grown in media containing GlcNAc as the sole carbon and nitrogen source. Only the knockout mutants and the ancestor strain of the Wisconsin lineage, Q176 (Backus *et al.*, 1946), were able to use the final product of chitin catabolism efficiently. This strain-specific phenotype suggests that the ability to use GlcNAc was lost during a mutagenesis programme that led to the producer strain Wisconsin 54-1255 (Elander, 1983).

In the published sequence of Wisconsin 54-1255, all genes presumed to be involved in GlcNAc catabolism, based on their well-characterized homologues in the yeast *C. albicans*, are present as intact ORFs (van den Berg *et al.*, 2008; Wendland *et al.*, 2009; Yamada-Okabe *et al.*, 2001). Moreover, transcript levels of the *P. chrysogenum* homologue of the *NAG1* gene showed no strain-specific expression pattern, indicating that GlcNAc catabolism is functional in Wisconsin 54-1255 and its descendants. When the expression of the homologue of *CreA*, a major regulator of carbon repression in *A. nidulans* (Arst & Cove, 1973; Dowzer & Kelly, 1989), was analysed in response to GlcNAc, increased transcript levels were observed in Q176 and deletion mutants. As indicated by the rescued strain lacking any detectable *CreA* transcript, increased *CreA* levels in disruption mutants are a direct or indirect consequence of *PcchiB1* deletion.

Chitinase PcChiB1 is associated with the cell wall

Microscopic analysis of the cellular localization of the PcChiB1–DsRed fusion protein revealed predominant

fluorescence at the fungal cell wall. To our knowledge, this is the first report of a class V chitinase associating with the cell wall. However, the primary structure of PcChiB1 contains no detectable motif for directing PcChiB1 to the cell wall: neither a chitin-binding motif, as found in the cell wall-located chitinase CTS1 from *Saccharomyces cerevisiae* (Kuranda & Robbins, 1991), nor a glycosylphosphatidylinositol (GPI) anchor for cell wall localization (Bowman *et al.*, 2006; Li *et al.*, 2007) can be predicted for the PcChiB1 sequence. Since PcChiB1 possesses a signal peptide for secretion (Perlman & Halvorson, 1983; von Heijne, 1986), we propose that cell wall fluorescence of PcChiB1–DsRed occurs when PcChiB1 is translocated to the cell wall just before being secreted by the secretory pathway of *P. chrysogenum*.

Further understanding of the function of PcChiB1 and other chitinases from *P. chrysogenum* can provide advantages in the fermentation of industrially important filamentous fungi. Manipulation of fungal morphology can contribute to cost-efficient production processes by lowering the energy required for aeration and stirring followed by filtering of fungal cells.

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