Impact of Velvet Complex on Transcriptome and Penicillin G Production in Glucose-Limited Chemostat Cultures of a β -Lactam High-Producing *Penicillium chrysogenum* Strain

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Abstract

The multicomponent global regulator Velvet complex has been identified as a key regulator of secondary metabolite production in *Aspergillus* and *Penicillium* species. Previous work indicated a massive impact of *PcvelA* and *PclaeA* deletions on penicillin production in prolonged batch cultures of *P. chrysogenum*, as well as substantial changes in transcriptome. The present study investigated the impact of these mutations on product formation and genome-wide transcript profiles under glucose-limited aerobic conditions, relevant for industrial production of β -lactams. Predicted amino acid sequences of *PcVelA* and *PcLaeA* in this strain were identical to those in its ancestor Wisconsin54-1255. Controls were performed to rule out transformation-associated loss of penicillin-biosynthesis clusters. The correct *PcvelA* and *PclaeA* deletion strains revealed a small reduction of penicillin G productivity relative to the reference strain, which is a much smaller reduction than previously reported for prolonged batch cultures of similar *P. chrysogenum* mutants. Chemostat-based transcriptome analysis yielded only 23 genes with a consistent differential response in the *PcvelA* Δ and *PclaeA* Δ mutants when grown in the absence of the penicillin G side-chain precursor phenylacetic acid. Eleven of these genes belonged to two small gene clusters, one of which contained a gene with high homology to the aristolochene synthase. These results provide a clear caveat that the impact of the Velvet complex on secondary metabolism in filamentous fungi is strongly context dependent.

Background

ASCOMYCETOUS FILAMENTOUS fungi produce a large range of structurally diverse, complex low-molecular-weight compounds known as secondary metabolites. Secondary metabolites are generally nonessential for survival and highly species or even strain specific (Keller et al., 2005) but may positively affect growth, physiology, or reproduction of the producing organism (Demain and Fang, 2000; Fox and Howlett, 2008; Luckner, 1991; von Dohren, 2004). In addition, several fungal secondary metabolites are of applied interest, for example, because of their role as toxins or their application as antibiotics (Demain and Adrio, 2008; Manzoni and Rollini, 2002; van der Beek and Roels, 1984).

The genes involved in fungal secondary metabolic pathways share a tendency towards physical clustering, with a

preference for subtelomeric regions (Galagan et al., 2005; van den Berg et al., 2008). Evidence is mounting that this spatial organization contributes to regulation of these pathways. Subtelomeric gene regulation was observed in *Aspergillus parasiticus*, in which chromosomal translocation of a secondary metabolite gene cluster resulted in deregulated expression (Chiou et al., 2002). Expression of the *A. parasiticus ver-1* and *nor-1* genes, involved in aflatoxin biosynthesis, was shown to depend strongly on chromosomal localization, thereby revealing a cellular strategy for activation or repression of entire biosynthetic gene clusters (Palmer and Keller, 2010; Shwab and Keller, 2008). Over the past years, several reports established epigenetic regulation of cryptic secondary metabolite clusters, in which coexpression of the clustered genes was directly affected by chromosomal remodeling. In *A. nidulans*, deletion of *cclA*, a *BRE2* ortholog involved in histone H3

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lysine 4 methylation, resulted in activation of the expression of secondary metabolite clusters (Bok et al., 2009).

The Velvet complex, an intensively studied multicomponent global regulator, is involved in morphogenesis as well as in the regulation of a wide range of secondary metabolite pathways in filamentous fungi (Bayram et al., 2008; Kosalkova et al., 2009). In *A. nidulans*, the core of the complex is composed of three subunits VeA, VelB, and LaeA (Bayram et al., 2008). The Velvet protein VeA has been identified as a positive transcriptional regulator of genes involved in secondary metabolism and as a negative regulator of asexual development (Dreyer et al., 2007; Kato et al., 2003; Krappmann et al., 2005). LaeA is a methyltransferase involved in activation of secondary metabolism genes through epigenetic control (Bayram et al., 2008; Keller et al., 2005; Reyes-Dominguez et al., 2010). VelB, a VeA-like protein, seems to be necessary for recruiting the regulator of sporogenesis VosA. Through VeA, the complex interacts with light responsive proteins, FhpA, LreA, and LreB or the α -importin KapA (Bayram et al., 2008).

Orthologs of the Velvet complex components in several fungi show a conservation of amino acid sequence and function, but also some degree of flexibility (Calvo, 2008; Hoff et al., 2010). As previously reported (Dreyer et al., 2007), in *P. chrysogenum* the predicted protein sequence encoded by the ORF Pc13g13200 exhibited 61% sequence similarity and 48% sequence identity with *A. nidulans* VelA. The predicted protein sequence of Pc16g14010 showed a 55% sequence identity to the amino acid sequence of *A. nidulans* LaeA. In *P. chrysogenum*, *PclaeA* (Pc16g14010), and *PcvelA* (Pc13g13200) knock-down mutants have been constructed in two strain backgrounds with different penicillin biosynthesis ability: Wisconsin54-1255 (an early strain in the improvement program) and P2niaD (a penicillin high-producing strain derived from the Panlabs strain improvement program) (Newbert et al., 1997). The *PclaeA* deletion resulted in transcriptional downregulation of all genes involved in penicillin biosynthesis and a drastic reduction of penicillin production (by circa 80%) (Hoff et al., 2010; Kosalkova et al., 2009). Deletion of *PcvelA* in the high-producing strain had a similar impact (Hoff et al., 2010). Moreover, transcriptome analysis of *PclaeA* and *PcvelA* deletions in strain P2niaD revealed a widespread impact, with over 10% of the genome being affected by these deletions, with a clear overrepresentation of genes involved in secondary metabolite pathways and in fungal development (Hoff et al., 2010). These observations are in line with studies on other filamentous fungi, in which deletion of *veA* or *laeA* was similarly shown to lead to downregulation of the production of several secondary metabolites (Calvo, 2008; Dreyer et al., 2007; Horwood et al., 2004; Wiemann et al., 2010).

Previous studies on the impact of the Velvet complex in *P. chrysogenum* were performed in prolonged batch cultures. Time course analysis revealed that the impact of *PcvelA* and *PclaeA* mutations was most pronounced after prolonged incubation (Hoff et al., 2010), but the physiological status of these cultures was not precisely defined. Industrial production of β -lactam antibiotics is performed in sugar-limited, aerobic fed-batch cultures (Menezes et al., 1994). The aim of the present study is to characterize the genetic basis of the Velvet complex in the *P. chrysogenum* high penicillin producing strain DS17690 and to investigate its impact on physiology, penicillin production, and transcriptional regulation

under industrially relevant conditions. To this end, we studied the impact of *PcvelA* and *PclaeA* deletions in aerobic, glucose-limited chemostat cultures of the penicillin high-producing strain *P. chrysogenum* DS17690.

Methods

Strains

Penicillium chrysogenum strains used in this study are listed in Table 1. DS17690 is a high penicillin producing strain, resulting from the DSM classical strain improvement program (DSM Anti-Infectives, Delft, The Netherlands). *P. chrysogenum* strains DS63171 (*hdfA* Δ *PcvelA* Δ) and DS67261 (*hdfA* Δ *PclaeA* Δ) were derived from strain DS54465 (*hdfA* Δ) (Snoek et al., 2009), which has a high frequency of homologous recombination. Requests for academic use of the *P. chrysogenum* strains, under a material transfer agreement, should be addressed to Prof. R.A.L. Bovenberg (DSM Anti-Infectives, P.O. Box 425, Delft NL-2600 AK, The Netherlands).

Deletion cassette construction

All *P. chrysogenum* gene fragments were amplified from genomic DS17690 DNA using Phusion Hot-Start polymerase (Finnzymes, Landsmeer, The Netherlands). Vector pDONR™ P4-P1R (Invitrogen, Breda, The Netherlands) was used to clone the 782-bp 5'-flanking region of the Pc16g14010 (*PclaeA*) gene. This fragment was PCR amplified with primers attB4F5 Pc16g14010HindIII and attB1R4 Pc16g14010 (Table 2). The vector and the fragment were recombined with BP clonases (Invitrogen) yielding pDONR41-5'FR 16g14010. pDONR™ P2-P3R (Invitrogen) was used to clone the 981-bp 3'-flanking region of the Pc16g14010 gene. This fragment was PCR amplified with the primer pair attB2F Pc16g14010/attB3R Pc16g14010 HindIII. The vector and the fragment were recombined with BP clonase yielding pDONR23-3'FR 16g14010. pDONR™ 221 Gateway® vector (Invitrogen) was used to clone the *A. nidulans* acetamidase-gene (*amdS*) gene under control of the *A. nidulans* *gpdA* promoter and *penDE* terminator, which were amplified from plasmid pBlueAMDS (Nijland et al., 2010) with primers attB1F AMDS and attB2R AMDS. The three fragments were then combined with the pDEST™ P4-R3 in the LR reaction of the Multisite Gateway® Three-Fragment Vector Construction Kit (Invitrogen)

TABLE 1. *P. CHRYSOGENUM* STRAINS USED IN THIS STUDY

Strain	Genotype/ description	Reference
DS17690	High penicillin producer	(Harris et al., 2007)
Wisconsin 54-1255/ ATCC28089	Ancestor of DS17690	(Macdonald et al., 1964)
DS54465	<i>hdfA</i> Δ	(Snoek I.S. et al., 2009)
DS50652	[<i>pcbAB-pcbC-penDE</i>] Δ	(Nijland et al., 2010)
DS63171	<i>hdfA</i> Δ Pc13g13200/ <i>PcvelA</i> Δ - <i>amdS</i>	This work
DS67261	<i>hdfA</i> Δ Pc16g14010/ <i>PclaeA</i> Δ - <i>amdS</i>	This work

TABLE 2. PRIMERS USED IN THIS STUDY

Primer	Sequence (5'–3')
attB4F5 Pc16g14010HindIII	GGGGACAACCTTTGTATAGAAAAGTTGAAGCTTCAACCTCTAGTTACCGGTAGCGCG
attB1R4 Pc16g14010	GGGGACTGCTTTTTTGTACAAACTTGGCGTTCGAGGCGTGGGATGCCTG
attB2F Pc16g14010	GGGGACAGCTTTCTTGTACAAAGTGGGTGAAGCATAGCAATCGACCGCC
attB3R Pc16g14010 HindIII	GGGGACAACCTTTGTATAATAAAGTTGGTTGGTCTACAATCCGGCGTTGGG
attB1F AMDS	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGCAGGAATTCGAGCTCTGTAC
attB2R AMDS	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGCTCGTACCATGGGTTGAG
F-amds	GAAAGTCCAGACGCTGCCTGCG
R-amds	CCCTGGTGGCATATGTTAGCTG
F14010	GTGCTATGGCTAACTGGTACTCG
R14010	TTTCGCGCTTGATAGATGTGCAG
F γ -actin gDNA	TTCTTGGCCTCGAGTCTGGCGG
R γ -actin gDNA	GTGATCTCCTTCTGCATACGGTCCG
F-IGR Pc20g07090	GTTCTATAGGACGTAGCTCCGC
R-IGR Pc20g07090	AAATCAGCTCTACTAGCGATCCGC
F-pcbAB	CACTTGACGTTGCGCACCGGTC
R-pcbAB	CTGGTGGGTGAGAACCTGACAG
F-pcbC	AGGGTTACCTCGATATCGAGGCG
R-pcbC	GTCGCCGTACGAGATTGGCCG
PVF	CCTTCGCCGACTGAGGAGTACGGAGTAGCTTGCGGTGACTTTTCATTC
PVR	TCTTAGACGCTCCGGAGCATAGAAGTTTCGCGGATTGATGTTGTTATCCAGATATTC
OVF	ATGGCCAAACAGACCATCTCTCATGCCACCTC
OVR	CCTTCGCCGACTGATGTACTCGACGGAGGAGCTTTCATGACTATTTTG
A3F	TTTTTGTCAAGCTTACATATGCCACCAGGGCTAC
A3R	ATGGATGGATCCATAACTTCGTATAATGTATGCTATACGAAGTTATGT TGAGTGGTATGGGGCCATCC
A5F	CTGGAATTGTTTAAACGCGGCCGCCGCTGCAGGATAACTTCGTATAGCATAC ATTATACGAAGTTATGACTCTTTCTGGCATGCGGAGAGAC
A5R	TTCTGGAGAAGCTTGTCCCAGAGCTCGTTCATGTTAACAG
TCF	CGAGGAGCACCTGCAGGCCGACGCCGACCAACACCGCC
TCR	CCGCCAGTGTTTAACTAGCGGCCGCATGGCGCGCCGTATTGGGTGTTACGGAGCA TTCAC
TWF	TGCAGCGCGTTAGAATAC
TWR	CGGATCCTTCGCCGACTGA
F13g13200	CAGTACCGAGTCCATGTATGCCGGG
R13g13200	GGGGGAAGTTTGTGTGGTACCTGG
RTamdS	GCCACAGGTGACTCTGGATGG
FpgdA	GTCTCTCCGCATGCCAGAAAG
veA Fw	CACCATGGCCAACAGACCATCTCTCATGC
veA Rv	TTATGTACTCGACGGAGGAGCTTTC
LaeA Fw	CACCATGTCTTACCGAGAGTCATCTGGTTCCTTTC
LaeA Rv	TTATTCCTCGACTGGTTTTTCGCGCTTG

resulting in the destination vector (pDest43-KO 16g14010). This vector contains flanking *HindIII* restriction sites that were used to cut out the deletion cassette. The Pc16g14010/*PclaeA* deletion cassette was transformed in DS54465 (*hdfAΔ*) protoplasts using the acetamidase gene (*amdS*) as selection marker (Kolar et al., 1988), resulting in strain (*hdfAΔ PcvclAΔ*).

For the mutation of the Pc13g13200 (*PcvclA*) gene, the promoter and open reading frame (ORF) were PCR amplified using the primer pairs PVF/PVR and OVF/OVR, respectively

(Table 2). Both fragments contained a 14-bp tail to facilitate directional and efficient cloning, via the STABY method (Eurogentec, Maastricht, The Netherlands). Two variants of the standard STABY vector, pSTC1.3, were constructed for individual cloning of both fragments. pSTamdSL was constructed by insertion of a 1.8-kb fragment of the *amdS* expression cassette in pSTC1.3. The fragment of 1.8 kb that contains the last 2/3 of *amdS* was PCR amplified from the plasmid pHELY-A1 (van den Berg et al., 2004) using primers

A3F and A3R (Table 2). The fragment and the plasmid pSTC1.3 were then digested with the restriction enzymes *HindIII*-*BamHI* and the two fragments were ligated overnight using T4 ligase (Invitrogen) as recommended by the STABY-protocol (Eurogentec) yielding the plasmid pSTamdSL. Similarly, the plasmid pSTamdSR was constructed by inserting an overlapping 2.4-kb fragment covering the *A. nidulans* *gpdA* promoter and the first 2/3 of the *amdS* expression cassette. The fragment was PCR amplified using pHELY-A1 as template and the primers A5F and A5R. In the process the *EcoRV* site found in the *PgpdA* sequence was removed. Subsequently, the PCR fragment and pSTC1.3 were digested with *HindIII*-*PmeI*. The two fragments were ligated as previously mentioned. Next, a strong terminator was inserted in front of the *PgpdA*-*amdS*. To do so, the 0.7-kb *trpC* terminator was PCR amplified using primers TCF and TCR (Table 2) from pAN7-1 (Punt et al., 1987) and introduced via the *SbfI*-*NotI* sites of *PgpdA*-*amdS* yielding the plasmid pSTamdSR. Both vectors contained an overlapping but nonfunctional fragment of the fungal selection marker *amdS* gene, encoding acetamidase and allowing recipient cells that recombine the two fragments into a functional selection marker to grow on agar media with acetamide as the sole nitrogen source (Tilburn et al., 1983). Plasmid pSTamdSL and pSTamdSR were transformed to chemically competent CYS21 *Escherichia coli* cells (Eurogentec). Transformants were used to PCR amplify the gene specific sequences fused to the nonfunctional *amdS* fragments using oligonucleotides TWF and TWR. The obtained PCR fragments were combined and used to transform *P. chrysogenum* DS54465 (*hdfAΔ*) (Snoek et al., 2009) protoplasts resulting in strain DS67261 (*hdfAΔ PclaeAΔ*).

Confirmation of the mutations by Southern blotting

Genomic DNA of four transformants and the DS54465 were isolated using the E.Z.N.A. Fungal DNA kit (Omega Bio-tek, Amsterdam, The Netherlands). Genomic DNA (2.5 µg) was digested using the *NdeI* restriction enzyme, separated on a 0.8% agarose gel, and blotted onto a Zeta-Probe membrane (BioRad, Hercules, CA) (Sambrook et al., 1989). The 5'-flanking region of the *Pc16g14010* gene was used as probe, which was labeled with digoxigenin using the PCR DIG Probe Synthesis Kit (Roche, Paris, France) according to the manufacturer's instructions. Hybridization was done overnight at 42°C in hybridization buffer (50% formamide, 5× SSC, 2% blocking reagent (Roche), 0.1% Na-lauroylsarcosine, 0.02% SDS) and the membranes were washed twice with 2× SSC, 0.1% SDS for 15 min, and twice with 0.2× SSC, 0.1% SDS. Digoxigenin-labeled probes were detected by chemiluminescence using CPD-star (Roche).

Confirmation of the mutations by PCR

To confirm the correct modification of the gene *Pc13g13200* (*PcvelA*) with the use of a bipartite vector, the primer pairs and F13g13200-RtamdS, F13g13200-R13g13200, and FgpdA-R13g13200 (Table 2) were used. To confirm the deletion in the strain *PclaeAΔ*, both the native *Pc16g14010* gene and the *Pc16g14010::amdS* deletion were amplified using primers F14010 and R14010 (Table 2 and Figure 1).

Penicillin gene cluster copy number determination by quantitative PCR analysis

To analyze the number of penicillin biosynthetic gene clusters in the transformants (*hdfAΔ PcvelAΔ*) and DS67261 (*hdfAΔ PclaeAΔ*), γ -actin (with the primers F γ -actin gDNA and R γ -actin gDNA) (Table 2) and an intergenic target (using the primers F-IGR *Pc20g07090* and R-IGR *Pc20g07090*) (Table 2) were used as reference templates in qPCR. The primers for *pcbAB* (F-*pcbAB* and R-*pcbAB*) and *pcbC* (F-*pcbC* and R-*pcbC*) (Table 2) were used to assess the cluster copy number in genomic DNA. *P. chrysogenum* strains DS54465 (*hdfAΔ*), Wisconsin54-1255, and DS50652 (lacking penicillin biosynthesis gene cluster) (Table 1) were used as controls containing 8, 1, and 0 penicillin gene clusters, respectively. The gene copy numbers were analyzed on a MiniOpticon™ system (BioRad) using the BioRad CFX manager software in which the C(t) values were determined automatically by regression. The SensiMix™ SYBR mix (Bioline, Alphen aan den Rijn, The Netherlands) was used as a master mix for qPCR with 0.4 µM primers and 40 ng gDNA in a 25-µL reaction volume. The copy numbers were calculated from duplicate experiments.

Sequencing of DS17690 *PcvelA* and *PclaeA* alleles

PcvelA and *PclaeA* genes were PCR amplified from DS17690 genomic DNA with the primers pairs *veA* Fw-*veA* Rv and *laeA* Fw-*laeA* Rv, respectively (Table 2). Genomic DNA was isolated as mentioned earlier. The PCR products were sequenced by the Sanger method at Baseclear (Leiden, The Netherlands). The resulting sequences were compared to the corresponding Wisconsin54-1255 sequences using Clustal W software (Larkin MA et al., 2007).

Media and culture conditions

P. chrysogenum strains were grown in a glucose-limited defined mineral medium with the following composition per liter: 0.8 g KH_2PO_4 , 3.5 g $(\text{NH}_4)_2\text{SO}_4$, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 7.5 g glucose, and 10 ml of trace elements solution. The trace element solution contained $15 \text{ g} \cdot \text{L}^{-1}$ $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, $0.5 \text{ g} \cdot \text{L}^{-1}$ $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $2 \text{ g} \cdot \text{L}^{-1}$ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $2 \text{ g} \cdot \text{L}^{-1}$ $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $4 \text{ g} \cdot \text{L}^{-1}$ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and $0.5 \text{ g} \cdot \text{L}^{-1}$ $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Penicillin G production was induced by adding $0.58 \text{ g} \cdot \text{L}^{-1}$ of phenylacetic acid (PAA) to the medium. The necessary amount of KOH was added to set the medium pH at 5.5.

All strains were grown in aerobic, glucose-limited chemostat cultures in a 3-L bioreactor (Applikon, Schiedam, The Netherlands) at pH6.5 and at a dilution rate of 0.03 h^{-1} as previously described (Harris et al., 2009b). Chemostat cultures were assumed to be in steady state when, after at least 5 volume changes of continuous cultivation under the same conditions, culture dry weight, and off gas CO_2 analyses differed by less than 4% over two consecutive volume changes (Daran-Lapujade et al., 2009).

Analytical methods

Biomass dry weight was measured in duplicate, by filtering 10 mL of culture sample over preweighed glass fiber filters (Type A/E, Pall Life Sciences, East Hills, NY). The filters were washed with demineralized water, dried in a microwave oven for 20 min at 600 W, and subsequently weighted.

FIG. 1. Strain construction scheme. Construction and characterization of the strains DS63171 (*PcvelA*Δ/*Pc13g13200*) (A, B) and DS67261 (*Pc16g14010PclaeA*Δ) (C, D). (A) Schematic representation of DS63171 (*Pc13g13200/PcvelA*Δ) strain construction using bipartite vectors. The arrows represent the primer pairs used to verify the correct gene insertion, with respective fragment size. (B) PCR confirmation of *PcvelA* deletion and integration of *amdS* marker in strain DS63171 (*hdfA*Δ *PcvelA*Δ). (C) Schematic representation of DS67261 (*hdfA*Δ *PclaeA*Δ) strain construction. The arrows represent the primer pairs used to verify the correct gene insertion, with respective fragment size. (D) PCR confirmation of *amdS* integration in strain DS67261 (*hdfA*Δ *PclaeA*Δ) and comparison to *PclaeA* gene in reference strain DS17690. L denotes the ladder used.

Glucose and PAA concentrations in media and culture supernatants were analyzed by HPLC (Waters Alliance 2695 Separation Module supplied with a Waters 2487 Dual Absorbance Detector and a Waters 2410 Refractive Index Detector; Waters, Milford, MA) containing a Biorad HPX87H column (BioRad) eluted at 60°C with 0.5 mM H₂SO₄ at a flow rate of 0.6 mL·min⁻¹. Quantitative ¹H-NMR was used to measure extracellular concentration of penicillin G, ACV, and IPN from *P. chrysogenum* cultures. Quantitative ¹H-NMR experiments were performed at 600 MHz on a Bruker Avance 600 spectrometer (Bruker, Wormer, The Netherlands). To a known quantity of filtrate, a known quantity of internal standard (maleic acid), dissolved in phosphate buffer, was added prior to lyophilization. The residue was dissolved in D₂O and measured at 300 K. The delay between scans (30 s) was more than five times T₁ of all compounds, so the ratio between the integrals of the compounds of interest and the integral of the internal standard was an exact measure for the quantity of the penicillins.

Transcriptome analysis

Chemostat sampling was performed by rapidly filtering 60 mL of culture broth over a glass fiber filter (Type A/E, Pall Life Sciences). The filter with mycelium was immediately wrapped in aluminum foil, quenched in liquid nitrogen, and stored at -80°C. Samples were processed as previously described (Harris et al., 2009a; van den Berg et al., 2008). Acquisition and quantification of array images and data filtering

were performed by using Affymetrix GeneChip Operating Software (GCOS version 1.2). All arrays were globally scaled to a target value of 100, using the average signal from all genes (global scaling). The arrays were analyzed as previously described (Harris et al., 2009a). To assess statistically significant changes in expression, replicate arrays experiment were compared by using the software Significance Analysis of Microarray (SAM version 2.0) (Tusher et al., 2001). The fold-change was set to 2.5 and a maximum false discovery rate of 7% was used. Gene clusters were evaluated for enrichment in MIPS (Munich Information Center for Protein Sequences) categories (version 1.3) by employing hypergeometric distribution with a *p*-value cutoff of 10⁻³ (Harris et al., 2009a; Knijnenburg et al., 2009). The transcriptome data analyzed in this study have been deposited at the Genome Expression Omnibus database <http://www.ncbi.nlm.nih.gov/geo/> under the accession number GSE31633. The transcriptome data of the Wisconsin54-1255 were derived from the GEO series GSE9825.

Results

Velvet complex genes are conserved in Penicillium chrysogenum

The ascomycetous fungus *Penicillium chrysogenum* is phylogenetically closely related to *Aspergillus* species (van den Berg et al., 2008; Verweij et al., 1995). Based on the extensive molecular analysis of the *A. nidulans* Velvet complex (Bayram

TABLE 3. SIMILARITY OF VELVET COMPLEX ORTHOLOG PROTEINS IN *P. CHRYSOGENUM*

<i>A. nidulans</i>	<i>P. chrysogenum</i>	Score	E-value	Similarity (%)
LreA	Pc20g08380	641	0	60
LreB	Pc22g02540	264	3.00 ^{E-71}	55
FphA	Pc06g00040	1168	0	70
KapA	Pc21g01970	1034	0	97
LaeA	Pc16g14010	PcLaeA	2.00 ^{E-136}	75
VeA	Pc13g13200	PcVelA	4.00 ^{E-119}	61
VelB	Pc22g22320		4.00 ^{E-80}	63
VosA	Pc22g06890		6.00 ^{E-110}	66

A BlastP was performed using *Aspergillus nidulans* query to search translation of *P. chrysogenum* ORFeome (van den Berg et al., 2008).

et al., 2008; Calvo, 2008; Fischer, 2008; Kato et al., 2003; Kim et al., 2002; Sprote and Brakhage, 2007), a genome-wide search was performed on the *P. chrysogenum* protein database (comprising 13,670 proteins; Genbank accession number: AM920416 to AM920464) (van den Berg et al., 2008) for sequences orthologous to the *A. nidulans* Velvet complex proteins. This search yielded clear orthologs for all eight subunits of the *A. nidulans* Velvet complex (Table 3).

Our analysis confirmed the previously reported (Dreyer et al., 2007) absence in PcVelA (Pc13g13200) of a PEST sequence, a motif implicated in targeted protein degradation, and clear difference in the nuclear export and localization motives. The predicted protein sequence of PcLaeA (Pc16g14010) (55% sequence identity to the *A. nidulans* LaeA amino acid sequence) exhibited four amino acids substitution in the methyltransferase domain (199-TGIWSIEVANKFPGSFV-215) compared to the *A. nidulans* sequence (positions 203 S → A, 210 F → Y, 212 G → D, and 213 S → A).

To investigate a possible impact of classical strain improvement (Newbert et al., 1997) on *PcvelA* and *PclaeA*, the sequences of these genes were compared between the β -lactam high producing strain DS17690 and its parental strain Wisconsin54-1255. Two single nucleotide differences were identified in DS17690 *PcvelA*, in positions 343 (C^{Wisconsin} → T^{DS17690}) and 585 (C^{Wisconsin} → T^{DS17690}) and one in DS17690 *PclaeA* in position 1012 (G^{Wisconsin} → A^{DS17690}). However, these mutations did not alter the predicted amino acid sequences of the PcLaeA and PcVelA proteins.

Construction of *PcvelA* and *PclaeA* knock-down strains

To further investigate the role of the Velvet complex as a global regulator in penicillin-producing *P. chrysogenum* cultures, *PcvelA* and *PclaeA* mutants were constructed by protoplast transformation of strain DS54465 (DS17690 *hdfAΔ*) (Snoek et al., 2009). Correct integration of the *amdS* cassettes was confirmed by Southern blot (data not shown) and PCR (Fig. 1). Preliminary experiments with a *PcvelA* mutant in this strain background indicated drastic reduction (by ca. 90%) of penicillin G production (data not shown). However, subsequent analysis showed that this reduced productivity coincided with the loss of all but one of its six to eight penicillin gene clusters. Previous studies have shown the instability of the number of penicillin gene clusters in high-producing strains, caused by recombination of the direct repeats in these clusters (Fierro et al., 1995; Nijland et al., 2010). Indeed, protoplast transformation-associated loss of penicillin gene cluster amplicons has been

used in the construction of a “cluster-free” *P. chrysogenum* strain (DS50661) (Harris et al., 2009a). Construction of new *PcvelAΔ* and *PclaeAΔ* mutants in the high penicillin producing strain therefore involved a rigorous copy number evaluation of the penicillin biosynthetic genes by quantitative PCR. Strains DS63171 (*hdfAΔ PcvelAΔ*) and DS67261 (*hdfAΔ PclaeAΔ*) were therefore analyzed by q-PCR, using the strains DS54465 (*hdfAΔ*) (six to eight penicillin biosynthetic clusters), Wisconsin54-1255 (one penicillin biosynthetic cluster), and DS50562 (zero penicillin biosynthetic cluster) as references. Furthermore, the comparison of the number of penicillin clusters of the strain DS54465 (*hdfAΔ*) (this study) and its ancestor DS17690 (Douma et al., 2011) did not reveal any intermediate loss of cluster between the two strains. Deletion strains DS63171 (*hdfAΔ PcvelAΔ*) and DS67261 (*hdfAΔ PclaeAΔ*) did not showed significant changes in penicillin biosynthetic gene cluster number relative to DS54465 and DS17690 (Fig. 2) and therefore were used in further analysis.

Impact of *PcvelA* and *PclaeA* mutations on physiology and penicillin G production in glucose-limited chemostats

To analyze the impact of the Velvet complex on β -lactam production under industrially relevant conditions, the reference strain *P. chrysogenum* DS17690, the *PcvelAΔ* strain DS63171 and the *PclaeAΔ* strain DS67261 were grown in glucose-limited chemostat cultures with and without addition of phenylacetate (PAA), side-chain precursor for penicillin G synthesis. The main noticeable macroscopic difference between the Velvet complex mutants and the reference strain was a higher propensity of both mutants to grow on the solid surfaces of the bio-reactor. However, microscopic analysis did not reveal a difference in hyphal morphology between the strains (data not shown).

When grown in the presence of PAA, both mutants exhibited a significant (*t*-test, $p < 0.01$) decrease of the penicillin G production rate that, however, did not exceed 30% relative to the DS17690 strain (Table 4). The decreased production of penicillin G was not accompanied by a reduction of the rate of PAA consumption, which was not significantly different in the mutant and reference strains (*t*-test, $p > 0.01$). Biomass yields on glucose (Y_{SX}) and specific rates of glucose consumption ($q_{Glucose}$) did not differ significantly among the three strains. Although the titer of the penicillin intermediate isopenicillin N, which is also produced in the absence of the side-chain precursor PAA (Harris et al., 2009a), exhibited

FIG. 2. Copy number of the penicillin biosynthesis cluster. Determination of the penicillin biosynthesis cluster copy number in the deletion strains *P. chrysogenum* DS63171 (*hdfA*Δ *PcvlA*Δ), DS67261 (*hdfA*Δ *PclaeA*Δ) and control strains Wisconsin54-1255, DS54465, and DS17690. Real-time quantitative-PCR using specific primer pairs for penicillin biosynthetic genes *pcbAB* and *pcbC* (Table 2) were performed. Copy number data were normalized using *actA* (encoding γ -actin). Error bars presented standard deviation from at least independent duplicate experiments.

some fluctuations, these did not reveal a clear impact of the gene deletions (Table 4).

Chemostat-based transcriptome analysis: data quality and overall responses

The relevance of the Velvet complex as a global regulator in aerobic, glucose-limited chemostat cultures was analyzed by a full transcriptome analysis on the *PclaeA*Δ, *PcvlA*Δ, and

reference strains. DNA-microarray analysis was performed on mRNA isolated from glucose-limited chemostat cultures ($D=0.03\text{ h}^{-1}$) of each of the three strains, grown in the presence and in the absence of PAA. For each strain, transcriptome analysis was performed from at least two independent replicate chemostat cultures. The average coefficient of variation for transcriptome data from replicated cultures was below 20%, in accordance with previous chemostat-based transcriptome analyses in *P. chrysogenum* (Douma et al., 2011; Gombert et al., 2011; Harris et al., 2009a, 2009b; Koetsier et al., 2010; Snoek et al., 2009; van den Berg et al., 2008).

Four pair-wise comparisons (two mutant strains and two cultivation regimes) were executed to assess the impact of the *PclaeA* and *PcvlA* deletions on the *P. chrysogenum* transcriptome. This analysis identified 550 transcripts (corresponding to 4% of the entire *P. chrysogenum* genome) whose levels in the reference strain and at least one of the mutants differed under at least one condition (Fig. 3 and Supplementary Table S1). The transcript data of the Velvet complex mutants were compared to data from the DS17690 strain and not from the DS54465 (*hdfA*Δ). As previously shown by Snoek et al. (2009), the difference in gene expression between the two strains was extremely small, as only 26 genes (0.18% of the *P. chrysogenum* genes) were deemed significantly changed. Moreover we did not observe differential expression of any of the 26 genes between DS54465 (*hdfA*Δ), DS63171 (*hdfA*Δ *PcvlA*Δ) and DS67261 (*hdfA*Δ *PclaeA*Δ). Finally, 18 genes (out of 26) retained their differential expression relative to the DS17690. Based on these comparisons we concluded that transcript data derived from the DS17690 strain were suitable to execute the comparative analysis with the strains DS63171 (*hdfA*Δ *PcvlA*Δ) and DS67261 (*hdfA*Δ *PclaeA*Δ).

In a separate analysis, we compared our results with previously published transcript data obtained with batch cultures of *P. chrysogenum* *PcvlA* mutant in P2niaD strain background. Although Hoff and coauthors (2010) investigated the impact of *PcVelA* in *P. chrysogenum*, the two studies differed in several points. Although we analyzed the effect on

TABLE 4. GROWTH AND PHYSIOLOGICAL CHARACTERISTICS OF *P. CHRYSOGENUM* STRAINS (DS17690, DS63171 (*HDFAA PCVELAA*) AND DS67261 (*HDFAA PCLAEAA*))

Strain	^a PAA	^b γ_{SX} (g/g)	^c $q_{Glucose}$ (mmol/g/h)	^d $q_{Side\ chain}$ (mmol/g/h)	^e $q_{Penicillin\ G}$ (mmol/g/h)	^f IPN (mM)	References
DS17690	–	0.37 ± 0.00	0.43 ± 0.01	NA	NA	0.10 ± 0.07	(Harris et al., 2006), this study
	+	0.35 ± 0.01	0.48 ± 0.02	0.024 ± 0.002	0.020 ± 0.001	0.005 ± 0.00	(Harris et al., 2006), this study
DS63171 (<i>PcvlA</i> Δ)	–	0.39 ± 0.01	0.42 ± 0.01	NA	NA	0.03 ± 0.04	This study
	+	0.34 ± 0.02	0.49 ± 0.04	0.030 ± 0.002	0.016 ± 0.001	0.04 ± 0.02	This study
DS67261 (<i>PclaeA</i> Δ)	–	0.35 ± 0.03	0.49 ± 0.07	NA	NA	0.10 ± 0.02	This study
	+	0.35 ± 0.01	0.50 ± 0.01	0.031 ± 0.008	0.014 ± 0.001	0.02 ± 0.01	This study

Physiological and metabolite analysis of indicated *P. chrysogenum* strains grown on glucose-limited chemostat cultivations ($D=0.03\text{ h}^{-1}$, $T=25^{\circ}\text{C}$, $\text{pH}=6.5$) in the presence or absence of the side-chain precursor phenylacetate (PAA). Data are presented as averages ± mean deviation from at least two independent replicate cultures.

^aPresence or absence of phenylacetate.

^bBiomass yield on glucose (g of biomass per g of glucose).

^cBiomass specific glucose consumption rate (mmol of glucose consumed/g biomass per hour).

^dBiomass specific phenylacetate consumption rate (mmol of phenylacetate consumed/g biomass per hour).

^eBiomass specific penicillin G production rate (mmol of penicillin G produced per g biomass per hour).

^fExtra cellular concentration of Isopenicillin N (mmol per liter).

NA, not applicable.

FIG. 3. Transcriptome analysis of *P. chrysogenum* strains DS63171 (*hdfAA PcvelAA*) and DS17690 and of DS67261 (*hdfAA PclaeAA*). Results of the pair-wise comparison of the *P. chrysogenum* strains DS63171 (*hdfAA PcvelAA*) and DS17690 and of DS67261 (*hdfAA PclaeAA*) and DS17690 in presence and absence of PAA. Circles indicate a strain grown under a specific condition. Red arrows represent the number of genes with a higher transcript level and green arrows the genes that showed a lower transcript level versus the strain DS17690. The total number of differently expressed genes is mentioned in between brackets.

cultures that produced or not penicillinG, Hoff et al. (2010), instead of PAA as a side chain, used phenoxyacetate (POA) to produce a different type of penicillin, PenV, and compared the transcriptome in producing conditions only. In addition, the genes deemed differentially expressed by Hoff et al. (2010) were not made available (Hoff et al., 2010). Therefore we compared the set of genes that showed a different transcript level in glucose-limited chemostat cultures of the *PcvelAA* strain with added PAA (generated in this study) with the pair-wise comparison of the 96-h time point of prolonged batch cultures of a *PcvelAA* mutant in the high-producing P2niaD strain background, grown in the presence of POA (Hoff et al., 2010) ($|\text{fold change}| > 2.5$). Strikingly, less than 1% of the responsive genes were found to be common to the two studies (Fig. 4; Supplementary Table S2).

Transcriptional coresponses to *PcvelA* and *PclaeA* deletions

Because *PcVelA* and *PcLaeA* are both core components of the Velvet complex, we compared the transcriptome of the *PclaeAA* and *PcvelAA* mutants derived from biomass samples grown in glucose-limited chemostat cultures. Only a small fraction (7%, 37/550) of the transcriptional response was shared between the two deletion mutants (Fig. 3). With the exception of the acetamidase (*amdS*) deletion marker and the gene Pc22g15510 (*hdfA*), which was deleted in the strain in which the deletions were introduced (Snoek et al., 2009), no genes were differently expressed in all four two-way comparisons. The *amdS* gene was not taken into account in further analysis as it is not a *P. chrysogenum* native gene (Table 5). A closer look at the expression signal of both *PclaeA* and *PcvelA* confirmed the absence of transcript and eventually the correct

deletion in the strains DS67261 (*hdfAA PclaeAA*) and DS63171 (*hdfAA PcvelAA*), respectively.

A Fisher Exact test did not reveal any significant enrichment of functional categories among the remaining 550 differentially regulated genes. 23 genes were downregulated in both mutants relative to the reference strain in cultures that were grown in the absence of added PAA (Table 5). Of this gene set 11 belong to two groups that showed chromosomal positional linkage. The first group comprised six genes (Pc06g02040–Pc06g02100) of unknown function. The second set of five linked genes (Pc12g06310–Pc12g06370) included genes that, according to their annotation, are likely to be involved in secondary metabolism (Keller et al., 2005). The protein Pc12g06310 shared very high similarity (97%) with the *Ari1* from *Penicillium roqueforti*, an aristolochene synthase (Proctor and Hohn, 1993). Aristolochene is the first intermediate in the synthesis of a sesquiterpene pathogenesis-related toxin, for which the complete biosynthetic pathway remains unidentified.

Two other genes displayed interesting annotations. Pc16g12580 shared high similarity to a blue-light receptor and was upregulated strongly in both deletion strains relative to the reference strain (3.1- and 9.9-fold change for *PcvelAA* and *PclaeAA*, respectively) when grown in the absence of PAA. In *A. nidulans*, the VelB/VeA/LaeA complex connects light to developmental regulation and control of secondary metabolism (Bayram et al., 2008; Fischer, 2008; Sprote and Brakhage, 2007; Sarikaya et al., 2010; Stinnett et al., 2007). Upregulation of Pc16g12580 might therefore be involved in some compensatory response to the disruption of the integrity of the Velvet complex. Finally, in presence of PAA the putative regulatory gene Pc21g08860 (that exhibits strong similarity to *acvA* gene expression regulator Pc804 like protein) was upregulated 3.6-fold (Table 5), which suggests that a negative effect of the inactivation of the Velvet complex may be partially compensated for by another regulatory mechanism.

Impact of *PclaeA* and *PcvelA* mutations on expression of secondary metabolism genes

To further assess the impact of the *PcvelA* and *PclaeA* deletions on regulation of secondary metabolism in glucose-limited chemostat cultures of *P. chrysogenum*, we specifically analyzed the transcript profiles of genes that, based on sequence similarity and/or functional analysis data, can be implicated in secondary metabolism. This targeted analysis also included the eight *P. chrysogenum* Velvet complex orthologs (Table 3). None of the eight Velvet complex orthologs (with the obvious exception of the deleted gene itself) showed drastic transcriptional changes in the deletion strains, irrespective of the inclusion of PAA in the growth media (Fig. 5). To investigate whether the intensive strain improvement program from which *P. chrysogenum* DS17690 originates might have affected expression of these genes, transcript levels of Velvet complex genes, including *PcvelA* and *PclaeA* were compared in chemostat cultures to the ancestor strain Wisconsin54-1255. This analysis did not provide evidence for changes in the expression levels of Velvet genes during strain improvement (Fig. 5).

During the first analysis of the *P. chrysogenum* genome sequence (van den Berg et al., 2008), 51 genes were annotated as nonribosomal peptide synthases (NRPS), polyketide synthases

FIG. 4. Comparative transcript analysis of *P. chrysogenum* *PcvelA* mutants in prolonged batch and chemostat cultivations. Comparison of genes that showed at least twofold change in prolonged batch cultivations with POA of *PcvelAΔ* versus *Pcku70Δ* as reported in Hoff et al. (2010) with genes that showed differential expression in glucose chemostat cultures of DS63171 (*PcvelAΔ*) relative to the reference DS17690 as reported in this study. The fold changes from the data reported in Hoff et al. (2010) were calculated by comparing the 96-h points in the *PcvelAΔ* and *Pcku70Δ*. Red arrows represent the number of genes with a higher transcript level in the *PcvelAΔ* mutant than in its respective reference and green arrows represent the genes that showed a lower transcript level in the *PcvelAΔ* mutant than in its respective reference. The total number of differentially expressed genes is mentioned in between brackets.

(PKS), NRPS-like enzymes, PKS-like enzymes, and NRPS-PKS hybrids. The paradigm of NRPS in *P. chrysogenum* is the gene *acvA* (*pcbAB*) that encodes for δ -(L- α -aminoacyl)-L-cysteine-D-valine synthetase, which catalyzes the first step of penicillin biosynthesis. In both mutants, irrespective of the presence of PAA, expression of *acvA* was not substantially altered, consistent with the small reduction of penicillin G productivity in the *PcvelA* and *PclaeA* mutants (Table 4). Similarly, expression of the other two clustered genes necessary for penicillin G synthesis, isopenicillin N synthase (*pcbC*) and isopenicillin N acyltransferase (*penDE*) was not strongly affected by the *PcvelA* and *PclaeA* deletions (Fig. 5). This result is in total contrast with the previous studies in which the deletions of *PcvelA* and *PclaeA* were accompanied with a strong downregulation of not only *pcbAB* but also *penDE* and *pcbC*.

Of the NRPS (-like), PKS (-like), and NRPS-PKS hybrids that are not involved in penicillin G biosynthesis, a large fraction (34%, 17 genes) were not expressed in any of the cultures. Another 54% (27 genes) were not differentially expressed, leaving only six genes (12%) (one NRPS, four PKS, and one NRPS-like) with differential expression in at least one pair wise comparison between either the *PcvelA* and *PclaeA* mutant and the DS17690 reference strain. These six genes did not exhibit consistent profiles as four were

downregulated in one of the mutants while the other two were upregulated (Fig. 6).

Discussion

Inactivation of the Velvet complex genes *PcvelA* and *PclaeA* has previously been shown to have a drastic effect on penicillin production by *P. chrysogenum*. In two different *P. chrysogenum* strains, deletion of *PcvelA* and/or *PclaeA* resulted in a massive reduction of penicillin production and a strong downregulation of the expression of genes involved in penicillin biosynthesis (Hoff et al., 2010; Kosalkova et al., 2009). Whereas the two previous studies were performed in prolonged batch cultures, the present study was performed in aerobic, glucose-limited cultures at a fixed specific growth rate of 0.03 h^{-1} , which is near to the optimum for penicillin G productivity in the strain background used (Hasegawa et al., 1985). Under these conditions, deletion of either *PclaeA* or *PcvelA* had only a moderate effect on penicillin production. Moreover, we did not observe the strong downregulation of penicillin biosynthesis genes and morphological changes reported previously for prolonged batch cultures (Hoff et al., 2010; Kosalkova et al., 2009). This main discrepancy could not be explained by mutations because the sequence analysis revealed no difference between the amino acid sequences of

TABLE 5. TRANSCRIPT ANALYSIS OF *P. CHRYSOGENUM* DS17690, DS63171 (HDFΔΔ *Pcvel*ΔΔ) AND DS67261 (HDFΔΔ *Pclae*ΔΔ) STRAINS

Absence of phenylacetate (PAA)		Fold change versus DS17690	
Genes upregulated in both DS63171 (<i>Pcvel</i> ΔΔ) and in DS67261 (<i>Pclae</i> ΔΔ) relative to DS17690 (9 genes)		DS63171 (<i>Pcvel</i> ΔΔ)	DS67261 (<i>Pclae</i> ΔΔ)
Similarity to			
Pc06g00230	hypothetical protein contig61.tfa_1250cg— <i>A. fumigatus</i>	12.0	4.1
Pc16g12580	hypothetical blue-light-inducible protein bli-3— <i>N. crassa</i>	3.1	9.9
Pc21g01220	alcohol dehydrogenase (NADP+) ALR— <i>S. scrofa</i>	3.5	14.7
Pc21g21440	hypothetical protein	3.8	4.5
Pc22g06430	hypothetical protein B5O22.140— <i>N. crassa</i>	3.4	6.5
Pc22g07910	hypothetical protein YGR086c— <i>S. cerevisiae</i>	5.5	11.4
Pc22g13930	glutathione-dependent formaldehyde dehydrogenase FDH— <i>M. marinus</i>	5.0	24.1
Pc23g00530	ankyrin repeat protein E4_2—Synthetic construct	3.3	4.1
Pc49g00070	hypothetical protein— <i>P. marneffeii</i>	3.4	20.9
Genes downregulated in both DS63171 (<i>Pcvel</i> ΔΔ) and in DS67261 (<i>Pclae</i> ΔΔ) relative to DS17690 (23 genes)			
Pc06g02040	hypothetical protein 15_scaffold_1.tfa_520cg— <i>F. graminearum</i>	−3.6	−10.0
Pc06g02050	hypothetical protein An08g11990— <i>A. niger</i>	−6.3	−17.1
Pc06g02070	hypothetical protein contig46_part_ii.tfa_1990wg— <i>A. fumigatus</i>	−10.8	−11.4
Pc06g02080	hypothetical protein An06g02320— <i>A. niger</i>	−5.2	−6.3
Pc06g02080	hypothetical protein An06g02320— <i>A. niger</i>	−6.7	−6.6
Pc06g02090	hypothetical protein	−5.9	−6.0
Pc06g02100	hypothetical protein	−6.3	−3.2
Pc12g02730	vegetative incompatibility factor het-e1— <i>P. anserina</i>	−3.3	−4.6
Pc12g06310	aristolochene synthase Ari1— <i>P. roqueforti</i>	−7.4	−4.5
Pc12g06320	hydroquinone oxidase mcrA— <i>S. lavendulae</i>	−4.8	−3.6
Pc12g06330	hypothetical short chain alcohol dehydrogenase cta— <i>C. sativus</i>	−5.7	−3.4
Pc12g06340	hypothetical cytochrome P450 monooxygenase p450-4— <i>G. fujikuroi</i>	−3.5	−3.5
Pc12g06370	trichothecene 3-O-acetyltransferase TRI101— <i>F. sporotrichioides</i>	−4.9	−4.0
Pc12g08290	antifungal protein precursor paf— <i>P. chrysogenum</i>	−5.4	−22.6
Pc13g08150	hypothetical protein An04g03190— <i>A. niger</i>	−3.2	−6.2
Pc20g07120	hypothetical protein An06g00470— <i>A. niger</i>	−3.5	−4.3
Pc20g13730	hypothetical protein An01g11500— <i>A. niger</i>	−4.2	−19.8
Pc20g13740	hypothetical protein BAB55393.1— <i>H. sapiens</i>	−3.5	−14.2
Pc21g01210	hypothetical amine transporter SPCC18.02— <i>S. pombe</i>	−3.5	−12.4
Pc21g22240	cholesterol 7alpha-monooxygenase CYP7— <i>O. cuniculus</i>	−3.0	−7.5
Pc22g15510	ATP-dependent DNA helicase II subunit Ku70— <i>M. musculus</i>	−8.8	−8.8
Pc22g17420	hypothetical protein contig_1_153_scaffold_12.tfa_500cg— <i>A. nidulans</i>	−3.0	−7.6
Pc22g21310	hypothetical protein 192_scaffold_1.tfa_150wg— <i>F. graminearum</i>	−4.9	−9.6
Presence of phenylacetate (PAA)		Fold change versus DS17690	
Genes upregulated in both DS63171 (<i>Pcvel</i> ΔΔ) and in DS67261 (<i>Pclae</i> ΔΔ) relative to DS17690 (3 genes)		DS63171 (<i>Pcvel</i> ΔΔ)	DS67261 (<i>Pclae</i> ΔΔ)
Pc06g00720	cellulosomal scaffoldin anchoring protein C— <i>A. cellulolyticus</i>	6.6	3.7
Pc13g10790	hypothetical protein An16g03760— <i>Aspergillus niger</i>	3.4	3.5
Pc21g08860	acvA gene expression regulator Pc804 like protein An13g01050— <i>A. niger</i>	3.6	2.9
Genes downregulated in both DS63171 (<i>Pcvel</i> ΔΔ) and in DS67261 (<i>Pclae</i> ΔΔ) relative to DS17690 (2 genes)			
Pc20g01520	hypothetical protein mg07210.1— <i>M. grisea</i>	−4.2	−3.4
Pc22g15510	ATP-dependent DNA helicase II subunit Ku70— <i>M. musculus</i>	−7.7	−7.7

Genes with consistent, significantly different transcript levels in comparisons of *Pcvel*Δ and *Pclae*Δ deletion mutants with the reference strain *P. chrysogenum* DS17690, grown in glucose-limited cultures in the presence and absence of PAA. These genes represent the overlap of the different pair-wise comparisons in Figure 3.

FIG. 5. I. Expression value of the genes encoding subunits of the Velvet complex and the penicillin biosynthetic enzymes in *P. chrysogenum* strain Wisconsin541255, DS17690, DS63171 (*hdfAA PcvlAA*) and DS67261 (*hdfAA PcvlAA*). The heatmap represents the item-wise Z-normalized expression value of the gene transcript levels. The numerical data represent the average hybridization intensity values of the corresponding gene probe sets on the Affymetrix array DSM_PENa520255F. Values represent the mean $\pm$ SD of data from at least duplicate experiments.

PclaeA and *PcvlA* from the different strains tested in the compared studies. Our results could indicate that, although the Velvet complex can act as a key regulator of penicillin biosynthesis, this role is strongly context dependent.

Secondary metabolites are characteristically produced during and after the shift from exponential growth to stationary phase, and it appears that it is under these conditions that the Velvet complex exerts its main effect on penicillin biosynthesis. Indeed, Hoff et al. (2010) showed a much stronger and wider impact of the Velvet complex on transcriptional regulation during the later phases of prolonged batch cultures (presumably corresponding with the stationary phase) than during the exponential growth phase. The present study indicates that, even at a low specific growth rate and under glucose-limited conditions, the role of the Velvet complex is much less pronounced than under essentially nongrowing conditions.

The small impact of the *PclaeA* and *PcvlA* deletions in glucose-limited, aerobic chemostat cultures indicates that the Velvet complex only plays a limited role in the activation of penicillin biosynthesis under industrially relevant conditions. This raises the question which other factor(s) contribute(s) to the efficient activation of penicillin biosynthesis under industrial conditions. A recent study in *A. nidulans* identified a multicopy suppressor of an inactive Velvet complex when overexpression of *rsmA* was shown to restore activation of sterigmatocystin biosynthetic genes (Shaaban et al., 2010). The gene *rsmA* encodes a putative bZIP transcription factor. We therefore investigated whether a *P. chrysogenum* *rsmA* ortholog might be involved in activation of penicillin biosynthesis. Open reading frame *Pc12g16510* was found to share 65%

similarity with *rsmA*. However, transcript levels of *Pc12g16510* were sevenfold higher in *P. chrysogenum* Wisconsin54-1255 than in the penicillin high-producing strain DS17690, which argues against a role as activator of penicillin biosynthetic genes. Moreover, *Pc12g16510* was not upregulated in the *PclaeA* and *PcvlA* mutants.

Further analysis of the transcriptome data suggested another interesting candidate for Velvet-independent activation of penicillin biosynthesis genes. The gene *Pc21g08860* was upregulated in glucose-limited chemostat cultures of *PcvlA* and *PclaeA* mutants relative to the reference strain DS17690 (3.6- and 2.9-fold, respectively) but only in cultures supplemented with the penicillin G side-chain precursor PAA. *Pc21g08860* is identical to the *P. chrysogenum* gene labeled Pc804, previously implicated in activation of penicillin biosynthesis via its binding to the *acvA* promoter (Cali et al., 2004, 2005). Further analysis will reveal the exact role of *Pc21g08860* in the regulation of penicillin production.

Conclusion

The present study presents a clear indication for the context dependency of transcriptional regulation of secondary metabolism in *P. chrysogenum* and, in particular, the role of the Velvet complex in this process. Transcriptome analysis of reference and mutant strains under a much wider range of different nutrient limitation regimes in chemostat cultures may contribute to the intellectually challenging and economically relevant goal of defining and, ultimately, controlling the role of the Velvet complex and other regulators in secondary metabolite production in *P. chrysogenum* and other filamentous fungi.

FIG. 6. Expression of secondary metabolism genes. Heat map of transcript levels of genes encoding NRPS (and -like), PKS (and -like) and hybrid NRPS-PKS in strain Wisconsin541255, DS17690, DS63171 (*hdfAΔ PcvelAΔ*) and DS67261 (*hdfAΔ PcvelAΔ*) grown aerobic glucose-limited chemostat cultures with and without PAA. The expression data were derived from hybridization intensity of Affymetrix DNA microarray (DSM PENa520255F). The transcript data were experiment wise Z-normalized. Genes are grouped accordingly to their expression profile when compared to the strain DS17690 in upregulated ($FC > 2$) [I], downregulated ($FC < -2$) [II], not changed ($-2 < FC < 2$) [III], and below detection (expression level lower than 12) [IV]. Secondary metabolism group are indicated by the following code: NRPS are indicated in green, the PKS in purple, the NRPS-like in yellow, the PKS-like in blue and the RPS-PKS hybrid in pink.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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