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Published in:
Applied and environmental microbiology

DOI:
[10.1128/AEM.01529-12](https://doi.org/10.1128/AEM.01529-12)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Weber, S. S., Polli, F., Boer, R., Bovenberg, R. A. L., & Driessen, A. J. M. (2012). Increased Penicillin Production in *Penicillium chrysogenum* Production Strains via Balanced Overexpression of Isopenicillin N Acyltransferase. *Applied and environmental microbiology*, 78(19), 7107-7113.
<https://doi.org/10.1128/AEM.01529-12>

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Increased Penicillin Production in *Penicillium chrysogenum* Production Strains via Balanced Overexpression of Isopenicillin N Acyltransferase

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Intense classical strain improvement has yielded industrial *Penicillium chrysogenum* strains that produce high titers of penicillin. These strains contain multiple copies of the penicillin biosynthesis cluster encoding the three key enzymes: δ -(1- α -aminoadipyl)-L-cysteiny-D-valine synthetase (ACVS), isopenicillin N synthase (IPNS), and isopenicillin N acyltransferase (IAT). The phenylacetic acid coenzyme A (CoA) ligase (PCL) gene encoding the enzyme responsible for the activation of the side chain precursor phenylacetic acid is localized elsewhere in the genome in a single copy. Since the protein level of IAT already saturates at low cluster copy numbers, IAT might catalyze a limiting step in high-yielding strains. Here, we show that penicillin production in high-yielding strains can be further improved by the overexpression of IAT while at very high levels of IAT the precursor 6-aminopenicillic acid (6-APA) accumulates. Overproduction of PCL only marginally stimulates penicillin production. These data demonstrate that in high-yielding strains IAT is the limiting factor and that this limitation can be alleviated by a balanced overproduction of this enzyme.

Since the discovery of penicillin by Alexander Fleming (6), classical strain improvement has been the main method to improve penicillin production. One of the most important phenomena in high-yielding *Penicillium chrysogenum* strains is the amplification of the penicillin biosynthetic gene cluster between tandem repeats (5, 18, 21). Other changes are the up-regulation of genes involved in side chain activation, α -aminoadipic acid, and valine and cysteine biosynthesis (23). The penicillin biosynthesis cluster in *P. chrysogenum* consists of three genes, *pcbAB*, *pcbC*, and *penDE* (Fig. 1A), encoding the enzymes that catalyze the key biosynthetic conversions for penicillin production (7).

Penicillin biosynthesis (Fig. 1B) starts with the condensation of three amino acids, L- α -aminoadipic acid, L-cysteine, and L-valine, into the tripeptide δ -(1- α -aminoadipyl)-L-cysteiny-D-valine (LLD-ACV). This step is catalyzed by the nonribosomal peptide synthetase δ -(1- α -aminoadipyl)-L-cysteiny-D-valine synthetase (ACVS) encoded by *pcbAB* (1, 11–13). Next, the β -lactam ring is formed by isopenicillin N synthase (IPNS) encoded by the *pcbC* gene (4, 13). After isopenicillin N enters the microbody, the L- α -aminoadipic acid side chain is replaced by an activated phenyl- or phenoxyacetyl group yielding penicillin G or V, respectively. This conversion is catalyzed by acyl-coenzyme A: isopenicillin N acyltransferase (IAT), encoded by *penDE* (3, 17, 22, 25). IAT is capable of substituting L- α -aminoadipic acid with phenylacetic acid (PAA) or phenoxyacetic acid (POA) only when these precursors are activated to their coenzyme A (CoA) thioesters. One of the main enzymes capable of carrying out this reaction is phenylacetic acid CoA ligase (PCL) (14, 16). The *phl* (Pc22g14900) gene is not part of the penicillin biosynthetic gene cluster (for a review, see reference 24), but the PCL protein also localizes to the microbody lumen that contains the IAT enzyme.

Recently, a “reverse engineering” study of the amplification of the penicillin biosynthetic gene cluster was carried out using a

series of isogenic strains derived from a former industrial production strain, *P. chrysogenum* DS17690. These isogenic strains differed only in the number of biosynthetic gene clusters, and they showed transcription levels of the biosynthetic genes that increased almost linearly with the gene cluster copy number while saturating only at very high copy numbers. Also, the protein levels of ACVS and IPNS correlated well with the transcript levels. On the other hand, the IAT protein levels increased only 2-fold when transcript levels increased within a range of 8-fold. This remarkable lack of correlation between the protein and transcript levels of the IAT enzyme suggested that the amount of active IAT is limiting in high-yielding strains. This was further evidenced by the accumulation of isopenicillin N in strains harboring high copy numbers (19). Alternatively, side chain activation could be a limiting factor in these high-yielding strains, but under those circumstances the intracellular accumulation of 6-aminopenicillic acid (6-APA) is expected rather than the formation of isopenicillin N because of the hydrolase properties of the IAT enzyme. Here we have investigated the effect of the overproduction of IAT (*penDE*) and PCL (*phl*) in *P. chrysogenum* strains equipped with different copy numbers of the biosynthetic gene cluster. The data demonstrate that the level of IAT is limiting in high-yielding strains and that increased levels of penicillin can be obtained upon the balanced overproduction of the *penDE* gene.

Received 14 May 2012 Accepted 26 July 2012

Published ahead of print 3 August 2012

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doi:10.1128/AEM.01529-12

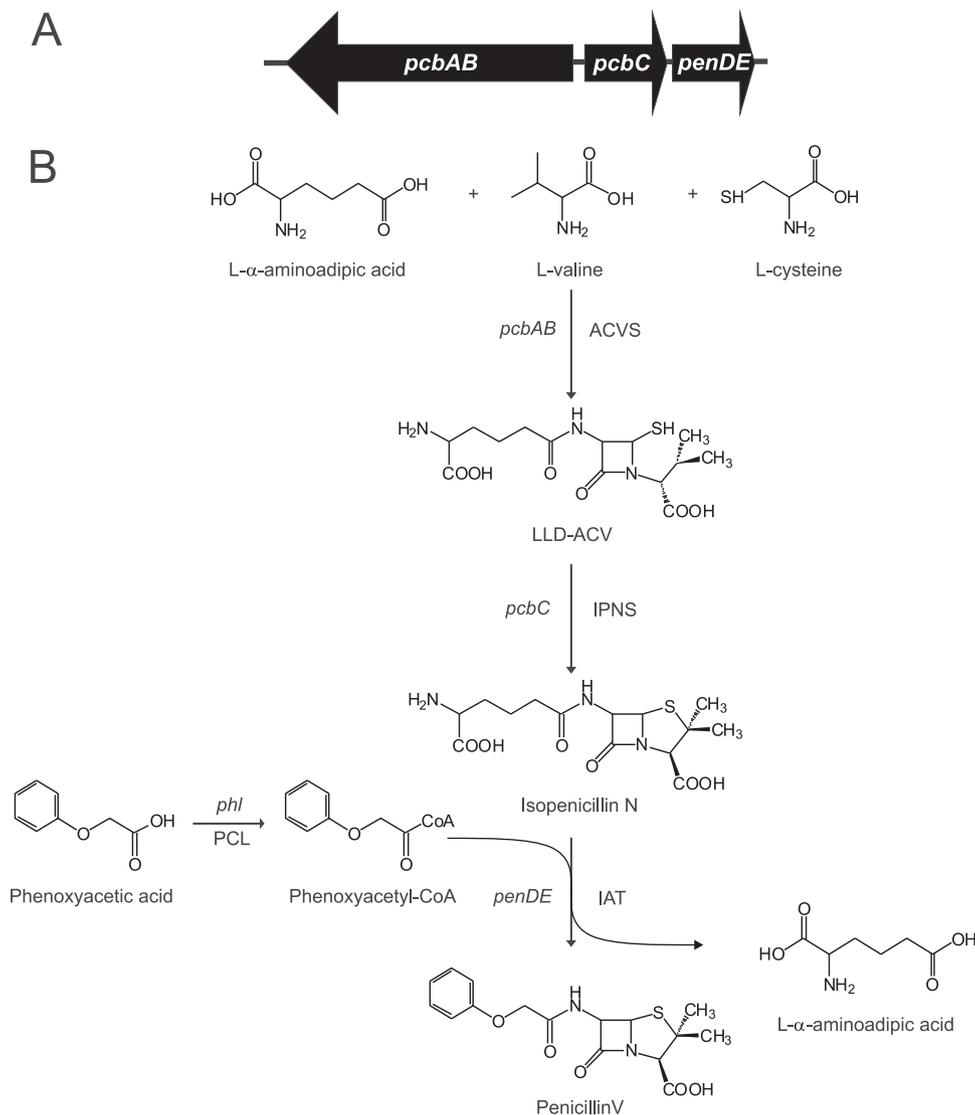


FIG 1 (A) Penicillin biosynthesis cluster: *pcbAB* coding for ACVS, *pcbC* coding for IPNS, and *penDE* coding for IAT. (B) Schematic representation of the penicillin biosynthetic pathway; the names of the enzymes and their corresponding genes are indicated next to the arrows.

MATERIALS AND METHODS

Fungal strains, media, and culture conditions. *Penicillium chrysogenum* DS17690 and its derivatives were kindly provided by DSM, The Netherlands. Spores immobilized on rice were inoculated in YGG medium containing (in g/liter): KCl, 10.0; glucose, 20.0; yeast nitrogen base (YNB), 6.66; citric acid, 1.5; K_2HPO_4 , 6.0; and yeast extract, 2.0. After inoculation, cultures were incubated for 24 h in a rotary incubator at 200 rpm at 25°C. On day 0, the mycelium was diluted 7 times in penicillin production medium (PPM) containing the following (in g/liter): glucose, 5.0; lactose, 75; urea, 4.0; Na_2SO_4 , 4.0; $\text{CH}_3\text{COONH}_4$, 5.0; K_2HPO_4 , 2.12; KH_2PO_4 , 5.1; and phenoxycetic acid, 2.5. After dilution, the mycelium was supplemented with a trace element solution (pH 6.3) and grown for 7 days in a rotary incubator at 200 rpm at 25°C.

Transformation to *P. chrysogenum*. Transformation was performed as described in reference 15. Primers for the amplification of the genes of interest *penDE* and *phl* can be found in Table 1. Protoplasts were prepared and transformation was performed using a linearized fragment of pSW070, pSW071, or pSW072 containing the gene *penDE*, *penDE*-SKL, or *phl* isolated from genomic DNA, or a linearized fragment from

pSW073 containing *penDE* isolated from cDNA, all under the control of the highly expressed *pcbC* promoter. The cells were cotransformed with pBlue-AMDS, containing the acetamidase gene (*amdS*), which was used as a selection marker on plates with acetamide as the sole nitrogen source.

gDNA extraction. Genomic DNA (gDNA) was isolated after 48 h of growth in YGG medium using a modified yeast genomic DNA isolation protocol (8) in which the fungal mycelium is broken in a FastPrep FP120 system (Qbiogene) (20).

TABLE 1 Oligonucleotides used for amplification of *penDE* or *phl*

Primer	Sequence (5'–3')
IAT-fw	ATGCGGATCCATGCTTCACATCCTCTGTCAAGG
IAT-rv	AGTCCC GGTC AAAGCCTGGCGTTGAGCGC
IAT-SKL-rv	AGTCCC GGTC AAAGCCTGCTGTTGAGCGCAG
PCL-fw	ATGCGGATCCATGGTTTTTTTACCTCCAAAGGAG
PCL-rv	AGTCCC GGTTAGATCTTGCTACCAAGCCTTC

TABLE 2 Oligonucleotides used during qRT-PCR analysis

Primer	Sequence (5′–3′)
<i>penDE</i> -fw	CATCCTCTGTCAAGGCACTCC
<i>penDE</i> -rv	CCATCTTTCCTCGATCACGC
<i>phl</i> -fw	CTGGGTATGGAGACAGCTGCCG
<i>phl</i> -rv	CGTGCCTCGACTCCAGGGAGC
γ-Actin-fw	CTGGCGGTATCCACGTCACC
γ-Actin-rv	AGGCCAGAATGGATCCACCG

Total RNA extraction and cDNA amplification. Total RNA samples were taken after 5 days of growth in penicillin production medium. Total RNA was isolated using TRIzol (Invitrogen), with additional DNase treatment by the Turbo DNA-free kit (Ambion). Total RNA was measured with a NanoDrop ND-1000 and set at a concentration of 500 ng per cDNA reaction. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) in a final volume of 10 μl.

qPCR expression analysis and gene copy number determination. The primers for analysis of *penDE*, *phl*, and γ-actin (Pc20g11630) can be found in Table 2. A negative reverse transcriptase (RT) control reaction was used to exclude gDNA contamination in isolated total RNA. Copy numbers using gDNA and expression levels using cDNA were analyzed, in duplicate, with a MiniOpticon system (Bio-Rad). The SensiMix SYBR mix (Bioline) was used as a master mix for quantitative PCR (qPCR) with 0.4 μM primers. The following thermocycler conditions were used: 95°C for 10 min, followed by 40 cycles of 95°C for 15s, 60°C for 30s, and 72°C for 30s.

Determination of metabolite concentrations. The extracellular concentrations of phenylacetic acid and penicillin V in the culture medium were determined using high-pressure liquid chromatography (HPLC) analysis, using an isocratic flow of acetonitrile at 245 g/liter, KH₂PO₄ at 640 mg/liter, and H₃PO₄ at 340 mg/liter. Peaks were separated on a Shim-pack XR-ODS 2.2 (Shimadzu) at a flow rate of 0.5 ml/min and detected at a wavelength of 254 nm. Production levels were corrected for small growth differences by dry weight determination. Determination of 6-APA was performed using an orbitrap mass spectrometer (Thermo Fisher Scientific Exactive). Samples were separated on a Shim-pack XR-ODS 2.2 (Shimadzu) column using an isocratic flow of 0.3 ml/min of acetonitrile at 7.86 g/liter and formic acid at 6.1 g/liter. Molecular masses were identified with the electrospray in negative mode. Quantification of masses was performed using the Excalibur (Thermo Fisher Scientific) software.

Western blot analysis. Cell extracts of *P. chrysogenum* were isolated after 5 days of growth. An aliquot of 1 ml of the culture was mixed with 1 ml 25% trichloroacetic acid (TCA) and frozen at –20°C. After defrosting on ice, cells were pelleted by centrifugation (10,000 × g for 10 min) and washed twice using cold (–20°C) 80% acetone. The pellet was air dried and solubilized in 200 μl solubilization buffer containing 1% sodium dodecyl sulfate (SDS) and 0.1 M NaOH, followed by the addition of 50 μl 5× SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer. Samples were boiled for 5 min and centrifuged for 10 min at 10,000 × g. The supernatants were used for SDS-PAGE analysis. Western blots were stained with polyclonal antibodies raised against IAT.

RESULTS

***penDE* or *phl* gene copy number and overexpression analysis.** In a previous study we have shown that the protein levels of the key enzyme acyl-coenzyme A: isopenicillin N acyltransferase (IAT) do not correlate with the transcript levels of the *penDE* gene. An 8-fold increase in penicillin biosynthesis gene cluster number resulted in a corresponding increase in transcript levels of all genes located on the cluster. While the protein levels of ACVS and IPNS increased accordingly (4- to 5-fold), the IAT protein level increased only 2-fold and saturated already at low gene copy num-

bers. This suggested that IAT might be the limiting factor in penicillin V production by high-yielding strains (19). To examine this phenomenon, a targeted overexpression of the *penDE* gene was initiated using the *P. chrysogenum* strains DS47274 (one cluster copy), DS47273 (three clusters), and DS17690 (seven or eight clusters) (9). For this purpose, additional copies of the native *penDE* gene, as well as a mutant *penDE* gene encoding an IAT with an improved peroxisomal targeting signal (C-terminal protein sequence SKL instead of ARL) (10), or a *penDE* gene without introns were introduced into these strains in a random manner, via the nonhomologous end-joining pathway (NHEJ). Next, the *penDE* gene copy number was determined using isolated genomic DNA and quantitative real-time PCR using specific primers for the *penDE* gene based on the genome sequence of *P. chrysogenum* Wisconsin 54-1255 (23). The γ-actin gene was used as a reference. In the selected strains, the copy numbers of *penDE*, including the number of copies already present, were elevated from 2 up to 30 copies depending on the host strain used (Fig. 2A). In the remainder of the manuscript, the strains are designated DSyyyyy^{penDEX}, with yyyyy as the original DS strain designation and X the number of copies of the *penDE* gene, including the copies localized in the biosynthetic gene cluster.

For the determination of the transcript levels of *penDE*, total RNA was isolated and the above-mentioned primers for *penDE* and γ-actin were used for qRT-PCR analysis. The transcript levels of *penDE* in all isogenic host strains increased 400- to 1,000-fold when more than 15 additional copies of *penDE* were introduced, while at lower numbers of additional copies of the *penDE* gene, transcription was only moderately elevated (Fig. 2A). Overall, the transcript level increased nonlinearly with the number of *penDE* copies to very high levels of overexpression. In addition, extra copies of the *phl* gene (encoding PCL) were introduced into the three host strains (DS47274, DS47273, and DS17690) using the NHEJ pathway. In all of these strains, the *phl* gene is present as a single copy, but this could be increased up to 16 copies following the transformation (Fig. 2B). These strains were designated DSyyyyy^{phlX}, with yyyyy as the original DS strain and X the number of copies of the *phl* gene, including the endogenously present copy. The transcript levels of *phl* increased almost linearly from 3-fold (DS47274^{phl2}) to 40-fold (DS47274^{phl17}) (Fig. 2B).

Penicillin V production by *penDE* or *phl* overproducing strains. To analyze whether the additional copies of the *penDE* gene influence the penicillin V production, the medium broth was analyzed after 5 and 7 (not shown) days of growth for penicillin V. In the DS47273 (three clusters) and DS17690 (seven or eight clusters) background strains, introduction of one or two additional copies (strains DS47273^{penDE4}, DS47273^{penDE5}, DS17690^{penDE10}, and DS17690^{penDE10–11}) of *penDE* results in a significant increase up to 160 to 200% in penicillin V production. However, with the introduction of more than 20 additional copies (DS47273^{penDE24} and DS17690^{penDE30}), this increase in penicillin V production levels dropped dramatically. When the DS47274 strain (one cluster) was used as the host, addition of a few (DS47274^{penDE2} and DS47274^{penDE4}) or many (DS47274^{penDE15–16}) copies of the *penDE* gene hardly affected the penicillin V production (Fig. 3A). This observation is consistent with the notion that in those low-copy-number strains, penicillin production is limited by the presence of only a single copy of the biosynthetic gene cluster.

The same analysis was performed on the strains that contained additional copies of the *phl* gene using the DS47273 (three clus-

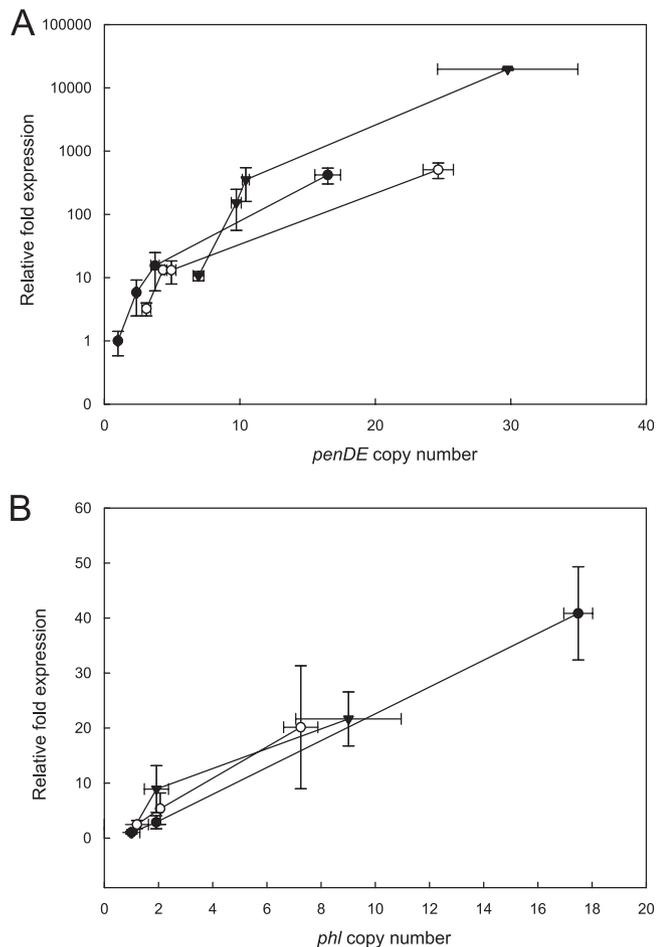


FIG 2 (A) Transcript level of *penDE* in transformed strains as a function of the copy number of the *penDE* gene. Closed circles correspond to strains DS47274 (one cluster), DS47274^{penDE2}, DS47274^{penDE4}, and DS47274^{penDE15-16}. Open circles show strains DS47273 (three clusters), DS47273^{penDE4}, DS47273^{penDE5}, and DS47273^{penDE24}, and closed triangles display DS17690 (seven or eight clusters), DS17690^{penDE10}, DS17690^{penDE10-11}, and DS17690^{penDE30}. (B) Transcript level of *phl* in transformed strains as a function of the copy number of the *phl* gene. Closed circles correspond to strains DS47274 (one cluster), DS47274^{phl2}, and DS47274^{phl17}. Open circles correspond to strains DS47273, DS47273^{phl2}, and DS47273^{phl7}, and closed triangles correspond to DS17690 (seven or eight clusters), DS17690^{phl2}, and DS17690^{phl9}. Results show that in all background strains the transcript levels of *phl* increase almost linearly when additional copies of *phl* are introduced.

ters) and DS17690 (seven or eight clusters) strains as the hosts. A slight increase in penicillin V production was noted only when multiple copies of the *phl* gene were introduced (DS47273^{phl7} and DS17690^{phl9}) (Fig. 3B), but in comparison to the *penDE* gene results, the overall effects are relatively marginal. The data demonstrate that the *phl*-encoded CoA ligase is not limiting for penicillin V production.

IAT protein levels in high-yielding *P. chrysogenum* strains. The IAT protein levels in the *penDE* overexpressing strains (5 days of growth) were analyzed by immunoblotting using IAT-specific polyclonal antibody. Cells were lysed, and proteins were collected using trichloroacetic acid (TCA) precipitation and analyzed by SDS-PAGE and Western blotting. With all host strains (DS47274, DS47273, and DS17690), there was an almost linear increase in the

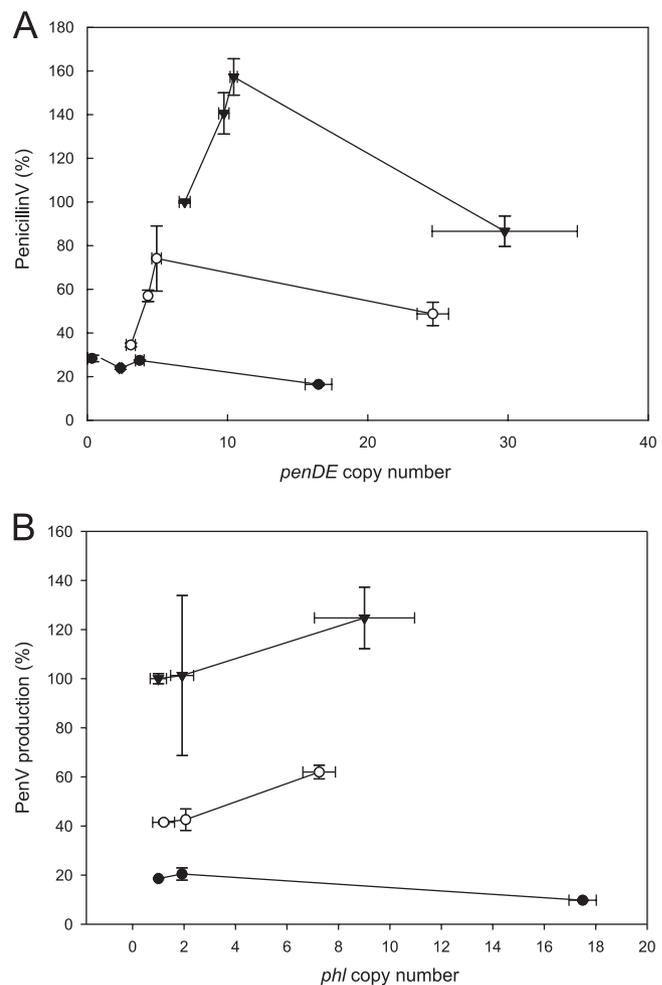


FIG 3 (A) Penicillin V production by strains with an increased copy number of *penDE*. Closed circles correspond to strains DS47274 (one cluster), DS47274^{penDE2}, DS47274^{penDE4}, and DS47274^{penDE15-16}. Open circles show strains DS47273 (three clusters), DS47273^{penDE4}, DS47273^{penDE5}, and DS47273^{penDE24}, and closed triangles display DS17690 (seven or eight clusters), DS17690^{penDE10}, DS17690^{penDE10-11}, and DS17690^{penDE30}. (B) Penicillin V production by strains with an increased copy number of the *phl* gene. Closed circles correspond to strains DS47274 (one cluster), DS47274^{phl2}, and DS47274^{phl17}. Open circles correspond to strains DS47273, DS47273^{phl2}, and DS47273^{phl7}, and closed triangles correspond to DS17690 (seven or eight clusters), DS17690^{phl2}, and DS17690^{phl9}.

IAT protein level at the lower number of *penDE* copies (1 to 3), but levels saturated at the very high copy numbers (15 to 30) (Fig. 4).

Fermentation characteristics of *penDE* overproducing strains. To determine if the availability of the side chain precursor is a limiting factor in batch cultures of the high-yielding strains with additional *penDE* copies, fermentation experiments were performed with the DS17690 parental strain and derived strains in which a further 2.5 g/liter of phenoxyacetic acid (POA) was added after 3 days of growth. After 5 days of growth, both the parental DS17690 and derived (DS17690^{penDE10}, DS17690^{penDE10-11}, and DS17690^{penDE30}) strains showed a 20 to 30% increase in penicillin V production when additional POA is added (Fig. 5). This demonstrates that in the newly constructed *penDE* overexpressing strains, production is not limited by the availability of phenoxyacetic acid.

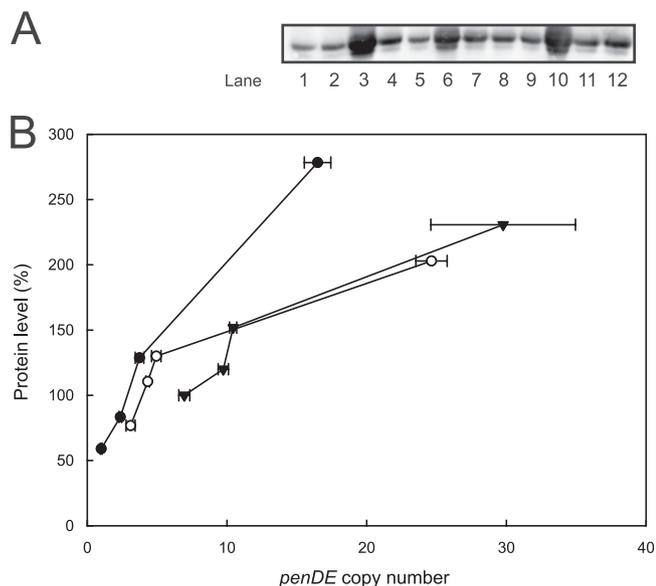


FIG 4 IAT protein levels in strains containing additional copies of *penDE*. (A) Protein levels of IAT as determined by Western blot analysis. Lanes: 1, DS47274 (one cluster); 2, DS47274^{penDE2}; 3, DS47274^{penDE15-16}; 4, DS47274^{penDE4}; 5, DS47273 (three clusters); 6, DS47273^{penDE24}; 7, DS47273^{penDE4}; 8, DS47273^{penDE5}; 9, DS17690 (seven or eight clusters); 10, DS17690^{penDE30}; 11, DS17690^{penDE10}; and 12, DS17690^{penDE10-11}. (B) Quantitation of the IAT levels as a function of the *penDE* gene copy number. Closed circles correspond to strains DS47274 (one cluster), DS47274^{penDE2}, DS47274^{penDE4}, and DS47274^{penDE15-16}. Open circles show strains DS47273 (three clusters), DS47273^{penDE4}, DS47273^{penDE5}, and DS47273^{penDE24}, and closed triangles display DS17690 (seven or eight clusters), DS17690^{penDE10}, DS17690^{penDE10-11}, and DS17690^{penDE30}.

Since IAT also catalyzes the hydrolytic reaction from penicillin V to 6-APA (2), the extracellular levels of 6-APA were determined after 5 and 7 (data not shown) days of growth using liquid chromatography-mass spectrometry (LC-MS). With more than 20 copies of *penDE*, a marked (4- to 5-fold) increase in extracellular 6-APA levels was noted in the DS47273 (three clusters) and

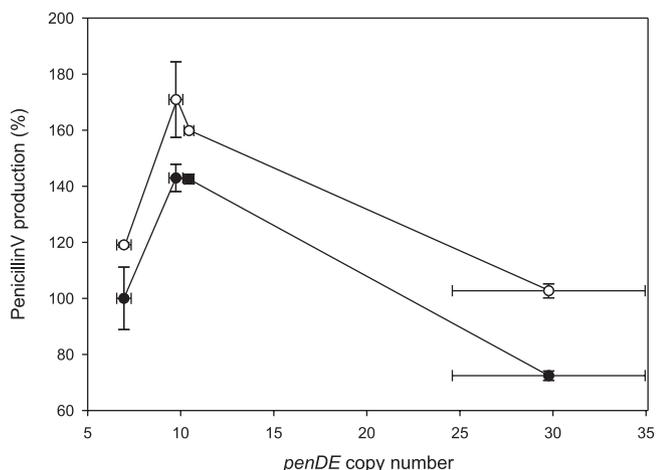


FIG 5 Penicillin V production in the presence and absence of the extra addition of POA by strains with an increased copy number of the *penDE* gene. Closed circles correspond to DS17690 (seven or eight clusters), DS17690^{penDE10}, DS17690^{penDE10-11}, and DS17690^{penDE30} without the addition of POA, and open circles display DS17690^{penDE10}, DS17690^{penDE10-11}, and DS17690^{penDE30} with the addition of POA.

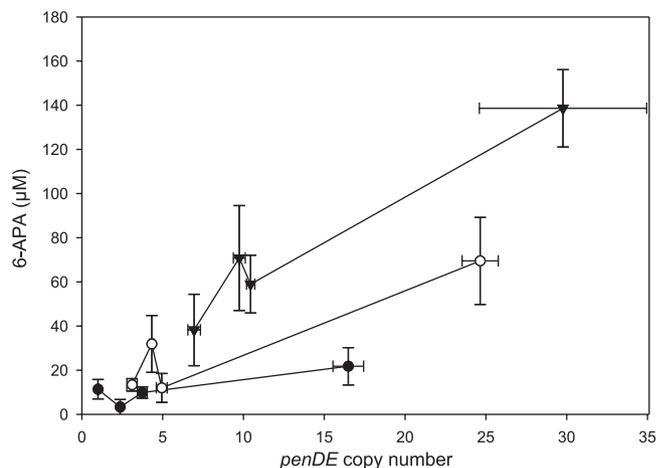


FIG 6 6-APA production by strains with an increased copy number of the *penDE* gene. Closed circles correspond to strains DS47274 (one cluster), DS47274^{penDE2}, DS47274^{penDE4}, and DS47274^{penDE15-16}. Open circles show strains DS47273 (three clusters), DS47273^{penDE4}, DS47273^{penDE5}, and DS47273^{penDE24}, and closed triangles display DS17690 (seven or eight clusters), DS17690^{penDE10}, DS17690^{penDE10-11}, and DS17690^{penDE30}.

DS17690 (seven or eight clusters)-derived strains (Fig. 6). This was not apparent at the lower copy numbers (4 to 11), which suggests that the decrease in penicillin V production at high IAT levels is caused by a conversion of penicillin V or isopenicillin N into 6-APA. It should be noted that in the DS47274 (one cluster)-derived strains, in which penicillin production is limited by the lower level of expression of the biosynthetic gene cluster, only low levels of 6-APA were formed irrespective of the number of *penDE* copies (2 to 15). Therefore, significant levels of 6-APA are formed only when the penicillin production levels are high and IAT activity is in excess.

DISCUSSION

The increase in industrial β-lactam production by *P. chrysogenum* has been mainly the result of an intense classical strain improvement (CSI) program that, among other things, resulted in a large increase in the copy number of the entire penicillin biosynthesis cluster that readily undergoes multiplication in between tandem repeats (5). Previously, we have studied a series of isogenic strains that differed in the copy numbers of the penicillin biosynthesis cluster to reveal potential bottlenecks in high-yielding strains (19). The study showed the accumulation of substantial levels of isopenicillin N in strains harboring high numbers of the biosynthetic gene cluster. Furthermore, the level of IAT protein saturated already at low cluster copy numbers, suggesting that this enzyme is limiting for penicillin production in high-yielding strains. However, in such cells the expression of *phl* was also reduced, likely because of the depletion of the precursor phenyl- or phenoxyacetic acid that also acts as an inducer for *phl* expression. Thus, it could not be excluded that penicillin production in high-yielding strains is limited by the availability of CoA-activated phenoxy- or phenylacetic acid, although under such circumstances accumulation of 6-APA and 8-hydroxypenicillanic acid (8-HPA) which is formed upon reaction of 6-APA with CO₂, would be expected rather than the accumulation of IPN. To resolve this issue, we have introduced additional copies of the *penDE* and *phl* genes into three different host strains containing 1 (DS47274), 3 (DS47273), or 8

(DS17690) copies of the penicillin biosynthesis cluster. In the approach, the copy number of *penDE* was elevated with few (1 or 2) and many (15 or more) copies, while for the *phl* gene, present in all these strains as a single-copy gene, the copy number could be increased up to 16-fold. The results clearly show that substantially higher levels (up to 2-fold) of penicillin were produced upon the introduction of additional copies of the *penDE* gene provided that the copy number does not become too high, i.e., that it remains less than 10. In contrast, the increase in the *phl* gene copy number only marginally affected penicillin production. Moreover, introduction of additional copies of *penDE* or *phl* in the DS47274 strain that contains only a single biosynthetic gene cluster did not result in an increased production of penicillin V. Apparently, the levels of the other biosynthetic enzymes limit production. In this study, we also introduced an IAT variant with an improved peroxisomal targeting signal and a variant in which the introns in the *penDE* gene were removed. However, this did not result in a specific improvement beyond that observed by overexpression of the native *penDE* gene (data not shown).

With some of the clones, high copy numbers of the *penDE* gene, of 15 up to 30, were obtained. In these strains, IAT was overexpressed to very high levels, showing that the *pcbC* promoter can boost the *penDE* transcript levels to much higher levels than its native promoter. Apparently, the increased transcript levels overcome the possible bottlenecks in either the translation, maturation, targeting, or microbody translocation of IAT. In this respect, a change of PTS1 targeting signal for microbody-localized proteins from the less optimal ARL to SKL (10) did not further improve the strains, thus making targeting a less obvious candidate for the limiting step. Also, maturation does not seem to be limiting for IAT expression, as the immunoblot analysis of IAT revealed only low levels of unprocessed IAT in the high-*penDE*-copy-number strains. Unprocessed IAT was essentially absent in the low-copy-number strains. Possibly the mRNA stability and/or degradation is a factor that limits the IAT production in low-copy-number strains. Surprisingly, in the strains with very high numbers of the *penDE* gene, the penicillin V production levels decreased with a concomitant increase of 6-APA. The latter suggests the uncontrolled removal of the side chain of penicillin. This is likely due to a loss in balance between the expression levels of IAT and those of ACVS and IPNS, with the excess of IAT causing a loss of penicillin V production because of the hydrolase activity of IAT on penicillin V and/or IPN. Interestingly, with strain DS47274, which contains a single copy of the biosynthetic gene cluster, these very high numbers of *penDE* copies did not result in significant 6-APA production, consistent with the notion that in this strain the availability of the biosynthetic enzymes is limiting for production. Thus, 6-APA production upon the high overexpression of *penDE* must be linked to high levels of intracellular IPN or penicillin V. Since the affinity of IAT to convert IPN or penicillin V into 6-APA is low (2), this reaction will occur to a significant extent only at high intracellular concentrations of IPN or penicillin V. Therefore, at these high production levels, export of the final product out of the microbody and/or the cell might become limiting, or alternatively the activity of PCL becomes limiting in providing sufficient activated side chain to the IAT enzyme, resulting in the intracellular accumulation of IPN.

To summarize, the introduction of additional copies of the *penDE* gene encoding IAT into high-penicillin-yielding strains results in a substantial increase in penicillin production. However,

this expression must be balanced with that of the other biosynthetic enzymes; i.e., these high levels of penicillin production are not obtained when the IAT levels are too low or too high. In the latter case, IAT utilizes some of the IPN or penicillin V as a substrate, resulting in reduced penicillin V production and an accumulation of 6-APA. This study provides a rational approach to increase the penicillin production levels in industrial strains.

ACKNOWLEDGMENTS

This project was carried out within the research program of the Kluyver Centre for Genomics and Industrial Fermentation, which is part of the Netherlands Genomic Initiative/Netherlands Organization for Scientific Research.

We acknowledge the DSM Biotechnology Center for providing *Penicillium chrysogenum* DS strains.

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