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*Published in:*
Applied and environmental microbiology

*DOI:*
10.1128/AEM.01561-19

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BIOTECHNOLOGY

# Impact of Classical Strain Improvement of *Penicillium rubens* on Amino Acid Metabolism during β-Lactam Production

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**ABSTRACT** To produce high levels of β-lactams, the filamentous fungus *Penicillium rubens* (previously named *Penicillium chrysogenum*) has been subjected to an extensive classical strain improvement (CSI) program during the last few decades. This has led to the accumulation of many mutations that were spread over the genome. Detailed analysis reveals that several mutations targeted genes that encode enzymes involved in amino acid metabolism, in particular biosynthesis of L-cysteine, one of the amino acids used for β-lactam production. To examine the impact of the mutations on enzyme function, the respective genes with and without the mutations were cloned and expressed in *Escherichia coli*, purified, and enzymatically analyzed. Mutations severely impaired the activities of a threonine and serine deaminase, and this inactivates metabolic pathways that compete for L-cysteine biosynthesis. Tryptophan synthase, which converts L-serine into L-tryptophan, was inactivated by a mutation, whereas a mutation in 5-aminolevulinate synthase, which utilizes glycine, was without an effect. Importantly, CSI caused increased expression levels of a set of genes directly involved in cysteine biosynthesis. These results suggest that CSI has resulted in improved cysteine biosynthesis by the inactivation of the enzymatic conversions that directly compete for resources with the cysteine biosynthetic pathway, consistent with the notion that cysteine is a key component during penicillin production.

**IMPORTANCE** *Penicillium rubens* is an important industrial producer of β-lactam antibiotics. High levels of penicillin production were enforced through extensive mutagenesis during a classical strain improvement (CSI) program over 70 years. Several mutations targeted amino acid metabolism and resulted in enhanced L-cysteine biosynthesis. This work provides a molecular explanation for the interrelation between secondary metabolite production and amino acid metabolism and how classical strain improvement has resulted in improved production strains.

**KEYWORDS** *Penicillium rubens*, classical strain improvement, mutation, amino acid metabolism, cysteine biosynthesis, penicillin production

Over the past 70 years, the filamentous fungus *Penicillium rubens* (previously named *Penicillium chrysogenum*) has been used as a major industrial producer of penicillin, which is one of the β-lactam antibiotics. To date, penicillins are still widely used against bacterial infections. Since the initial discovery of penicillin in 1928 by Alexander Fleming, classical strain improvement (CSI) was initiated on *Penicillium* strains during the Second World War, and after several decades of mutagenesis and selection, this resulted in industrial strains of *P. rubens* that are used today. Importantly, during CSI, the gene copy number of the main β-lactam-producing enzymes increased, which is a main contributor to the improved yield. However, CSI has also led to a massive accumulation of mutations (1, 2), and for many of these mutations, the exact impact on penicillin production remains unknown.

**Citation** Wu M, Crismaru CG, Salo O, Bovenberg RAL, Driessen AJM. 2020. Impact of classical strain improvement of *Penicillium rubens* on amino acid metabolism during β-lactam production. Appl Environ Microbiol 86:e01561-19. https://doi.org/10.1128/AEM.01561-19.

**Editor** Irina S. Druzhinina, Nanjing Agricultural University



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**Received** 10 July 2019
**Accepted** 16 November 2019
**Accepted manuscript posted online** 22 November 2019
**Published** 21 January 2020

Recently, a comparative genomic analysis was carried out on the genome sequences of three *P. chrysogenum* strains that are part of a main lineage of CSI, namely, strains NRRL1951 (a wild-type-like natural isolate), Wisconsin54-1255 (laboratory reference strain and a derivative of NRRL1951), and DS17690 (a high-yielding strain and a derivative of the Wisconsin lineage) (1). The analysis revealed that about 215 mutations occurred in genes of Wisconsin54-1255 compared to the progenitor NRRL1951, while an additional 869 mutations occurred in genes of strain DS17690 compared to its progenitor Wisconsin54-1255. Statistical analysis revealed that the mutations were spread over the genome and widely distributed among different functional categories, such as transport and metabolism, transcription, the cell cycle, energy production and conversion, and secondary metabolite biosynthesis. Given the large number of mutations, there is no specific class of genes that appears to be affected in particular.

From both genomic analysis as well as earlier genetic studies, it is clear that the CSI program has led to improved penicillin production by a number of events. This includes the amplification of the penicillin biosynthetic gene cluster (BGC) (3), the altered expression of certain genes involved in amino acid metabolism (2, 4), the reduced production of unrelated secondary metabolites (5), as well as an increased proliferation of microbodies where some of the critical enzymatic steps in penicillin biosynthesis are localized (6). However, further investigation of the mutations that accumulated during CSI also revealed mutations that likely critically affected the functionality of enzymes involved in amino acid metabolism. The three precursors for penicillin biosynthesis, i.e., L-$\alpha$-aminoadipic acid, cysteine, and valine, are derived from the general amino acid biosynthetic pathways (7). Cysteine biosynthesis is interconnected to sulfur metabolism. The focus of this study was to determine the impact of the mutations on the enzyme activity of proteins involved in amino acid and sulfur metabolism. From the genomic analysis, seven genes in amino acid metabolism were identified with mutations obtained during CSI. Through functional analysis of the catalytic activities of the purified wild-type and mutated proteins, the potential impacts of these identified mutations on amino acid metabolism were mapped. The data show that many of the mutations served to optimize the biosynthesis of cysteine, a key amino acid in penicillin biosynthesis.

## RESULTS

**CSI mutations in genes involved in amino acid metabolism.** In a recent genomic analysis of a lineage of *P. rubens* strains (1), we identified a large set of genes that were mutated during the CSI program. The analysis included the progenitor NRRL1951 that through CSI was converted into an intermediate strain, Wisconsin54-1255, with a single penicillin BGC (CSI stage I). The latter was further developed into the high-penicillin-yielding strain DS17690 through CSI, and among others, this resulted in a massive amplification of the penicillin BGC (CSI stage II). The latter has been a major factor in the improved penicillin production, but to what extent the mutations have contributed to the improved strain characteristics in penicillin production is still poorly understood. Seven of the 10 mutated genes encode enzymes involved in amino acid metabolism (1), and these were further analyzed in this work (Table 1). By using multiple-sequence alignment (NCBI) and the SusPect tool (8), enzyme functions were predicted, as was the possible impact of the mutations on catalytic activity. A higher SusPect score suggests a greater likelihood of a deficiency in the enzyme activity. Multiple-sequence alignment was used to determine if the point mutations localize to highly conserved regions. Only two of the seven amino acid metabolism genes (*Pc20g08350* and *Pc22g13500*) were found to be mutated during CSI stage I, whereas the others were mutated in CSI stage II. Most of these mutations are predicted to be located in highly conserved domains, especially the mutation in *Pc16g03260* that is predicted to be in the highly conserved pyridoxal 5′-phosphate (PLP) binding site.

Bioinformatics analysis indicated that all seven mutated genes are annotated to be associated with cysteine biosynthesis (Fig. 1). Only *Pc20g08350* is directly involved in cysteine biosynthesis, as it encodes a putative *O*-acetyl-L-serine (OAS) sulfhydrylase

**TABLE 1** Genes involved in amino acid metabolism with mutations introduced during CSI stages I and II

| Gene ID | Base change | Amino acid mutation | EC no. | Enzyme function | CSI stage | Type of region | SusPect score |
|---|---|---|---|---|---|---|---|
| *Pc20g04020* | 1144A>T | T382S | 4.2.3.1 | Threonine synthase | II | Conserved | 13 |
| *Pc13g07730* | 1070G>A | R357H | 4.3.1.19 | Threonine deaminase | II | Highly conserved | 29 |
| *Pc16g03260* | 136A>C | K46Q | 4.3.1.17 | Serine deaminase | II | PLP binding site | 95 |
| *Pc20g08350* | 1069A>G | T357A | 2.5.1.47 | *O*-Acetyl-L-serine sulfhydrylase | I | Highly conserved | 20 |
| *Pc22g13500* | 796G>T | A266S | 2.3.1.37 | 5-Aminolevulinate synthase | I | Conserved | 23 |
| *Pc21g03590* | 94G>A | E32K | 4.2.1.20 | Tryptophan synthase | II | Highly conserved | 45 |
| *Pc13g06360* | 590C>T | A197V | 3.1.3.7 | 3′(2′),5′-Bisphosphate nucleotidase | II | Highly conserved | 37 |
| *Pc12g10550* | 1384C>T | V462I | 1.1.1.95 | D-3-Phosphoglycerate dehydrogenase | I | Disordered | 8 |
| *Pc20g00530* | 357C>T | | 1.1.1.95 | D-3-Phosphoglycerate dehydrogenase | II | | |
| *Pc23g00640* | 521G>A | A174V | 4.3.1.19 | Threonine deaminase | II | Highly conserved | 51 |
| | | | 4.2.1.20 | Tryptophan synthase | | | |

(OASS). *Pc13g06360* encodes a 3′(2′),5′-bisphosphate nucleotidase involved in sulfur assimilation, while the other five genes are predicted to be involved in threonine, glycine, and serine metabolism. The latter pathways all connect to cysteine biosynthesis; thus, the mutations may indirectly affect this process. *Pc20g04020* encodes a threonine synthase (TS) involved in threonine biosynthesis, while the other genes are involved in the degradation of threonine, glycine, and serine. *Pc13g07730* and *Pc16g03260* encode threonine/serine deaminases that utilize L-threonine and L-serine, while *Pc22g13500* encodes a 5-aminolevulinate synthase (5-ALAS) that converts glycine into 5-aminolevulinate. *Pc21g03590* encodes a tryptophan synthase that utilizes L-serine as a substrate. The reactions catalyzed by these enzymes are depicted in Fig. S1 in the supplemental material.

Three more genes involved in amino acid metabolism were identified with mutations (Table 1), but these were not further studied as either the mutations did not result in amino acid substitutions or the respective genes are not expressed. *Pc12g10550* and *Pc20g00530* are annotated as encoding 3-phosphoglycerate dehydrogenases that catalyze the first committed and rate-determining step in the phosphoserine pathway of

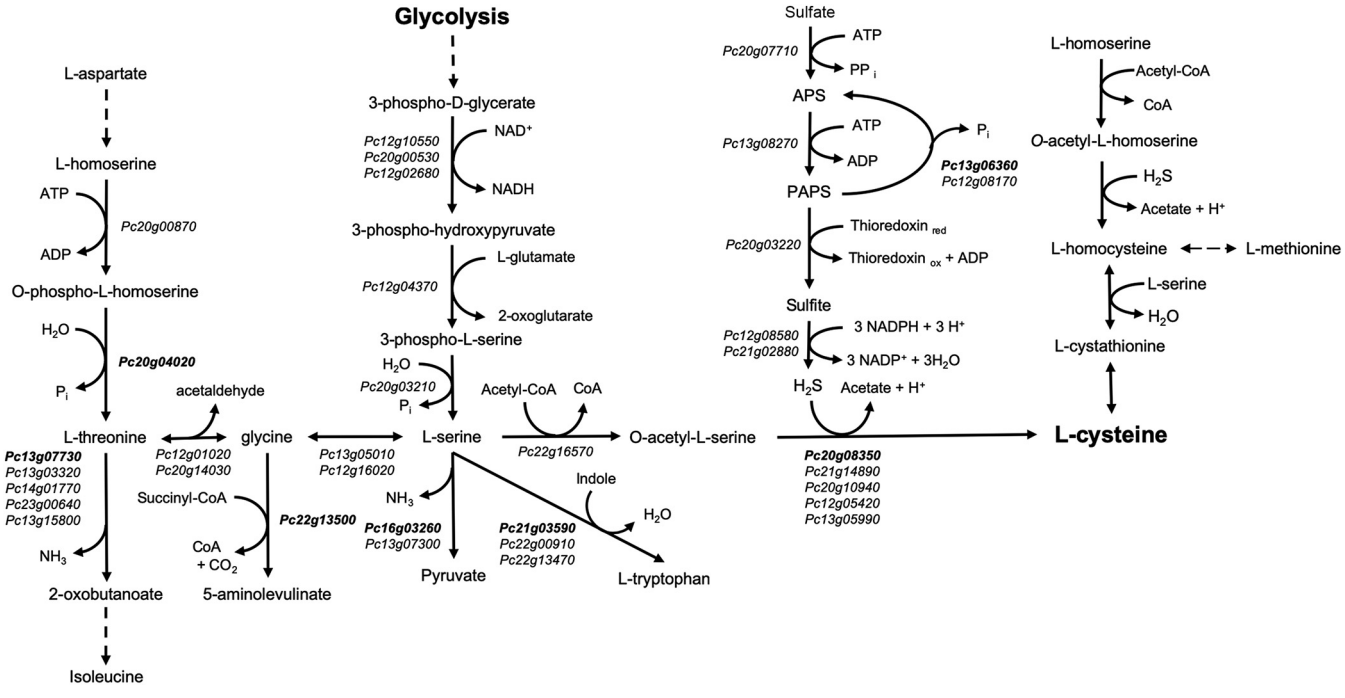


**FIG 1** Amino acid and sulfur metabolism in *Penicillium rubens*. Genes indicated in boldface type were analyzed in this study and contained mutations collected during CSI.

serine biosynthesis. The enzyme converts 3-phospho-D-glycerate (3PG) and $NAD^+$ into 3-phosphonooxypyruvate and NADH. The mutation in the *Pc20g00530* gene is silent, as it does not change the amino acid isoleucine at position 119. In *Pc12g10550*, the mutation site is located in a predicted disordered region yielding a very low SusPect score. A further mutation was found in the *Pc23g00640* gene, predicted to encode a threonine deaminase or tryptophan synthase $\beta$, which are both PLP-dependent enzymes. However, sequence alignment shows that the putative PLP binding site contains a histidine instead of a strictly conserved lysine, which implies that the enzyme cannot use PLP (9). The exact function of this enzyme therefore remains unknown. Importantly, this gene is not expressed in Wisconsin54-1255 and DS17690 (Table S1) and therefore was not further analyzed in this study.

To validate the predicted enzyme functions of the selected group of enzymes and to assess the impact of the mutations on their catalytic activity, the genes of various wild-type and mutant proteins (Table 1) were expressed in *Escherichia coli* BL21(DE3) and encoded C- or N-terminal 6×His-tagged proteins. Proteins were subjected to purification by Ni-nitrilotriacetic acid (NTA) affinity chromatography (Fig. 2) and further characterized enzymatically. Only in the case of the threonine deaminase mutant was the protein overexpressed and purified as a C-terminal fusion to maltose binding protein (MBP), as discussed below.

**Threonine synthase (Pc20g04020).** *Pc20g04020* is the only gene in *P. rubens* that is predicted to encode a threonine synthase (TS). This enzyme catalyzes the conversion of *O*-phospho-L-homoserine (OPHS) into L-threonine and phosphate (Fig. S1a). L-Threonine can be further converted into other amino acids such as L-cysteine. During CSI stage II, the gene was mutated, causing an amino acid change from threonine to serine at position 382. Multiple-sequence alignment with a related fungal threonine deaminase indicates that the mutation is in a region that is not highly conserved, and in related fungal species, variants exist that already have a serine at this position instead of threonine. Furthermore, the impact of the substitution may be rather limited, as both amino acids contain a hydroxyl group in the side chain and hence have a low SusPect score. Transcriptome data for *P. rubens* strains Wisconsin54-1255 and DS17690 grown in shaken flasks with and without the penicillin side chain precursor phenylacetic acid showed that the *Pc20g04020* gene is expressed, while the expression level is slightly higher in the DS17690 strain than in Wisconsin54-1255 (Table S1).

Both the wild type and the T382S mutant were overexpressed in *E. coli* BL21(DE3) and purified (Fig. 2A). Next, the proteins were tested for TS activity, and indeed, conversion of *O*-phospho-L-homoserine into L-threonine and phosphate was observed. Kinetic analysis revealed that the T382S mutant was fully active, showing only a somewhat reduced $K_m$ for *O*-phospho-L-homoserine and an elevated $V_{max}$ value, resulting in a $k_{cat}$ that was only slightly higher than that of the wild type (Table 2). Threonine synthase activity in some organisms, such as in *Arabidopsis thaliana* (10, 11) or *Lemna paucicostata* (12), is stimulated by *S*-adenosylmethionine (SAM). Also, feedback inhibition by L-cysteine has been reported, for instance, for the threonine synthases of *Bacillus subtilis* (13) and *Glycine max* (14). Therefore, we examined if the mutation affected the above-mentioned processes. The activity of neither the wild-type nor the mutant enzyme was stimulated by SAM (up to 1 mM) or inhibited by L-cysteine (up to 5 mM) (data not shown). Taken together, these data suggest that the *Pc20g04020* gene encodes a threonine synthase enzyme and that the point mutation (T382S) collected during CSI marginally affected enzyme activity. However, since the transcriptional level of the *Pc20g04020* gene in the DS17690 strain is elevated relative to that in Wisconsin54-1255, we conclude that CSI has resulted in elevated threonine synthase activity.

**L-Serine/L-threonine deaminase (Pc13g07730/Pc16g03260).** *Pc13g07730* is annotated as encoding an L-threonine deaminase (TD), while the *Pc16g03260* gene product might function as an L-serine deaminase (SD). These enzymes deaminate L-threonine and L-serine, resulting in the production of 2-oxobutanoate and pyruvate, respectively

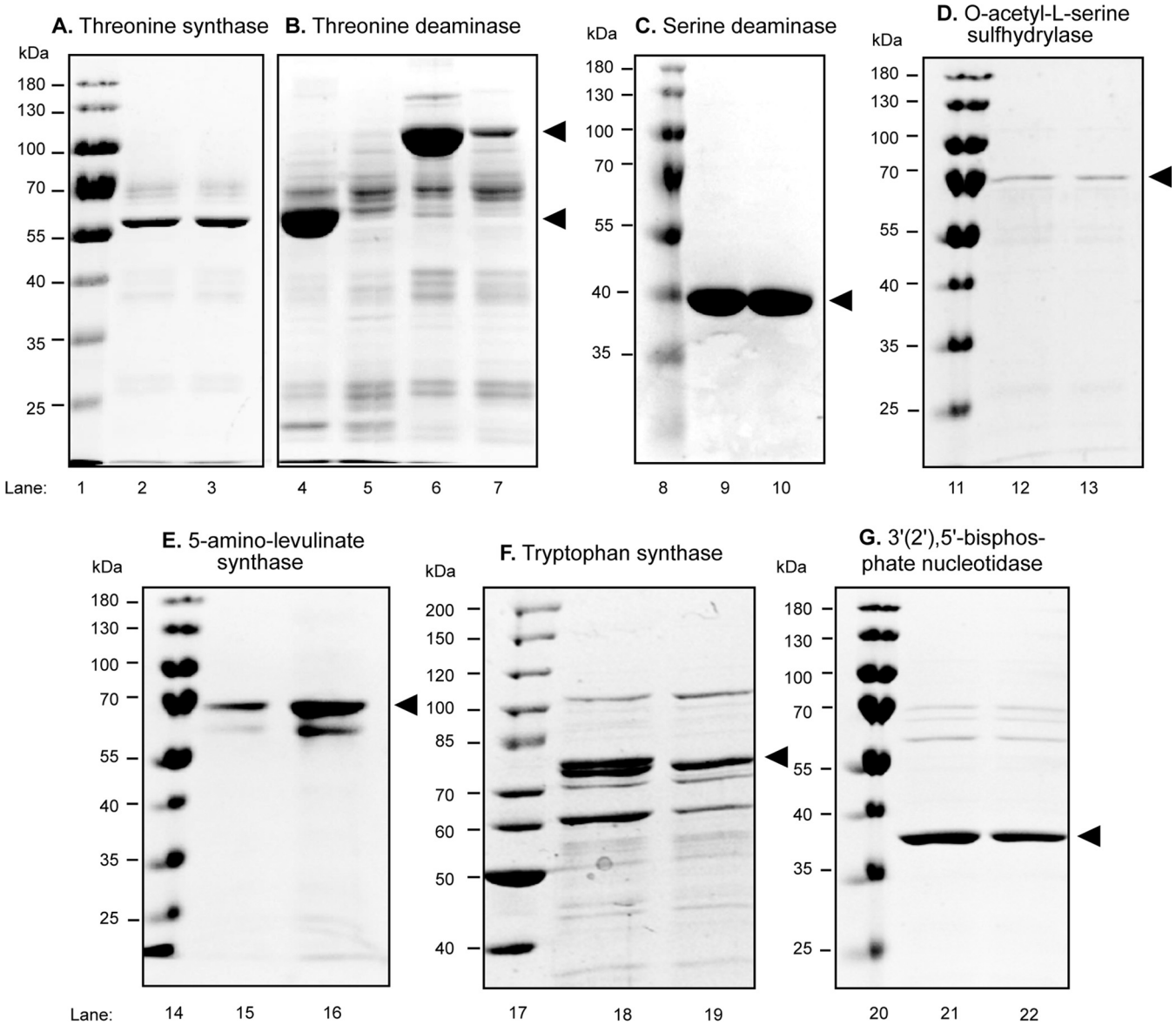


**FIG 2** SDS-PAGE gels of purified wild-type and mutant proteins. (A) Threonine synthase (lane 1, mass marker; lane 2, wild type; lane 3, T382S mutant); (B) threonine deaminase (lane 4, wild type; lane 5, R357H mutant; lane 6, wild-type and MBP fusion; lane 7, R357H mutant and MBP fusion); (C) serine deaminase (lane 8, mass marker; lane 9, wild type; lane 10, K46Q mutant); (D) *O*-acetyl-L-serine sulfhydrylase (lane 11, mass marker; lane 12, wild type; lane 13, T357A mutant); (E) 5-aminolevulinate synthase (lane 14, mass marker; lane 15, wild type; lane 16, A266S mutant); (F) tryptophan synthase (lane 17, mass marker; lane 18, wild type; lane 19, E32K mutant); (G) 3′(2′),5′-bisphosphate nucleotidase (lane 20, mass marker; lane 21, wild type; lane 22, A197V mutant). The arrows indicate the expected molecular masses of the respective enzymes in the SDS-PAGE gel based on the molecular weight marker.

(Fig. S1b). Potentially, these enzymes compete with the L-cysteine biosynthetic pathway for the above-mentioned substrates. Further bioinformatics screening revealed five other potential threonine/serine deaminase orthologs in the *P. rubens* genome (Fig. 1 and Table S1), two of which are annotated as acetylornithine deacetylases and one of

**TABLE 2** Kinetic parameters of wild-type and mutant threonine synthases (Pc20g04020)

| Enzyme[a] | Mean sp act ($\mu$mol/min/mg) ± SD | Mean $V_{max}$ ($\mu$mol/min/mg) ± SD | Mean $K_m$ (mM) ± SD | Mean $k_{cat}$ ($s^{-1}$) ± SD | Mean $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) ± SD |
|---|---|---|---|---|---|
| TS | 0.25 ± 0.01 | 0.28 ± 0.01 | 0.36 ± 0.07 | 0.28 ± 0.01 | 0.80 ± 0.10 |
| TS T382S | 0.30 ± 0.01 | 0.35 ± 0.02 | 0.90 ± 0.16 | 0.35 ± 0.01 | 0.39 ± 0.04 |

[a]Kinetic parameters were determined by varying the OPHS concentration from 0.25 to 10 mM.

**TABLE 3** Specific activities of threonine deaminase (Pc13g07730) and serine deaminase (Pc16g03260) wild-type and mutant enzymes

| Enzyme | Mean sp act ($\mu$mol/min/mg) ± SD[a] | |
|---|---|---|
| | L-Threonine | L-Serine |
| Threonine deaminase | | |
| Wild type | 31.2 ± 0.8 | 1.16 ± 0.02 |
| R357H mutant | 0.79 ± 0.01 | 0.01± 0.04 |
| Serine deaminase | | |
| Wild type | 7.36 ± 0.19 | 1.64 ± 0.01 |
| K46Q mutant | ND | ND |

[a]ND, not detectable.

which might have a different function, as detailed below (Pc23g00640), but is not expressed.

PLP is the active form of vitamin $B_6$ and is a coenzyme in a variety of enzymatic reactions. Threonine and serine deaminases are both PLP-dependent enzymes, where PLP will covalently bind through a Schiff base linkage to a conserved lysine but also interacts with other amino acyl sites. Based on sequence analysis, the C-3 hydroxyl group of PLP likely will be hydrogen bonded to the side chain of N77 (Fig. S2, gray), while the phosphate group of PLP will be coordinated by the main-chain amides from the tetraglycine loop ($^{198}$GGGGL$^{202}$) (Fig. S2, gray) (9).

The above-mentioned deaminase genes were mutated during CSI stage II. The R357H mutation in *Pc13g07730* is in a highly conserved domain leading to an intermediate SusPect score. This suggests a potential defect in activity. On the other hand, the K46Q mutation in *Pc16g03260* concerns a conserved lysine, which is part of the PLP cofactor binding site, as evident from multiple-sequence alignment (Fig. S2). Indeed, the SusPect score indicates a severe catalytic defect by the K46Q mutation. Transcriptome data show that both genes are expressed when *P. rubens* is grown in shaken flasks (Table S1). Notably, the expression level of *Pc13g07730* is lower in the high-penicillin-yielding strain DS17690 than in Wisconsin54-1255.

Both wild-type and mutant proteins were expressed as His-tagged proteins in *E. coli* BL21(DE3) and purified (Fig. 2B and C). In the case of the Pc16g03260 protein, the yields of purified protein were comparable for the wild type and the K46Q mutant. However, in the case of the Pc13g07730 protein, the R357H mutation dramatically reduced the protein yield. This low expression level suggests that this mutant is highly unstable in *E. coli* and points to a folding defect (Fig. 2B). In order to obtain sufficient amounts of the threonine deaminase for enzyme activity assays, the *Pc13g07730* gene (wild type and mutant) was expressed as a C-terminal fusion with maltose binding protein (MBP) using a pBAD expression system. MBP enhanced the expression and solubility of the threonine deaminase mutant. This resulted in the purification of both proteins, although the yield of the mutant was only ~14% compared to that of the wild type (Fig. 2B).

Next, the purified enzymes were tested for serine and threonine deaminase activity using L-threonine and L-serine as the substrates, resulting in the production of 2-oxobutanoate and pyruvate, respectively. Pc13g07730 showed much higher L-threonine deaminase activity than Pc16g03260, while the latter showed slightly higher L-serine deaminase activity (Table 3). Both enzymes are more active with L-threonine than with L-serine. The specific threonine deaminase activity of the Pc13g07730 R357H mutant protein was reduced by almost 40-fold, while its serine deaminase activity was almost undetectable. The Pc16g03260 K46Q mutant was completely inactive.

The results of protein expression and enzyme assays show that the point mutation (R357H) severely reduces the stability and activity of the Pc13g07730 protein. Assuming that protein stability is also reduced in *P. rubens*, the mutation resulted in a near-to-complete loss of serine and threonine deaminase activity. Likewise, the K46Q mutation

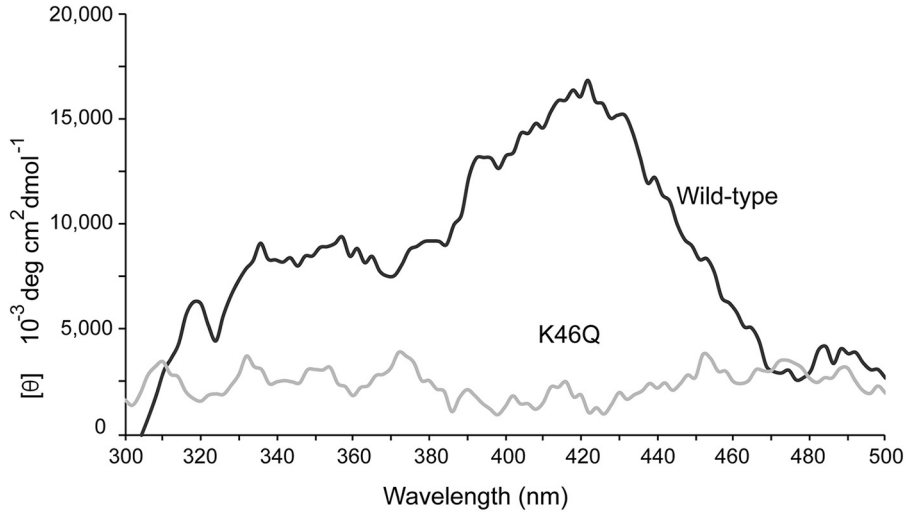


**FIG 3** CD spectra of the serine deaminase wild type and K46Q mutant encoded by the *Pc16g03260* gene. When bound to the apoenzyme, the cofactor PLP yields a typical CD spectrum with positive ellipticities at 330 and 415 nm. Serine deaminase enzyme (1 mg/ml) was suspended in a solution containing 10 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, and 0.1 mM DTT.

in Pc16g03260 leads to a complete loss of deaminase activity. To verify that K46 is indeed critical for covalent binding to PLP, circular dichroism (CD) spectroscopy was performed on the wild-type and mutant proteins. The results show that the K46Q mutant of Pc16g03260 no longer binds PLP, as the typical absorption peaks at 330 and 415 nm reminiscent of the presence of PLP (Fig. 3) were absent. Taken together, these data indicate that L-threonine and L-serine degradation is severely impacted by the mutations in Pc16g03260 and Pc13g07730, which potentially allows for a greater flux of these amino acids into L-cysteine biosynthesis. Because of the expression of alternative threonine/serine deaminases, however, the respective pathways are likely still operational.

***O*-Acetyl-L-serine sulfhydrylase (Pc20g08350).** Multiple-sequence alignment and protein modeling by Phyre² (15) predict that *Pc20g08350* encodes a putative cystathionine gamma-synthase (CGS), which catalyzes the conversion of *O*-acetyl-L-homoserine and L-cysteine into L-cystathionine and acetate. *Pc20g08350* has collected a point mutation (T357A) during CSI stage I (from NRRL1951 to Wisconsin54-1255), and this mutation concerns a highly conserved residue. The SusPect score is intermediate. The average transcript levels suggest that the gene is expressed (Table S1). The Pc20g08350 wild-type and T357A mutant proteins were expressed in *E. coli* BL21(DE3) and purified (Fig. 2D). Next, the wild-type protein was tested with different potential substrates. Pc20g08350 was able to convert *O*-acetyl-L-serine and hydrogen sulfide into L-cysteine and acetate, a reaction that is part of the direct sulfhydrylation pathway for L-cysteine biosynthesis (Fig. S1c). Importantly, the enzyme was found to be inactive with *O*-acetyl-L-homoserine and L-cysteine for the production of L-cystathionine or with *O*-acetyl-L-homoserine and hydrogen sulfide for the production of L-homocysteine. The latter two reactions belong to the transsulfuration pathway. These data identify Pc20g08350 as an *O*-acetyl-L-serine sulfhydrylase (OASS) instead of the annotation as a CGS or *O*-acetyl-L-homoserine sulfhydrylase (OAHS). This further suggests that in *P. rubens*, L-cysteine can be formed through the direct sulfhydrylation pathway (16). However, the reported molecular masses of the putative OASS proteins of 59 and 68 kDa differ from what is observed in this study, which is 65.6 kDa. Also, the $K_m$ of 1.3 mM for OAS in the previous report differs from the $K_m$ value found for Pc20g08350 in this study, which is about 21 mM (Table 4). Unfortunately, the enzyme in the previous report was not linked to a specific gene.

Next, we examined the activity of the T357A mutant of Pc20g08350, which revealed an increase in activity by about 40% (Table 4). Since both the $k_{cat}$ and $k_{cat}/K_m$ were

**TABLE 4** Kinetic parameters of *O*-acetyl-L-serine sulfhydrylase (Pc20g08350) wild-type and mutant enzymes

| Enzyme[a] | Mean sp act (μmol/min/mg) ± SD | Mean $V_{max}$ (μmol/min/mg) ± SD | Mean $K_m$ (mM) ± SD | Mean $k_{cat}$ ($s^{-1}$) ± SD | Mean $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) ± SD |
|---|---|---|---|---|---|
| Wild type | 0.052 ± 0.001 | 0.074 ± 0.007 | 21.5 ± 5.6 | 0.08 ± 0.01 | 0.0039 ± 0.0005 |
| T357A mutant | 0.073 ± 0.002 | 0.110 ± 0.013 | 25.3 ± 7.2 | 0.12 ± 0.01 | 0.0049 ± 0.0007 |

[a]Kinetic parameters were determined by varying the OAS concentration from 5 to 60 mM.

higher for the mutant than for the wild type, the enzyme catalytic efficiency was improved by the mutation, which in turn may lead to improved L-cysteine biosynthesis in the CSI-optimized *P. rubens* strains. The elevated activity of the Pc20g08350 mutant may arise from reduced substrate inhibition (17) for the OASS enzyme, as reported previously for the enzymes from *E. coli* (18), *Spinacia oleracea* (19), and *Salmonella enterica* serovar Typhimurium (20). However, neither OAS nor $Na_2S$ (both up to 50 mM) inhibited the purified enzyme. *O*-Acetylserine sulfhydrylase is also referred to as cysteine synthase. Therefore, these results identify the *Pc20g08350* gene as a cysteine synthase. *Pc21g14890*, *Pc20g10940*, *Pc12g05420*, and *Pc13g05990* are also predicted to function as cysteine synthases. The first three genes are all expressed, while the *Pc13g05990* gene is not expressed (Table S1), but none of the genes obtained were mutated during CSI. Our study provides direct evidence that the direct sulfhydrylation pathway is active in *P. rubens* and also that multiple enzymes will contribute to this cysteine biosynthesis pathway.

**5-Aminolevulinate synthase (Pc22g13500).** 5-Aminolevulinate synthase (5-ALAS) is a PLP-dependent enzyme that catalyzes the conversion of glycine and succinyl-CoA into 5-aminolevulinate, coenzyme A, and carbon dioxide (Fig. S1d). The corresponding enzyme in *P. rubens* is encoded by the *Pc22g13500* gene and collected an A266S point mutation during stage I of CSI. Multiple-sequence alignment indicates that the point mutation is in a less conserved region, and the low SusPect score suggests that its effect on enzyme activity might be minor. However, the gene is expressed (Table S1) and could potentially impact glycine levels in the cell. Thus, its activity may affect L-cysteine production, and therefore, the impact of the mutation was examined by enzyme overexpression, purification, and activity assays. As shown in Table 5, the enzyme exhibited the expected activity, and the kinetics were not affected by the A266S mutation (Fig. 2E). These data demonstrate that the mutation did not impact the activity and thus likely does not affect L-cysteine production.

**Tryptophan synthase (Pc21g03590).** Tryptophan synthase (TrpS) is a PLP-dependent enzyme that consists of an $\alpha_2\beta_2$ complex and catalyzes the last two steps of tryptophan biosynthesis in bacteria, plants, and fungi. It converts L-serine and indole-3-glycerol phosphate (IGP) into L-tryptophan and glyceraldehyde. Each of the subunits is responsible for catalyzing an individual step, which can be performed by isolated subunits. The $\alpha$ subunits catalyze the reversible formation of indole and glyceraldehyde-3-phosphate (G3P) from IGP. The $\beta$ subunits catalyze the irreversible condensation of indole and serine to form tryptophan in a PLP-dependent reaction (Fig. S1e). Sequence alignment suggests that the TrpS gene of *P. rubens* specifies both the $\alpha$ and $\beta$ subunits of the tryptophan synthase. Also, the enzyme harbors the expected PLP binding sites. During CSI stage II, TrpS collected a point mutation (E32K) at a highly conserved position. Sequence alignment and a high SusPect score suggest that the mutation is located in the $\alpha$ subunit and likely compromises enzyme function. Transcriptional data

**TABLE 5** Kinetic parameters of 5-aminolevulinate synthase (Pc22g13500) wild-type and mutant enzymes

| Enzyme[a] | Mean sp act (μmol/min/mg) ± SD | Mean $K_m$ (mM) ± SD | Mean $V_{max}$ (μmol/min/mg) ± SD | Mean $k_{cat}$ ($s^{-1}$) ± SD | Mean $k_{cat}/K_m$ ($s^{-1}$ $mM^{-1}$) ± SD |
|---|---|---|---|---|---|
| Wild type | 0.340 ± 0.021 | 6.6 ± 0.8 | 0.351 ± 0.008 | 0.404 ± 0.009 | 0.062 ± 0.005 |
| A266S mutant | 0.335 ± 0.025 | 6.4 ± 1.0 | 0.346 ± 0.009 | 0.398 ± 0.011 | 0.062 ± 0.005 |

[a]Kinetic parameters were determined by varying the glycine concentration from 1 to 120 mM.

**TABLE 6** Kinetic parameters for the wild-type and mutant tryptophan synthases (Pc21g03590)[a]

| Enzyme | Mean sp act (μmol/min/mg) ± SD | Mean $K_m$[b] (mM) ± SD | Mean $V_{max}$ (μmol/min/mg) ± SD |
|---|---|---|---|
| TrpS | 25.62 ± 1.83 | 8.92 ± 0.6 | 28.51 ± 0.46 |
| TrpS E32K | ND | ND | ND |

[a]ND, not detectable.
[b]$K_m$ was determined by varying the L-serine concentration from 2 to 100 mM.

suggest that TrpS is overexpressed in the high-penicillin-yielding *P. rubens* strain DS17690 relative to the Wisconsin strain (Table S1).

Wild-type and mutant TrpS proteins were overexpressed in *E. coli* BL21(DE3) and partially purified by Ni-NTA affinity chromatography. SDS-PAGE showed two distinct bands with a molecular mass in the expected 75-kDa range, whereas in the mutant, only the lower band was present (Fig. 2F). In addition, the sample showed several lower-mass degradation products. Due to the commercial unavailability of the substrate IGP, the forward reaction could not be tested. However, when the enzyme was tested for the conversion of indole and serine into L-tryptophan, pronounced activity was evident with the wild type, while the mutant was completely inactive (Table 6). The complete lack of L-tryptophan biosynthesis activity by the TrpS mutant may result in an improved flux of serine into L-cysteine biosynthesis. Interestingly, the expression of the paralog Pc22g00910 is severely reduced in the high-yielding strain DS17690, magnifying this effect, while the expression of Pc22g13470 remained and likely serves to maintain a sustainable level of L-tryptophan synthesis for growth.

**3′(2′),5′-bisphosphate nucleotidase (Pc13g06360).** 3′(2′),5′-Bisphosphate nucleotidase catalyzes the hydrolytic conversion of adenosine 3′-phosphate 5′-phosphosulfate (PAPS) into adenosine 5′-phosphosulfate (APS) and phosphate as well as the conversion of adenosine 3′,5′-bisphosphate (PAP) into AMP and phosphate. These reactions are essential for sulfur metabolism. The *Pc13g06360* gene is annotated to specify a 3′(2′),5′-bisphosphate nucleotidase, as verified by sequence alignment with a family of these enzymes. The *Pc13g06360* gene was mutated during CSI stage II, causing an alanine-to-valine substitution at position 197. This point mutation is in a highly conserved region, so it might potentially affect enzyme function. Importantly, transcriptional data show that the *Pc13g06360* gene is about 2-fold downregulated in the DS17690 strain compared to Wisconsin54-1255 (Table S1). This might give rise to reduced levels of 3′(2′),5′-bisphosphate nucleotidase activity.

To verify the enzyme function of Pc13g06360 and the impact of the mutation, the enzymes were expressed in *E. coli* and purified (Fig. 2G). Next, the activities of the wild-type and mutant proteins were tested under the same conditions, i.e., with fixed amounts of the substrate (0.5 mM) and purified enzyme (5 μg). The specific activity of the wild type was 0.094 ± 0.006 μmol/min/mg, while the specific activity of the mutant was 0.08 ± 0.006 μmol/min/mg. These data suggest that the mutation had only a minor effect on enzyme activity, but taken together with the reduced expression in the DS17690 strain, it appears that the overall activity is reduced in the high-yielding strain. This might result in less consumption of PAPS, and thus, more of this substrate would be available for L-cysteine biosynthesis.

## DISCUSSION

During the past several decades, classical strain improvement of industrial *P. rubens* strains has led to the appearance of many unknown beneficial mutations that collectively resulted in improved penicillin production in large-scale industrial fermentors. Amino acid metabolism is a key process in β-lactam production, as it serves to supply the three amino acid building blocks, L-aminoadipate, L-cysteine, and L-valine, to the L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine synthetase (ACVS) responsible for tripeptide formation. Here, we describe the effect of CSI on amino acid metabolism that targeted seven genes by mutations.

**TABLE 7** Impact of CSI on cysteine biosynthesis-related enzymes

| Gene ID | Mutation | Enzyme | Impact(s) on enzyme activity | Potential impact on cysteine biosynthesis |
|---|---|---|---|---|
| *Pc20g04020* | T382S | Threonine synthase | Slight increase in activity | Increased L-threonine production |
| *Pc13g07730* | R357H | Threonine deaminase | Highly unstable, low expression | Inactivation of L-threonine- and L-serine-metabolizing pathway |
| *Pc16g03260* | K46Q | Serine deaminase | Complete loss of activity | Inactivation of L-threonine- and L-serine-metabolizing pathway |
| *Pc20g08350* | T357A | *O*-Acetyl-L-serine sulfhydrylase | Slight increase in activity | Increased production of L-cysteine |
| *Pc22g13500* | A266S | 5-Aminolevulinate synthase | No change | No change |
| *Pc21g03590* | E32K | Tryptophan synthase | Inactivation | Inactivation of L-serine-metabolizing pathway |
| *Pc13g06360* | A197V | 3′(2′),5′-Bisphosphate nucleotidase | Slight decrease in activity | Increased supply of sulfur for cysteine biosynthesis |

To assess the impact of the CSI mutations on the function of the enzymes identified with mutations obtained during CSI, wild-type and mutant proteins were overexpressed in *E. coli*, purified, and subjected to enzyme assays. Three of the mutated genes are involved in the branched pathways for L-threonine and L-serine degradation, and these either lost their enzyme activities or are severely impaired in enzyme stability (Table 7). These identified genes were all mutated during the second stage of CSI (i.e., from Wisconsin54-1255 to DS17690), and thus, this process occurred in parallel with the massive amplification of the penicillin BGC. The mutated *Pc16g03260* gene that encodes a serine/threonine deaminase completely lost its enzyme function due to a point mutation (K46Q) in the PLP binding site that caused a loss of covalent PLP cofactor binding. The mutation in the *Pc13g07730* gene resulted in a highly unstable serine/threonine deaminase. This is apparent from protein expression in *E. coli*. Although the stability of expression was not tested in *Penicillium*, instability will likely lead to a significantly reduced activity. Finally, the *Pc21g03590* gene, which encodes a tryptophan synthase (TrpS) that utilizes L-serine, is severely inactivated by the mutation introduced during CSI. Furthermore, the orthologous *Pc22g00910* gene that is also annotated as a TrpS enzyme was significantly downregulated in the DS17690 strain (see Table S1 in the supplemental material). Therefore, in addition to the transcriptional effects, the inactivation of the three genes likely results in an increased flux of L-threonine and L-serine into the L-cysteine biosynthetic pathway, as there will be less consumption of these amino acids in metabolic pathways that use the same intermediates as the substrate. However, these branching pathways can be inactivated only partially, as there is a regular demand for amino acids for growth. This is likely ensured by the presence of various paralogs that are unaffected by mutagenesis, as also depicted in Fig. 1. Furthermore, the mutated threonine synthase enzyme, encoded by the *Pc20g04020* gene, showed slightly increased activity, which also will contribute to L-cysteine production. Overall, the mutational impact on amino acid metabolism is consistent with the increased demand for L-cysteine in the DS17690 strain that was induced by the amplification of the penicillin BCG.

CSI further impacted L-cysteine biosynthesis through the sulfate uptake pathway. The enzyme encoded by the *Pc13g06360* gene, 3′(2′),5′-bisphosphate nucleotidase, catalyzes the hydrolytic conversion of PAP(S) into APS or AMP and phosphate and thereby counteracts the pathway that drives the formation of sulfide from extracellular sulfate for assimilation into cysteine. The 3′(2′),5′-bisphosphate nucleotidase mutant showed slightly reduced activity, but importantly, the transcriptional data revealed that the genes involved in sulfur metabolism by converting sulfate into sulfide are all upregulated in strain DS17690 compared to Wisconsin54-1255 (Table S1), whereas the mutated *Pc13g06360* gene is about 2-fold downregulated. Previously, it was reported that the expression of *sutB*, a major sulfate permease gene, is elevated under sulfur starvation conditions in *P. rubens* Wisconsin54-1255. The expression of *sutB* is deregulated in a high-penicillin-yielding strain even when cells are grown in the presence of excess sulfate, and this will facilitate the more efficient uptake of sulfate and incorporation of sulfur into cysteine and penicillin (21). Taken together, the above-mentioned mutations and regulatory effects likely resulted in an improved diversion of the flux of sulfide for cysteine biosynthesis.

**TABLE 8** Plasmids and primers used for mutagenesis

| Gene | Plasmid | Primer for site-directed mutagenesis PCR[a] |
|---|---|---|
| *Pc20g04020* | pET28b | FW 5′-TCCAACGGTGGTTTCC-3′<br>RV 5′-TTTCAGTTCGTTCAGCC-3′ |
| *Pc13g07730* | pET28b/pBAD | FW 5′-ATGAACTGATCGCTATCACC-3′<br>RV 5′-GGTTCGGGTCCGGAG-3′ |
| *Pc16g03260* | pET28b | FW 5′-CAATCTCGTGGTATCGG-3′<br>RV 5′-GAAAGAACCAGACGGCTGC-3′ |
| *Pc20g08350* | pET28b | FW 5′-ACTCCGGACCTGAAACGTATC-3′<br>RV 5′-TTTCAGCAGCGGGTTAC-3′ |
| *Pc22g13500* | pET28b | FW 5′-GCTACCCTGGGTTCTAAAATG-3′<br>RV 5′-CAGGGTAGCGTCGTTAG-3′ |
| *Pc13g06360* | pET28b | FW 5′-TTGGTCAGGGTGCTACCATC-3′<br>RV 5′-CAACAGCAGAGATCATCTGACC-3′ |
| *Pc21g03590* | pET28b | FW 5′-AAAACCCGTGACATCCTGCTG-3′<br>RV 5′-TTCAACACGCGGGAAACCAG-3′ |

[a]FW, forward; RV, reverse.

In this study, we validated their predicted enzymatic functions. Cysteine synthase is predicted to be encoded by five different genes in *P. rubens*. The *Pc20g08350* gene harboring a mutation collected in CSI stage I showed only minor changes in enzyme kinetics, and its expression level was basically unaltered during CSI stage II. However, the *Pc21g14890*, *Pc20g10940*, and *Pc12g05420* genes are all upregulated during CSI stage II, while *Pc13g05990* is not expressed (Table S1). A proteome analysis of *P. rubens* (4) revealed that the *Pc21g14890* gene is 2-fold overrepresented in Wisconsin54-1255 compared to its progenitor NRRL1951 strain. Moreover, transcriptional data indicate that the *Pc12g10550* gene and its ortholog *Pc20g00530*, both of which are annotated as 3-phosphoglycerate dehydrogenase genes, are expressed at significantly higher levels in the DS17690 strain than in Wisconsin54-1255 (Table S1). Both genes collected a point mutation during CSI with an unknown effect on catalytic activity or expression. Their enzymes catalyze the first committed and rate-determining step in the phosphoserine pathway of serine biosynthesis, i.e., the conversion of 3-phospho-D-glycerate (3PG) and $NAD^+$ into 3-phosphonooxypyruvate and NADH. It is uncertain if the mutations indeed affected activity, as the mutation in the *Pc20g00530* gene is silent, while the mutation in the *Pc12g10550* gene is located in a predicted disordered region. The higher expression levels, however, may also contribute to an elevated production of L-cysteine.

In conclusion, our study demonstrates that the CSI program has led to mutations that affect cysteine biosynthesis, more specifically inactivating or reducing the enzymatic reactions that compete with cysteine biosynthesis for intermediates. Since transcriptional data have demonstrated that CSI has led to the upregulation of genes involved in cysteine biosynthesis, our data further underscore that cysteine biosynthesis has been one of the key targets in CSI to improve penicillin production. There are many other genes involved in primary metabolism and transport that obtained mutations during CSI and for which the function is poorly understood. Further exploration will help gain deeper insight into how classical strain improvements led to the industrial *P. rubens* strains used today and may provide leads for how to optimize this process further by rational engineering.

## MATERIALS AND METHODS

**Strains and plasmids.** The genes of seven enzymes involved in amino acid metabolism and sulfur metabolism that obtained mutations during CSI were synthesized as codon optimization versions for *E. coli* by Integrated DNA Technologies. The respective genes were cloned into the pET28b or pBAD expression plasmid and transformed into *E. coli* DH5$\alpha$ competent cells. Next, the respective mutants were obtained by site-directed mutagenesis using the PCR primers listed in Table 8, and mutations were

verified by sequencing (Macrogen, Europe). Finally, all constructs were transformed into *E. coli* BL21(DE3) for protein expression and purification. *P. rubens* DS17690 was kindly provided by DSM Sinochem B.V., now Centrient B.V.

**Protein production and purification.** For protein production, *E. coli* BL21(DE3) cells were grown overnight in LB medium containing 50 $\mu$g/ml kanamycin in a shaker at 37°C at 200 rpm. Fresh LB medium (100 ml) supplemented with 50 $\mu$g/ml kanamycin was inoculated with the culture grown overnight to an initial optical density at 600 nm ($OD_{600}$) of 0.05, and cells were grown at 37°C at 200 rpm to an $OD_{600}$ of about 0.5 to 0.6. The culture was transferred to 18°C and incubated for 1 h with shaking at 200 rpm, whereupon the inducer isopropyl-$\beta$-D-1-thiogalactopyranoside (IPTG) was added to the medium to a final concentration of 0.1 to 1 mM. Growth was continued overnight. The cells were then harvested by centrifugation at 4,000 rpm for 15 min. The pellet was resuspended in 2 ml lysis buffer (50 mM HEPES, 3 M NaCl, 1 M dithiothreitol [DTT], protease inhibitor cocktail tablet mini [cOmplete; Sigma-Aldrich], 10 mM imidazole), and cells were lysed by sonication (MSE Soniprep 150). The cell lysate was then centrifuged at 17,000 $\times$ *g* for 15 min at 4°C. The cleared supernatant was applied to a Ni-NTA column according to the manufacturer's instructions. After loading, the column was washed with a solution containing 50 mM HEPES, 300 mM NaCl, and 20 or 50 mM imidazole, and bound protein was eluted with the same buffer containing an elevated imidazole concentration (150 to 300 mM range depending on the protein). The eluate was diluted with specific buffers (50 mM Tris-HCl, phosphate, or HEPES buffer [pH 7 to 8]) and concentrated using an Amicon Ultra centrifugal filter (Millipore, USA) in order to remove the imidazole. Enzyme purity was judged by SDS-PAGE, and the protein concentration was determined using the detergent-compatible (DC) method.

**Enzyme activity measurements.** The L-threonine synthase (TS) activity was determined by the use of a malachite green reagent method described previously (11). The reaction mixture contained 50 mM HEPES buffer (pH 7.5), 50 $\mu$M PLP, different concentrations of *O*-phospho-L-homoserine (OPHS) purchased from Sigma-Aldrich, and purified enzyme (~5 $\mu$g/ml). After incubation at 25°C for 15 min, the reaction was terminated by the addition of the malachite green reagent, and after 10 min, the released inorganic phosphate was detected at 630 nm in a 96-well microplate reader. Specific activity is defined as the amount of inorganic phosphate (in micromoles) formed per minute per milligram of purified TS enzyme. $V_{max}$ is the maximum rate of the reaction when the enzyme is saturated with the substrate. Kinetic parameters ($K_m$ and $k_{cat}$) were determined with 10 different OPHS concentrations ranging from 0.25 to 10 mM and were extracted from the data with OriginPro 9.0 software (OriginLab).

The L-serine/L-threonine deaminase (SD/TD) activity of Pc16g03260 and Pc13g07730 was determined by a coupled assay using lactate dehydrogenase (LDH) (22). The reaction mixture (total of 200 $\mu$l) contained 50 mM Tris-HCl (pH 7.4), 50 $\mu$M pyridoxal 5′-phosphate (PLP) (Sigma-Aldrich), 50 mM L-serine or L-threonine, 0.5 mM NADH, and 10 U LDH (Sigma-Aldrich). The reaction was started by the addition of purified protein (1 $\mu$g SD or 1.5 $\mu$g TD with L-serine; 0.15 $\mu$g SD or 0.055 $\mu$g TD with L-threonine), and the decrease of NADH at 340 nm was monitored over time in a 96-well microplate reader (BioTek PowerWave microplate spectrophotometer) for 10 min at 25°C. Specific activities are expressed as micromoles of NADH consumed per minute per milligram of purified protein.

The *O*-acetyl-L-serine sulfhydrylase (OASS) enzyme activity was determined by measuring the absorbance at 560 nm of the reaction product L-cysteine with an acid ninhydrin solution (16, 23). The reaction mixture contained 10 mM DTT, 50 $\mu$M PLP, different concentrations of *O*-acetyl-serine (OAS), and purified protein (0.2 mg/ml) in 100 mM potassium phosphate buffer (pH 7.2). To start the reaction, 5 mM $Na_2S$ (dissolved in 0.1 M potassium phosphate buffer, pH 6.8) was added, followed by incubation at 25°C for 15 min. Reactions were stopped by the addition of 200 $\mu$l of an acid ninhydrin solution (250 mg ninhydrin in 4 ml HCl and 16 ml glacial acetic acid). The samples were boiled for 10 min and cooled in an ice bath, followed by the addition of 400 $\mu$l 95% ethanol to form a pink complex that was measured at 560 nm in a 96-well microplate reader. Specific activity is defined as micromoles of cysteine produced per minute per milligram of purified OASS enzyme. Kinetic parameters ($K_m$ and $k_{cat}$) were determined with 10 different OAS concentrations ranging from 5 to 60 mM.

The 5-aminolevulinate synthase (ALAS) enzyme activity was determined by monitoring the absorbance of the product of the reaction, CoA, as a conjugate with Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB)] (24). Briefly, a 50-$\mu$l reaction mixture containing 20 mM HEPES buffer (pH 7.5), different concentrations of glycine, 0.5 mM succinyl-CoA, 40 $\mu$M PLP, and 0 to 5 $\mu$g of purified protein was incubated in a 96-well microplate at 25°C for 10 min. The reaction was terminated with 50 $\mu$l of stop solution (50 mM Tris-HCl [pH 8], 6 M guanidine-HCl). Next, 50 $\mu$l of Ellman's reagent (50 mM Tris-HCl [pH 8], 1 mM DTNB, and 1 mM EDTA) was added to the reaction mixture. Absorbance values were measured at 412 nm. One unit of specific activity is defined as 1 $\mu$mol CoA produced per min per mg of the purified protein under the specified conditions. Kinetic parameters ($K_m$ and $k_{cat}$) were determined with 10 different glycine concentrations ranging from 1 to 120 mM. The purified proteins were highly unstable in solution both on ice and at room temperature, which is a characteristic of ALAS enzymes. The half-life is very short (about 45 min) in solutions on ice; however, the protein can be kept at −80°C without a loss of activity for several weeks.

Tryptophan synthase (TrpS) enzyme activity was determined by monitoring the difference in absorptions between indole and L-tryptophan at 290 nm at pH 7.5 at 25°C (25). The reaction mixture contained (total of 200 $\mu$l) 100 mM Tris-HCl (pH 7.5), indole (1 mM), different concentrations of L-serine, 50 $\mu$M PLP, 180 mM NaCl, 0.2 mM DTT, 1 mM EDTA, and purified protein (85 $\mu$g). The absorbance values were monitored with a 96-well microplate reader at 290 nm at 25°C for 20 min. One unit of specific activity is defined as 1 $\mu$mol tryptophan converted by indole per min per mg of purified protein under

the specified conditions. The $K_m$ was determined with 10 different L-serine concentrations ranging from 2 to 100 mM.

The activity of 3′(2′),5′-bisphosphate nucleotidase was determined by a colorimetric method that detects inorganic phosphate with malachite green (26). A 100-$\mu$l reaction mixture contained 50 mM Tris-HCl buffer (pH 8), 2 mM $MgCl_2$, 2 mM KCl, 0 to 5 $\mu$g purified protein, and 0.5 mM adenosine 3′-phosphate 5′-phosphosulfate (PAPS). After 15 min of incubation at 25°C, the malachite green reagent was added as described above for L-threonine synthase, and released inorganic phosphate was quantitated by measuring the absorbance at 630 nm. One unit of specific enzyme activity is expressed as 1 $\mu$mol inorganic phosphate produced per min per mg of the purified protein under the specified conditions.

**Microarray analysis.** Samples for microarray analysis were taken after 5 days of growth of strain DS17690 in penicillin production medium. After filtration using a Büchner funnel, the samples were quickly frozen using liquid nitrogen. Samples were processed as described previously (2) but with the following modification: double-stranded cDNA synthesis was carried out using 10 $\mu$g of total RNA and the components of the One Cycle cDNA synthesis kit (Affymetrix). The double-stranded cDNA was purified (GeneChip sample cleanup module; Qiagen) before *in vitro* transcription and labeling (GeneChip IVT labeling kit; Affymetrix). Acquisition and quantification of microarray images and data filtering were performed using GeneChip Command Console software (AGCC) (Affymetrix, Santa Clara, USA). Arrays were globally scaled to a target value of 100, using the average signal from all probe sets. The arrays were analyzed as previously described (27). Significant changes in expression in the replicate array experiments were assessed statistically by using Significance Analysis of Microarray (SAM version 1.21) software.

**Other analytical procedures.** CD spectroscopy was performed as described previously, using a Jasco 715 spectropolarimeter (28).

Experimental and Affymetrix expression data will be made available upon request.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.5 MB.

## ACKNOWLEDGMENTS

This work was funded by the China Scholarship Council (to M. Wu) and BeBASIC, an international public-private partnership (to C. G. Crismaru and O. Salo).

We thank DSM Sinochem Pharmaceuticals (Delft, The Netherlands) for kindly providing the *P. rubens* DS17690 strain. We thank Stefan Weber, Jean-Marc Daran, and Jack T. Pronk for the Affymetrix expression analysis.

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