Amplification and disruption of the phenylacetyl-CoA ligase gene of *Penicillium chrysogenum* encoding an aryl-capping enzyme that supplies phenylacetic acid to the isopenicillin N-acyltransferase

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A gene, *phl*, encoding a phenylacetyl-CoA ligase was cloned from a phage library of *Penicillium chrysogenum* AS-P-78. The presence of five introns in the *phl* gene was confirmed by reverse transcriptase-PCR. The *phl* gene encoded an aryl-CoA ligase closely related to *Arabidopsis thaliana* 4-coumaroyl-CoA ligase. The Phl protein contained most of the amino acids defining the aryl-CoA (4-coumaroyl-CoA) ligase substrate-specificity code and differed from acetyl-CoA ligase and other acyl-CoA ligases. The *phl* gene was not linked to the penicillin gene cluster. Amplification of *phl* in an autonomous replicating plasmid led to an 8-fold increase in phenylacetyl-CoA ligase activity and a 35 % increase in penicillin production. Transformants containing the amplified *phl* gene were resistant to high concentrations of phenylacetic acid (more than 2.5 g/l). Disruption of the *phl* gene resulted in a 40 % decrease in penicillin production and a similar reduction of phenylacetyl-CoA ligase activity. The disrupted mutants were highly susceptible to phenylacetic acid. Complementation of the

disrupted mutants with the *phl* gene restored normal levels of penicillin production and resistance to phenylacetic acid. The phenylacetyl-CoA ligase encoded by the *phl* gene is therefore involved in penicillin production, although a second aryl-CoA ligase appears to contribute partially to phenylacetic acid activation. The Phl protein lacks a peptide-carrier-protein domain and behaves as an aryl-capping enzyme that activates phenylacetic acid and transfers it to the isopenicillin N acyltransferase. The Phl protein contains the peroxisome-targeting sequence that is also present in the isopenicillin N acyltransferase. The peroxisomal colocalization of these two proteins indicates that the last two enzymes of the penicillin pathway form a peroxisomal functional complex.

Key words: aryl-capping enzyme, isopenicillin N acyltransferase (IAT), *Penicillium chrysogenum*, phenylacetic acid, phenylacetyl-CoA ligase.

INTRODUCTION

The pathway for penicillin biosynthesis has been largely elucidated [1,2], although the molecular mechanisms underlying some of the reactions, e.g. the activation of PAA (phenylacetic acid) and the amino acid epimerization reactions that are catalysed by ACV (δ -L- α -aminoadipyl-L-cysteinyl-D-valine) synthetase, remain obscure [3]. The *Penicillium chrysogenum* genes *pcbAB*, *pcbC* and *penDE*, encoding the enzymes that catalyse the three central penicillin biosynthetic reactions, have been characterized [4–8].

In the last reaction of the penicillin biosynthetic pathway, the α -aminoadipyl side chain of isopenicillin N is removed and replaced by a phenylacetate (or phenoxyacetate) residue [5]. This reaction is catalysed by the IAT (isopenicillin N acyltransferase), a complex enzyme with several catalytic activities [9–11]. The IAT is able to attach PAA efficiently to 6-APA (6-aminopenicillanic acid), but requires that the PAA is activated previously in the form of a thioester with CoA (PAA-CoA, phenylacetyl-CoA) [10,11].

The mechanism of activation of PAA has remained obscure for decades despite its industrial interest. There was early evidence for the existence of a PAA-activating activity [12,13]. Although partial purification of the enzyme was achieved [13], the enzyme was very labile and no significant characterization of its biochemical properties could be made.

An acetyl-CoA synthase gene (*acuA* or *facA*) able to activate several fatty acids *in vitro* was reported [14]. However, when the

gene *acuA* (*facA*) encoding the acetyl-CoA synthase [15,16] was mutated, it did not affect penicillin biosynthesis [16], suggesting that the authentic PAA-CoA ligase was a different enzyme.

Gledhill et al. [17] described in a patent application the purification of the PAA-CoA ligase and sequenced the N-terminal end of the protein. A DNA fragment containing the putative PAA-CoA ligase gene was cloned from *P. chrysogenum* BW1901. However, the patent work did not provide genetic evidence to prove that the cloned gene corresponded to the authentic PAA-CoA ligase. Gene disruption in *P. chrysogenum* is difficult to perform because of frequent ectopic integration of the exogenous DNA during disruption experiments, but gene inactivation is required to prove unequivocally the involvement of a specific gene in a biosynthetic reaction. We have previously optimized gene disruption in *P. chrysogenum* [18] using the two-marker strategy [19].

Taking into account the precursors involved in the biosynthesis of benzylpenicillin, its pathway adjusts to the model of hybrid polyketide–non-ribosomal-peptide secondary metabolites. Activation of short-chain fatty acids and aromatic acids and their sequential condensation with amino acids during the biosynthesis of hybrid polyketide–polypeptide secondary metabolites is a subject of great interest. The modules of NRPSs (non-ribosomal peptide synthetases) are involved in amino acid activation [20]. Fatty acids and organic acids are activated as acyladenylates and later transferred to a PCP (peptide-carrier protein) domain equivalent to the ACP (acyl-carrier protein) domain of polyketide synthases. In

Abbreviations used: 6-APA, 6-aminopenicillanic acid; ArCP, aromatic-acid-carrier protein; CP, complex production; DP, defined production; DPhl, disrupted in *phl*; DTT, dithiothreitol; IAT, isopenicillin N acyltransferase; NRPS, non-ribosomal peptide synthetase; PAA, phenylacetic acid; PAA-CoA, phenylacetyl-CoA; PCP, peptide-carrier protein; RT, reverse transcriptase.

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Table 1 Plasmids used in the present study

Plasmid	Characteristics	Origin
pULJL43	Integrative vector. Contains the <i>ble</i> resistance marker under control of the fungal <i>pcbC</i> promoter.	[43]
pAMF9L	Autonomous replicating plasmid (AMA1 sequence). Contains the <i>P. chrysogenum</i> <i>pyrG</i> gene as selective marker.	[44]
pAMF9E	Derivative of pAMF9L containing the <i>phl</i> gene in a 6.6 kb <i>EcoRI</i> DNA fragment.	Present study
pZE1.2	Derivative of the <i>E. coli</i> vector pZero carrying the 6.6 kb <i>EcoRI</i> DNA fragment containing the <i>phl</i> gene.	Present study
pBle3	Integrative plasmid designed for <i>phl</i> gene disruption by double recombination.	Present study
pZph6	Integrative plasmid carrying a 4 kb <i>StuI</i> / <i>SacII</i> fragment that includes the <i>phl</i> gene. Used for complementation studies.	Present study

Table 2 Oligonucleotides used in this work

Oligonucleotides	Nucleotide sequence	Use
Ocla5	5'-TATTACCCCGAGGATTG-3'	Intron 1, spore PCR analysis, probe
Oaa14	5'-AACACCGCCAGTCTGTGAAC-3'	Intron 1, spore PCR analysis
Oaa5	5'-GCCCGGCGAACAGCATTG-3'	Intron 2, intron 3
Oaa11	5'-GGAAGGAGACCAAGCGCAAC-3'	Intron 2
Oaa2	5'-AACAGAGACGTCACGGAATCT-3'	Intron 3
Oaa10	5'-GAGACAGCTGCCGATTCTCTC-3'	Intron 4
Oaa9	5'-CACAATTCGTGCCTCGACT-3'	Intron 4
Oaa12	5'-GGCATCGAGCAGTGTTAT-3'	Intron 5
Ocla3	5'-TACCCCGACATTTT-3'	Intron 5, probe
QC1	5'-GGTCAGAATGGGATAAGCTTTGGCCGTGTG-3'	Site-directed mutagenesis
QC2	5'-CAAACACGGCCAAAGCTTATCCATTCTGACC-3'	Site-directed mutagenesis

this regard, the exact role of the protein encoded by the PAA-CoA ligase gene is unknown.

It was, therefore, necessary to perform a detailed genetic analysis of the possible involvement of this gene in PAA activation and in penicillin biosynthesis to clarify the molecular mechanism of this important step of β -lactam antibiotic biosynthesis.

MATERIALS AND METHODS

Strains and culture conditions

P. chrysogenum Wis 54-1255, a low-penicillin-producing strain with a single copy of the penicillin gene cluster [21], was used as the parental strain. *P. chrysogenum* Wis 54-1255 *pyrG*, a uridine auxotroph blocked in the *pyrG* gene [22], was used as the recipient strain in transformation procedures. *P. chrysogenum* *npe10 pyrG*, a deletion strain containing multiple copies of the entire penicillin gene cluster [23,24], and *P. chrysogenum* AS-P-99, a strain containing multiple copies of the penicillin gene cluster [21], were used to study the genome localization of the *phl* gene.

Competent cells of *Escherichia coli* DH5 α strain were used for high-efficiency transformation, amplification and isolation of plasmid DNA. It was grown in LB (Luria-Bertani) medium with ampicillin (100 μ g/ml), chloramphenicol (30 μ g/ml) or kanamycin (50 μ g/ml).

P. chrysogenum spores were obtained from plates of Power medium [23] ($n = 18$) grown for 5 days at 28 °C. Seed cultures were initiated by inoculating fresh spores from one sporulated Petri dish into CI (complex inoculation) medium (20 g/l corn steep solids, 10 g/l yeast extract, 58 mM sucrose, 50 mM calcium carbonate, pH 5.7) or DI (defined inoculation) medium [18] after incubation for 24 h. Penicillin production was studied in cultures in CP (complex production) medium [4 g/l potassium phenylacetate, 20 g/l Pharmamedia (Traders Protein, Memphis, TN, U.S.A.), 50 g/l lactose, 0.03 M ammonium sulphate and 0.05 M calcium carbonate, pH 6.6] or DP (defined production) medium [18]. Cultures (100 ml in 500 ml flasks) were inoculated

with 5% seed cultures and incubated in an orbital shaker (at 250 rev./min for up to 96 h at 25 °C). The mycelia were harvested by filtration, washed with 1 vol. of 0.9 g/l NaCl, and stored at –80 °C until use. The resistance to PAA was studied in DP medium with 0.5, 1.0, 1.5, 2.0, 2.5 and 3 g/l potassium phenylacetate or potassium phenoxyacetate. In all experiments, PAA or phenoxyacetic acid were neutralized to pH 8.0 with concentrated KOH before use.

Transformation of *P. chrysogenum* protoplasts

Protoplasts of *P. chrysogenum* Wis 54-1255 *pyrG* were obtained as described by Díez et al. [22] and were transformed according to the procedure of Cantoral et al. [25]. Transformant clones were selected by complementation of the uridine auxotrophy or phleomycin resistance. Uridine and phleomycin were added at final concentrations of 100 μ g/ml and 30 μ g/ml respectively when necessary. The plasmids that were used are listed in Table 1.

Isolation of genomic DNA of *P. chrysogenum* strains and phage library constructions

Genomic DNA was isolated from *P. chrysogenum* cultures in MPPY (40 g/l glucose, 3 g/l NaNO₃, 2 g/l yeast extract, 0.5 g/l KCl, 0.5 g/l MgSO₄ · 7H₂O, 0.01 g/l FeSO₄ · 7H₂O, adjusted to pH 6.0) [26], supplemented with 100 μ g/ml uridine when necessary, as described previously [26a]. A *P. chrysogenum* AS-P-78 genomic library in the vector LambdaGEM-12 (Promega) [27] was used to clone the *phl* gene. The oligonucleotides used as primers in different experiments are listed in Table 2.

Southern hybridization and nucleic acid manipulations

Genomic DNA (2–4 μ g) was digested and the fragments were separated on a 0.7% agarose gel and blotted on to Hybond-N⁺ membranes (Amersham Biosciences) using a vacuum system (Pharmacia VacuGene). Digoxigenin labelling, hybridization and detection were performed with the Genius kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Hybridizations were done by standard procedures [27b]

at 42 °C using 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1 % (w/v) dodecylsarcosine, 0.02 % (w/v) SDS, 40 % (w/v) formamide and 2 % (w/v) blocking reagent. The hybridization signals were visualized with BCIP (5-bromo-4-chloroindol-3-yl phosphate) and NBT (Nitro Blue Tetrazolium) following standard procedures [27a]. PstI-digested λ bacteriophage DNA labelled with digoxigenin was used as size marker.

cDNA synthesis and intron analysis

cDNA was obtained from RNA extracted with the RNeasy kit (Qiagen) from mycelia grown for 48 h in CP medium. To elucidate the presence of putative introns in the DNA sequence, the region containing the expected intron sites was amplified by RT (reverse transcriptase)-PCR (Invitrogen) using the appropriate primers listed in Table 2. The amplified cDNA region was sequenced to confirm the presence of the introns. Automatic sequencing was performed with the AutoRead™ System (Pharmacia).

Site-directed mutagenesis and PCR analysis of spore DNA

In vitro mutagenesis was performed with the QuikChange® site-directed mutagenesis kit (Stratagene). Primers QC1 and QC2 (Table 2) were used to delete one nucleotide, creating a HindIII site.

PCR amplification of DNA from spores was used for analysis of the transformants. Spores were broken in distilled water with glass beads (425–600 μ m diameter, Sigma) by vortex-mixing for 15 s. The beads were removed by centrifugation at 8000 g for 10 min, and 20 μ l of the supernatant was used as DNA template solution for PCR.

Isolation of disrupted mutants by *phl* gene replacement

The *phl* gene was disrupted by the double marker gene replacement technique [18,19] using the construction pBble3 in which the *phl* was inactivated by a double mutation: (i) a *pyrG* gene insertion (in the opposite orientation) within the 5' region of the region of the gene, and (ii) a frameshift mutation downstream of the *pyrG* insertion site to avoid the possibility of the formation of a truncated Phl from some internal promoter.

HPLC determination of benzylpenicillin

Benzylpenicillin in the cultures was determined using a Waters Systems HPLC equipped with a 2487 dual absorbance detector, using a 4.6 mm × 250 mm RPC18 Lichrospher® 100 (Merck) column with a flow rate of 0.8 ml/min. Samples were analysed using, as a mobile phase, buffer A [15 mM ammonium formate, pH 5.0, and 5 % (v/v) acetonitrile] and buffer B [buffer A/acetonitrile, 1:4 (v/v)]. The benzylpenicillin was quantified by determining the absorbance at 214 nm.

In vitro assay for PAA-CoA ligase

Protein extract (500 μ g; quantified by the Bradford method [27b]) diluted in extraction buffer [30 mM Tris/HCl, pH 8.5, 1 mM DTT (dithiothreitol) and 100 μ g/ml Pefabloc (Sigma) in glycerol] was mixed with 10 mM PAA, 5 mM ATP, 10 mM magnesium chloride, 1 mM CoA sodium salt and 0.75 mM DTT at pH 8.0 in a total volume of 0.3 ml and was incubated at 28 °C for 30 min. The same reaction volume of methanol was added to precipitate the proteins. The PAA-CoA formed was determined using a Waters Systems HPLC, as described above, with a flow rate of 0.9 ml/min using, as a mobile phase, solvent A (10 mM ammonium phosphate and 5 mM tetrabutylammonium hydrogen sulphate, pH 5.5) and solvent B (100 % acetonitrile). The PAA-CoA formed was quan-

tified by determining the absorbance at 260 nm, using pure PAA-CoA (Sigma) as standard, and is given as μ g per mg of protein extract.

Computer analyses

Computer analyses of nucleotide and amino acid sequences were performed with the Lasergene® software package (DNASTAR Madison, WI, U.S.A.), BLASTX2 and BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple alignments were made with CLUSTALW (<http://www.ebi.ac.uk/clustalw/>). GeneDoc (<http://www.psc.edu/biomed/genedoc>) was used for editing the multiple sequence alignments. Putative conserved domains were detected with the Protein Families Database of Alignments and Hidden Markov Models (PFAM; <http://www.sanger.ac.uk/Software/Pfam/>).

RESULTS

Isolation of bacteriophages containing the *phl* gene from a *P. chrysogenum* library

A 2105-bp DNA fragment was amplified by PCR from the genomic DNA of *P. chrysogenum* using oligonucleotides Ocl3 and Ocl5 as primers. The amplified DNA fragment was sequenced and was shown to be identical with that of *P. chrysogenum* BW1901 reported in the patent application [17]. This DNA fragment was used as a probe to screen a LambdaGEM-12 genomic library of *P. chrysogenum* AS-P-78. Two positive bacteriophages were isolated and purified by four consecutive rounds of hybridization. A 17-kb NotI-hybridizing DNA band of bacteriophage F2 was subcloned in the pZero vector; the resulting plasmid was named pZ1.2. Restriction endonuclease mapping and hybridization with the 2105-bp PCR probe allowed the insertion of the PCR-amplified fragment in the central region of the 17-kb fragment in this bacteriophage. A 6.6-kb EcoRI fragment was subcloned (new plasmid pZE1.2) and a region of 3770 bp was sequenced on both strands (Figure 1).

An open reading frame of 2076 bp that contained five putative introns was identified. The presence of the five introns corresponding to positions 277–340 (64 bp), 723–822 (100 bp), 1093–1148 (56 bp), 1311–1377 (67 bp) and 1689–1743 (55 bp), numbered from the ATG of the gene, was confirmed by RT-PCR using RNA isolated from a 48 h culture of *P. chrysogenum* Wis 54-1255 grown in CP medium, and the specific oligonucleotides listed in Table 1 as primers. The gene was named *phl* (phenylacetyl-CoA ligase).

The *phl* gene encodes a protein similar to other aryl-CoA ligases

The protein encoded by the *phl* gene consisted of 578 amino acids and showed a molecular mass of 62.6 kDa. Computer analysis of the amino acid sequence revealed that it corresponded to an aryl-CoA ligase of the family of acyladenylate-forming enzymes (see the Discussion). The Phl protein showed approx. 30 % identical amino acids with four different 4-coumaroyl-CoA ligases of *Arabidopsis thaliana* and other plants. These enzymes catalyse the activation of 4-coumaric acid to 4-coumaroyl-CoA, the starter unit of phenylpropanoid metabolism in plants [28]. The highest similarity of the Phl protein was with proteins of unknown function deduced from the genomes of *Aspergillus nidulans* (65 % identity), *Neurospora crassa* (54 % identity), *Aspergillus fumigatus* (30 % identity), *Gibberella zeae* and *Magnaporthe grisea* (approx. 30 % identity) (Figure 2). The presence of Phl protein in *Asp. nidulans* is consistent with the ability of that fungus to produce penicillin. The Phl-like protein in the other fungi is

Figure 1 The *phl* gene is present as a single copy in different *P. chrysogenum* strains

(A) *P. chrysogenum* DNA fragment cloned in phage F1.2 and in plasmid pZE1.2 (9.9 kb). The *phl* gene is indicated with a shaded arrow. The 0.9-kb *phl* fragment used as probe in hybridization experiments is indicated with a solid bar. Restriction endonuclease sites: A, ApaI; Ba, BamHI; C, ClaI; E, EcoRI; N, NotI; S, SalI; Sa, SacII; Sm, SmaI; St, StuI. (B) Hybridization of the DNA of three different strains of *P. chrysogenum* with the *phl* probe. Lane 1, parental Wis 54-1255 strain; lane 2, deletion mutant Wis *npe10*; lane 3, penicillin-overproducer AS-P-99 strain. The DNA was digested with different enzyme mixtures: S-B, SalI/BglII; Sm, SmaI; St-Sa, StuI/SacII. λ M, size markers [λ bacteriophage digested with PstI hybridized with labelled λ Pst as probe (λ P)]. (C) Hybridization of SmaI-digested DNA of the three *P. chrysogenum* strains with the *phl* (panel 1) or SalI-digested DNA of the three strains with the *penDE* (panel 2), showing that the *phl* gene is present in the three strains, whereas the *penDE* gene is absent in the deletion mutant Wis *npe10* and amplified (thick band) in strain AS-P-99. Lanes 1, 2, and 3, DNA of the three *P. chrysogenum* strains as in (B).

Figure 2 Conserved motifs and amino acids involved in the substrate-specificity code of aryl-CoA ligases

(A) Conserved motifs in *phl* and the acyladenylate protein family. The consensus motifs C, D, E, F, G, I were as proposed by Martín [8], Marahiel et al. [30] and Turgay et al. [31]. (B) Amino acids involved in the substrate-specificity code of aryl-CoA ligases [37] numbered according to the positions in the *P. chrysogenum* Phl protein. Note the amino acid conservation (shaded) in the *P. chrysogenum* Phl protein in comparison with the four coumaroyl-CoA ligases (4CL1–4CL4) of *A. thaliana*. Protein At4g62340 (bottom line) is an *A. thaliana* acyladenylate enzyme but not a true 4-coumaroyl-CoA ligase, and is shown for comparative purposes. *P. tomentosa*, *Populus tomentosa*.

probably related to the detoxifying activity of this protein (see below).

Interestingly, the Phl protein showed no significant similarity to PAA-CoA ligases described in several PAA-degrading bacteria (see the Discussion).

Aryl-capping enzymes: the encoded PAA-CoA ligase lacks a PCP domain and contains a C-terminal peroxisome-targeting motif

Aryl-capping enzymes have been shown to be involved in the biosynthesis of peptide siderophores and other secondary metabolites starting with an aryl group [29]. However, it was unclear if biosynthesis of phenylacetylpenicillin responds to this model. The Phl protein contained all the consensus motifs of acyladenylating enzymes of type A, including the five motifs involved in substrate recognition, designated as: C (adenylate-forming motif); D; E; F (ATP-binding motif); and G, H and I (putative CoA binding) [30,31]. These motifs also occur in the amino-acid-activating modules of the NRPSs [3]. The C and E motifs in Phl are partially modified with respect to the consensus motif of NRPS, suggesting that the Phl protein has a different substrate specificity.

In contrast with the modules of NRPSs, the Phl protein lacks a PCP domain, i.e. the phosphopantothenic acid attachment site [32] that is present in other acyladenylate-forming enzymes.

The Phl protein shows a C-terminal SKI (Ser-Lys-Ile) motif that adjusts to the consensus microbody (peroxisome) targeting sequence (see the Discussion). Targeting of the Phl to peroxisomes (microbodies) is very interesting because the last enzyme of the penicillin pathway, the IAT, is also targeted to microbodies [33].

The *phl* gene is present in a single copy and is not located in the 50-kb amplified region that contains the penicillin gene cluster

The presence and the number of copies of the *phl* gene was tested by Southern hybridization with a 0.94-kb probe internal to the *phl* gene in three relevant strains: *P. chrysogenum* Wis 54-1255 that contained a single copy of the penicillin gene cluster Wis 54-1255, *npe10* (a deletion derivative of *P. chrysogenum* Wis 54-1255 lacking the 50-kb region containing the *pen* cluster) and a high-penicillin-producing strain, *P. chrysogenum* AS-P-99, that contains multiple copies of the 50-kb region, including the entire penicillin gene cluster.

Results showed that the three strains contained a single 3.0-kb *Sma*I-hybridizing band of the same intensity (Figure 1B), indicating that the *phl* gene is not located in the 50-kb region deleted in strain *npe10* and that it is not amplified in strain AS-P-99, in contrast with the other penicillin biosynthesis genes *pcbAB*, *pcbC* and *penDE*, as shown by comparative hybridization with a probe internal to the *penDE* gene (Figure 1C). As observed in Figure 1(C), strain *npe10* lacks the *penDE* gene; moreover, this gene is amplified in tandem repeats in strain AS-P-99 (as shown by the thick band of the same size as for Wis 54-1255; see the Discussion).

However, a second *phl*-related gene was shown by hybridization with the entire *phl* gene under low-stringency conditions (see below).

Amplification of *phl* leads to increased penicillin production

The role of the *phl* gene in relation to penicillin biosynthesis and PAA resistance was studied by gene amplification and gene deletion. For amplification studies, plasmid pAMF9E, carrying the *phl* gene expressed from its own promoter and the *pyrG* marker in an autonomous replicating plasmid (AMA1 replicon), was used to transform *P. chrysogenum* Wis 54-1255 *pyrG*⁻, and transfor-

Figure 3 Amplification of the *phl* gene results in increased benzylpenicillin production

(A) Construction (pAMF9E) used to amplify the *phl* gene in an autonomous replicating plasmid containing the AMA1 sequence (*ars*) from *Asp. nidulans*. Restriction endonuclease sites: *E*, EcoRI; *S*, *Sall*; and *Sm*, *Sma*I. (B) HPLC of benzylpenicillin measured directly in culture broths of the parental strain Wis (solid line) and the PhlAR⁺ transformant (broken line). (C) Direct HPLC determination of benzylpenicillin in culture broths of the parental Wis 54-1255 strain (solid line) and the disrupted Dphl1 mutant (broken line). Benzylpenicillin was eluted at 23 min. Note the significant decrease in penicillin production in the disrupted mutant. The second mutant Dphl2 showed the same reduction in penicillin production as Dphl1 (results not shown).

Table 3 Benzylpenicillin production at 72 h and 96 h in defined DF medium by the parental strain Wis 54-1255 and transformant PhlAR⁺ at PAA concentrations of 1.5, 2.0 and 2.5 g/l

Data for penicillin concentrations are means $\pm$ S.D. for three separate flasks.

Strain	PAA concentration (g/l)	Benzylpenicillin production (μ g/mg dry weight)	
		72 h	96 h
Wis 54-1255	1.5	3.8 $\pm$ 0.20	5.2 $\pm$ 0.40
PhlAR ⁺	1.5	4.2 $\pm$ 0.25	5.4 $\pm$ 0.35
Wis 54-1555	2.0	3.3 $\pm$ 0.25	5.1 $\pm$ 0.15
PhlAR ⁺	2.0	4.1 $\pm$ 0.25	5.8 $\pm$ 0.18
Wis 54-1255	2.5	2.8 $\pm$ 0.18	4.0 $\pm$ 0.18
PhlAR ⁺	2.5	4.7 $\pm$ 0.20	6.1 $\pm$ 0.20

ants were selected by complementation of the *pyrG* auxotrophy (Figure 3A). Eight randomly selected transformants were analysed by Southern hybridization using a probe internal to the *phl* gene. They all contained the transforming *phl* gene without rearrangement.

Results showed that all transformants produced increased penicillin levels (between 20 and 60 % higher than the untransformed strain at 48 h, and between 35 and 80 % at 72 h of incubation) in CP medium. One of the transformants, named PhlAR⁺, was used for further analysis. This transformant consistently overproduced penicillin, as shown by HPLC and bioassay analysis (Table 3 and Figure 3B). Results of three different fermentations in defined DP medium with increasing PAA concentrations (1.5–2.5 g/l) showed that the transformant overproduced penicillin (30–35 % increase), particularly at high PAA concentrations.

Figure 4 Disruption strategy and Southern hybridization showing the inactivation of *phl* in two different transformants

(A) Construction pBble3 in which the *phl* gene (shaded arrow) was disrupted *in vitro* by insertion of the *pyrG* gene (white arrow) and by creating a frameshift mutation (*) (see text). Restriction endonuclease sites: E, EcoRI; S, SalI; Sm, SmaI. (B) Recombination (double crossing-over with the genome); letter codes as in (A). (C) Organization of the *phl* region in the disrupted mutants; letter codes as in (A). (D) Southern hybridization of the DNA of the parental strain Wis 54-1255 (lane 1), two disrupted mutants DPhl1 and DPhl2 (lanes 2 and 3) and an abnormal non-disrupted transformant (lane 4) showing ectopic integration. Lanes 1–4 were hybridized with the *phl* probe. Lane λ M, control size markers (λ digested with PstI), was hybridized with a labelled λ probe (λ P). The sizes (in kb) of the hybridizing bands are indicated. (E) PCR amplification using primers Ocl5 and Oaa14, showing that the two disrupted mutants contain the same amplification band (lanes 1 and 2, arrow), different from that of the non-disrupted Wis 54-1255 (lane W).

Disruption of the *phl* gene results in decreased penicillin production

Targeted inactivation of the *phl* gene in *P. chrysogenum* Wis 54-1255 was performed by double crossing-over (gene replacement) between the endogenous copy and the pBble3 construction in which the *phl* gene was disrupted by inserting the *pyrG* in a SalI site in opposite orientation to the *phl* gene (Figure 4). Furthermore, a frameshift mutation was introduced at nucleotide position 257 in pBble3, to prevent translation of the 3' region of *phl* if expressed from any putative promoter located in the *pyrG* insert.

A total of 689 clones of the 1858 *pyrG*⁺ transformants (selected by complementation of the uridine auxotrophy) tested were phleomycin-sensitive, indicating that a double cross-over had occurred. All 689 clones were tested by PCR amplification of the *phl* gene

Table 4 Benzylpenicillin production at 72 and 96 h in culture broths in DF medium of the parental strain Wis 54-1255 and the DPhl1 transformant disrupted in the *phl* gene

Data for penicillin concentrations are means $\pm$ S.D. for three separate flasks.

Strain	PAA concentration (g/l)	Benzylpenicillin production (μ g/mg dry weight)	
		72 h	96 h
Wis 54-1255	0.5	3.2 $\pm$ 0.32	4.9 $\pm$ 0.30
DPhl1	0.5	2.8 $\pm$ 0.25	4.0 $\pm$ 0.22
Wis 54-1255	1	3.7 $\pm$ 0.20	5.1 $\pm$ 0.15
DPhl1	1	2.3 $\pm$ 0.25	3.6 $\pm$ 0.35
Wis 54-1255	1.5	3.8 $\pm$ 0.28	5.4 $\pm$ 0.4
DPhl1	1.5	2.3 $\pm$ 0.20	3.4 $\pm$ 0.25

region using primers Ocl5 and Oaa14 to detect whether the *phl* gene had been disrupted (by introduction of the *pyrG* insert present in the exogenous construction).

Two independent clones containing the disrupted *phl* gene, named DPhl1 (disrupted in *phl* 1) and DPhl2, were isolated. These two transformants were confirmed by Southern hybridizations using probes internal to the *phl* (Figure 4D) and the *pyrG* genes. The *phl*-hybridizing band (4.4 kb SmaI) was 1.4 kb larger than the band in the control Wis 54-1255 strain, a size increase that corresponded exactly to the size of the *pyrG* insert (Figure 4D, lanes 2 and 3). A large number of non-disrupted transformants that contained the intact original 3.0-kb band and the pBble3 cassette integrated in a non-homologous site were also observed (Figure 4D, lane 4). The two disrupted transformants showed the same hybridization pattern and an identical PCR product (Figure 4E), although the double recombination may have occurred in different places within the homologous region.

The nucleotide sequences of the PCR products showed the presence of the frameshift mutation in the *phl* gene and confirmed the introduction of the *pyrG* without rearrangement of the construction.

The two disrupted mutants DPhl1 and DPhl2 produced 40 % less penicillin than the parental strain at PAA concentrations of 1.5 and 1.0 g/l (Table 4 and Figure 3C). The effect of *phl*-gene disruption on penicillin production was dependent upon the concentration of PAA used because the *phl* disruption affected significantly the resistance of the strains to PAA (see below).

Amplification of *phl* leads to high resistance to PAA, and disruption of this gene results in PAA-supersensitive strains

The resistance to PAA of the DPhl1 and DPhl2 mutants and the PhlAR⁺ transformant containing the amplified *phl* gene was tested at PAA concentrations of 1.0, 1.5, 2.0, 2.5 and 3.0 g/l in DP medium.

Under these conditions, the parental Wis 54-1255 was resistant up to 2.0 g/l PAA. This strain showed very poor growth at 2.5 g/l PAA and failed to grow at concentrations of 3 g/l PAA. By contrast, the PhlAR⁺ strain grew well at 3 g/l PAA.

On the other hand, the two *phl*-disrupted mutants were susceptible to PAA concentrations of 1.5 g/l and failed to grow at 2.0 g/l. These results indicate that the PAA-CoA ligase encoded by the *phl* gene is a PAA-detoxifying enzyme (see the Discussion). Similar results were obtained when phenoxyacetic acid was used instead of PAA, suggesting that the PAA-CoA ligase was able to detoxify *P. chrysogenum* from the accumulation of both PAA and phenoxyacetic acid.

Figure 5 PAA-CoA formed *in vitro* by the PAA-CoA ligase

(A) PAA-CoA formed *in vitro* by the PAA-CoA ligase of the parental strain Wis 54-1255 and the disrupted DPhl1. (B) PAA-CoA formed *in vitro* by the PAA-CoA ligase of the parental strain Wis 54-1255 and the PhlAR⁺ transformant containing multiple copies of the *phl* gene. Pure PAA-CoA was used as standard (elution time 23.5 min). A stock solution of 100 µg/ml PAA-CoA was used to determine the standard curve. The reactions were performed with 500 µg of protein (crude extract) per reaction (0.3 ml volume) in all cases. Note the different scales between panels (A) and (B).

PAA-CoA ligase activity in the disrupted mutants: evidence for a second *phl*-related gene

Since the *phl*-disrupted mutants were still able to produce reduced levels of benzylpenicillin, the PAA-CoA ligase was measured in the different strains using an HPLC analysis that quantified the PAA-dependent and CoA-dependent conversion of PAA into PAA-CoA. Results showed that both the DPhl1 and DPhl2 mutants had 39.1 % less PAA-CoA ligase activity (average of five determinations) than the undisrupted Wis 54-1255 control strain (Figure 5A). These results confirm that the *phl* gene encodes PAA-CoA ligase activity, and, at the same time, indicate that there is a second gene in *P. chrysogenum* that encodes the remaining PAA-CoA ligase activity.

A search was then made for a *phl*-like gene by hybridization with the 0.62-kb *phl* probe. Under very-low-stringency conditions, a second 6.0-kb hybridizing band was detected (results not shown), which probably encodes a second aryl-CoA ligase.

On the other hand, amplification of the *phl* gene in the PhlAR⁺ strain resulted in an 8-fold increase in the PAA-CoA formed (Figure 5B). This large increase indicates that several copies of the autonomous replicating plasmid were expressed. However, the increase in penicillin production was not proportional to this increase in PAA-CoA ligase (Figure 3), suggesting that there is a limitation in other biosynthetic steps (e.g. enzymes involved in isopenicillin N formation, or IAT) in the Wis 54-1255 strain that contains a single copy of the penicillin gene cluster.

Complementation of the *phlD1* mutant restores full penicillin production and PAA resistance

The DPhl1 strain was complemented with the pZphl6 plasmid. The introduction of the *phl* construction in the plasmid without rearrangement was confirmed by Southern hybridization (Figure 6). Three transformants with the correct hybridization pattern were tested for PAA resistance and penicillin production. Comple-

Figure 6 Complementation of the disrupted mutants with the *phl* gene restores PAA resistance

(A) Complementation of the PhlD1 mutant with the *phl* gene (construction pZphl6). Restriction endonuclease sites: B, BglII; C, ClaI; S, Sall; and Sm, SmaI. (B) Hybridization of DNA of strains Wis 54-1255 (lane W), DPhl1 (lane DPhl1) and one complemented clone (lane C) with the *phl* probe. λM, size markers (λ bacteriophage digested with PstI) hybridizing with labelled λ phase (λP). (C) The complemented clone (C) recovered full resistance to PAA. The penicillin production of the complemented clone was restored to the level of that of Wis 54-1255 (W).

mented transformants regained resistance to 2 g/l PAA (Figure 6), and the levels of penicillin were restored to those of the parental Wis 54-1255 cultures.

DISCUSSION

The penicillin gene cluster consists of three genes (*pcbAB*, *pcbC* and *penDE*) with the same arrangement in different penicillin-producing fungi [7,34,35]. A 50-kb DNA region encoding the entire cluster is deleted in the *P. chrysogenum npe10* mutant, and the same region is amplified in tandem copies in the strains that produce high levels of penicillin [21,23]. As shown in the present paper, hybridization studies demonstrated that *phl* is present in a single copy in DNA digested with different restriction enzymes; the hybridizing bands were, in all cases, the same size, suggesting the presence of an identical *phl* gene and adjacent regions in all strains. The *phl* gene is present in *P. chrysogenum npe10* that lacks the 50-kb amplified region, including the penicillin gene cluster. This finding indicates that the PAA-CoA ligase gene has a different evolutionary origin from the other penicillin genes and does not appear to have been transferred horizontally, as has been proposed for the penicillin gene cluster [1].

The PAA-CoA ligase belongs to the acyladenylate protein family [31]. These proteins activate the acyl or aryl acids to acyl-AMP or aryl-AMP respectively using ATP. After activation, the carboxy group is transferred to the thiol group of CoA, forming a thioester and releasing AMP.

All members of the acyladenylate family show a highly conserved AMP-binding domain [PROSITE (<http://www.expasy.org/prosite/>) ID PS00455]. However, they differ in their substrate specificity (e.g. chain length and presence of an aromatic ring) and cellular localization. The most well-characterized aryl-CoA ligases are the 4-coumaroyl-CoA ligases [36,37]. The substrate specificity of two of the four putative 4-coumaroyl-CoA ligases of *A. thaliana* has been studied in detail by Schneider et al. [38]. These authors have defined a substrate-specificity code (binding pocket) for 4-coumaroyl-CoA ligases based on the amino acids occurring in positions 252–256, 293, 320, 322, 323, 346, 348, 354–356 and 540 of these proteins. The catalytic Lys⁵⁴⁰ is always invariant [38]. The *P. chrysogenum* Phl protein and the highly conserved *Asp. nidulans* homologous protein belong to the aryl-CoA ligases of the 4-coumaroyl-CoA ligase type (Figure 2B). The other *Asp. nidulans* Phl-related proteins show some differences in the binding pocket and may serve to activate other substrates.

Aryl-capping enzymes that add aryl side chains to non-ribosomal peptides such as siderophores (e.g. enterobactin, pyochelin and yersiniabactin) and antitumour drugs (e.g. bleomycin and epothilone) have been reported previously [29]. The acyladenylate enzymes share several motifs (ATP-binding motifs) with the A domains (amino-acid-activating) of NRPS but, in contrast with the latter enzymes, the PAA-CoA ligase and other acyladenylate enzymes lack the phosphopantetheine-binding PCP domain. Thus the Phl protein is essentially an aryl-capping enzyme, because it lacks the PCP domain.

In some cases (e.g. EntE, involved in dihydroxybenzoic acid activation for enterobactin biosynthesis), the aryl-capping enzyme transfers the activated aromatic acid to a separate PCP [named ArCP (aromatic-acid-carrier protein)] domain (in this case the EntB protein). In other cases, the activated aryl group is transferred to a specific ArCP domain that forms part of a larger NRPS, e.g. the salicylic-acid-activating aryl-capping protein of the PchD (pyochelin cluster) transfers the activated salicylic acid to an ArCP domain of pyochelin synthetase I [29]. In *P. chrysogenum*, the first acceptor of the activated PAA-CoA is the IAT.

The IAT contains a PAA-CoA-acceptor site with a thioesterase active centre (GKS³⁰⁹RGSTL) that is likely to be involved in cleavage of the thioester bond of PAA-CoA [10] to transfer the PAA to the non-ribosomal-peptide-derived 6-APA [2]. Indeed, a Ser³⁰⁹ → Ala mutation proved that Ser³⁰⁹ in the thioesterase centre is essential for IAT activity [39]. Transfer of the PAA molecule to the IAT has been confirmed by electrospray MS [40].

The C-terminal sequence of the Phl protein is characteristic of peroxisomal enzymes. The IAT of *P. chrysogenum* is also known to be located in microbodies (peroxisomes) [33]. This enzyme and Phl may therefore form a functional complex located in peroxisomes. In summary, the Phl protein acts as a peptide aryl-capping enzyme that transfers the activated PAA to the IAT in the peroxisomes.

Disruption of the *phl* gene confers increased sensitivity to PAA or phenoxyacetic acid, and increasing the *phl* copy number results in higher levels of resistance to these compounds. This result supports observations of the role of other PAA-degrading enzymes on the detoxification of PAA [41]. Increasing the *phl* gene expression level is therefore a useful tool to enhance PAA resistance and penicillin biosynthesis. Indeed, selection of PAA-resistant clones has been reported as a method to isolate increased-penicillin-producing strains [42].

Disruption of the *phl* gene reduced benzylpenicillin production but did not block completely penicillin biosynthesis. Since we measured benzylpenicillin specifically using HPLC, this result could not be explained by the accumulation of isopenicillin N or other hydrophilic penicillins. The remaining benzylpenicillin pro-

duction suggests that a second Phl-like protein exists in *P. chrysogenum*. The gene encoding this second protein was detected by hybridization at reduced stringency. In the genome of *Asp. nidulans* there is a *phl* gene (65 % identity) and three other putative acyl-CoA ligase genes, and similar genes might be present in *P. chrysogenum*, although the substrate specificity and affinity for PAA of those acyl-CoA ligases remains to be studied in this organism.

An interesting observation was that Phl is clearly different from bacterial PAA-degrading enzymes such as those of *Pseudomonas putida*, *Azoarcus evasii*, *Bacillus halodurans*, *Stenophomonas* sp. and *E. coli* (which all have identities of 7–10 %). This finding indicates that, in contrast with bacterial aryl-CoA ligases, the Phl protein has probably evolved to serve a biosynthetic role, rather than a degradative function.

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REFERENCES

- Aharonowitz, Y., Cohen, G. and Martín, J. F. (1992) Penicillin and cephalosporin biosynthetic genes: structure, regulation and evolution. *Annu. Rev. Microbiol.* **46**, 461–495.
- Martín, J. F., Gutiérrez, S. and Demain, A. L. (1997) β -Lactams. In *Fungal Biotechnology* (Anke, T., ed.), pp. 91–127. Chapman & Hall, Weinheim, Germany.
- Martín, J. F. (2000) α -Aminoadipyl-cysteinyI-valine synthetases in β -lactam producing organisms. *J. Antibiotics* **53**, 1008–1021.
- Barredo, J. L., Cantoral, J. M., Álvarez, E., Díez, B. and Martín, J. F. (1989) Cloning, sequence analysis and transcriptional study of the isopenicillin N synthase of *Penicillium chrysogenum* AS-P-78. *Mol. Gen. Genet.* **216**, 91–98.
- Barredo, J. L., van Solingen, P., Díez, B., Álvarez, E., Cantoral, J. M., Kattevilder, A., Smaal, E. B., Groenen, M. A. M., Veenstra, A. E. and Martín, J. F. (1989) Cloning and characterization of the acyl-coenzyme A: 6-aminopenicillanic-acid-acyltransferase gene of *Penicillium chrysogenum*. *Gene* **83**, 291–300.
- Díez, B., Gutiérrez, S., Barredo, J. L., van Solingen, P., van der Voort, L. H. M. and Martín, J. F. (1990) The cluster of penicillin biosynthetic genes. *J. Biol. Chem.* **265**, 16358–16365.
- Montenegro, E., Fierro, F., Fernández, F. J., Gutiérrez, S. and Martín, J. F. (1992) Resolution of chromosomes III and VI of *Aspergillus nidulans* by pulsed-field gel electrophoresis shows that the penicillin biosynthetic pathway genes *pcbAB*, *pcbC*, and *penDE* are clustered on chromosome VI (3.0 megabases). *J. Bacteriol.* **174**, 7063–7067.
- Martín, J. F. (2000) Molecular control of expression of penicillin biosynthesis genes in fungi: regulatory proteins interact with a bidirectional promoter region. *J. Bacteriol.* **182**, 2355–2362.
- Tobin, M. B., Fleming, M. D., Skatrud, P. L. and Miller, J. R. (1990) Molecular characterization of the acyl-coenzyme A: isopenicillin N acyltransferase gene (*penDE*) from *Penicillium chrysogenum* and *Aspergillus nidulans* and activity of recombinant enzyme in *Escherichia coli*. *J. Bacteriol.* **172**, 5908–5914.
- Álvarez, E., Meesschaert, B., Montenegro, E., Gutiérrez, S., Díez, B., Barredo, J. L. and Martín, J. F. (1993) The isopenicillin N acyltransferase of *Penicillium chrysogenum* has isopenicillin N amidohydrolase, 6-aminopenicillanic acid acyltransferase and penicillin amidase activities, all of which are encoded by the single *penDE* gene. *Eur. J. Biochem.* **215**, 323–332.
- Fernández, F. J., Cardoza, R. E., Montenegro, E., Velasco, J., Gutiérrez, S. and Martín, J. F. (2003) The isopenicillin N acyltransferase of *Aspergillus nidulans* and *Penicillium chrysogenum* differ in their ability to maintain the 40 kDa $\alpha\beta$ heterodimer in an undissociated form. *Eur. J. Biochem.* **270**, 1958–1968.
- Brunner, R. and Rohr, M. (1975) Phenacyl:coenzyme A ligase. *Methods Enzymol.* **43**, 476–481.
- Kogekar, R. G. and Deshpande, V. N. (1983) Biosynthesis of penicillin *in vitro*: part II – purification and properties of 6-aminopenicillanic acid-phenylacetyl-CoA/ phenoxyacetyl-CoA transferase. *Ind. J. Biochem. Biophys.* **20**, 208–212.
- Martínez-Blanco, H., Reglero, A., Fernández-Valverde, M., Ferrero, M. A., Moreno, M. A. and Luengo, J. M. (1992) Isolation and characterization of the acetyl-CoA synthetase from *Penicillium chrysogenum*. *J. Biol. Chem.* **276**, 5475–5481.

- 15 Gouka, R. J., van Hartingsveldt, W., Bovenberg, R. A., van Zeijl, C. M., van den Hondel, C. A. and van Gorcom, R. F. (1993) Development of a new transformant selection system for *Penicillium chrysogenum*: isolation and characterization of the *P. chrysogenum* acetyl-coenzyme A synthetase gene (*facA*) and its use as a homologous selection marker. *Appl. Microbiol. Biotechnol.* **38**, 514–519
- 16 Martínez-Blanco, H., Orejas, M., Reglero, Á., Luengo, J. M. and Peñalva, M. A. (1993) Characterisation of the gene encoding acetyl-CoA synthetase in *Penicillium chrysogenum*: conservation of intron position in plectomycetes. *Gene* **130**, 265–270
- 17 Gledhill, L., Greaves, P. A. and Griffin, J. P. (1997) A process for preparing an enzyme for *Penicillium chrysogenum* having phenylacetate-coenzyme A activity. International Patent WO 97/02349
- 18 Casqueiro, J., Gutiérrez, S., Bañuelos, Ó., Hijarrubia, M. J. and Martín, J. F. (1999) Gene targeting in *Penicillium chrysogenum*: disruption of the *lys2* gene leads to penicillin overproduction. *J. Bacteriol.* **181**, 1181–1188
- 19 Liu, G., Casqueiro, J., Bañuelos, Ó., Cardoza, R. E., Gutiérrez, S. and Martín, J. F. (2001) Targeted inactivation of the *mecB* gene encoding cystathionine- γ -lyase shows that the transsulfuration pathway is required for high level cephalosporin biosynthesis in *Acremonium chrysogenum* C10 but not for methionine-induction of the cephalosporin genes. *J. Bacteriol.* **183**, 1765–1772
- 20 Mootz, H. D. and Marahiel, M. (1999) Design and application of multimodular peptide synthetases. *Curr. Opin. Biotechnol.* **10**, 341–348
- 21 Fierro, F., Barredo, J. L., Díez, B., Gutiérrez, S., Fernández, F. J. and Martín, J. F. (1995) The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6200–6204
- 22 Díez, B., Álvarez, E., Cantoral, J. M., Barredo, J. L. and Martín, J. F. (1987) Selection and characterization of *pyrG* mutants of *Penicillium chrysogenum* lacking orotidine-5-phosphate decarboxylase and complementation by the *pyr4* gene of *Neurospora crassa*. *Curr. Genet.* **12**, 277–282
- 23 Fierro, F., Montenegro, E., Gutiérrez, S. and Martín, J. F. (1996) Mutants blocked in penicillin biosynthesis show a deletion of the entire penicillin gene cluster at a specific site within a conserved hexanucleotide sequence. *Appl. Microbiol. Biotechnol.* **44**, 597–604
- 24 Cantoral, J. M., Gutiérrez, S., Fierro, F., Gil-Espinoso, S., van Liempt, H. and Martín, J. F. (1993) Biochemical characterization and molecular genetics of nine mutants of *Penicillium chrysogenum* impaired in penicillin biosynthesis. *J. Biol. Chem.* **268**, 737–744
- 25 Cantoral, J. M., Díez, B., Barredo, J. L., Alvarez, E. and Martín, J. F. (1987) High-frequency transformation of *Penicillium chrysogenum*. *Bio/Technology* **5**, 494–497
- 26 Anné, J. (1977) Somatic hybridization between *Penicillium* species after induced fusion of their protoplasts. In *Dissertationes de Agricultura* 25, PhD thesis, pp. 39–117, KU Leuven, Belgium
- 26a Fierro, F., Gutiérrez, S., Díez, B. and Martín, J. F. (1993) Resolution of four chromosomes in penicillin-producing filamentous fungi: the penicillin gene cluster is located on chromosome II (9.6 Mb) in *Penicillium notatum* and chromosome I (10.4 Mb) in *Penicillium chrysogenum*. *Mol. Gen. Genet.* **241**, 573–578
- 27 Fernández, F. J., Gutiérrez, S., Velasco, J., Montenegro, E., Marcos, A. T. and Martín, J. F. (1994) Molecular characterization of three loss-of-function mutations in the isopenicillin N acyltransferase gene (*penDE*) of *Penicillium chrysogenum*. *J. Bacteriol.* **176**, 4941–4948
- 27a Sambrook, J. and Russel, D. W. (2001) *Molecular Cloning: a Laboratory Manual*, 3rd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 27b Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 28 Schröder, J. (1999) Probing plant polyketide biosynthesis. *Nat. Struct. Biol.* **6**, 714–716
- 29 Quadri, L. (2000) Assembly of aryl-capped siderophores by modular peptide synthetases and polyketide synthases. *Mol. Microbiol.* **37**, 1–12
- 30 Marahiel, M. A., Stachelhaus, T. and Mootz, H. D. (1997) Modular peptide synthetases and polyketide synthases. *Chem. Rev.* **97**, 2651–2673
- 31 Turgay, K., Krause, M. and Marahiel, M. A. (1992) Four homologous domains in the primary structure of *GrsB* are related to domains in a superfamily of adenylate forming enzymes. *Mol. Microbiol.* **6**, 529–546
- 32 Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., Lacelle, M., Marahiel, M. A., Reid, R., Khosla, C. and Walsh, C. T. (1996) A new enzyme superfamily: the phosphopantetheinyl transferases. *Chem. Biol.* **3**, 923–936
- 33 Müller, W. H., Bovenberg, R. A. L., Groothuis, M. H., Kattevilder, F., Smaal, E. B., van der Voort, L. H. M. and Verkleij, A. J. (1992) Involvement of microbodies in penicillin biosynthesis. *Biochim. Biophys. Acta* **1116**, 210–213
- 34 Laich, F., Fierro, F., Cardoza, R. E. and Martín, J. F. (1999) Organization of the gene cluster for biosynthesis of penicillin in *Penicillium nalgiovense* and antibiotic production in cured dry sausages. *Appl. Environ. Microbiol.* **65**, 1236–1240
- 35 Laich, F., Fierro, F. and Martín, J. F. (2002) Production of penicillin by fungi growing on food products: identification of a complete penicillin gene cluster in *Penicillium griseofulvum* and a truncated cluster in *Penicillium verrucosum*. *Appl. Environ. Microbiol.* **68**, 1211–1219
- 36 Lozoya, E., Hoffmann, H., Douglas, C., Schulz, W., Schell, D. and Hahlbrock, K. (1988) Primary structures and catalytic properties of isoenzymes encoded by the two 4-coumarate:CoA ligase genes in parsley. *Eur. J. Biochem.* **176**, 661–667
- 37 Schneider, K., Hövel, K., Witzel, K., Hamberger, B., Schomburg, D., Kombrink, E. and Stüble, H. P. (2003) The substrate specificity-determining amino acid code of 4-coumarate:CoA ligase. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 8601–8606
- 38 Schneider, K., Kienow, L., Schmelzer, E., Colby, T., Bartsch, M., Miersch, O., Wasternack, C., Kombrink, E. and Stüble, H. P. (2005) A new type of peroxisomal acyl-coenzyme A synthetase from *Arabidopsis thaliana* has the catalytic capacity to activate biosynthetic precursors of jasmonic acid. *J. Biol. Chem.* **280**, 13962–13972
- 39 Tobin, M. B., Cole, S. C. J., Kovacevic, S., Miller, J. R., Baldwin, J. E. and Sutherland, J. D. (1994) Acyl-coenzyme A: isopenicillin N acyltransferase from *Penicillium chrysogenum*: effect of amino acid substitutions at Ser²²⁷, Ser²³⁰ and Ser³⁰⁹ on proenzyme cleavage and activity. *FEMS Microbiol. Lett.* **121**, 39–46
- 40 Aplin, R. T., Baldwin, J. E., Roach, P. L., Robinson, C. V. and Schofield, C. J. (1993) Investigations into the post-translational modification and mechanism of isopenicillin N:acyl-CoA acyltransferase using electrospray mass spectrometry. *Biochem. J.* **294**, 357–363
- 41 Rodríguez-Sáiz, M., Díez, B. and Barredo, J. L. (2005) Why did the Fleming strain fail in penicillin industry? *Fungal Genet. Biol.* **42**, 464–470
- 42 Barrios-González, J., Montenegro, E. and Martín, J. F. (1993) Penicillin production by mutants resistant to phenylacetic acid. *J. Ferm. Bioeng.* **76**, 455–458
- 43 Gutiérrez, S., Díez, B., Montenegro, E. and Martín, J. F. (1991) Characterization of the *Cephalosporium acremonium pcbAB* gene encoding α -aminoacyl-cysteine-valine synthetase, a large multidomain peptide synthetase: linkage to the *pcbC* gene as a cluster of early cephalosporin-biosynthetic genes and evidence of multiple functional domains. *J. Bacteriol.* **173**, 2354–2365
- 44 Fierro, F., Kosalková, K., Gutiérrez, S. and Martín, J. F. (1996) Autonomously replicating plasmids carrying the AMA1 region in *Penicillium chrysogenum*. *Curr. Genet.* **29**, 482–489