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Inactivation of the *lys7* Gene, Encoding Saccharopine Reductase in *Penicillium chrysogenum*, Leads to Accumulation of the Secondary Metabolite Precursors Piperideine-6-Carboxylic Acid and Pipecolic Acid from α-Aminoadipic Acid

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Pipecolic acid serves as a precursor of the biosynthesis of the alkaloids slaframine and swainsonine (an antitumor agent) in some fungi. It is not known whether other fungi are able to synthesize pipecolic acid. *Penicillium chrysogenum* has a very active α-aminoadipic acid pathway that is used for the synthesis of this precursor of penicillin. The *lys7* gene, encoding saccharopine reductase in *P. chrysogenum*, was target inactivated by the double-recombination method. Analysis of a disrupted strain (named *P. chrysogenum* SR1$^-$) showed the presence of a mutant *lys7* gene lacking about 1,000 bp in the 3′-end region. *P. chrysogenum* SR1$^-$ lacked saccharopine reductase activity, which was recovered after transformation of this mutant with the intact *lys7* gene in an autonomously replicating plasmid. *P. chrysogenum* SR1$^-$ was a lysine auxotroph and accumulated piperideine-6-carboxylic acid. When mutant *P. chrysogenum* SR1$^-$ was grown with L-lysine as the sole nitrogen source and supplemented with DL-α-aminoadipic acid, a high level of pipecolic acid accumulated intracellularly. A comparison of strain SR1$^-$ with a *lys2*-defective mutant provided evidence showing that *P. chrysogenum* synthesizes pipecolic acid from α-aminoadipic acid and not from L-lysine catabolism.

Pipecolic acid is an important compound that serves as a substrate for some nonribosomal peptide and polyketide synthetases, resulting in the formation of secondary metabolites with interesting novel pharmacological activities, e.g., immunosuppressors and antitumor agents (31, 32). In *Metarhizium anisopliae* and the fungal parasite *Rhizoctonia leguminicola*, pipecolic acid formed by the catabolism of L-lysine is used to form the octahydroindolizine alkaloids slaframine and swainsonine (34–36). Swainsonine is known to have antitumor activity (10, 21).

Pipecolic acid is a product of lysine catabolism in several organisms, including mammals, plants, bacteria, and fungi (18). In mammals, L-lysine can be degraded by two distinct routes, the saccharopine pathway and the L-pipecolate pathway. Although in humans the saccharopine pathway is the primary route of degradation of lysine in most tissues, in the brain the L-pipecolate pathway is the most active; indeed, the accumulation of pipecolic acid is one of the first biochemical abnormalities detected in patients with pipecolatemia and Zellweger syndrome (12, 22).

The first evidence that in some fungi pipecolic acid is derived from the pathway of the biosynthesis of lysine came from the work of Aspen and Meister (2), who showed that certain lysine auxotrophs of *Aspergillus nidulans* appear to be able to convert α-aminoadipic acid to pipecolic acid.

Penicillium chrysogenum has a very active stem of the lysine pathway that also provides α-aminoadipic acid for the biosynthesis of a δ-α-L-aminoadipyl-L-cysteinyl-D-valine tripeptide (3, 9, 29). High levels of α-aminoadipic acid accumulate in *P. chrysogenum* strains with *lys2* disruptions (9).

Although there is evidence that certain microorganisms can synthesize pipecolic acid, it is not known whether this activity occurs in *P. chrysogenum*. In this article, we report for the first time the ability of *P. chrysogenum lys7* disruption mutants to produce pipecolic acid in relatively large amounts.

MATERIALS AND METHODS

Strains and culture conditions. *P. chrysogenum* Wis 54-1255, a strain that produces low levels of penicillin and that has a single copy of the penicillin gene cluster, was used as the parental strain in this study (14). *P. chrysogenum* TDX195 is a lysine auxotroph that was obtained by targeted inactivation of the *lys2* gene, encoding α-aminoadipate reductase (9). *P. chrysogenum* HS$^-$ is a lysine auxotroph that was defective in homocitrate synthase and that was obtained by targeted inactivation of the *lys1* gene (3). *Escherichia coli* DH5α was used for high-frequency DNA transformation and amplification.

P. chrysogenum spores were obtained from plates of Power medium (8) grown for 5 days at 28°C. The mycelium of these strains grown in liquid MPPY medium (16) was collected by filtration through Nytal filters. After incubation for 30 h, seed cultures were initiated by using fresh spores to inoculate defined MDFP medium without phenylacetate (9). MDFP medium (100 ml in 500-ml flasks) inoculated with 10% seed cultures was incubated in an orbital shaker (250 rpm; 25°C). Czapek minimal medium was used in phenotype assays. The lysine auxotroph mutants were grown in the presence of 1.75 mM lysine (8).

Transformation of *P. chrysogenum* protoplasts. Protoplasts of *P. chrysogenum* were obtained as described by Fierro et al. (15). Transformation of protoplasts was performed as described by Cantoral et al. (6) and Díez et al. (11). Transformant clones were selected by phleomycin resistance or by complementation of the lysine auxotrophy. Phleomycin was added to the plates at a final concentration of 30 μg/ml (19.2 μM).

Plasmids and probes. pC43, an integrative plasmid that contains the *ble* gene (bleomycin or phleomycin resistance) of *Streptoalloteichus hindustanus* under the control of the *pcbC* gene promoter of *P. chrysogenum* and the *CYC1* terminator of *Saccharomyces cerevisiae*, was used to subclone the phleomycin resistance

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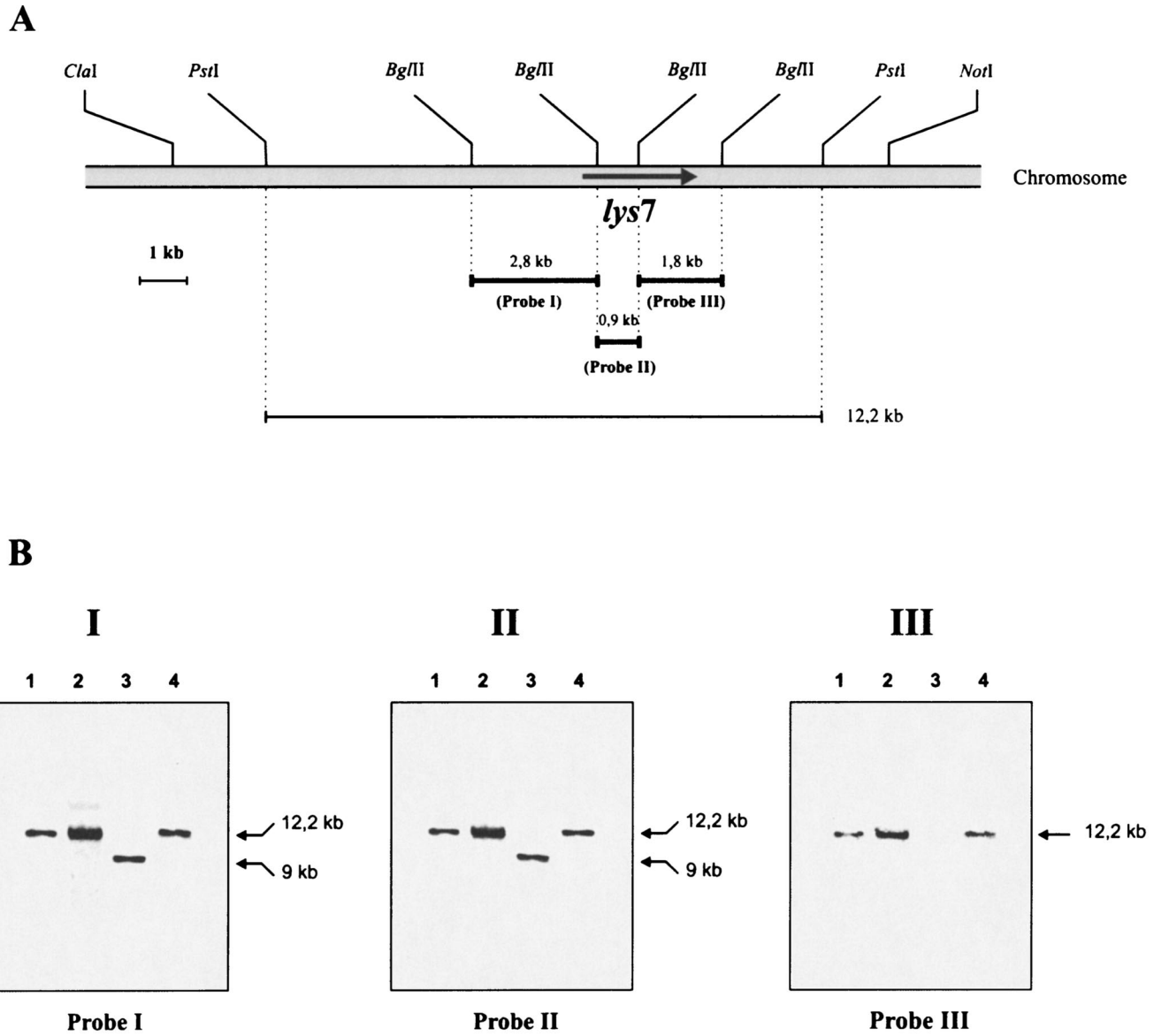

FIG. 1. Disruption of the *lys7* gene of *P. chrysogenum* and molecular analysis of transformants. (A) Restriction map of the chromosomal region containing the *lys7* gene in *P. chrysogenum* Wis 54-1255. The three probes used in the Southern blot hybridizations are indicated by thick lines. The 12.2-kb *Pst*I fragment in the DNA of the parental strain is shown by a thin line. (B) Southern blot hybridizations of *Pst*I-digested genomic DNAs with a 2.8-kb *Bgl*II fragment containing the 5′ end of the *lys7* gene (probe I), a 0.9-kb *Bgl*II fragment internal to the *lys7* gene (probe II), and a 1.8-kb *Bgl*II fragment containing the 3′ end of the *lys7* gene (probe III). Lane 1, parental strain Wis 54-1255; lanes 2 and 4, two randomly selected prototroph transformants; lane 3, lysine auxotroph transformant *P. chrysogenum* TDL7-111. The sizes of the hybridizing bands are indicated on the right. Note that in hybridizations with probe III, the 9-kb band is missing in strain TDL7-111, indicating that the 3′ end of the *lys7* gene has been deleted in this mutant.

cassette (S. Gutiérrez, personal communication). pF4L7 is an integrative plasmid that contains a 16-kb *Not*I DNA fragment carrying the *lys7* gene, encoding saccharopine reductase. p10T1 is an autonomously replicating plasmid bearing the left arm of the *AMA1* sequence of *A. nidulans* (16) and the *P. chrysogenum lys7* gene under the control of its own promoter region (30). pDL7, an integrative plasmid designed to perform the disruption of the *lys7* gene by the double-recombination method, contains a 10-kb *Cla*I/*Not*I DNA fragment and the phleomycin resistance cassette. It was constructed by subcloning an end-filled 5.9-kb *Cla*I/*Bgl*II* fragment (the asterisk indicates filled ends) with the upstream region of the *lys7* gene obtained from pF4L7 into the *Hin*dIII*/*Cla*I site of plasmid pC43. The resulting plasmid was named pDL7-A. Then, a 4.1-kb *Bgl*II*/*Not*I site downstream of the *lys7* gene obtained from pF4L7 was ligated to the *Eco*RI*/*Not*I site of pDL7-A. The plasmid obtained was named pDL7.

Three probes were used in Southern experiments: a 2.8-kb *Bgl*II fragment (5′ end of the *lys7* gene), a 0.9-kb *Bgl*II fragment (internal to the *lys7* gene), and a 1.8-kb *Bgl*II fragment (3′ end of the *lys7* gene).

Isolation of genomic DNA. Spores from *P. chrysogenum* were used to inoculate MPPY medium (16) supplemented with 1.75 mM lysine when necessary. The medium was incubated in an orbital shaker at 250 rpm for 24 to 48 h at 25°C. Then, mycelium was recovered, filtered through Nytal filters, and lyophilized. Samples of lyophilized mycelium (500 mg) were treated with 0.5 ml of 0.18 M Tris-HCl (pH 8.2)–10 mM EDTA– 1% sodium dodecyl sulfate and extracted with 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1) for 30 min at 50°C. The phenol-chloroform-isoamyl alcohol treatment was repeated until the interface was clear. Total DNA was precipitated with 2.5 volumes of ethanol and 0.1 volume of 3 M acetate (pH 3.2) and resuspended in Tris-EDTA buffer (33).

Southern hybridization and nucleic acid manipulations. Samples of 2 to 4 μg of plasmid or genomic DNA of *P. chrysogenum* were digested with appropriate

endonucleases. DNA fragments were separated in 0.8% agarose gels and blotted onto Hybond-N^+ (Amersham Pharmacia Biotech) membranes by using a vacuum system (Pharmacia VacuGene). Digoxigenin labeling, hybridization, and detection were performed with a digoxigenin high prime system (Roche) according to the manufacturer's instructions. Hybridizations were done at 65°C with a buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% laurylsarcosine, 0.02% sodium dodecyl sulfate, and 2% blocking reagent. All other nucleic acid manipulations were carried out by standard methods (33).

Enzyme activities. Cultures of the various *P. chrysogenum* mutants were grown in defined MDFP medium (9). Cells collected at various fermentation times were frozen in liquid nitrogen and stored at −80°C until use. Cell extracts were obtained by grinding frozen mycelium samples with glass beads and a mortar. The disrupted cells (0.5 to 1 g) were resuspended in 0.8 to 1 ml of 50 mM Tris-HCl–1 mM EDTA (pH 7.5) and centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant was used in enzymatic assays. Protein concentrations were determined by the Bradford assay.

Saccharopine reductase reverse activity was determined (30) by measuring piperideine-6-carboxylic acid (P6C) formation in a reaction mixture containing 4 mM saccharopine, 0.5 mM NADP, and 50 μg of total protein in 100 mM Tris-HCl (pH 9.0). Control reactions were carried out without NADP in the reaction mixture. The reaction mixtures were incubated for 1 h at 30°C, and the reactions were terminated by the addition of 200 μl of 5% tricloroacetic acid (in ethanol). The P6C formed was quantified by derivatization of a 500-μl reaction product with 750 μl of *ortho*-aminobenzaldehyde (OAB) (1 mg/ml in 2% ethanol); after incubation for 30 min at 37°C, the absorbance at 465 nm was determined. The activity was expressed as units per milligram of protein. One unit of saccharopine reductase was defined as the activity forming 1 nanomole of P6C per min. The molar extinction coefficient for P6C-OAB is 2,800 liters/mol × cm.

Analysis of lysine, saccharopine, pipecolic acid, and α-aminoadipic acid intracellular pools by HPLC. Analysis of lysine, saccharopine, pipecolic acid, and α-aminoadipic acid was performed by a reverse-phase high-pressure liquid chromatography (HPLC) method based on precolumn derivatization with 9-fluorenylmethyl chloroformiate (FMOC) as described by Sim and Perry (34). Cell extracts in 500 mM borate buffer (pH 7.7) were obtained as indicated above. Proteins were precipitated by the addition of 1 volume of methanol and centrifuged for 20 min at 14,000 × *g*. The supernatants were derivatized with FMOC. Five hundred microliters of 10 mM FMOC dissolved in acetone was added to 500 μl of sample, and the mixture was incubated for 1 min at room temperature. After the addition of 2 ml of cyclohexane and vortexing, the upper, organic phase was discarded. Cyclohexane extraction was repeated twice, and 100 μl of the extracted aqueous layer was injected into the HPLC column. The same procedure was followed with standard samples of lysine, saccharopine, and pipecolic acid and a mixture of the three compounds (2 μg of each).

The derivatized amino acids were resolved by HPLC (Beckman System Gold) through a μBondapak C_{18} column (Waters) with mobile phases of 50 mM sodium acetate buffer (pH 4.2) and acetonitrile. The flow rate was 1 ml/min.

P6C was quantified spectrophotometrically by monitoring the reaction with OAB as indicated above.

Mass spectrometry. Mass spectra for both pipecolic acid and FMOC-pipecolate were determined by using an LCT-TOF mass electrospray system (Micromass Instruments, S.A., Barcelona, Spain) in the electrospray and APCI ionization modes.

RESULTS

Disruption of the *lys7* gene of *P. chrysogenum* Wis 54-1255. In order to study whether *P. chrysogenum* is able to synthesize pipecolic acid by flux channeling of α-aminoadipic acid semialdehyde into pipecolic acid, we obtained mutants lacking saccharopine reductase by disrupting the *lys7* gene. Plasmid pDL7, containing a 10-kb *Cla*I/*Not*I genomic fragment with the phleomycin resistance cassette replacing the *lys7* gene, was used to transform parental strain *P. chrysogenum* Wis 54-1255. A total of 3,000 phleomycin-resistant transformants were obtained in several transformation experiments, and their phenotypes were tested. Since saccharopine reductase is involved in the lysine biosynthesis pathway in *P. chrysogenum*, inactivation of the *lys7* gene should lead to lysine auxotrophy. One clone out of about 3,000 transformants showed lysine auxotrophy.

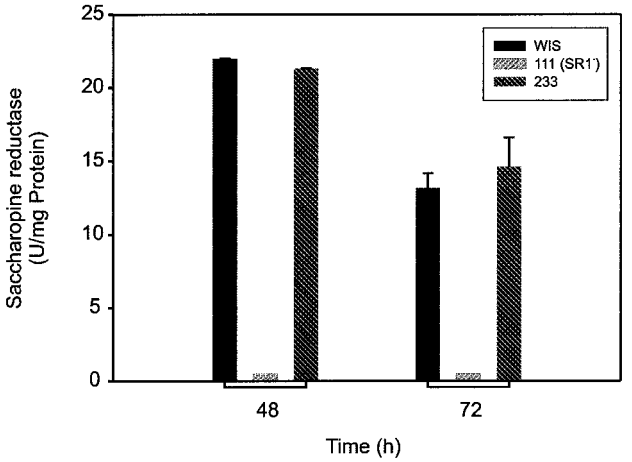

FIG. 2. Saccharopine reductase activity in cell extracts of *P. chrysogenum* TDL7-111. Parental strain Wis 54-1255 (WIS) and a randomly selected prototroph transformant (*P. chrysogenum* TDL7-233 [designated 233]) were used as controls. The strains were grown in MDFP medium, and mycelium was collected at 48 and 72 h. Standard errors are indicated by error bars. Note that *P. chrysogenum* TDL7-111 (111), which has a disruption of the *lys7* gene, lacks saccharopine reductase activity (renamed $SR1^-$).

To determine whether the selected lysine auxotroph (*P. chrysogenum* TDL7-111) had a disruption of the *lys7* gene, Southern analysis was performed with three different fragments of the *lys7* gene as probes: a 2.8-kb *Bgl*II fragment (5′ end of the *lys7* gene), a 0.9-kb *Bgl*II fragment (internal to the *lys7* gene), and a 1.8-kb *Bgl*II fragment (3′ end of the *lys7* gene). A hybridization band of 12.2 kb was expected for *Pst*I-digested genomic DNA of parental strain *P. chrysogenum* Wis 54-1255 with any of the three probes (Fig. 1A). In addition to the mutant *P. chrysogenum* TDL7-111, two prototroph transformants (TDL7-233 and TDL7-913) that were not lysine auxotrophs were randomly selected and tested for the hybridization pattern with each probe. The results showed that the three probes hybridized with a 12.2-kb band in parental strain *P. chrysogenum* Wis 54-1255 (Fig. 1B, lanes 1) and both prototroph transformants (Fig. 1B, lanes 2 and 4). However, this band was replaced by a 9-kb band in the lysine auxotroph *P. chrysogenum* TDL7-111 when the 2.8-kb *Bgl*II fragment probe (5′ end of the *lys7* gene) or the 0.9-kb *Bgl*II fragment probe (internal to the *lys7* gene) was used (Fig. 1B, panels I and II, lanes 3). Interestingly, in the lysine auxotroph *P. chrysogenum* TDL7-111, there was no hybridization when a 1.8-kb *Bgl*II fragment probe (3′ end of the *lys7* gene) was used, indicating that the recombinant strain lacked the 3′ region of the *lys7* gene (about 1,000 bp) and the downstream region (Fig. 1B, panel III, lane 3). These results indicated that the *lys7* gene had been disrupted in *P. chrysogenum* TDL7-111.

In addition, an analysis of the reversion rate showed that no revertants were obtained in a population of 5.2×10^7 spores. The auxotrophic phenotype of *P. chrysogenum* TDL7-111 was stable, as expected for a deletion mutant.

The disruption mutant *P. chrysogenum* TDL7-111 lacks saccharopine reductase activity. In order to confirm that the disruption of the *lys7* gene was the cause of the lysine auxotrophy, saccharopine reductase activity was measured in extracts of *P.*

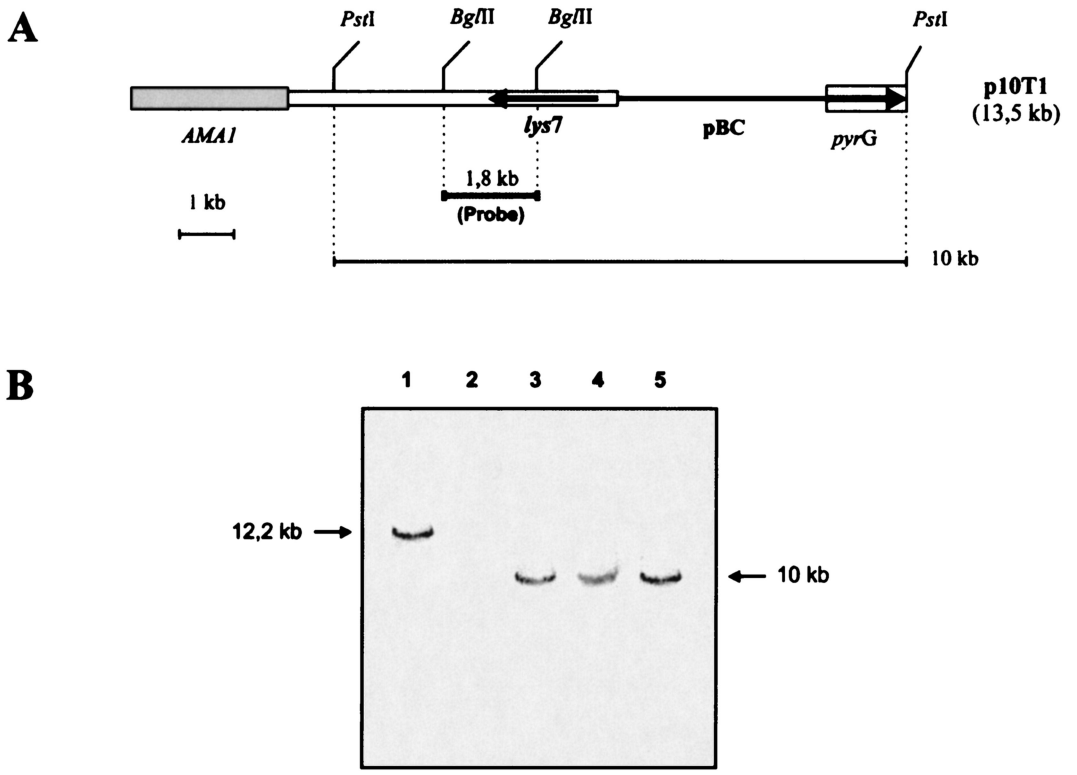

FIG. 3. Complementation of mutant *P. chrysogenum* SR1$^-$ with plasmid p10T1. (A) Physical map of autonomously replicating plasmid p10T1 bearing the *lys7* gene under the control of its own promoter region. A 1.8-kb *Bgl*II fragment containing the 3′ end of the *lys7* gene (thick line) was used as a probe. (B) Southern blot hybridization of *Pst*I-digested genomic DNA from three randomly selected prototroph transformants (T1, T2, and T3 [lanes 3, 4, and 5, respectively]); untransformed *P. chrysogenum* Wis 54-1255 and *P. chrysogenum* SR1$^-$ were used as controls (lanes 1 and 2, respectively). The sizes of the hybridizing bands are indicated on the left and the right. The 12.2-kb hybridizing band corresponds to the DNA fragment containing the endogenous *lys7* gene in *P. chrysogenum* Wis 54-1255. The 10-kb hybridizing band corresponds to the *lys7* gene in transforming plasmid p10T1 (panel A).

chrysogenum TDL7-111. Parental strain *P. chrysogenum* Wis 54-1255 and prototroph transformant *P. chrysogenum* TDL7-233 were used as controls. Saccharopine reductase was present at the same levels in parental strain *P. chrysogenum* Wis 54-1255 and prototroph transformant *P. chrysogenum* TDL7-233 (Fig. 2). However, no saccharopine reductase activity was detected in *P. chrysogenum* TDL7-111 (with the *lys7* gene disrupted). This result confirmed that *P. chrysogenum* TDL7-111 had a disruption of the *lys7* gene, encoding saccharopine reductase, and that this disruption was the cause of the lysine auxotrophy. To simplify the nomenclature, this transformant was renamed SR1$^-$ (for saccharopine reductase negative).

Transformation of *P. chrysogenum* SR1$^-$ with the *lys7* gene complemented the lysine auxotrophy. To test whether *lys7* inactivation was responsible for the lysine auxotrophy, *P. chrysogenum* SR1$^-$ was transformed with plasmid p10T1, containing the *P. chrysogenum lys7* gene. Hundreds of prototroph transformants were obtained in a single experiment. Comparative Southern hybridizations were performed with DNAs from three randomly selected prototroph transformants (named T1, T2, and T3), from mutant *P. chrysogenum* SR1$^-$, and from parental strain *P. chrysogenum* Wis 54-1255 and with a 1.8-kb *Bgl*II fragment (3′ end of the *lys7* gene) as a probe. The results showed a hybridizing 10.0-kb *Pst*I band in genomic DNA from transformants T1, T2, and T3 (Fig. 3B, lanes 3, 4, and 5) that corresponded to an exogenous (from p10T1) nonreorganized *lys7* gene (Fig. 3A). Parental strain *P. chrysogenum* Wis 54-1255 contained the 12.2-kb hybridizing band (Fig. 3B, lane 1), which was absent from *P. chrysogenum* SR1$^-$ (Fig. 3B, lane 2) and from transformants T1, T2, and T3 (Fig. 3B, lanes 3, 4, and 5). These results confirmed that the *lys7* gene was responsible for complementation of the lysine auxotrophy of *P. chrysogenum* SR1$^-$.

The *lys7* gene restored saccharopine reductase activity in mutant *P. chrysogenum* SR1$^-$. Saccharopine reductase activities in crude extracts of transformants T1, T2, and T3 (obtained by complementation of strain SR1$^-$), in mutant *P. chrysogenum* SR1$^-$, and in parental strain *P. chrysogenum* Wis 54-1255 were measured. The results showed that saccharopine reductase activity was present in all strains studied, except for mutant *P. chrysogenum* SR1$^-$ (Fig. 4). Transformants T1, T2, and T3 showed levels of saccharopine reductase activity about two- to threefold higher than those in parental strain *P. chrysogenum* Wis 54-1255 at both 48 and 72 h (Fig. 4). This increase in activity probably was due to the presence of the *lys7* gene in the autonomously replicating plasmid (25 to 50 copies per cell) (16). These results confirmed that the *lys7* gene was strictly required to restore saccharopine reductase activity in mutant *P. chrysogenum* SR1$^-$.

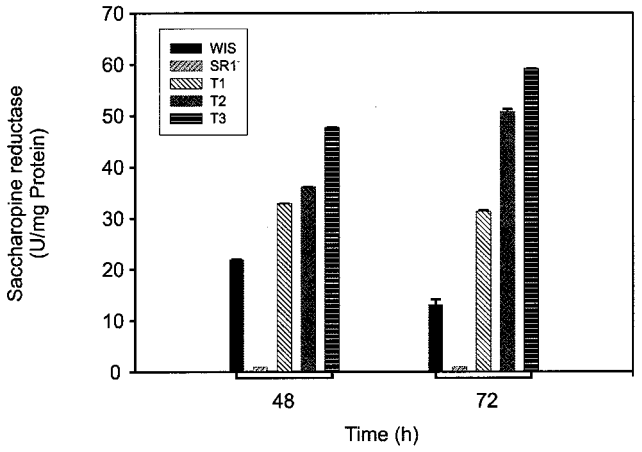

FIG. 4. Saccharopine reductase activity in cell extracts of transformants T1, T2, and T3 derived from *P. chrysogenum* SR1$^-$ by complementation with p10T1. Parental strain Wis 54-1255 (WIS) and *P. chrysogenum* SR1$^-$ were used as controls (untransformed). All strains were grown in MDFP medium, and cells were collected at 48 and 72 h. Standard errors are indicated by error bars. Note that the three transformants recovered saccharopine reductase activity. The high level of saccharopine reductase activity in the transformants was due to the large number of copies of *lys7* in autonomously replicating plasmid p10T1.

Pipecolic acid is formed in mutant SR1$^-$ but does not originate from lysine catabolism in the parental strain. To investigate the synthesis of pipecolic acid in *P. chrysogenum*, intracellular pools of pipecolic acid, saccharopine, L-lysine, and α-aminoadipic acid in extracts of cells grown for 48 and 72 h in MDFP medium with L-lysine (25 mM) as the sole nitrogen source were analyzed by HPLC. The results (Fig. 5) showed that parental strain *P. chrysogenum* Wis 54-1255 accumulated saccharopine through a lysine catabolic pathway (13) but did not synthesize pipecolic acid when grown with L-lysine as the sole nitrogen source, indicating that pipecolic acid is not formed from lysine catabolism in *P. chrysogenum*.

However, HPLC analysis of extracts of *P. chrysogenum* SR1$^-$ showed that this strain, when grown with L-lysine as the sole nitrogen source, accumulated saccharopine as well as a small peak corresponding to pipecolic acid (Fig. 5). Because saccharopine reductase is inactive in *P. chrysogenum* SR1$^-$, it was important to measure the intracellular concentration of P6C in the same crude extracts. The results showed that *P. chrysogenum* SR1$^-$, when grown with L-lysine as the sole nitrogen source, accumulated levels of P6C higher than those in control strain Wis 54-1255 (Table 1), suggesting that P6C could be the main precursor in pipecolic acid synthesis in *P. chrysogenum*.

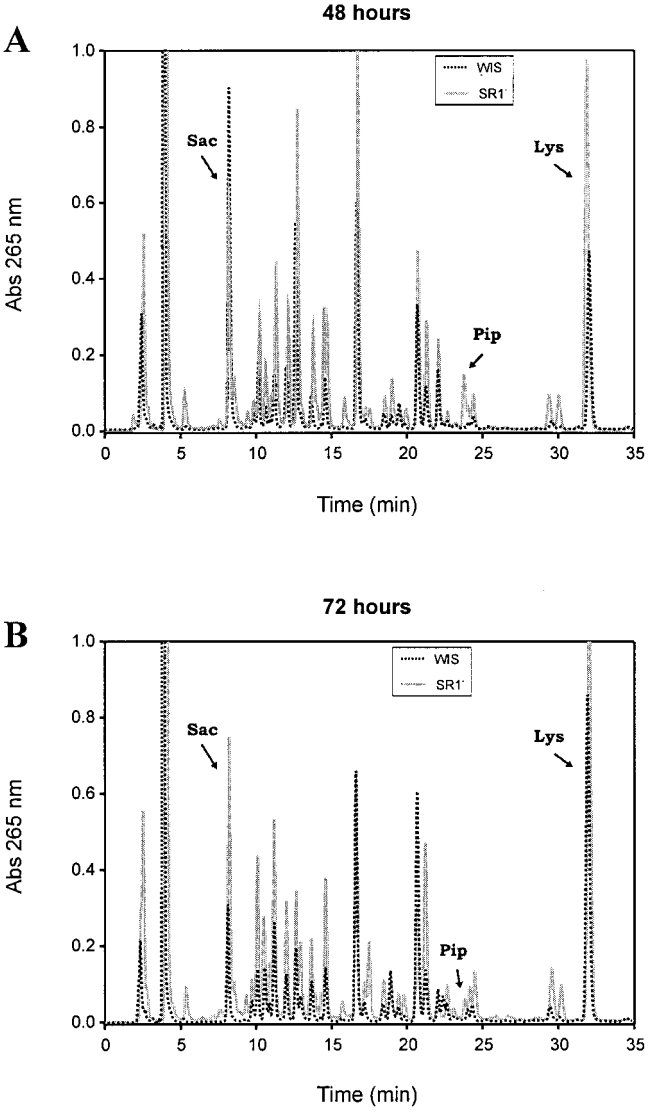

FIG. 5. Intracellular concentrations of pipecolic acid and saccharopine. HPLC analysis of intracellular concentrations of pipecolic acid (Pip) and saccharopine (Sac) was carried out with extracts of cells grown in MDFP medium supplemented with L-lysine (25 mM) at 48 (A) and 72 (B) h. Note that the parental strain, Wis 5-255 (WIS), accumulated saccharopine but did not form pipecolic acid, whereas strain SR1$^-$ accumulated saccharopine and pipecolic acid under the same conditions. Abs, absorbance.

TABLE 1. Intracellular concentration of P6C in *P. chrysogenum* Wis 54-1255 and *P. chrysogenum* SR1$^-$ in MDFP medium with L-lysine and α-aminoadipic acid[a]

P. chrysogenum strain	P6C formed (nmol/mg of protein) at the indicated h of culturing in the presence of:			
	L-Lysine		L-Lysine + α-Aminoadipic acid	
	48	72	48	72
Wis-54 1255	38	37	75	80
SR1$^-$	286	168	295	364

[a] L-Lysine was used as the sole nitrogen source at 25 mM. It is strictly required for growth of the SR1$^-$ (*lys7* disruption) strain.

DL-α-Aminoadipic acid significantly increases pipecolic acid accumulation in *P. chrysogenum* SR1$^-$. If pipecolic acid is synthesized from P6C, then the addition of DL-α-aminoadipic acid to culture broth should stimulate the intracellular accumulation of pipecolic acid. Therefore, a fermentation was carried out with *P. chrysogenum* Wis 54-1255 and mutant SR1$^-$ in MDFP medium with L-lysine (25 mM; to satisfy the lysine requirement of mutant SR1$^-$) and DL-α-aminoadipic acid (25 mM) as nitrogen sources. HPLC analysis of the intracellular concentration of pipecolic acid showed (Fig. 6) that under

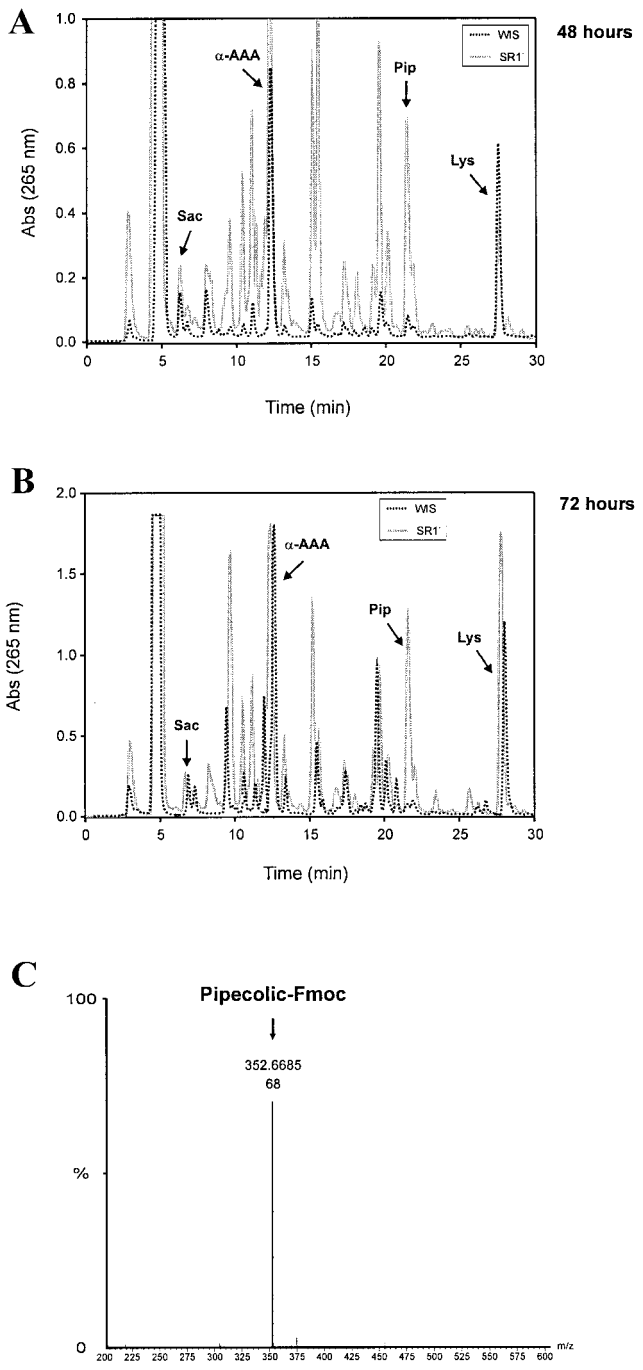

FIG. 6. Effects of DL-α-aminoadipic acid on the intracellular concentrations of pipecolic acid and saccharopine. (A and B) HPLC analysis of the intracellular concentrations of pipecolic acid (Pip) and saccharopine (Sac) was carried out with extracts of cells grown in MDFP medium with L-lysine (25 mM) and supplemented with DL-α-aminoadipic acid (25 mM) at 48 (A) and 72 (B) h. Note that the parental strain, Wis 54-1255 (WIS), accumulated saccharopine and very low levels of pipecolic acid, whereas strain SR1$^-$ accumulated saccharopine and very high levels of pipecolic acid under the same conditions at both 48 and 72 h. α-AAA, α-aminoadipic acid. (C) Mass spectrum of the compound eluting in the Pip peak of panel B. Note the molecular mass of 352.6. Abs, absorbance.

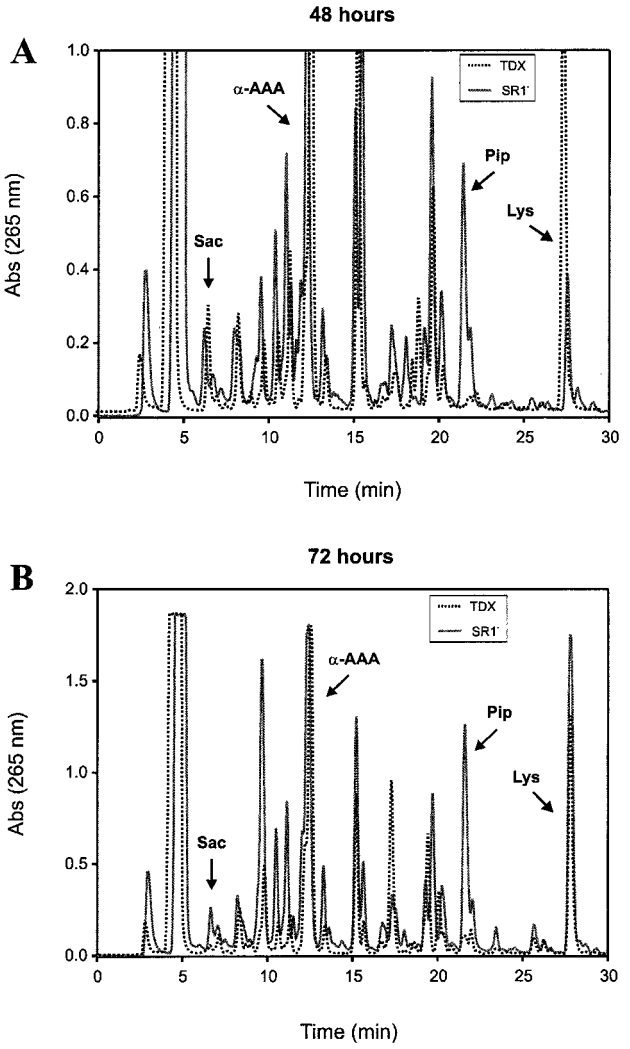

FIG. 7. Lack of pipecolic acid formation in the *lys2* disruption mutant. HPLC analysis of intracellular concentrations of pipecolic acid (Pip) and saccharopine (Sac) was carried out with extracts of TDX195 (TDX) and SR1$^-$ cells grown in MDFP medium with L-lysine (25 mM) and supplemented with DL-α-aminoadipic acid (25 mM) at 48 (A) and 72 (B) h. Note that strain SR1$^-$ accumulated pipecolic acid, whereas strain TDX195 did not form it under the same conditions. α-AAA, α-aminoadipic acid. Abs, absorbance.

these conditions, a small peak corresponding to pipecolic acid was obtained in crude extracts of *P. chrysogenum* Wis 54-1255. However, in *P. chrysogenum* SR1$^-$, the addition of L-lysine and DL-α-aminoadipic acid resulted in a high level of pipecolic acid in cells grown for both 48 and 72 h. These results strongly suggested that pipecolic acid is synthesized from α-aminoadipic acid in *P. chrysogenum*.

Mass spectra of accumulated pipecolic acid. To confirm the identity of the accumulated compound, the mass spectra of the pipecolic acid peak shown in Fig. 6B were compared with those of authentic pipecolic acid (Fluka Chemika, Buchs, Switzerland) and a pipecolic acid derivative. The mass spectra of the main compound in the pipecolic acid peak showed an M^+ ion of 352.6 (Fig. 6C) that corresponded to protonated FMOC-pipecolate and revealed the presence in nonderivatized cell

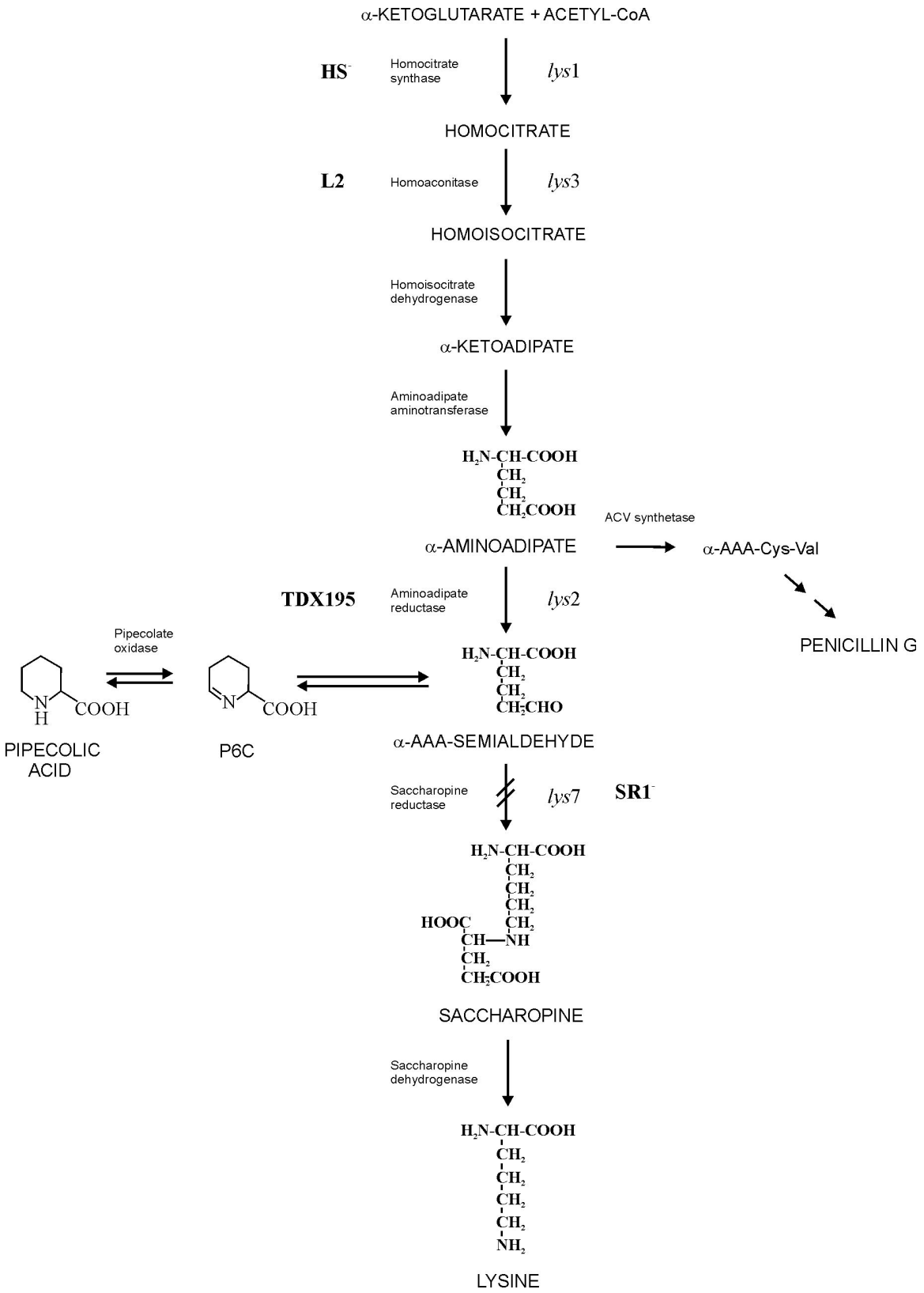

FIG. 8. Biosynthesis of lysine and penicillin G in *P. chrysogenum*. The flow chart shows the formation of pipecolic acid and the interconversion of pipecolic acid to lysine. Pipecolate oxidase catalyzes the conversion of pipecolic acid to P6C, which is in equilibrium with α-aminoadipate-δ-semialdehyde. The chemical structures of these compounds are shown. The conversion step blocked in *P. chrysogenum* SR1$^-$ is indicated by an arrow with hatch marks through the stem. HS$^-$ and TDX195 in bold type indicate the steps blocked in *P. chrysogenum* mutants HS$^-$ and TDX195. CoA, coenzyme A; L2, *P. chrysogenum* L2, a *lys3* gene mutant; ACV, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; α-AAA, α-aminoadipic acid.

extracts of *P. chrysogenum* SR1$^-$ of a compound with a mass of 129.14, which corresponds exactly to the mass of pipecolic acid.

Pipecolic acid is synthesized from α-aminoadipic acid through P6C. To prove that pipecolic acid is synthesized from α-aminoadipic acid through P6C in *P. chrysogenum*, the intracellular accumulation of pipecolic acid in *P. chrysogenum* SR1$^-$ (lacking saccharopine reductase) was compared to that in *P. chrysogenum* TDX195 (lacking α-aminoadipate reductase, the enzyme involved in the conversion of α-aminoadipic acid to α-aminoadipate-δ-semialdehyde). Strain TDX195 is unable to form P6C from α-aminoadipic acid (7–9). HPLC analysis of the intracellular concentration of pipecolic acid showed (Fig. 7) that, as expected, *P. chrysogenum* SR1$^-$ accumulated large amounts of pipecolic acid under these culture conditions. However, under the same conditions, strain TDX195 (unable to form P6C from α-aminoadipic acid) did not form pipecolic acid. These results confirmed that pipecolic acid is synthesized from α-aminoadipic acid through P6C in *P. chrysogenum* (Fig. 8), with particularly high levels in strain SR1$^-$, which is defective in saccharopine reductase.

DISCUSSION

Pipecolic acid, a six-carbon cyclic imino acid, is an important precursor for the biosynthesis of secondary metabolites in microorganisms and plants. The formation of this compound is related to lysine metabolism in various organisms, including plants, mammals, fungi, and bacteria (2, 12, 17, 19, 20, 22, 26, 27).

The α-aminoadipic acid pathway is very active in *P. chrysogenum*. α-Aminoadipic acid is a well-known precursor of penicillin (29) and cephalosporins and cephamycins (1, 28).

Very important questions are whether pipecolic acid is synthesized in *P. chrysogenum* and how it is formed. In other filamentous fungi, such as *M. anisopliae* and *R. leguminicola*, pipecolic acid is reported to be formed from lysine catabolism (34–36). However, Aspen and Meister (2) proposed that, in *A. nidulans*, pipecolic acid is formed from α-aminoadipic acid (i.e., from an intermediate of lysine biosynthesis). As shown in this work, several experimental data support the conclusion that in *P. chrysogenum*, pipecolic acid is obtained from α-aminoadipic acid and not from lysine catabolism. When strain Wis 54-1255 was grown with L-lysine as the sole nitrogen source, no accumulation of pipecolic acid occurred. However, under these conditions, *M. anisopliae* and *R. leguminicola* accumulated pipecolic acid (34–36). When *P. chrysogenum* SR1$^-$, lacking saccharopine reductase, was grown with L-lysine (required for growth by this auxotroph), a small peak corresponding to pipecolic acid was formed; under these conditions, strain SR1$^-$ accumulated P6C, which is the putative precursor of pipecolic acid. When cultures of strain SR1$^-$ were supplemented with α-aminoadipic acid (in addition to L-lysine), the addition of α-aminoadipic acid to the cultures resulted in a high level of pipecolic acid. These results strongly support the conclusion that in *P. chrysogenum*, pipecolic acid is derived from α-aminoadipic acid rather than from lysine catabolism. An important finding was the observation that strain TDX195, which is unable to form P6C from α-aminoadipic acid because it lacks α-aminoadipate reductase (9), did not accumulate pipecolic acid when it was grown with L-lysine and α-aminoadipic acid. This finding confirmed that in *P. chrysogenum*, pipecolic acid is formed from α-aminoadipic acid (Fig. 8).

The parental strain *P. chrysogenum* Wis 54-1255 does not accumulate P6C; therefore, little—if any—pipecolic acid is formed. A minor catabolic pathway is known to convert lysine to P6C in *P. chrysogenum* (13; E. Martín de Valmaseda and J. F. Martín, unpublished results). This catabolic pathway may be more active in *M. anisopliae* and *R. leguminicola*, thus explaining the observed differences between these fungi and *P. chrysogenum*.

Saccharopine reductase (EC 1.5.1.10), also called saccharopine dehydrogenase (glutamate forming) or α-aminoadipate-δ-semialdehyde glutamate reductase, is an NADPH-dependent enzyme that catalyzes the penultimate step in the so-called α-aminoadipate pathway for the biosynthesis of lysine in fungi—the reversible conversion of glutamate and α-aminoadipate-δ-semialdehyde to saccharopine (4, 5, 23–25). The directed disruption of the *lys7* gene, encoding saccharopine reductase, leads to the accumulation of P6C, a cyclic form of α-aminoadipic acid semialdehyde which is later converted to pipecolic acid (Fig. 8).

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REFERENCES

1. **Aharonowitz, Y., G. Cohen, and J. F. Martín.** 1992. Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation and evolution. Annu. Rev. Microbiol. **46:**461–495.
2. **Aspen, A. J., and A. Meister.** 1962. Conversion of α-aminoadipic acid to L-pipecolic acid by *Aspergillus nidulans*. Biochemistry **1:**606–611.
3. **Bañuelos, O., J. Casqueiro, S. Steidl, S. Gutiérrez, A. Brakhage, and J. F. Martín.** 2002. Subcellular localization of the homocitrate synthase in *Penicillium chrysogenum*. Mol. Gen. Genet. Genomics **266:**711–719.
4. **Bhattacharjee, J. K.** 1992. Evolution of α-aminoadipate pathway for the synthesis of lysine in fungi, p. 47–80. *In* R. P. Mortlock (ed.), Evolution of metabolic function. CRC Press, Inc., Boca Ratón, Fla.
5. **Bhattacharjee, J. K.** 1985. α-Aminoadipate pathway for the biosynthesis of lysine in lower eukaryotes. Crit. Rev. Microbiol. **12:**131–151.
6. **Cantoral, J. M., B. Díez, J. L. Barredo, E. Alvarez, and J. F. Martín.** 1987. High frequency transformation of *Penicillium chrysogenum*. Bio/Technology **5:**494–497.
7. **Casqueiro, J., S. Gutiérrez, O. Bañuelos, F. Fierro, J. Velasco, and J. F. Martín.** 1998. Characterization of the *lys2* gene of *Penicillium chrysogenum* encoding α-aminoadipic acid reductase. Mol. Gen. Genet. **259:**549–556.
8. **Casqueiro, J., O. Bañuelos, S. Gutiérrez, M. J. Hijarrubia, and J. F. Martín.** 1999. Intrachromosomal recombination between direct repeats in *Penicillium chrysogenum*: gene conversion and deletion events. Mol. Gen. Genet. **261:**994–1000.
9. **Casqueiro, J., S. Gutiérrez, O. Bañuelos, M. J. Hijarrubia, and J. F. Martín.** 1999. Gene targeting in *Penicillium chrysogenum*: disruption of the *lys2* gene leads to penicillin overproduction. J. Bacteriol. **181:**1181–1188.
10. **Dennis, J. W.** 1986. Effects of swainsonine and polyinosinic:polycytidylic acid on murine tumor cell growth and metastasis. Cancer Res. **46:**5131–5136.
11. **Díez, B., E. Álvarez, J. M. Cantoral, J. L. Barredo, and J. F. Martín.** 1987. Isolation and characterization of *pyr*G mutants of *Penicillium chrysogenum* by resistance to 5′-fluoroorotic acid. Curr. Genet. **12:**277–282.
12. **Dodt, G., D. G. Kim, S. A. Reimann, B. Reuber, K. MacCabe, S. Gould, and S. Mihalik.** 2000. L-pipecolic acid oxidase, a human enzyme essential for the degradation of L-pipecolic acid, is most similar to the monomeric sarcosine oxidases. Biochem. J. **345:**487–494.
13. **Esmahan, C., E. Alvarez, E. Montenegro, and J. F. Martín.** 1994. Catabolism of lysine in *Penicillium chrysogenum* leads to formation of 2-aminoadipic acid, a precursor of penicillin biosynthesis. Appl. Environ. Microbiol. **60:** 1705–1710.
14. **Fierro, F., J. L. Barredo, B. Díez, S. Gutiérrez, F. J. Fernández, and J. F. Martín.** 1995. The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. Proc. Natl. Acad. Sci. USA **92:**6200–6204.

15. **Fierro, F., S. Gutiérrez, B. Díez, and J. F. Martín.** 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.
16. **Fierro, F., K. Kosalková, S. Gutiérrez, and J. F. Martín.** 1996. Autonomously replicating plasmids carrying the *AMA1* region in *Penicillium chrysogenum*. Curr. Genet. **29:**482–489.
17. **Fothergill, J. C., and J. R. Guest.** 1977. Catabolism of L-lysine by *Pseudomonas aeruginosa*. J. Gen. Microbiol. **99:**139–155.
18. **Fujii, T., M. Mukaihara, H. Agematu, and H. Tsunekawa.** 2002. Biotransformation of L-lysine to L-pipecolic acid catalysed by L-lysine 6-aminotransferase and pyrroline-5-carboxylate reductase. Biosci. Biotechnol. Biochem. **66:**622–627.
19. **Fujii, T., Y. Aritoku, H. Agematu, and H. Tsunekawa.** 2002. Increase in the rate of L-pipecolic acid production using *lat*-expressing *Escherichia coli* by *lys*P and *yei*E amplification. Biosci. Biotechnol. Biochem. **66:**1981–1984.
20. **Goncalves-Butruille, M., P. Szajner, E. Torigoi, A. Leite, and P. Arruda.** 1996. Purification and characterization of the bifunctional enzyme lysine-ketoglutarate reductase-saccharopine dehydrogenase from maize. Plant Physiol. **110:**765–771.
21. **Humphries, M. J., K. Matsumoto, S. L. White, and K. Olden.** 1986. Oligosaccharide modification by swainsonine treatment inhibits pulmonary colonization by B16-F10 murine melanoma cells. Proc. Natl. Acad. Sci. USA **83:**1752–1756.
22. **Ijlst, L., I. de Kromme, W. Oostheim, and R. J. Wanders.** 2000. Molecular cloning and expression of human L-pipecolate oxidase. Biochem. Biophys. Res. Commun. **270:**1101–1105.
23. **Johansson, E., J. J. Steffens, M. Emptage, Y. Lindqvist, and G. Schneider.** 2000. Cloning, expression, purification and crystallization of saccharopine reductase from *Magnaporthe grisea*. Acta Crystallogr. Sect. D **56:**662–664.
24. **Johansson, E., J. J. Steffens, Y. Lindquist, and G. Schneider.** 2000. Crystal structure of saccharopine reductase from *Magnaporthe grisea*, an enzyme of the alpha-aminoadipate pathway of lysine biosynthesis. Struct. Fold Des. **8:**1037–1047.
25. **Jones, E. E., and H. P. Broquist.** 1966. Saccharopine, an intermediate of the aminoadipic acid pathway of lysine biosynthesis. III. Aminoadipic semialdehyde-glutamate reductase. J. Biol. Chem. **241:**3430–3434.
26. **Kinzel, J. J., and J. K. Bhattacharjee.** 1979. Role of pipecolic acid in the biosynthesis of lysine in *Rhodotorula glutinis*. J. Bacteriol. **138:**410–417.
27. **Kinzel, J. J., and J. K. Bhattacharjee.** 1982. Lysine biosynthesis in *Rhodotorula glutinis*: properties of pipecolic acid oxidase. J. Bacteriol. **151:**1073–1077.
28. **Martín, J. F.** 1998. New aspects of genes and enzymes for β-lactam antibiotic biosynthesis. Appl. Microbiol. Biotechnol. **50:**1–15.
29. **Martín, J. F.** 2000. α-Aminoadipyl-cysteinyl-valine synthetases in β-lactam producing organisms. From Abraham's discoveries to novel concepts of non-ribosomal peptide synthesis. J. Antibiot. **53:**1008–1021.
30. **Naranjo, L., E. Martín de Valmaseda, O. Bañuelos, P. López, J. Riaño, J. Casqueiro, and J. F. Martín.** 2001. Conversion of pipecolic acid into lysine in *Penicillium chrysogenum* requires pipecolate oxidase and saccharopine reductase: characterization of the *lys7* gene encoding saccharopine reductase. J. Bacteriol. **183:**7165–7172.
31. **Nielsen, J. B., M. J. Hsu, K. M. Byrne, and L. Kaplan.** 1991. Biosynthesis of the immunosuppressant immunomycin: the enzymology of pipecolate incorporation. Biochemistry **30:**5789–5796.
32. **Reynolds, K. A., and A. L. Demain.** 1997. Rapamycin, FK506, and ascomycin-related compounds, p. 497–520. *In* W. R. Strohl (ed.), Bio/technology of antibiotics. Marcel Dekker, Inc., New York, N.Y.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Sim, K. L., and D. Perry.** 1997. Analysis of swainsonine and its early metabolic precursors in cultures of *Metarhizium anisopliae*. Glycoconjugate J. **14:**661–668.
35. **Wickwire, B. M., C. M. Harris, T. M. Harris, and H. P. Broquist.** 1990. Pipecolic acid biosynthesis in *Rhizoctonia leguminicola*. I. The lysine saccharopine delta 1-piperideine-6-carboxylic acid pathway. J. Biol. Chem. **265:**14742–14747.
36. **Wickwire, B. M., C. Wagner, and H. P. Broquist.** 1990. Pipecolic acid biosynthesis in *Rhizoctonia leguminicola*. II. Saccharopine oxidase: a unique flavin enzyme involved in pipecolic acid biosynthesis. J. Biol. Chem. **265:**14748–14753.