Microbiology (2009), **155**, 000–000 **DOI** 10.1099/mic.0.031005-0

Transcriptional upregulation of four genes of the lysine biosynthetic pathway by homocitrate accumulation in *Penicillium chrysogenum*: homocitrate as a sensor of lysine-pathway distress

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Received 20 May 2009
Revised 16 July 2009
Accepted 17 August 2009

The lysine biosynthetic pathway has to supply large amounts of α-aminoadipic acid for penicillin biosynthesis in *Penicillium chrysogenum*. In this study, we have characterized the *P. chrysogenum* L2 mutant, a lysine auxotroph that shows highly increased expression of several lysine biosynthesis genes (*lys1*, *lys2*, *lys3*, *lys7*). The L2 mutant was found to be deficient in homoaconitase activity since it was complemented by the *Aspergillus nidulans lysF* gene. We have cloned a gene (named *lys3*) that complements the L2 mutation by transformation with a *P. chrysogenum* genomic library, constructed in an autonomous replicating plasmid. The *lys3*-encoded protein showed high identity to homoaconitases. In addition, we cloned the mutant *lys3* allele from the L2 strain that showed a G^{1534} to A^{1534} point mutation resulting in a Gly^{495} to Asp^{495} substitution. This mutation is located in a highly conserved region adjacent to two of the three cysteine residues that act as ligands to bind the iron–sulfur cluster required for homoaconitase activity. The L2 mutant accumulates homocitrate. Deletion of the *lys1* gene (homocitrate synthase) in the L2 strain prevented homocitrate accumulation and reverted expression levels of the four lysine biosynthesis genes tested to those of the parental prototrophic strain. Homocitrate accumulation seems to act as a sensor of lysine-pathway distress, triggering overexpression of four of the lysine biosynthesis genes.

INTRODUCTION

Lysine is synthesized by two different biosynthetic pathways in nature. Some organisms such as bacteria and plants use the so-called diaminopimelic acid pathway for biosynthesis of this amino acid. However, other organisms including filamentous fungi, yeast, some *Euglena* species (Bhattacharjee, 1985) and the bacteria *Thermus thermophilus* and *Pyrococcus horikoshii* (Kosuge & Hoshino, 1998; Kobashi *et al.*, 1999; Jia *et al.*, 2006) use the so-called α-aminoadipate pathway (Zabriskie & Jackson, 2000; Xu *et al.*, 2006).

Penicillium chrysogenum is used industrially for large-scale penicillin production. Penicillin derives from the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV), which is synthesized by a large non-ribosomal peptide synthetase (Aharonowitz *et al.*, 1993; Theilgaard *et al.*, 1997; Martín, 2000). One of the precursors of the ACV tripeptide is α-aminoadipic acid (Aharonowitz *et al.*, 1992), which is formed as an intermediate in lysine biosynthesis (Casqueiro *et al.*, 2001).

Lysine biosynthesis in filamentous fungi (Fig. 1) begins with the condensation, catalysed by homocitrate synthase (Bañuelos *et al.*, 1999), of α-ketoglutarate and acetyl-CoA to form homocitrate, which is later converted into homoisocitrate, α-ketoadipate and α-aminoadipate by the consecutive action of homoaconitase, homoisocitrate dehydrogenase and α-aminoadipate aminotransferase. α-Aminoadipate is the branch point where lysine and

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Abbreviation: IRP, iron regulatory protein.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is EU264159.

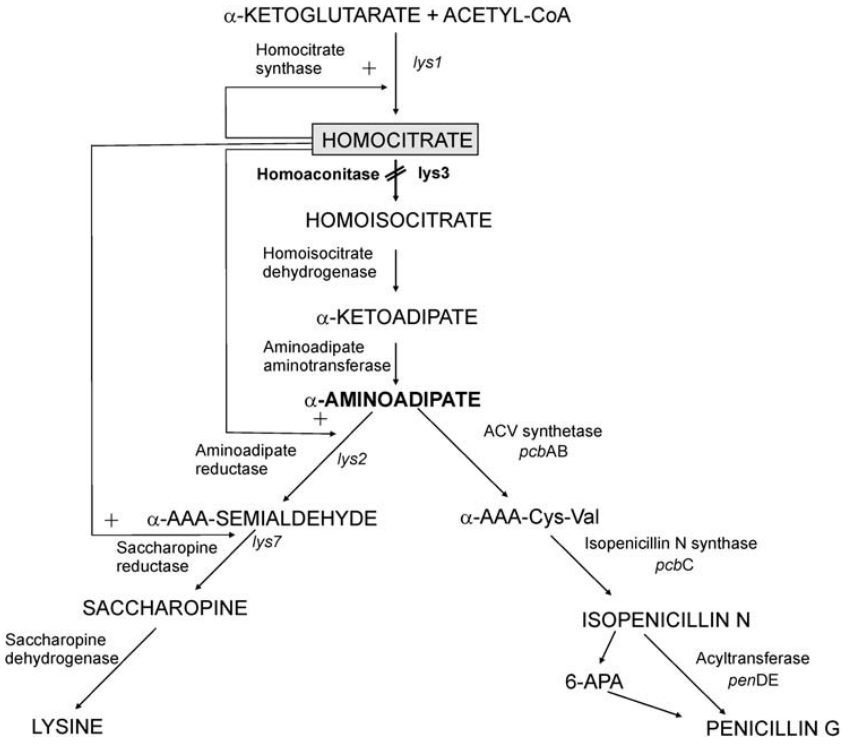

Fig. 1. Lysine biosynthesis pathway in *P. chrysogenum*. α-Aminoadipic acid is the branch point where lysine and penicillin biosynthesis diverge. The mutation identified in the *lys3* gene is indicated by the ≠ symbol. The homoaconitase is shown in bold type. The stimulatory effects observed on *lys1*, *lys2*, *lys3* and *lys7* transcript levels are indicated by the + ↓.

penicillin biosynthesis diverge (Jaklitsch *et al.*, 1986; Casqueiro *et al.*, 2001).

The regulation of the lysine biosynthetic pathway in *P. chrysogenum* is very important for the supply of precursors for penicillin biosynthesis (Demain, 1957; Friedrich & Demain, 1977; Luengo *et al.*, 1979, 1980; Casqueiro *et al.*, 1999a, 2001). However, very little is known about specific mechanisms controlling the lysine biosynthetic pathway in *P. chrysogenum* (Bañuelos *et al.*, 1999).

Mutants blocked in the early part of the lysine pathway are impaired in penicillin production because they are unable to form α-aminoadipate (Demain, 1983). Several years ago we isolated a lysine auxotroph named *P. chrysogenum* L2 blocked in α-aminoadipate biosynthesis, which accumulates homocitrate (Luengo *et al.*, 1980). This mutant is a single-clone derivative of a partial lysine auxotroph, *P. chrysogenum* L1, isolated by Masurekar *et al.* (1972).

In initial studies characterizing this mutant it was observed that it overexpresses several lysine biosynthesis genes (*lys1*, *lys2*, *lys3* and *lys7*) (see below). Therefore, it was unclear whether mutant L2 was altered in a lysine biosynthesis gene (since it is a lysine auxotroph) or in a regulatory gene controlling lysine biosynthesis. A pleiotropic regulatory factor of the lysine pathway (Lys14p) has been found in *Saccharomyces cerevisiae* (Feller *et al.*, 1997), but nothing is known about equivalent regulatory mechanisms in *P. chrysogenum*. It was therefore of great interest to characterize the mechanism that leads to overexpression of at least four lysine biosynthetic genes in the L2 mutant.

In this work, the *P. chrysogenum* L2 mutant was found to be defective in the homoaconitase step. We have characterized a gene (named *lys3*), cloned from the parental strain Wis 54-1255 by complementation of the L2 mutation that encodes a functional homoaconitase activity. In addition, we have cloned the *lys3* mutant allele from strain L2. The mutant Lys3 protein is altered in a region required to bind the [4Fe–4S] cluster characteristic of homoaconitases. Accumulation of homocitrate in the cells resulting from the *lys3* mutation seems to be responsible for the overexpression of the *lys* genes, since deletion of the *lys1* (homocitrate synthase) in the L2 strain restored normal expression levels to the mutant strain.

METHODS

Micro-organisms and culture media. *Escherichia coli* DH5α was used as host for routine DNA manipulation. *P. chrysogenum* Wis 54-1255 is a low penicillin-producing strain with a single copy of the penicillin gene cluster (Fierro *et al.*, 1995). The following strains are all Wis 54-1255 derivatives: *P. chrysogenum* L2, a lysine auxotroph, was obtained by selection of individual clones from a lysine bradytroph, L1 (Masurekar *et al.*, 1972; Luengo *et al.*, 1980); *P. chrysogenum* TD10-195 is a lysine auxotroph (Table 1) obtained by targeted inactivation of the *lys2* gene, encoding α-aminoadipate reductase (Casqueiro *et al.*, 1999a); *P. chrysogenum* HS^{-} is a lysine auxotroph defective in homocitrate synthase obtained by targeted inactivation of the *lys1* gene (Bañuelos *et al.*, 2002). All fungal strains were maintained on Power medium (Casqueiro *et al.*, 1999b) and spores were collected from cultures grown for 5 days at 28 °C. Mycelia of these strains grown in MPPY medium (Fierro *et al.*, 1993) were collected by filtration through Nytal filters. Czapek minimal

Table 1. Characteristics of *P. chrysogenum* strains used in this work

Strain	Genotype	Characteristics	Source
Wis 54-1255	Prototroph	Low penicillin producer; single copy of the penicillin gene cluster	Demain (1983)
L2	*lys*	Derivative of Wis 54-1255; lysine auxotroph; non-producer of penicillin; accumulates homocitrate	Luengo *et al.* (1980)
HS^-	*lys*	Derivative of Wis 54-1255; lysine auxotroph disrupted in the *lys1* gene	Bañuelos *et al.* (2002)
TD10-195	*lys*	Derivative of Wis 54-1255; lysine auxotroph disrupted in the *lys2* gene	Casqueiro *et al.* (1999a)
L2 + C	Prototroph	A derivative of *P. chrysogenum* L2 obtained by complementation of the L2 mutation with a DNA fragment of Wis 54-1255 bearing the *lys3* gene	This article

medium was used in phenotype assays. Lysine was added at a final concentration of 1.75 mM.

Autonomous replicating and integrative vectors. pAM9L (Fierro *et al.*, 1996) is an autonomous replicating plasmid carrying the left arm of the *AMA1* sequence of *Aspergillus nidulans* (Gems *et al.*, 1991) and the *P. chrysogenum pyrG* gene as selectable marker. Plasmid pLysF1 bears *lysF* from *A. nidulans*, which encodes the homoaconitase protein (Weidner *et al.*, 1997).

RNA isolation and intron analysis. Total RNA was isolated with the RNeasy kit (Qiagen) from *P. chrysogenum* Wis 54-1255 grown in MPPY for 24 h. To confirm the presence of a putative intron, the DNA regions around the expected splicing site were amplified by RT-PCR using *P. chrysogenum* mRNA from 24 h cultures as a template and as primers oligonucleotides 1 (5′-TGTCTCCGTGTTCGATG-TTGTTGA-3′) and 2 (5′-AGGTTCGCTGCGCTGGTGTTTT-3′). The DNA fragments amplified by RT-PCR were sequenced to confirm the splicing site.

Isolation of genomic DNA and Southern hybridization. Spores from *P. chrysogenum* were inoculated into MPPY medium (Fierro *et al.*, 1993), supplemented with lysine (1.75 mM) when necessary, and incubated in a New Brunswick Scientific orbital shaker at 200 r.p.m. for 48 h at 25 °C. The mycelium was collected by filtration through Nytal filters and lyophilized. Samples of lyophilized mycelium (500 mg) were treated with 0.5 ml 0.18 M Tris/HCl pH 8.2; 10 mM EDTA; 1 % (w/v) SDS and 0.5 ml phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.; phenol-CIA) and incubated for 30 min at 50 °C. Then, phenol-CIA treatment was repeated until the interface was clear. DNA was precipitated with ethanol and resuspended in TE buffer (Sambrook *et al.*, 1989).

Samples of 2–4 μg genomic DNA of *P. chrysogenum* were digested with appropriate endonucleases for Southern hybridizations. Genomic or plasmid DNA fragments were separated in 0.8 % agarose gels and blotted onto nylon membranes (Hybond-N+; Amersham-Biosciences) using a vacuum system (Pharmacia VacuGene). Digoxigenin labelling of DNA fragments, hybridization and detection were performed with the Genius kit (Roche) according to the manufacturer's instructions. Hybridizations were done at 65 °C using as buffer 5 × SSC; 0.1 % lauroyl sarcosine; 0.02 % (w/v) SDS and 2 % (w/v) blocking reagent.

Northern hybridization and quantification of hybridization signals. The DNA fragments used as probes for Northern analysis were: a 0.67 kb *Acc*I fragment of the *lys1* gene; a 2.7 kb *Nde*I fragment of the *lys2* gene; a 2.9 kb *Bgl*II–*Pst*I fragment of the *lys3* gene; and a 0.4 kb *Sac*I fragment of the actin A gene. All of them were homologous and were obtained from the previously cloned *P. chrysogenum* genes.

The probes were labelled by nick translation with [α-^{32}P]dCTP by standard methods (Sambrook *et al.*, 1989). The same amount of total RNA from each sample was transferred to Hybond-N+ nylon membranes. The membranes were prehybridized for 3 h at 42 °C in 50 % (v/v) formamide, 5 × Denhardt's solution, 5 × SSPE, 0.1 % (w/v) SDS and 500 μg salmon sperm DNA ml^{-1}. The hybridization was carried out overnight at the same temperature and with the same buffer as that used for prehybridization except that the salmon sperm DNA concentration used was 100 μg ml^{-1}. After the hybridization, the membranes were washed first with 2 × SSC, 0.1 % (w/v) SDS for 20 min at 42 °C, then with 0.1 × SSC, 0.1 % (w/v) SDS for 20 min at 42 °C and finally with 0.1 × SSC, 0.1 % (w/v) SDS for 10 min at 65 °C. The membranes were exposed to X-ray film for 7 days at –70 °C. The intensity of the hybridization bands was determined by using a phosphoimager scanner (Instant-Imager, Packard). Parallel hybridizations with an actin A probe were used as an internal control.

Transformation of *P. chrysogenum* protoplasts. Protoplasts of *P. chrysogenum* Wisconsin 54-1255 *pyrG* were obtained as described previously (Fierro *et al.*, 1993). Transformation of protoplasts was performed according to the procedure of Cantoral *et al.* (1987). Czapek plus ClK 0.7 M was used as protoplast regeneration medium (Díez *et al.*, 1987).

DNA sequencing and nucleic acid manipulations. For sequencing of the *lys3* gene, a 3.5 kb DNA fragment of the pAMPL2 insert was subcloned in both orientations in pBluescript KS(+) (Stratagene); a nested set of deletions from the resulting plasmid was generated by the Erase-a-Base procedure (Promega) by digestion with exonuclease III (Henikoff, 1984). The fragments were sequenced by the Sanger method, using the Autoread sequencing kit (Amersham-Biosciences). All other nucleic acid manipulations were performed by standard methods (Sambrook *et al.*, 1989).

PCR amplification. PCR amplifications were performed with *Pfu* DNA Polymerase (Stratagene) according to the manufacturer's instructions. The oligonucleotides used in PCRs were: 3, 5′-GAG CCC AGG AAC ACG GAA TCA C-3′; 4, 5′-CGG CAT GCG TCT GGG CTG GTT A-3′; 5, 5′-GCC GAC CCC GAT GCT CAA TAC G-3′; 6, 5′-AAT CCA TGT TCC ACG CCA GAC CAA T-3′; 7, 5′-ATG GTG CTC CTG CCA CCG TC -3′; 8, 5′-CTA TAC GGG AAG ACC GGC AG -3′.

RESULTS

The *P. chrysogenum* L2 mutant is deficient in homoaconitase

P. chrysogenum L2 is a lysine auxotroph that accumulates homocitrate (Luengo *et al.*, 1980). The L2 mutant grew in Czapek minimal medium when this medium was supplemented with α-aminoadipic acid, suggesting that the

mutation affects an enzyme of the first half of the lysine biosynthetic pathway at some point between homocitrate and α-aminoadipic acid. This mutant supplemented with 0.01 M lysine grows well, but produces very low levels of penicillin (Esmahan *et al.*, 1994). To characterize the L2 mutant, it was initially transformed with plasmid pLysF1 bearing *lysF* from *A. nidulans,* which encodes the homoaconitase protein (Weidner *et al.*, 1997). About 600 prototrophic transformants per microgram of DNA were obtained, suggesting that the *lysF* gene complements the mutation present in *P. chrysogenum* L2. Six of these transformants were analysed at the genetic level; their DNA was digested with *Bam*HI and *Hin*dIII and hybridized with a 0.8 kb *Xho*I fragment internal to the *A. nidulans lysF* gene. Results of the Southern analysis (Fig. 2b) showed a hybridization signal of 3.1 kb present in all prototrophic transformants (Fig. 2b, lanes 1–6), but not in the L2 strain; this band corresponds to the intact *lysF*-containing DNA fragment from *A. nidulans* (Fig. 2a). Under the high-stringency conditions used, the endogenous *lys3* (*lysF*) gene of *P. chrysogenum* L2 and Wis 54-1255 did not hybridize (Fig. 2b, lanes 7 and 8). These results confirmed that the restoration of prototrophy in complemented *P. chrysogenum* L2 transformants was due to *lysF* (encoding homoaconitase) and indicated that the L2 mutant was deficient in homoaconitase.

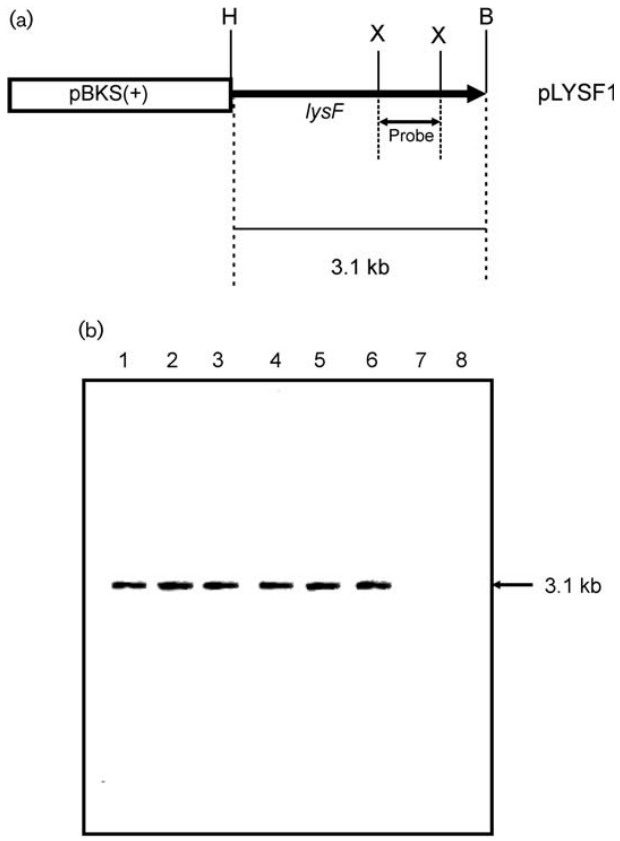

Fig. 2. (a) Diagram of the pLYSF1 plasmid containing the *A. nidulans lysF* gene (solid arrow) used to test complementation of the L2 mutation. The 3.1 kb *Bam*HI–*Hin*dIII fragment containing the intact *A. nidulans lysF* gene is shown. B, *Bam*HI; H, *Hin*dIII; X, *Xho*I. (b) Southern hybridization of *Bam*HI/*Hin*dIII-digested genomic DNA from six complemented (prototrophic) transformants (lanes 1–6), *P. chrysogenum* mutant L2 (lane 7) and wild-type *P. chrysogenum* Wis 54-1255 (lane 8). The probe used in the hybridizations is indicated by a thin double-headed arrow.

Cloning of a *P. chrysogenum* Wis 54-1255 gene complementing the L2 mutation

In order to study the initial observation of a possible regulatory effect of the L2 mutation, we decided to clone both the homoaconitase gene of the parental strain *P. chrysogenum* Wis 54-1255 and the allele of the L2 mutant.

To clone the *P. chrysogenum* homoaconitase gene, protoplasts of the L2 mutant were transformed with a genomic library constructed in the autonomous replicating (AMA1-based) plasmid pAM9L (Fierro *et al.*, 1996; Naranjo *et al.*, 2001). After protoplast regeneration, ten complemented transformants were obtained. To rescue the complementing plasmids, genomic DNA from each of the ten transformants was isolated and transformed into *E. coli*. Five out of the ten genomic DNAs used gave transformed *E. coli* colonies resistant to ampicillin (the antibiotic marker in the pAM9L vector). Restriction analysis of the plasmids present in *E. coli* showed that most of them had a plasmid of the same size (11.7 kb), which was named pAMPL2. This plasmid was retransformed into *P. chrysogenum* L2, giving about 2000 transformants per microgram of plasmid DNA, suggesting that plasmid pAMPL2 bears the *P. chrysogenum* L2 complementing gene.

lys3 encodes a protein homologous to homoaconitases

A 3350 bp region of the insert of pAMPL2 was sequenced on both strands. An open reading frame (ORF1) of 2408 bp was found. RT-PCR studies using oligonucleotides 1 and 2 (see Methods) as primers and RNA from 24 h cultures of *P. chrysogenum* Wis 54-1255 as template confirmed the presence of one intron. A DNA band of 537 bp was obtained by this technique. The amplified DNA band was sequenced on both strands, confirming that a 50 bp intron had been removed at the splicing sites corresponding to nucleotides 67–116 numbered from the ATG translation initiation codon.

The cloned gene encoded a protein of 785 amino acids with a deduced molecular mass of 84.49 kDa. The encoded protein showed high identity to other homoaconitases, particularly those of *A. nidulans* (*E*-value 0.0, 78 % homology, 68 % identity) (Weidner *et al.*, 1997), *Aspergillus fumigatus* (*E*-value 0.0, 80 % homology, 67 % identity), *Aspergillus niger* (*E*-value 0.0, 78 % homology, 67 % identity), *Penicillium marneffei* (*E*-value 0.0, 77 % homology, 67 % identity), *Saccharomyces cerevisiae*

(*E*-value 0.0, 71 % homology, 59 % identity) or *Schizosaccharomyces pombe* (*E*-value 0.0, 68 % homology, 54 % identity). These results suggest that the protein encoded by the cloned gene corresponds to the *P. chrysogenum* homoaconitase. The gene was named *lys3* according to the standard nomenclature proposed for the *P. chrysogenum lys* genes (Naranjo *et al.*, 2001).

The *lys3*-encoded protein also showed similarity to aconitases, particularly those from *Sch. pombe* (31 % identical amino acid residues), *Sac. cerevisiae* (30 %), human (29 %), *Drosophila* (27 %), *A. fumigatus* (26 %) and *P. chrysogenum* (25 %), but the percentage of similarity was lower than with the homoaconitase genes. Homoaconitases and aconitases carry out the same isomerization reaction, but aconitases recognize citrate, which has one carbon atom less than homocitrate, as substrate. Some aconitases are bifunctional proteins that act also as iron regulatory proteins (IRPs).

The *lys3*-encoded protein and also the homoaconitases from *A. fumigatus*, *A. nidulans*, *Sac. cerevisiae* and *Sch. pombe* showed the three conserved cysteine residues (Fig. 3a) at equivalent positions (C^{409}-68X-C^{478}-2X-C^{481} in the *P. chrysogenum* Lys3) that are required to bind a [4Fe–4S] iron–sulfur cluster characteristic of members of the aconitase, homoaconitase and IRP superfamily.

The L2 mutation produces a drastic increase in the transcript levels of several lysine biosynthesis genes

Initial observations indicated that the L2 strain showed an increased expression of some lysine biosynthesis genes. To investigate this phenomenon in detail, Northern blot analysis was done using RNA extracted from mycelia of different strains, *P. chrysogenum* L2 (impaired in the homoaconitase activity), *P. chrysogenum* L2 + C (strain L2 complemented with pAML2), and *P. chrysogenum* Wis 54-1255 (control parental strain) (see Table 1 for the genotype of each strain). The transcript level of *lys1* (homocitrate synthase), *lys2* (α-aminoadipate reductase), *lys3* (homoaconitase) and *lys7* (saccharopine reductase) was investigated using probes internal to these genes and compared to the actin A gene transcript as control. This study was repeated in three independent Northern experiments.

The results (Fig. 4a) showed that the mRNA level of *lys1* is about five times higher in *P. chrysogenum* L2 than in the parental strain Wis 54-1255. In the L2 + C complemented transformant (complemented with the *lys3* gene) the *lys1* transcript level reverted to the level observed in the parental strain Wis 54-1255.

Similarly, the transcript level of the *lys2* gene in the L2 mutant (Fig. 4b) was about eightfold higher than in the parental strain Wis 54-1255. In *P. chrysogenum* L2 + C (complemented transformant) the *lys2* transcript level reverted to the level obtained in the parental strain Wis 54-1255.

Analysis of the transcription of the *lys3* and *lys7* genes showed a three- to fourfold increase in the transcript level of this gene in *P. chrysogenum* L2 as compared with the parental strain Wis 54-1255 (Fig. 4c). These results indicate that the regulatory effect of the mutation present in *P. chrysogenum* L2 strain on the *lys2*, *lys3* and *lys7* transcript level is similar to that observed on the *lys1* transcript. 2

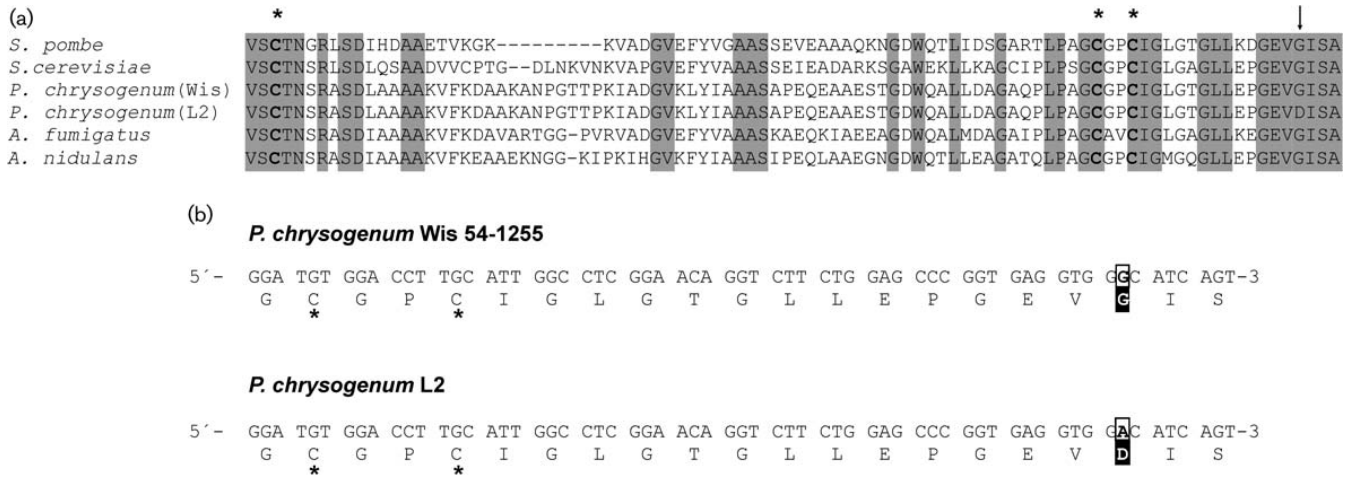

Fig. 3. (a) Alignment of the amino acid sequences of the central region of homoaconitases from *P. chrysogenum*, *Sac. cerevisiae*, *Sch. pombe*, *A. fumigatus* and *A. nidulans* and the mutant protein of *P. chrysogenum* L2. Note the strong conservation of several amino acid stretches, particularly of the regions adjacent to the three cysteine ligands of the [4Fe–4S] cluster. (b) Characterization of the *lys3* mutation in *P. chrysogenum* L2: nucleotide and deduced amino acid sequences of the region of the *lys3* containing the mutation. The nucleotide changes are boxed and the modified amino acid is shown in reverse type. 5

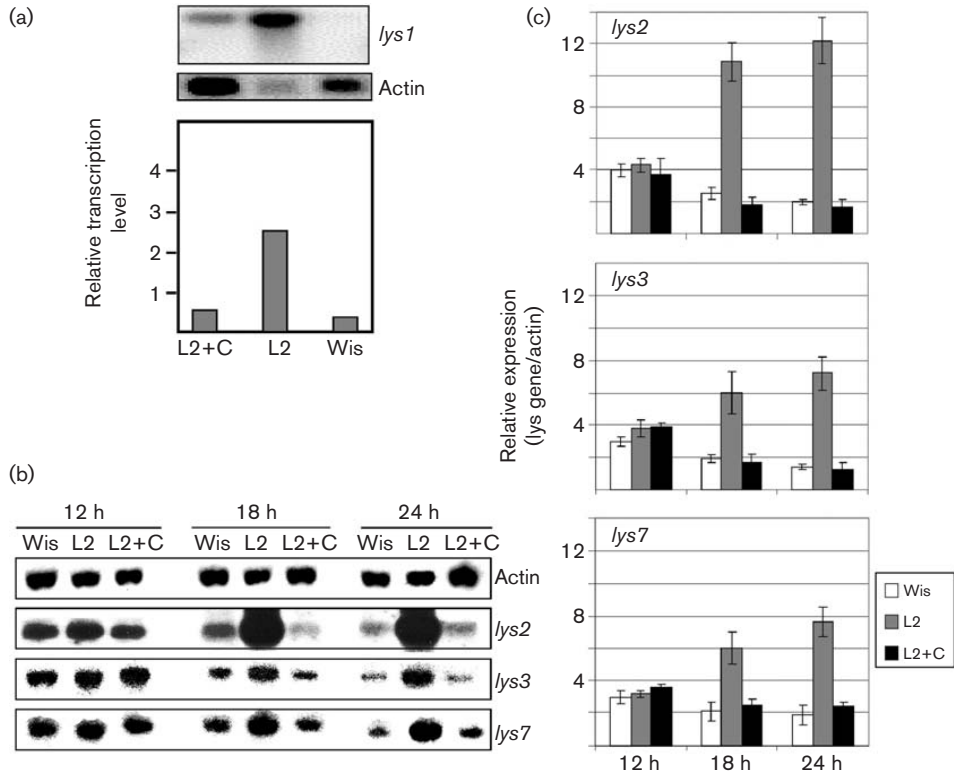

Fig. 4. Transcript levels of *lys1* (a), *lys2*, *lys3* and *lys7* (b) in *P. chrysogenum* L2; *P. chrysogenum* L2+C (complemented with *lys3*) and *P. chrysogenum* Wis 54-1255. The actin gene (*actA*) transcription level was used as control. The intensity of the RNA bands was quantified by densitometry (c). Note the high increase in expression of the four *lys* genes at 18 and 24 h cultures and the restoration to normal transcript levels in the complemented L2+C transformant.

6

Characterization of the *lys3* mutant allele present in mutant *P. chrysogenum* L2

To investigate the possible role of the Lys3 protein, the mutation present in the *lys3* gene of *P. chrysogenum* L2 was characterized. The *lys3* allele of strain L2 was amplified by PCR using a high-fidelity polymerase and oligonucleotides 3 and 4, and a DNA fragment of 2.5 kb that includes the *lys3* gene was obtained using as template the genomic DNA from *P. chrysogenum* L2. The 2.5 kb band was cloned into pBluescript KS(+) (giving rise to plasmid pL3D) and the insert was sequenced on both strands. Analysis of the mutant *lys3* sequence (Fig. 3b) showed a single point mutation: the G^{1534} had been converted into A^{1534}, leading to a change in the amino acid sequence from a glycine (G^{495}) to an aspartic acid (D^{495}).

To confirm that the nucleotide change found in strain L2 is a true mutation occurring in this strain, a small DNA region containing the putative mutation was amplified again with oligonucleotides 5 and 6 (see Methods), using as templates the DNAs of both mutant L2 and the parental strain Wis 54-1255. The same Gly495Asp mutation was found in the amplified DNA fragment from strain L2 but not from the parental strain. This point mutation is located in a highly conserved region surrounding one of the three cysteine residues (C^{409}-68X-C^{478}-2X-C^{481}) that are required to bind the iron–sulfur cluster essential for homoaconitase activity (Fig. 3a).

Transformation of *P. chrysogenum* L2 with the mutant *lys3* gene fails to complement the homoaconitase mutation

To prove that the detected *lys3* mutation was responsible for the lysine auxotrophy of strain L2, protoplasts of this strain were transformed with pL3D (containing the mutant *lys3* gene) with the help of pIBRC43 (conferring resistance to phleomycin; Kosalková *et al.*, 2009). Transformants were selected by resistance to phleomycin (30 μg ml^{-1}) in Czapek-phleomycin medium supplemented with 1.75 mM lysine.

Control transformants were created with plasmid pAMPL2 containing the wild-type *lys3* gene (see above). Prototrophic transformants were obtained with pAMPL2, but not with pL3D, indicating that the mutation found in the *lys3* gene is responsible for the lysine auxotrophy in the L2 strain.

Genetic disruption of the *lys1* gene, to prevent homocitrate formation

In order to elucidate if the upregulation of expression of the lysine biosynthesis genes is influenced by the reported accumulation of homocitrate (Luengo *et al.*, 1980), the *lys1* gene was disrupted in the *P. chrysogenum* L2 strain by double recombination (Fig. 5a). For this purpose we used the pDL1L2 plasmid, in which the *lys1* gene is interrupted by the *pyrG* gene with its own transcriptional terminator and flanked by a 1048 bp region upstream and a 6026 bp region downstream of *lys1*. This plasmid also contains a *ble* marker for the double marker selection procedure. After transformation of the *P. chrysogeneum* L2 *pyrG* derivative, prototrophic recombinants that did not require uridine and were also phleomycin-sensitive were selected. The correct gene replacement was analysed by Southern blotting (data not shown) and confirmed by PCR amplification (using primers 7 and 8, see Methods). These primers were designed to amplify the whole *lys1* gene (1600 bp). In the L2/*lys1* mutant the 3014 bp band amplified by PCR contained the *lys1* gene interrupted by the *pyrG* gene, whereas in the parental strain, the same primers amplified a 1600 bp band, which included the intact *lys1* gene (Fig. 5b). The authenticity of these bands was confirmed by sequencing.

The *P. chrysogenum* L2/*lys1* strain was fully blocked in lysine biosynthesis, in agreement with the fact that it is mutated in both *lys1* and *lys3* genes and required concentrations of about 1.75 mM L-lysine for growth on minimal medium plates (Fig. 5c). It grew poorly on this medium supplemented with 0.875 mM lysine. On the other hand, the L2 strain was able to grow on solid minimal medium supplemented with 0.875 mM lysine.

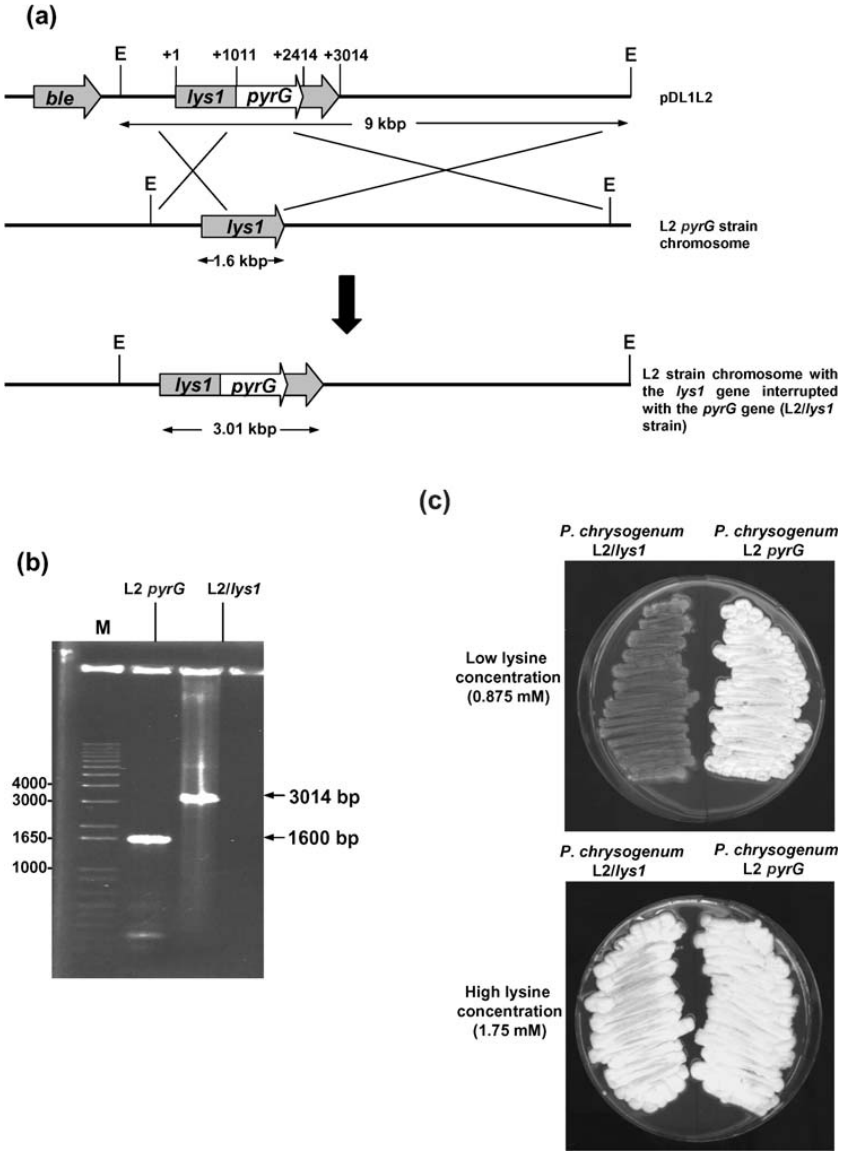

Fig. 5. Disruption of the *lys1* gene in the L2 mutant to prevent homocitrate formation. (a) Scheme of the disruption process. (b) Electrophoresis performed with the PCR product obtained after PCR amplification of the *lys1* gene using genomic DNA obtained from the L2 *pyrG* and L2/*lys1* strains and primers 7 and 8 (see Methods). (c) Growth of *P. chrysogenum* L2 (*pyrG*) and the double mutant *P. chrysogenum* L2/*lys1* on Czapek medium supplemented with 0.875 mM lysine and 5 mM uridine. The double mutant L2/*lys1* grows slowly in this medium, but it grows well at higher lysine (1.75 mM) concentrations.

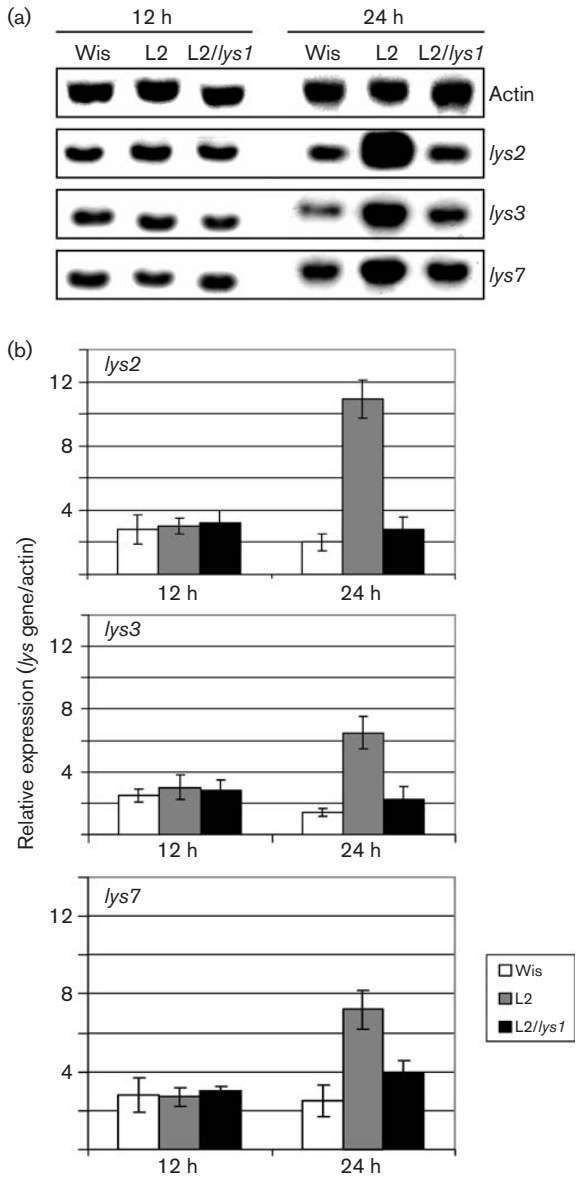

Fig. 6. Effect of disruption of the *lys1* gene on expression of *lys2*, *lys3* and *lys7* in the L2 strain. Note that disruption of *lys1* (preventing homocitrate synthesis) restores the expression level of the *lys* genes in the L2 mutant to that of the parental strain Wis 54-1255.

Disruption of *lys1*, preventing homocitrate formation, reverts the overexpression of the lysine biosynthetic genes

A comparison of the *lys2*, *lys3* and *lys7* transcript levels in the parental Wis 54-1255 strain, the L2 mutant and the L2/*lys1* recombinant in simultaneous cultures under the same experimental conditions revealed that disruption of the *lys1* gene resulted in restoration of the transcript levels of the L2/*lys1* mutants to those of the parental strain Wis 54-1255 (Fig. 6). Since the L2 and L2/*lys1* strains are derived from

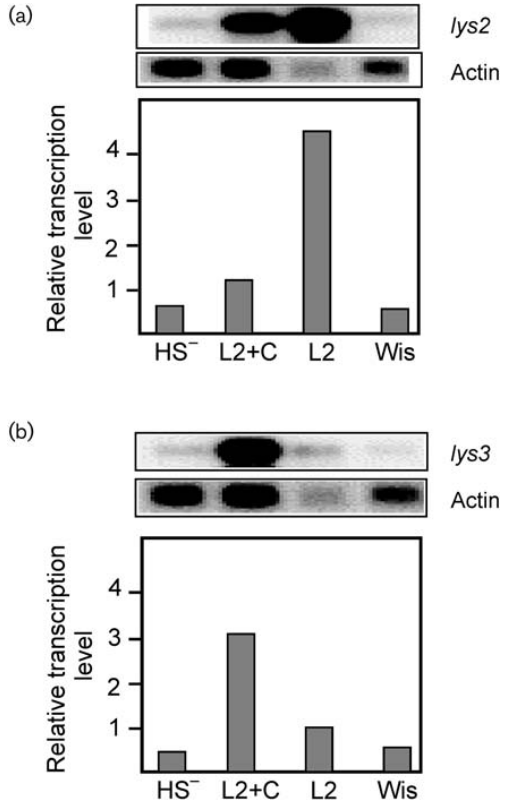

Fig. 7. Transcript levels in the *P. chrysogenum* HS⁻ strain (a lysine auxotroph, disrupted in *lys1*) compared with the parental strain Wis 54-1255 and with the L2 and the complemented L2+C strains. Note the lack of induction of the *lys2* and *lys3* transcripts in the HS⁻ strain. The high expression of *lys3* in the L2+C transformant (b) is due to the gene copy number (following complementation with *lys3*).

Wis 54-1255 and are isogenic for the rest of the genome, these results suggest that the accumulation of homocitric acid in the L2 strain, resulting from the mutation in the *lys3* (homoaconitase) gene, is required for the upregulation of the lysine biosynthetic genes (see Discussion).

DISCUSSION

Little is known about the regulation of α-aminoadipic acid biosynthesis despite its involvement in penicillin biosynthesis in *P. chrysogenum* (Aharonowitz *et al.*, 1992; Busch *et al.*, 2003). In wild-type strains the α-aminoadipate pool is limiting for penicillin production (Hönlinger & Kubicek, 1989; Lu *et al.*, 1992). A correlation between intracellular α-aminoadipate concentration and penicillin production has been reported in *P. chrysogenum* strains with different penicillin production ability (Jaklitsch *et al.*, 1986; Hijarrubia *et al.*, 2002). The gene that complements the *P. chrysogenum* L2 mutation (named *lys3*; Fig. 1) was cloned. The *lys3*-encoded protein is homologous to homoaconitases, showing a high degree of identity to homoaconitases

from *A. fumigatus*, *A. nidulans* (Weidner *et al.*, 1997), *Sac. cerevisiae* and *Sch. pombe*, and a lower similarity with aconitases from humans, *Drosophila*, *Sac. cerevisiae* and *Sch. pombe*. Aconitases and homoaconitases carry out a similar reaction in which an isomerization of one hydroxyl group in the substrate takes place. Aconitases and homoaconitases bind a [4Fe–4S] iron–sulfur nucleus (Beinert *et al.*, 1996) that is linked to three cysteine residues located at conserved positions in these proteins (Fig. 3). The presence of the iron–sulfur cluster is strictly required for the homoaconitase catalytic activity of the protein (Haile *et al.*, 1992; Philpott *et al.*, 1993). The same [4Fe–4S] prosthetic group occurs in the iron regulatory proteins (IRPs) (Hentze & Kühn, 1996).

Indeed, phylogenetic studies of homoaconitases, aconitases and IRPs suggest a strong evolutionary conservation of the three cysteine residues (Irvin & Bhattacharjee, 1998), since these three cysteine residues of the protein act as ligands of the iron–sulfur cluster (Robbins & Stout, 1989). In contrast to other iron–sulfur proteins functioning as electron carriers, the [4Fe–4S] cluster of aconitases reacts directly with the enzyme substrate (Beinert & Kennedy, 1989) and the same is probably true for homoaconitases (in this case the substrate is homocitrate).

Southern hybridization analysis revealed that there is only one copy of the homoaconitase gene, a fact that has been recently confirmed after publication of the *P. chrysogenum* Wis 54-1255 genome (van den Berg *et al.*, 2008). As shown in this work, the *lys3* gene encodes a putative homoaconitase but in the L2 mutant the point mutation Gly496Asp results in a mutant protein that is not enzymically active, as shown by the accumulation of homocitrate (Luengo *et al.*, 1980) and by the lysine auxotrophy of the L2 mutant. This change of glycine to aspartic acid (a negatively charged polar amino acid) is located in a conserved region flanking the cysteine ligands of the iron–sulfur cluster and appears to disturb the

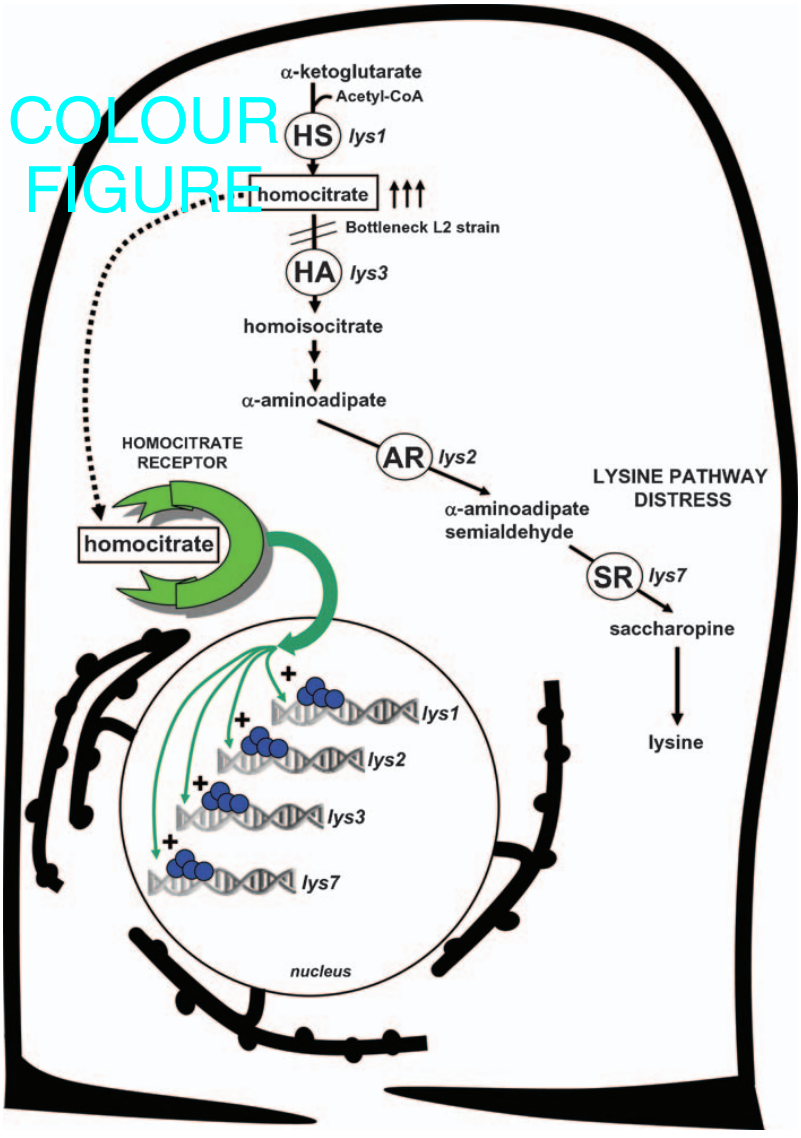

Fig. 8. Proposed model for the regulatory role of homocitrate in the lysine biosynthetic pathway. In this model, the excess of homocitrate generated in the L2 strain in the early step of the lysine biosynthetic pathway activates a regulatory protein that triggers a transcriptional response of the lysine biosynthetic genes *lys1* (encoding homocitrate synthase, HS), *lys2* (encoding aminoadipate reductase, AR), *lys3* (encoding homoaconitase, HA) and *lys7* (encoding saccharopine reductase, SR).

structure of the protein, preventing the formation of the active holoenzyme cluster.

The high transcript levels observed for the *lys1*, *lys2*, *lys3* and *lys7* genes in *P. chrysogenum* L2 might be due to the effect of the Lys3 mutant protein itself (acting as a regulatory protein) or to the accumulation of homocitrate or to a combination of both. The possibility that the overexpression of the *lys1*, *lys2*, *lys3* and *lys7* genes could be due to a deficiency of lysine in the cell was excluded because the overexpression effect was not observed in a lysine auxotroph defective in the homocitrate synthase (*P. chrysogenum* HS^-) (Fig. 7) or in another strain of *P. chrysogenum* (TD10-195), which is disrupted in the α-aminoadipate reductase (not shown).

The accumulation of homocitrate resulting from the lack of homoaconitase activity serves as a sensor of lysine-pathway distress and triggers the overexpression of at least four genes of the lysine biosynthetic pathway (*lys1*, *lys2*, *lys3* and *lys7*).

The lysine pathway in yeasts is regulated by a pathway-specific co-inducer-dependent transcriptional activation (Ramos *et al.*, 1988; Feller *et al.*, 1997; Becker *et al.*, 1998). In *Sac. cerevisiae* the reporter co-inducer of the lysine pathway is α-aminoadipic semialdehyde (AAS), which is an intermediate of the second part of the pathway (Ramos *et al.*, 1988). Expression of the lysine biosynthetic genes is induced by the regulatory protein Lys14p in the presence of the inducer AAS (Ramos *et al.*, 1988). Mutations in the *lys14* gene encoding the transcriptional activator protein cause yeasts to grow slowly in the absence of lysine. Lys14p is required for expression of the *lys9* gene [encoding saccharopine reductase (Borell *et al.*, 1984)]. Lys14p is a 90 kDa protein with a DNA-binding domain (Feller *et al.*, 1994) that interacts with many other proteins (Xu *et al.*, 2006).

The mechanism that we describe in this article is clearly different from the AAS-mediated control. The lack (or very low activity) of the homoaconitase in the L2 mutant causes a lysine-pathway stress that results in drastic overexpression of at least four genes of the lysine pathway. The stress is avoided by complementation with the wild-type *lys3*-encoding functional homoaconitase.

Our results suggest that the accumulation of homocitrate plays an important role in the regulation of lysine gene expression, since the overexpression of the four *lys* genes is clearly reduced when the formation of homocitrate is prevented by deletion of the homocitrate synthase gene (*lys1*).

An interesting question is why the lysine biosynthesis genes are regulated by homocitrate. It is likely that homocitrate (or homocitrate iron–sulfur cluster) levels are sensed by a regulatory protein that signals the need to activate the entire lysine biosynthetic pathway (Fig. 8). In this regard, homocitrate may be considered as a specific intracellular signal of lysine-pathway distress. The L2 mutant was a single-clone isolate of *P. chrysogenum* L1, a lysine bradytroph (leaky mutant) (Masurekar *et al.*, 1972). Leaky mutants having a partial deficiency in homoaconitase may react by overexpressing several genes of the lysine biosynthesis pathway. The characterization of the *lys3*-encoded protein opens the way for a rational manipulation of the lysine biosynthetic pathway in industrial *P. chrysogenum* strains to drive the lysine metabolic flux towards penicillin biosynthesis.

ACKNOWLEDGEMENTS

This work was supported by a grant from the European Union (QLK3-1999-00729). F. T. and L. N. received fellowships from the Agencia Española de Cooperación Iberoamericana (AECI). We thank A. Brakhage for providing plasmid pLysF1 and acknowledge the excellent technical assistance of J. Merino, B. Martín, A. Casenave and B. Aguado.

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