Journal of Industrial Microbiology & Biotechnology (2001) 27, 252–258

www.nature.com/jim

A moderate amplification of the *mec*B gene encoding cystathionine-γ-lyase stimulates cephalosporin biosynthesis in *Acremonium chrysogenum*

K Kosalková[1], AT Marcos[2] and JF Martín[1,2]

[1]*Area de Microbiología, Facultad de Ciencias Biológicas y Ambientales, Universidad de León, León 24071, Spain;* [2]*Instituto de Biotecnología INBIOTEC, Parque Científico de León, Avda. del Real, no. 1, León 24006, Spain*

L-cysteine is a precursor of the penicillin, cephalosporin and cephamycin families of β-lactam antibiotics. Cystathionine-γ-lyase (encoded by the *mec*B gene), an enzyme that splits cystathionine releasing cysteine, is required for high-level cephalosporin production in methionine-supplemented medium. By amplification of the *mec*B gene in *Acremonium chrysogenum* C10, several transformants were obtained that produced 10–40% higher levels of cephalosporin. All selected transformants contained at least two or three copies of the *mec*B gene as shown by Southern hybridization with a probe internal to *mec*B. Two of these transformants, *A. chrysogenum* T27 and *A. chrysogenum* T58, showed 4- to 10-fold higher cystathionine-γ-lyase activity than the control strain. Northern hybridizations indicated that the levels of the two *mec*B transcripts of 1.7 and 1.5 kb were greatly increased in transformants T27 and T58. Fermentor studies using controlled conditions confirmed that transformant T27 was a cephalosporin overproducer, reaching titers of nearly 2000 μg/ml of cephalosporin in Shen-defined medium that correlated with two- to fourfold higher cystathionine-γ-lyase levels than in the control strain. Transformant T58 containing five- to sixfold higher levels of cystathionine-γ-lyase in fermentor cultures showed a reduced growth rate and a slow cephalosporin accumulation rate. In conclusion, moderately increased levels of cystathionine-γ-lyase stimulated cephalosporin production but very high levels of this enzyme were deleterious for growth and cephalosporin biosynthesis. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 252–258.

Keywords: cephalosporin biosynthesis; cystathionine-γ-lyase; gene amplification; *mec*B; sulfur metabolism

Introduction

In yeasts and filamentous fungi, L-cysteine is synthesized by two different pathways: (i) from sulphate through the autotrophic pathways that involve condensation of H_2S with *O*-acetyl-serine [6], and (ii) from methionine through the reverse transsulfuration pathway [21]. L-cysteine is a precursor of the tripeptide δ-L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) [2,13,14] for the biosynthesis of the β-lactam antibiotics (penicillins, cephalosporins and cephamycins) [1,5]. In *Acremonium chrysogenum*, the supply of L-cysteine for cephalosporin biosynthesis was proposed to take place preferentially from methionine *via* the reverse transsulfuration pathway [15,19,20] and Ciba-Geigy workers provided preliminary information indicating that the enzyme cystathionine-γ-lyase that splits cystathionine into cysteine and α-ketobutyrate is a key enzyme in cephalosporin production [28].

Recently, we cloned and characterized the cystathionine-γ-lyase gene, *mec*B, of *A. chrysogenum* [12]. Disruption of the *mec*B gene provided clear evidence indicating that there is a single gene encoding this enzyme in *A. chrysogenum*. Knock-out mutants lacking the *mec*B gene were still able to produce cephalosporin C (cysteine is formed through the autotrophic pathway), but a functional *mec*B gene is required for high-level cephalosporin biosynthesis in methionine-supplemented cultures [11]. Amplification of the *mec*B gene is, therefore, an interesting strategy for improving cephalosporin biosynthesis since it should provide more cysteine for biosynthesis of the ACV tripeptide — a step that is believed to be limiting for cephalosporin biosynthesis [10]. However, the equilibrium between direct and reverse flows (Figure 1) in the transsulfuration pathway may be affected by overexpressing the *mec*B gene.

We report in this article that amplification of the *mec*B gene in *A. chrysogenum* C10 results in very high intracellular levels of cystathionine-γ-lyase and an increment in cephalosporin biosynthesis. However, a transformant with a five- to sixfold higher cystathionine-γ-lyase activity than the parental strain was crippled and showed a reduced growth rate and cephalosporin yields. The levels of cystathionine-γ-lyase appear, therefore, to be critical for cephalosporin biosynthesis in methionine-supplemented medium.

Materials and methods

Strains and plasmids

A. chrysogenum C10 (ATCC 48272), a high cephalosporin-producing strain provided by PanLabs [5,23], was used as host for gene amplification experiments. The *mec*B gene (encoding cystathionine-γ-lyase) used in this study was cloned previously from *A. chrysogenum* C10 [12].

Plasmid pULC43 used to amplify the *mec*B gene contains a bleomycin (phleomycin) resistance cassette as selective marker in *A. chrysogenum* and a chloramphenicol resistance gene for selection in *Escherichia coli* (instead of the ampicillin resistance

Correspondence: Dr JF Martín, Area de Microbiología, Facultad de Ciencias Biológicas y Ambientales, Universidad de León, León 24071, Spain
Received 30 January 2001; accepted 23 June 2001

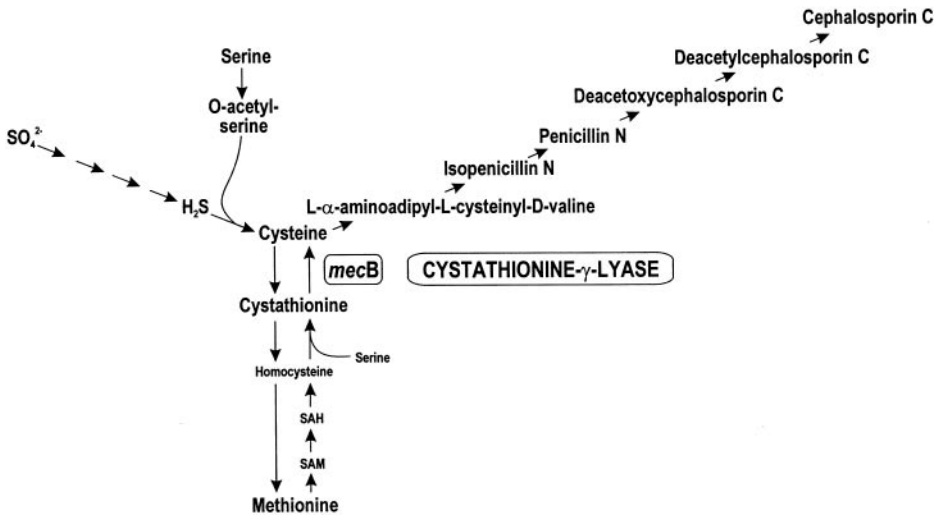

Figure 1 Biosynthetic pathways of cysteine from sulphate (autrophic pathway) and from methionine (reverse transsulfuration).

of β-lactamase to avoid interference with β-lactam biosynthesis) (S Gutiérrez and JF Martín, unpublished).

E. coli DH5α was used as host in DNA subcloning experiments.

Transformation of A. chrysogenum

Transformation of *A. chrysogenum* protoplasts was carried out as described previously [8]. Transformants were selected in tryptic soy agar (TSA; Difco, Detroit, MI) with sucrose (10.3%) supplemented with 10 μg/ml phleomycin [9].

DNA manipulation and Southern analysis

DNA manipulations were performed by standard methods [24]. Southern blots were carried out as described previously [12]. Probes were labeled by nick translation [24].

RNA extraction and Northern hybridizations

Total RNA of *A. chrysogenum* was extracted from cultures in Shen-defined production medium at different times. RNA was isolated from frozen mycelium disrupted by grinding in a mortar refrigerated with liquid nitrogen. The disrupted mycelium was mixed with RLT lysis buffer and the mixture was centrifuged at top speed in a microcentrifuge for 3 min; the supernatant was mixed with 1 vol of 70% ethanol and purified by passing it through an RNeasy spin column as described by the manufacturer (Qiagen, UK).

Northern hybridizations were performed according to Sambrook *et al* [24]. Probes were internal to the β-actin gene of *A. nidulans* (830 bp *Nco*I–*Kpn*I) and the *mec*B of *A. chrysogenum* (570 bp *Bgl*I fragments).

Shake flasks and fermentor cultures

Cultures of *A. chrysogenum* C10 (pULC43) (transformed with the control vector) and all the transformants with the *mec*B gene were grown in triplicate in 500-ml baffled flasks containing 100 ml of the defined inoculum medium [26] for 48 h at 25°C and 250 rpm in a New Brunswick Scientific (Edison, NJ) (INNOVA 4330) orbital shaker. These seed cultures (400 ml) were used to inoculate two 5-l Biostat B (Braun, Germany) fermentors containing 4 l of Shen-defined production (DP) medium [26]. The fermentations were run at 25°C and 350 rpm with an air flow of 4 l/min. The agitation speed in both fermentors was increased to 400 rpm at 32 h, 450 rpm at 44 h, 500 rpm at 48 h and 550 rpm at 68 h to prevent the decrease of dissolved oxygen below 30% of saturation. Samples were taken every 24 h. Aliquots were used for cephalosporin determination and the mycelium was washed with sterile saline solution and frozen in liquid nitrogen for enzyme assays.

Cephalosporin assays

Samples (in duplicate) were taken every 24 h from triplicate flasks (or fermentors) and β-lactam antibiotics were determined by bioassays with *E. coli* ESS 2231 (a β-lactam supersensitive strain) as the test strain as described previously [32].

The titer of cephalosporin was determined after addition of *Bacillus cereus* penicillinase (penicillin-specific; Difco) to remove penicillins from the broth followed by bioassay using *E. coli* ESS 2231. Antibiotics were measured in culture supernatants at dilutions of 1:20 and 1:100 and compared to controls of pure cephalosporin C.

Cell-free extracts and cystathionine-γ-lyase activity assay

Frozen mycelium (500 mg) was ground in a mortar cooled with liquid nitrogen and resuspended in 100 mM sodium phosphate buffer (pH 7.3) containing 1 mM EDTA (free acid) and 0.1 mM pyridoxal 5′-phosphate. Cell debris was removed by centrifugation at 15,000$\times g$ for 20 min at 4°C. The crude extract was passed through Sephadex G-25 columns (Pharmacia, Sweden) to remove cysteine and other low-molecular-weight compounds.

Cystathionine-γ-lyase activity was assayed by determining the conversion of L-cystathionine into L-cysteine in a reaction mixture containing 4 mM L-cystathionine, 5.5×10^{-2} mM pyrixodal 5′-phosphate, 7 mM EDTA, 2 mM dithiothreitol and 0.1 mg protein in a final volume of 0.5 ml. The reactions were incubated for 30 min at 30°C and were stopped by the addition of 1 ml of Gaitonde's reagent and boiled for 5 min. The precipitated proteins were removed by centrifugation and the cysteine in 1 ml of supernatant was determined by acid ninhydrin assays [7]. This assay is highly specific and gives essentially no

reaction with cystathionine or methionine. Total protein in cell extracts was determined by the Bradford method with ovoalbumin as a standard.

Results

Isolation of A. chrysogenum transformants with the mecB gene

The *mec*B gene was subcloned as a *Bam*HI–*Eco*RI fragment into the fungal integrative vector pULC43 that contains the bleomycin (phleomycin) resistance gene *ble* as selective marker resulting in plasmid pULC43*mec*B (Figure 2A). *A. chrysogenum* transformants T27, T33, T58, T62 and T81 were selected from about 100 transformants because they showed higher levels of phleomycin resistance (75–150 μg/ml). A high *ble* gene dosage and phleomycin resistance level may be associated with an increased copy number of the *mec*B gene since (at least in some cases) integration of the entire pULC43*mec*B in the genome may occur.

Copy number of the mecB gene in the transformants

To confirm that the selected transformants contained an increased number of copies of the *mec*B gene, total DNA (*Sac*I-digested) of the transformants and also of strains *A. chrysogenum* C10 (untransformed) and *A. chrysogenum* C10 pULC43 (transformed with the vector without insert) were hybridized with a 570-bp *Bgl*I fragment internal to the *mec*B gene as probe.

Results of the Southern analysis (Figure 2B) showed that all selected transformants contained one or more copies of the *mec*B gene in addition to the endogenous one that showed up clearly in the hybridization as a 7.0-kb *Sac*I fragment (arrow in Figure 2B). Some of the hybridizing bands may contain incomplete copies of the *mec*B gene as deduced from the small size of some bands (e.g., T62, lane 6 in Figure 2B).

Initial studies in shake flasks (not shown) indicated that transformants T27, T33 and T81 produced cephalosporin at levels 20–40% higher than the control *A. chrysogenum* C10 (pULC43) in methionine-supplemented Shen DP medium, whereas transformants T58 and T62 showed the same levels of cephalosporin as the parental strain. The best cephalosporin producer in these initial studies was transformant T27 (containing at least two additional copies of the *mec*B gene) that reached about 1000 μg of cephalosporin per milliliter at 96 h in Shen DP medium compared to 750 μg/ml for the control strains under the same conditions.

Cystathionine-γ-lyase levels in the transformants

The increased cephalosporin yields observed in shake flask cultures of some transformants might have been due to an increased cystathionine-γ-lyase activity or to an indirect effect on cephalosporin biosynthesis as a result of altering gene expression at the plasmid integration site in the genome.

Analysis in triplicate of cystathionine-γ-lyase activity of cultures of the different transformants showed that transformants T27 and T58 contained very high levels of cystathionine-γ-lyase, whereas transformants T62 and T81 contained normal levels of cystathionine-γ-lyase (Figure 3). Transformants T27 and T58 showed the highest copy number in the Southern hybridization experiments. Therefore, strains T27 and T58 were selected for further molecular and fermentation studies.

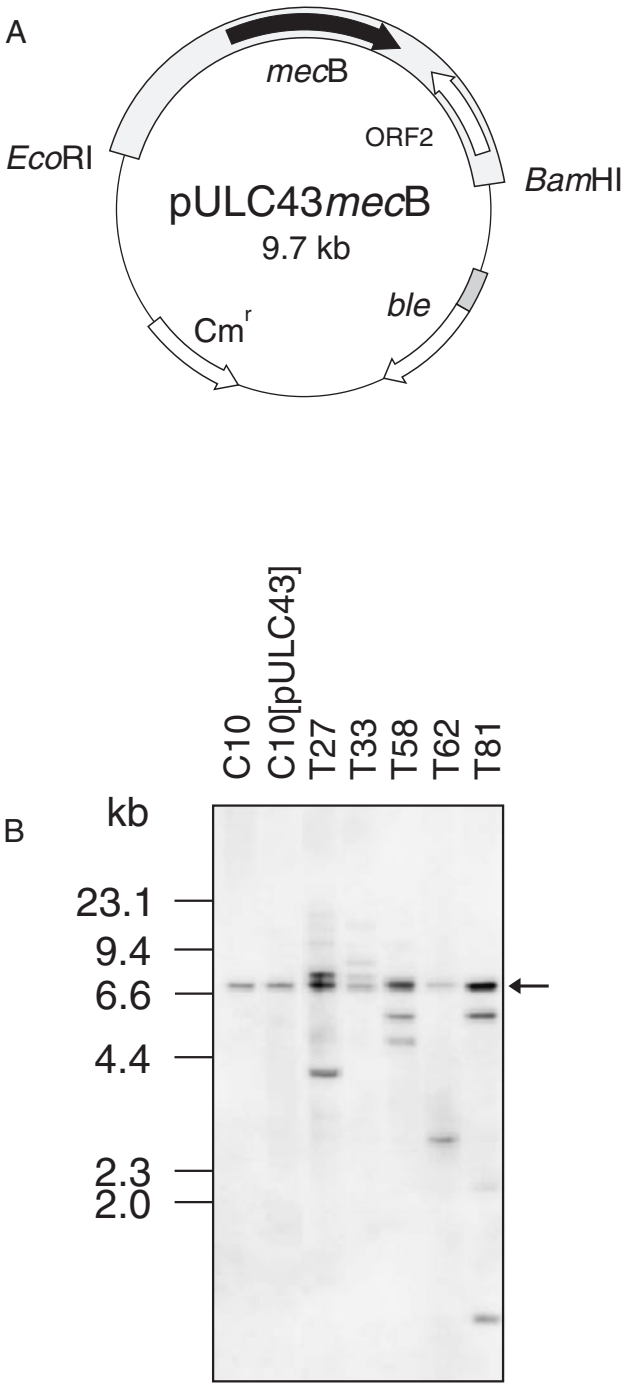

Figure 2 Amplification of the *mec*B gene in *A. chrysogenum* C10. (A) Integrative plasmid pULC43 *mec*B used for amplification of the cystathionine-γ-lyase gene. The boxed *Eco*RI–*Bam*HI fragment containing the *mec*B gene was cloned from *A. chrysogenum* [12]. This plasmid contains the bleomycin/phleomycin resistance (*ble*) cassette (with the *pcb*C promoter of *P. chrysogenum*) for selecting transformants in *A. chrysogenum* and the chloramphenicol resistance gene for selection in *E. coli*. (B) Hybridization pattern of *Sac*I-digested total DNA of the untransformed *A. chrysogenum* C10, the control strain (transformed with pULC43) and five selected transformants (T27 to T81). The endogenous *mec*B gene shows up as a 7.0-kb band (arrow on the right). Note that all transformants showed several hybridizing bands in addition to the endogenous 7.0-kb fragment. DNA size markers (in kilobase) are indicated on the left.

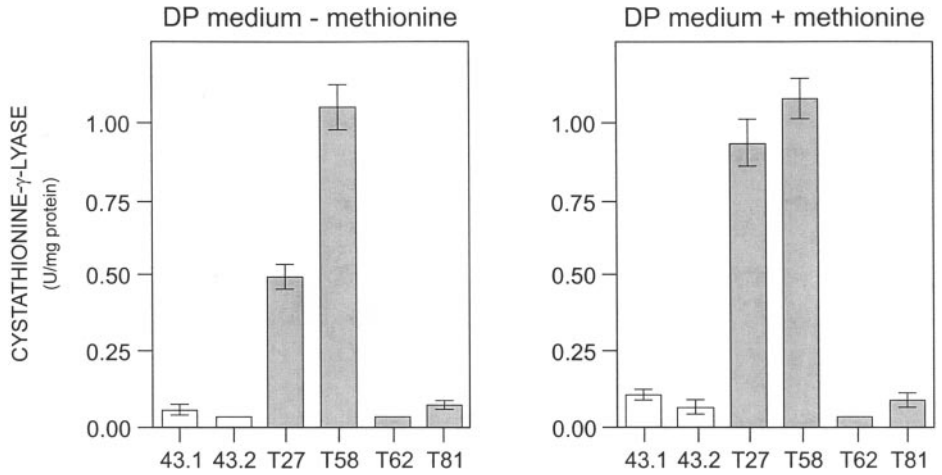

Figure 3 Cystathionine-γ-lyase activity in extracts of transformants grown without or with methionine. Transformants 43.1 and 43.2 are different clones with the control plasmid pULC43 (without insert). Vertical lines indicate standard mean deviation of four determinations (from duplicate reactions).

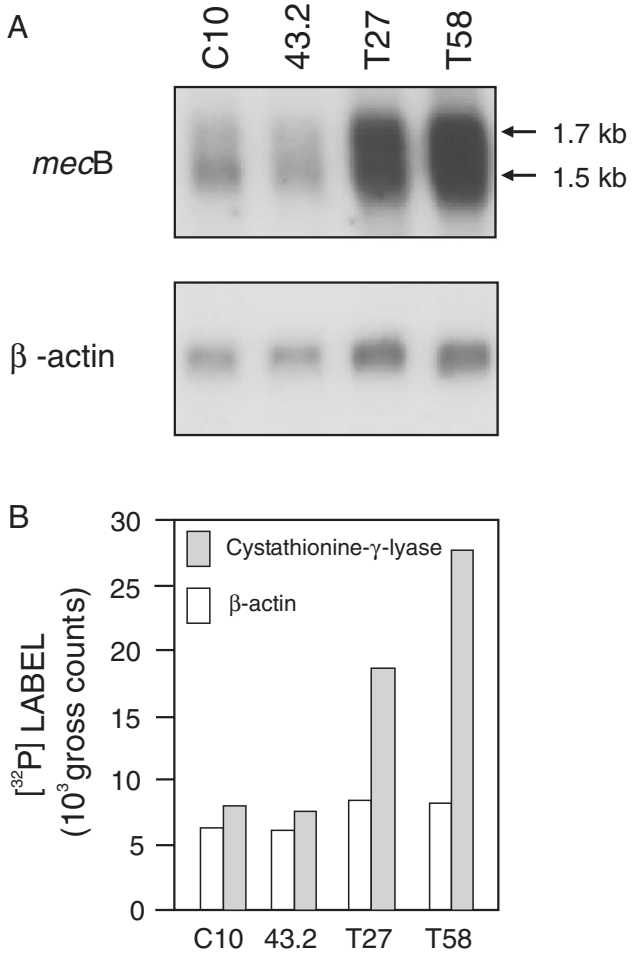

Figure 4 *mec*B transcript levels in *A. chrysogenum*. (A) Northern blots showing the increase of *mec*B transcripts in transformants T27 and T58. Lanes C10, *A. chrysogenum* C10; 43.2, control transformant with pULC43 (without insert); T27 and T58, *A. chrysogenum* transformants T27 and T58. Note that both the 1.5- and 1.7-kb transcripts are increased in transformants T27 and T58. Similar results were obtained in DP medium with or without methionine. (B) Quantification of the label in the upper panel (*mec*B and β-actin) using a phosphorimager (Instant Imager; Packard, Meriden, CT) instrument.

Increased expression of the mecB gene in transformants T27 and T58: formation of two transcripts

To confirm that the *mec*B gene was expressed efficiently in transformants T27 and T58, blots of total RNA of these transformants were hybridized with the 570-bp *Bgl*I probe (the same used in Southern hybridizations) internal to the *mec*B gene. Results showed (Figure 4) that transformants T27 and T58 contained a very high transcript level of the *mec*B gene both in methionine-free and in methionine-supplemented medium. The addition of methionine did not affect significantly the level of *mec*B mRNA.

*mec*B formed two transcripts, a major one of 1.5 kb and a minor one of 1.7 kb [12]. Both transcripts were amplified greatly in the T27 and T58 transformants compared to the control strains (Figure 4), indicating that both transcripts correspond to the *mec*B gene amplified in these transformants. Since in *A. chrysogenum* there is a single *mec*B gene, the short transcript (1.5 kb) may derive from the large one (see Discussion).

Cystathionine-γ-lyase and cephalosporin production in 5-liter fermentors

Parallel fermentations were performed in three identical 5-l fermentors (Biostat B) in defined Shen DP medium supplemented with methionine. The total β-lactams (isopenicillin N, penicillin N and cephalosporins) or just the cephalosporins were determined separately (Figure 5). Under the controlled conditions in the fermentors, yields of about 2000 μg/ml β-lactams were obtained with the T27 transformant, i.e., about 20–30% increase in cephalosporin titers at 72 and 96 h with respect to the parental strain (Figure 5, panels A and B). Results were similar when yields were expressed volumetrically (panel 5A) or as micrograms of antibiotic per milligram of dry weight (panel 5B). About 80% of the accumulated β-lactams was cephalosporin (Figure 5, panels C and D).

The behavior of strain T58 in the fermentors was intriguing. It grew more slowly than the control and the T27 strains, and the rate of cephalosporin accumulation was consistently lower than that obtained with the control strain requiring more than 144 h of culture to reach cephalosporin levels higher than those of the parental strain

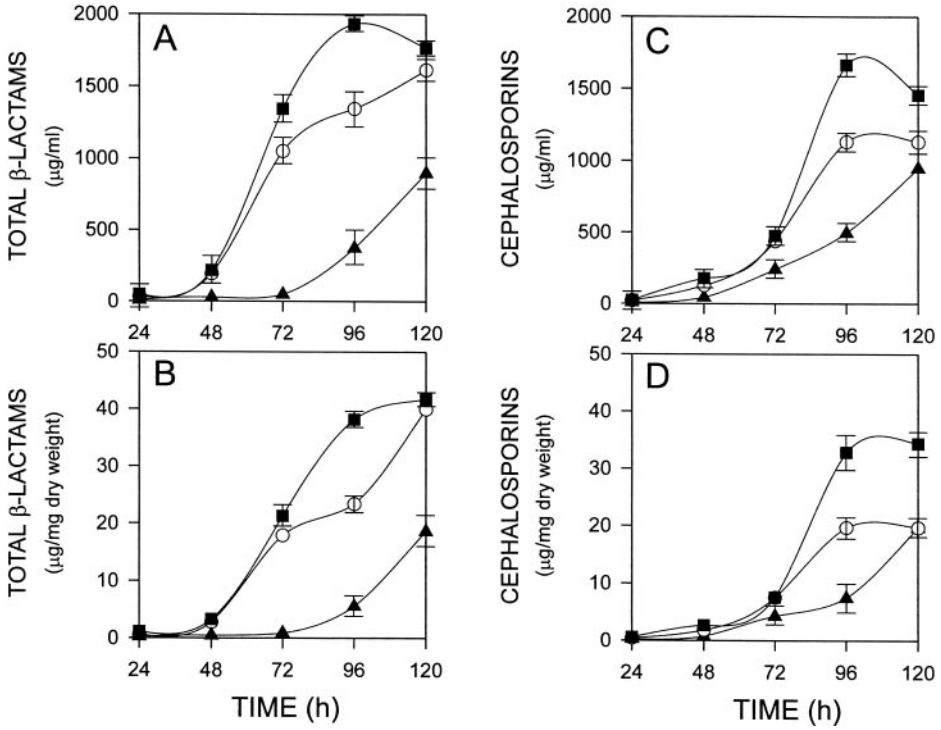

Figure 5 β-Lactam production by transformants of *A. chrysogenum* strain 43.2 (○), T27 (■) and T58 (▲). Total β-lactams (panels A and B) and cephalosporins (panels C and D) were determined separately. Results at the different times are the average of three data points from each fermentor.

(see Discussion). Fermentor studies were repeated three times with results similar to those shown in Figure 5.

To confirm that the *mec*B gene was well expressed under the fermentor conditions, the cystathionine-γ-lyase activity was determined in mycelium from samples taken at different times from the fermentor. There were very high levels of cystathionine-γ-lyase activity in the T27 and particularly in the T58 transformants compared to the parental untransformed strain (Figure 6). The level of cystathionine-γ-lyase in both strains was very high at 24 h and increased drastically at 48 h in the T58 transformants, but not in T27 transformants. These differences may explain the distinct behavior of both transformants (see Discussion).

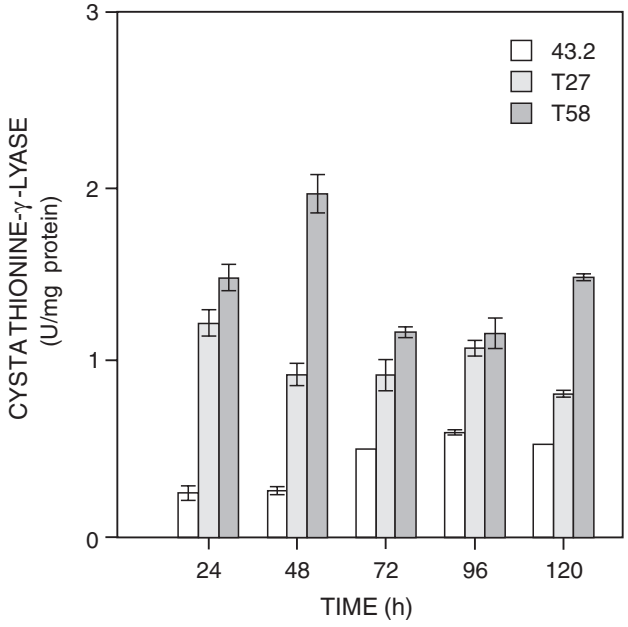

Figure 6 Specific activity of cystathionine-γ-lyase in cells taken at 24, 48, 72, 96 and 120 h from the fermentor cultures of *A. chrysogenum* transformants 43.2 (control), T27 and T58. Data are the averages of four determinations (from duplicate reactions). Vertical bars indicate the standard mean deviation.

Discussion

Amplification of limiting steps for the biosynthesis of microbial metabolites is a basic strategy in metabolic engineering [3]. This approach requires a detailed knowledge of the biosynthetic pathways and the regulatory mechanisms that control the biosynthesis of microbial metabolites. Lack of knowledge of the limiting steps is particularly worrying in the field of secondary metabolite biosynthesis [16,17,30]. One of the best known examples of limiting steps for secondary metabolite production is the poor conversion in *A. chrysogenum* of deacetylcephalosporin C to cephalosporin C by the DAC acetyltransferase due to low expression of the *cef*G gene [29]. Overexpression of the *cef*G gene from efficient fungal promoters resulted in high levels of cephalosporin production [9].

Another limiting step for high cephalosporin biosynthesis is the availability of cysteine that is formed either from sulphate [6] or from methionine by the reverse transsulfuration pathway. The supply of cysteine by hydrolysis of cystathionine was believed to be the main source of cysteine for cephalosporin biosynthesis [19,20]. Recent cloning [12] and disruption of the *mec*B gene [11] provided unequivocal evidence showing that the *mec*B gene is

required for high-level cephalosporin production in methionine-supplemented medium, although cephalosporin is still produced (from cysteine synthesized *via* the autotrophic pathway) in the *mec*B knock-out mutants.

As shown in this article, amplification of the *mec*B gene results in increased cephalosporin production in some of the transformants containing additional copies of the *mec*B gene. The correlation of the increased copy number and high endogenous levels of cystathionine-γ-lyase was quite good. Transformants T27 and T58 containing at least three copies of the *mec*B gene showed very high *mec*B transcript levels and high intracellular cystathionine-γ-lyase activity. The increased levels of cystathionine-γ-lyase of transformant T27 resulted in higher cephalosporin production both in shake flasks and fermentors, but the very high level of this enzyme accumulated in strain T58 is deleterious for growth and, therefore, for cephalosporin production in this strain. A high cystathionine-γ-lyase activity is likely to produce elevated intracellular levels of cysteine that are toxic for the cells [22]. Cellular thiols modulate the redox-regulated signal transduction in a variety of eucaryotic cells [25,27] including the β-lactam producer, *Penicillium chrysogenum* [4]. Usually, most of the free cysteine are maintained as part of the tripeptide glutathione (glutamyl–cysteinyl–glycine) to avoid alteration of the redox potential in the cell. Excess cysteine is also inhibitory for optimal activity of β-lactam-synthesizing enzymes [18]. Another possibility is that the excess α-ketoburyrate released by the cystathionine-γ-lyase may be toxic for branched chain amino acid biosynthesis.

Our results indicate that both transformants T27 and T58 contain a high level of the *mec*B transcript in agreement with the high levels of cystathionine-γ-lyase levels observed in these strains. The extra copies of *mec*B integrated in the transformants are functional and transcribed correctly. Transcription of the amplified *mec*B genes resulted in high levels of the 1.7- and 1.5-kb transcripts. The major *mec*B transcript in untransformed *A. chrysogenum* C10 strain corresponds to the 1.5-kb [12]. Since there is a single *mec*B gene in *A. chrysogenum* [12], it is likely that the 1.5 kb transcript is formed by specific processing of the 1.7-kb form and that the processing mechanism is saturated in the T27 and T58 transformants [31], although we cannot exclude other possibilities at this time. The possible mechanism of processing of the *mec*B mRNA is the subject of further research.

In summary, amplification of the *mec*B gene resulting in a moderate increase of cystathionine-γ-lyase activity enhances cephalosporin biosynthesis in *A. chrysogenum*, but excess levels of this enzyme appear to be deleterious for growth and cephalosporin production.

Acknowledgements

This work was supported by a grant from the CICYT (BIO97-0289-C02-01). We thank S. Gutiérrez for helpful discussions, and M. Mediavilla, B. Martín, R. Barrientos and M. Corrales for excellent technical assistance.

References

1 Aharonowitz Y, G Cohen and JF Martín. 1992. Penicillin and cephalosporin biosynthetic genes: Structure, organization, regulation and evolution. *Annu Rev Microbiol* 46: 461–496.

2 Aharonowitz Y, J Bergmeyer, JM Cantoral, G Cohen, AL Demain, U Fink, J Kinghorn, H Kleinkauf, A MacCabe, H Palissa, E Pfeifer, T Schwecke, H van Liempt, H von Döhren, S Wolfe and J Zhang. 1993. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase, the multienzyme integrating the four primary reactions in β-lactam biosynthesis, as a model peptide synthetase. *Biotechnology* 11: 807–810.

3 Bailey JE. 1991. Towards a science of metabolic engineering. *Science* 252: 1668–1675.

4 Cohen G, A Argaman, R Schreiber, M Mislovati and Y Aharonowitz. 1994. The thioredoxin system of *Penicillium chrysogenum* and its possible role in penicillin biosynthesis. *J Bacteriol* 176: 973–984.

5 Demain AL. 1983. Strain exchange between industry and academy. *ASM News* 49: 431.

6 Döbeli H and J Nüesch. 1980. Regulatory properties of *O*-acetyl-L-serine sulfhydrylase of *Cephalosporium acremonium*: Evidence of an isoenzyme and its importance in cephalosporin C biosynthesis. *Antimicrob Agents Chemother* 18: 111–117.

7 Gaitonde MK. 1967. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochem J* 104: 627–633.

8 Gutiérrez S, B Díez, E Alvarez, JL Barredo and JF Martín. 1991. Expression of the *pen*DE gene of *Penicillium chrysogenum* encoding isopenicillin N acyltransferase in *Cephalosporium acremonium*: Production of benzylpenicillin by the transformants. *Mol Gen Genet* 225: 56–64.

9 Gutiérrez S, J Velasco, AT Marcos, FJ Fernández, F Fierro, JL Barredo, B Díez and JF Martín. 1997. Expression of the *cef*G gene is limiting for cephalosporin biosynthesis in *Acremonium chrysogenum*. *Appl Microbiol Biotechnol* 48: 606–614.

10 Kennedy J and G Turner. 1996. δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. *Mol Gen Genet* 253: 189–197.

11 Liu G, J Casqueiro, O Bañuelos, RE Cardoza, S Gutiérrez and JF Martín. 2001. Targeted inactivation of the *mec*B 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.

12 Marcos AT, K Kosalková, RE Cardoza, F Fierro, S Gutiérrez and JF Martín. 2001. Characterization of the reverse transsulfuration *mec*B gene of *Acremonium chrysogenum* encoding a functional cystathionine-γ-lyase. *Mol Gen Genet* 264: 746–754.

13 Martín JF. 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.

14 Martín JF. 2000. Molecular control of expression of penicillin biosynthesis genes in fungi: Regulatory proteins interact with a bidirectional promoter region. *J Bacteriol* 182: 2355–2362.

15 Martín JF and Y Aharonowitz. 1983. Regulation of biosynthesis of β-lactam antibiotics. In: Demain AL, NA Solomon (Eds), Antibiotics Containing the β-Lactam Structure. Springer, New York, pp. 189–228.

16 Martín JF and P Liras. 1989. Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. *Annu Rev Microbiol* 43: 173–206.

17 Martín JF and P Liras. 1989. Enzymes involved in penicillin, cephalosporin and cephamycin biosynthesis. In: Fiechter A (Ed), Advances in Biochemical Engineering/Biotechnology, Vol. 39. Springer Verlag, Berlin, pp. 153–187.

18 Meeschaert B, J Cortés, E Alvarez, P Liras and JF Martín. 1987. An improved HPLC assay of the substrate and products of the isopenicillin N synthase. *Biotechnol Tech* 1: 137–140.

19 Nüesch J, HJ Treichler and M Liersch. 1973. The biosynthesis of cephalosporin C. In: Vanek Z, Z Hostalek and J Cudlin (Eds), Genetics of Industrial Microorganisms, Vol. 2. Academia, Prague, p. 309.

20 Nüesch J, J Heim and H-J Treichler. 1987. The biosynthesis of sulfur-containing β-lactam antibiotics. *Annu Rev Microbiol* 41: 51–57.

21 Paszewski A, R Natorff, M Piotrowska, J Brzywczy, M Sienko, M Grynberg, E Pizzinini and G Turner. 2000. Regulation of sulfur amino acid biosynthesis in *Aspergillus nidulans*: physiological and genetical aspects. In: Brunold C (Ed), Sulfur Nutrition and Sulfur Assimilation in Higher Plants. Paul Haupt, Bern, Switzerland, pp. 93–105.

22 Penninckx MJ and MT Elskens. 1993. Metabolism and functions of glutathionine in microorganisms. *Adv Microb Physiol* 34: 239–301.
23 Ramos FR, JM López-Nieto and JF Martín. 1986. Coordinate increase of isopenicillin N synthetase, isopenicillin N epimerase and deacetoxycephalosporin C synthetase in a high cephalosporin-producing mutant of *A. chrysogenum* and simultaneous loss of the three enzymes in a non-producing mutant. *FEMS Microbiol Lett* 35: 123–127.
24 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
25 Sen CK. 2000. Cellular thiols and redox-regulated signal transduction. *Curr Top Cell Regul* 36: 1–30.
26 Shen Y-Q, S Wolfe and AL Demain. 1986. Levels of isopenicillin N synthetase and deacetoxycephalosporin C synthetase in *Cephalosporium acremonium* producing high and low levels of cephalosporin C. *Biotechnology* 4: 61–64.
27 Tortorella D, CM Story, JB Huppa, EJ Wiertz, TR Jones, I Bacik, JR Bennink, JW Yewdell and HL Ploegh. 1998. Dislocation of type I membrane proteins from the ER to the cytosol is sensitive to changes in redox potential. *J Cell Biol* 142: 365–376.
28 Treichler HJ, M Liersch, J Nüesch and H Dobeli. 1979. Role of sulfur metabolism in cephalosporin C and penicillin biosynthesis. In: Sebek OK and AI Laskin (Eds), Genetics of Industrial Microorganisms. American Society for Microbiology, Washington, DC, p. 97.
29 Velasco J, S Gutiérrez, FJ Fernández, AT Marcos, C Arenós and JF Martín. 1994. Exogenous methionine increase levels of mRNAs transcribed from *pcb*AB, *pcb*C, and *cef*EF genes, encoding enzymes of the cephalosporin biosynthetic pathway, in *Acremonium chrysogenum*. *J Bacteriol* 176: 985–991.
30 Von Döhren H and U Grafe. 1997. General aspects of secondary metabolism. In: Kleinkauf H and H von Döhren (Eds), Products of Secondary Metabolism. VCH Verlag, Weinheim.
31 Wahle E and U Rüegsegger. 1999. 3′-End processing of pre-mRNA in eukaryotes. *FEMS Microbiol Lett* 23: 277–295.
32 Zanca DM and JF Martín. 1983. Carbon catabolite regulation of the conversion of penicillin N into cephalosporin C. *J Antibiot* 36: 700–708.