Targeted Inactivation of the *mecB* Gene, Encoding Cystathionine-γ-Lyase, Shows that the Reverse Transsulfuration Pathway Is Required for High-Level Cephalosporin Biosynthesis in *Acremonium chrysogenum* C10 but Not for Methionine Induction of the Cephalosporin Genes

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Targeted gene disruption efficiency in *Acremonium chrysogenum* was increased 10-fold by applying the double-marker enrichment technique to this filamentous fungus. Disruption of the *mecB* gene by the double-marker technique was achieved in 5% of the transformants screened. Mutants T6 and T24, obtained by gene replacement, showed an inactive *mecB* gene by Southern blot analysis and no cystathionine-γ-lyase activity. These mutants exhibited lower cephalosporin production than that of the control strain, *A. chrysogenum* C10, in MDFA medium supplemented with methionine. However, there was no difference in cephalosporin production between parental strain *A. chrysogenum* C10 and the mutants T6 and T24 in Shen's defined fermentation medium (MDFA) without methionine. These results indicate that the supply of cysteine through the transsulfuration pathway is required for high-level cephalosporin biosynthesis but not for low-level production of this antibiotic in methionine-unsupplemented medium. Therefore, cysteine for cephalosporin biosynthesis in *A. chrysogenum* derives from the autotrophic (SH_2) and the reverse transsulfuration pathways. Levels of methionine induction of the cephalosporin biosynthesis gene *pcbC* were identical in the parental strain and the *mecB* mutants, indicating that the induction effect is not mediated by cystathionine-γ-lyase.

β-Lactam biosynthesis in *Acremonium chrysogenum* (formerly known as *Cephalosporium acremonium*) begins with the nonribosomal condensation of the three precursor amino acids L-α-aminoadipic acid, L-cysteine, and L-valine (1, 21, 23). There are two ways to synthesize cysteine in filamentous fungi (35, 26). One pathway, the so-called autotrophic pathway, converts inorganic sulfur to cysteine via serine *O*-acetyltransferase and *O*-acetylserine sulfhydrilase (Fig. 1). In the second route, cysteine can be obtained via the reverse transsulfuration pathway, in which the sulfur atom of methionine is transferred to cysteine through *S*-adenosylmethionine, *S*-adenosylhomocysteine, homocysteine, and cystathionine as intermediates.

The predominant route of the cysteine supply for β-lactam biosynthesis depends on the producer microorganism (6). Cysteine for penicillin biosynthesis in *Penicillium chrysogenum* is obtained mainly from sulfate reduction (31). In this microorganism, the transsulfuration pathway has been considered to have a minor role in penicillin biosynthesis. On the other hand, the sulfur atom for β-lactam biosynthesis by *A. chrysogenum* is believed to derive preferentially from methionine via the reverse transsulfuration pathway (3, 24). However, there is no genetic evidence for, or against, this hypothesis.

DL-Methionine is known to stimulate cephalosporin biosynthesis in *A. chrysogenum* (7, 16). For many years it was unclear whether the stimulatory effect of DL-methionine was due to a precursor effect (one providing cysteine) or to an inducing effect (8, 20, 23). Sawada and coworkers established that methionine increases isopenicillin N synthase, deacetoxycephalosporin C synthase (30), and α-aminoadipyl-cysteinyl-valine synthetase activities (38) in *Acremonium* cultures. Later, Velasco et al. (35) showed that methionine induces the transcription of three of the four known cephalosporin biosynthetic genes.

Therefore, methionine has presumably a double effect on cephalosporin biosynthesis: (i) it could be the main supplier of cysteine via the reverse transsulfuration pathway and (ii) it has an induction effect on cephalosporin biosynthetic genes.

The *mecB* gene of *A. chrysogenum* has recently been cloned and characterized in our laboratory (19). This gene encodes a cystathionine-γ-lyase activity which splits cystathionine into cysteine and α-ketobutyrate. Several years ago it was proposed that cystathionine-γ-lyase is required for cephalosporin biosynthesis (34). In order to study the influence of the transsulfuration pathway on cephalosporin biosynthesis, *mecB* was inactivated by a novel disruption technique in this fungus and the effect of this inactivation on cephalosporin production was studied. Results show unequivocally that the splitting of cystathionine is essential for high-level production of cephalosporin but that reverse transsulfuration is not the only pathway for cysteine formation for cephalosporin since the *mecB*-disrupted mutants were still able to produce cephalosporin.

MATERIALS AND METHODS

Microbial strains. *A. chrysogenum* C10 (ATCC 48278), a strain producing high levels of cephalosporin C, released by Panlabs (6, 28), was used for *mecB* inactivation experiments. *Aspergillus nidulans* C47 (*mecB cysB*) and M63 (*mecB*) were obtained from A. Paszewski and used as controls to test for methionine auxotrophy following gene disruption. *Escherichia coli* ESS2231, a β-lactam-

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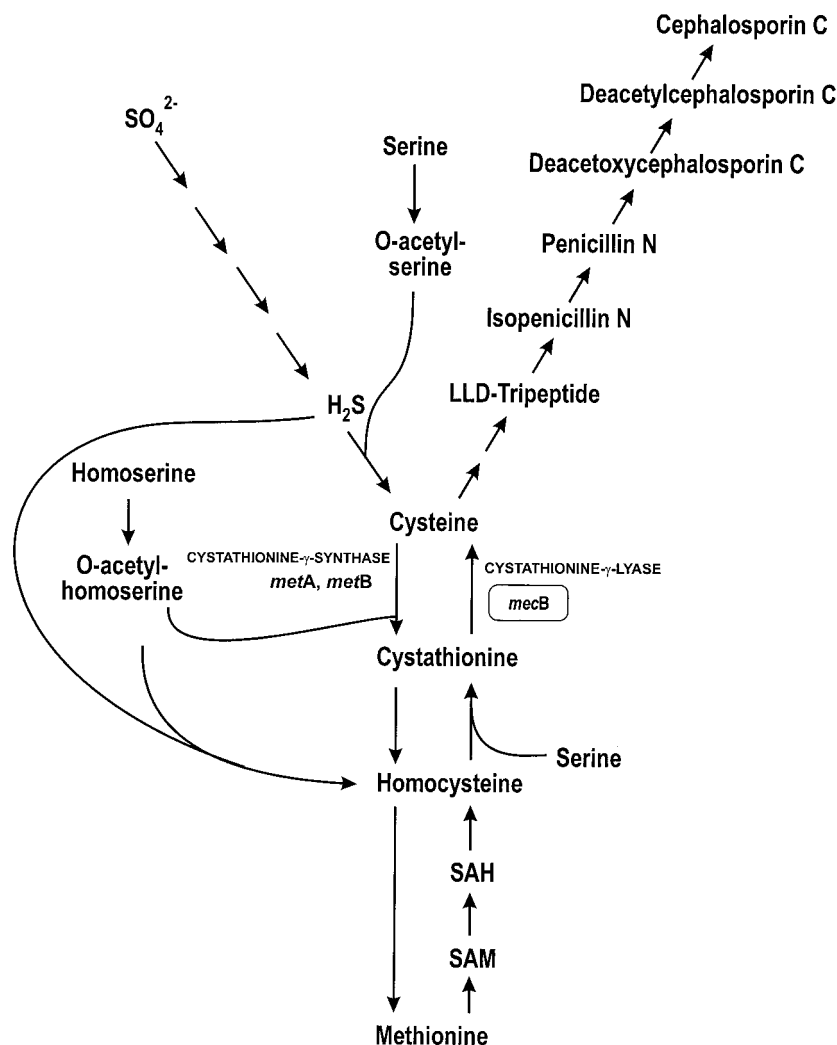
FIG. 1. Cysteine biosynthesis in *A. chrysogenum* from sulfate (autotrophic pathway) and from methionine (reverse transsulfuration pathway). The *mecB* gene encoding cystathionine-γ-lyase is shaded. This gene has been disrupted in transformants T6 and T24 (see the text). In the text, genes designated *met* belong to the methionine biosynthesis (direct transsulfuration) pathway and those named *mec* are involved in the reverse transsulfuration pathway. SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

supersensitive strain, was used for routine bioassays. *E. coli* DH5α competent cells were used in routine DNA manipulations.

Media and growth conditions for cephalosporin production. *A. chrysogenum* was grown in seed medium (32) for 48 h; 10 ml of seed culture was used to inoculate Shen's defined production (MDFA) medium (32) with or without supplementation with DL-methionine (3 g/liter). The cultures were incubated in triple-baffled flasks (500 ml; Bellco) containing 100 ml of medium at 25°C in a rotary shaker. Samples were taken every 24 h, and levels of cephalosporin antibiotics were determined by bioassays against *E. coli* ESS2231.

***A. chrysogenum* transformation and isolation of genomic DNA.** Transformation of *A. chrysogenum* protoplasts was carried out as described previously (12). Transformants were selected in tryptic soy agar (Difco) with sucrose (10.3%), supplemented with hygromycin at 30 μg/ml. Genomic DNA of *A. chrysogenum* was isolated as described previously (12).

Site-directed mutagenesis. In vitro mutagenesis was performed with a Quick-change mutagenesis kit (Stratagene, La Jolla, Calif.) by following the manufacturer's instructions. Oligonucleotides used in the formation of an *Eco*RI site in the *mecB* gene were Ia (5′-CCTTATGTGCAGAATTCGCTCGACCTC) and Ib (5′-GAGGTCGAGCGAATTCTGCACATAAGG).

Southern blotting and hybridization. Three-microgram samples of genomic DNAs from *A. chrysogenum* C10 and its transformants were digested with *Eco*RI and separated in 0.8% agarose gel. The gel was blotted onto Hybond-N mem-

brane (Amersham Pharmacia Biotech) as described by Sambrook et al. (29). Digoxigenin-alkaline phosphatase labeling, hybridization, and detection were done with a Genius kit (Boehringer Mannheim) according to the manufacturer's protocol. Hybridizations were performed at 68°C, and the blots were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 5 min at room temperature and twice with 0.1× SSC–0.1% sodium dodecyl sulfate for 15 min at 68°C.

Cell extracts and cystathionine-γ-lyase activity assay. Frozen mycelia (500 mg) collected every 24 h were ground in a mortar with liquid nitrogen and resuspended in 100 mM sodium phosphate buffer (pH 7.3) containing 1 mM EDTA and 0.1 mM pyridoxal 5′-phosphate. Cell debris was removed by centrifugation at 15,000 × *g* for 20 min at 4°C. Cystathionine-γ-lyase activity was assayed by determining the conversion of L-cystathionine into L-cysteine and α-ketobutyrate. The reaction mixture consisted of 4 mM L-cystathionine, 5.5 × 10^{-2} mM pyridoxal 5′-phosphate, 7 mM EDTA, 2 mM dithiothreitol, and 0.1 mg of protein in a final volume of 0.5 ml. The reaction mixtures were incubated for 30 min at 30°C, and the reactions 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 amount of cysteine in 1 ml of supernatant was determined by using the acid ninhydrin assay. This assay is highly specific and gives essentially no reaction with cystathionine or methionine (10). Total protein
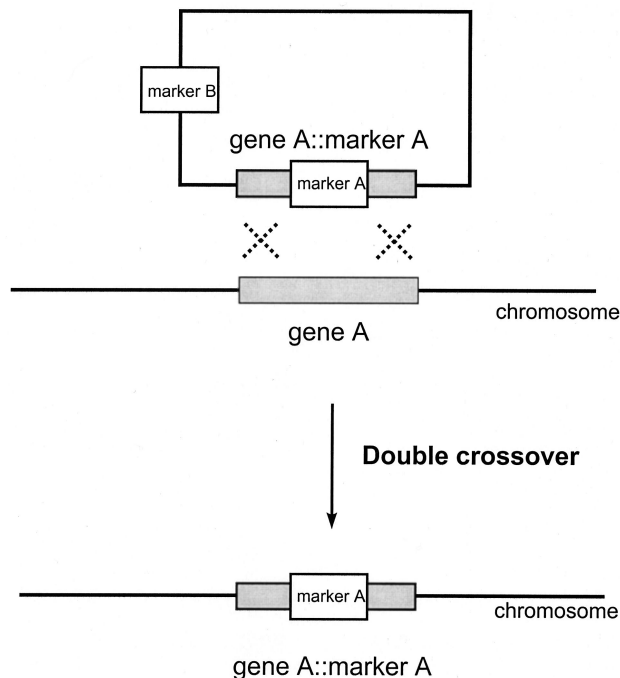
FIG. 2. Scheme of the double-marker enrichment technique for targeted gene disruption. Marker A is the transformation marker. Marker B is the enrichment marker (see the text). Transformants having gene A disrupted were selected as marker A resistant and marker B sensitive.

in cell extracts was determined by the Bradford method with ovalbumin as a standard.

RESULTS

Gene disruption in *A. chrysogenum* by the double-marker technique. Transforming DNA is integrated into the genome of *A. chrysogenum* at a very low efficiency, mainly via heterologous recombination (14, 36). The frequency of gene disruption in *A. chrysogenum* is one event every 200 transformants screened (36). Since target inactivation of the *mecB* gene, which encodes cystathionine-γ-lyase, would not generate any easily detectable phenotype (i.e., auxotrophy, resistance, color), we tried a technique to enrich the proportion of gene disruption events when inactivation is targeted to nonselectable genes (18). This technique, which has not been applied before to filamentous fungi, is based on the use of two selectable markers (Fig. 2). Marker A has two functions: (i) to

inactivate the desired gene and (ii) to serve as the transformation marker. When double recombination takes place, the second marker (marker B) is lost. Thus, transformants are selected as marker A resistant and then screened for marker B sensitivity.

To apply the double-marker technique, we constructed plasmid pCGL::hph (Fig. 3), which contains as marker A the hygromicin resistance gene (*hph*) under the control of the *gpd* gene promoter (27) and as marker B the phleomycin resistance (*ble*) under the control of the *pcbC* gene promoter (subcloned from pC43) (13).

A 7.7-kb *Bam*HI DNA fragment containing the *mecB* gene was inserted into the *Bam*HI site of pC43, which carries the *ble* gene (resistance to phleomycin). An *Eco*RI site was introduced in nucleotides 606 to 611 of the *mecB* gene by in vitro mutagenesis (see Materials and Methods), generating the pCGL plasmid. Finally, a hygromycin resistance (*hph*) cassette subcloned from pAN7-1E was inserted in the *Eco*RI position of the pCGL plasmid, generating the construct pCGL::hph (Fig. 3). This plasmid was used in the transformation of *A. chrysogenum* C10.

Southern blot analysis of transformants sensitive to phleomycin and resistant to hygromycin. About 600 transformants resistant to hygromycin were selected by transformation of *A. chrysogenum* C10 with pCGL::hph and screened for resistance to phleomycin. Ninety-nine out of 600 transformants were sensitive to phleomycin and resistant to hygromycin. There are two possibilities to explain the phenotype of these phleomycin-sensitive, hygromycin-resistant transformants: (i) phleomycin resistance is lost because double recombination took place or (ii) the transforming DNA is integrated into the genome through the phleomycin resistance cassette, thus losing its function.

To identify the transformants in which the endogenous *mecB* gene had been replaced by the inactivated gene, Southern blot analysis was performed in 35 Hyg[r] Phl[s] transformants. As shown in Fig. 4A, the genomic DNA of *A. chrysogenum* should give hybridization with an 11-kb *Eco*RI band when the 7.7-kb *Bam*HI fragment is used as a probe. When double recombination takes place within the homologous *mecB* region, the *ble* gene (conferring phleomycin resistance) is lost and the 11-kb hybridization band should change into two hybridization bands of 9 and 7 kb.

In order to study the genotypes of the transformants, the DNAs of 35 randomly selected Hyg[r] Phl[s] transformants were digested with *Eco*RI, blotted onto a nylon membrane, and hybridized with a 7.7-kb *Bam*HI fragment as the probe. South-
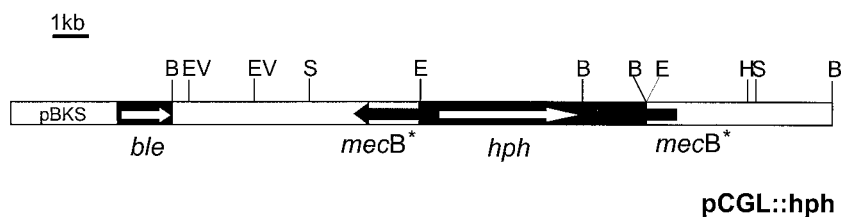

pCGL::hph

FIG. 3. Physical map of the pCGL::hph plasmid constructed for replacement of the *mecB* gene. The plasmid contains as marker A the hygromycin resistance cassette (*hph*) and as marker B the phleomycin resistance cassette (*ble*) (see the text for details of the resistance cassettes). B, *Bam*HI; EV, *Eco*RV; S, *Sst*I; E, *Eco*RI; H, *Hin*dIII.
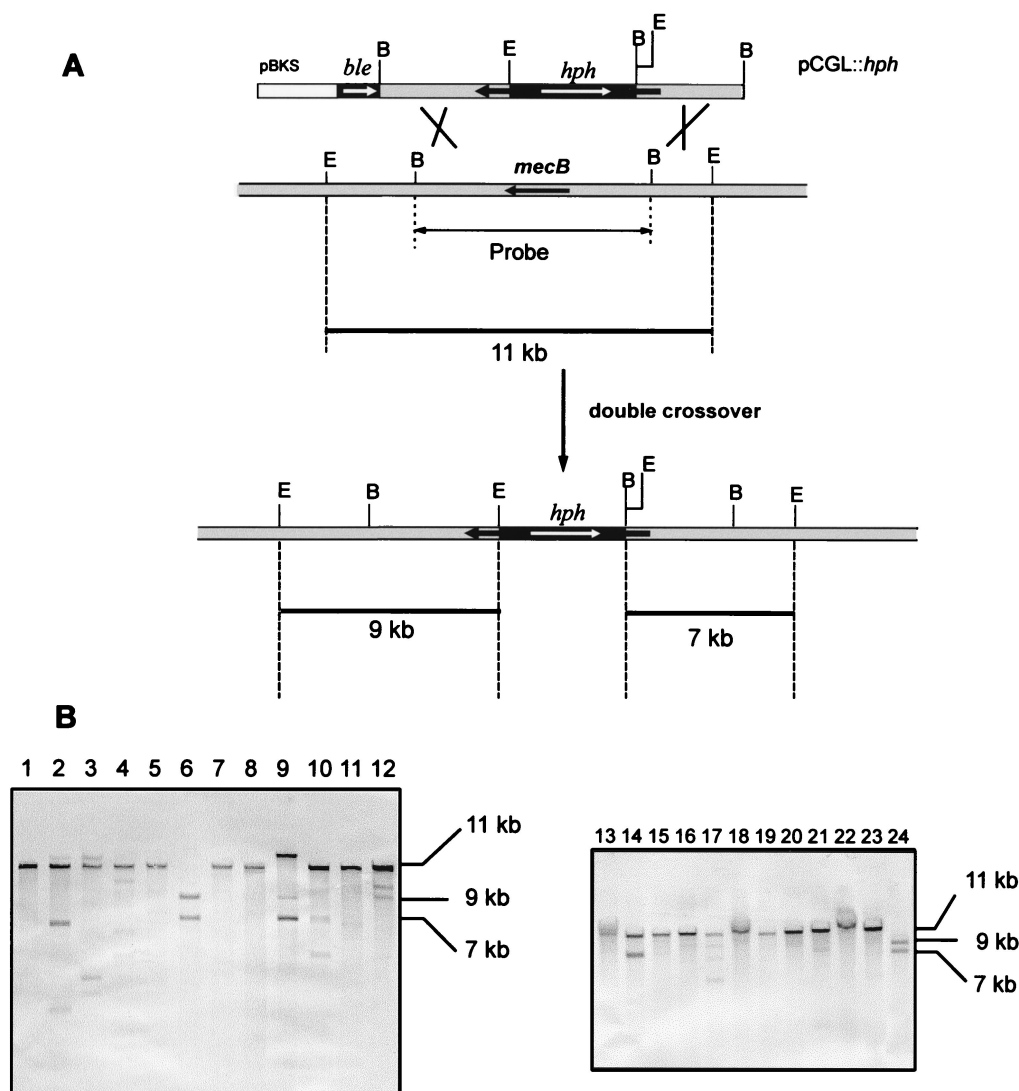
FIG. 4. Replacement of *mecB* by an inactive copy using the double-marker technique and molecular analysis of the transformants. (A) Disruption of *mecB* with the pCGL::hph plasmid. The 11-kb *Eco*RI fragment in the genome is changed into two fragments of 9 and 7 kb B, *Bam*HI; E, *Eco*RI. (B) Southern blot hybridization of *Eco*RI-digested genomic DNA (two Southern blots are shown) using as the probe the 7.7-kb *Bam*HI fragment containing *mecB*. Lane 1, *A. chrysogenum* C10; lanes 2 to 24 transformants T2 to T24. The sizes of the hybridization bands are indicated on the right.

ern hybridization results showed that the parental strain, *A. chrysogenum* C10 (Fig. 4B, lane 1), hybridized with an 11-kb band that was also present in most of the transformants. However, transformants T6 (Fig. 4B, lane 6) and T24 (Fig. 4B, lane 24) did not contain the 11-kb hybridization band, showing instead the predicted change for a gene disruption event (hybridizing bands of 9 and 7 kb).

Transformants T6 and T24 with the disrupted *mecB* gene lack cystathionine-γ-lyase activity. To study if transformants T6 and T24 had lost cystathionine-γ-lyase activity (encoded by *mecB*), fermentation in defined production medium was performed (see Materials and Methods) using strains *A. chrysogenum* C10, the transformants T6 and T24 (with a disrupted *mecB* region), and transformant T99 (unmodified *mecB* region). Results showed (Fig. 5) a typical profile of a primary metabolism enzyme for *A. chrysogenum* C10; a very similar

level of cystathionine-γ-lyase activity was obtained with transformant T99. However, transformants T6 and T24 showed no detectable cystathionine-γ-lyase activity. These results indicated that those transformants that suffered the canonical recombination process lost the *mecB* gene in the gene replacement process. These results confirmed that there is a single copy of the *mecB* gene in *A. chrysogenum*, as was proposed before on the basis of Southern hybridization evidence (19).

MecB-inactivated mutants of *A. chrysogenum* are not auxotrophs. Transformants T6 and T24, deficient in cystathionine-γ-lyase activity, lack the reverse transsulfuration pathway, but they are not auxotrophs, i.e., they are able to synthesize cysteine from sulfate and convert cysteine to methionine to meet the methionine requirement of the cell (Fig. 5, inset). The control *Aspergillus nidulans* strain C47 (*mecB cysB*), a double auxotroph defective in the autotrophic as well as in the reverse
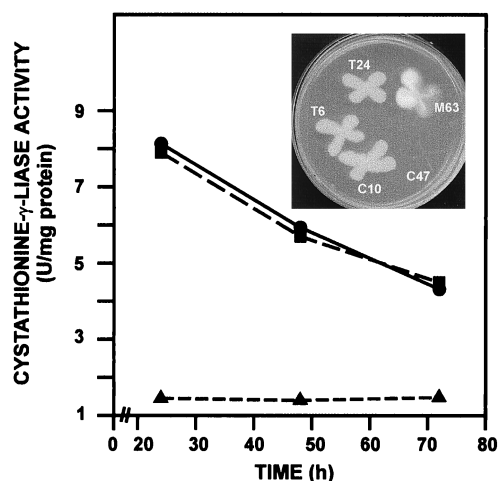
FIG. 5. Specific cystathionine-γ-lyase activity in *A. chrysogenum* C10 (●), mutant T99 (■), and mutants T6 and T24 (▲); the last two mutants showed no activity. (Inset) Growth of the T6 and T24 mutants in minimal Czapek medium. The parental strain, *A. chrysogenum* C10, is also a prototroph. *Aspergillus nidulans* C47 (*mecB cysB*), used as control, is unable to grow in minimal medium since it lacks both cysteine biosynthesis pathways, whereas *Aspergillus nidulans* M63 (*mecB*) behaves like the *A. chrysogenum* transformants T6 and T24.

transsulfuration pathway, was unable to grow in minimal Czapek medium. These results indicate that cystathionine-γ-lyase (catalyzing the splitting of cystathionine in the reverse transsulfuration pathway) is different from cystathionine-γ-synthase, which catalyzes the formation of cystathionine from cysteine and *O*-acetylserine in direct transsulfuration (conversion of cysteine to methionine); mutants lacking cystathionine-γ-synthase are known to be methionine auxotrophs, and this is not the case with transformants T6 and T24.

Inactivation of the *mecB* gene decreases cephalosporin production in methionine-supplemented cultures. The effect of inactivation of the *mecB* gene on cephalosporin biosynthesis was studied in cultures of the transformants T6 and T24 in triplicate flasks, and the same fermentation was repeated three times. Fermentations to evaluate the effect of inactivation of *mecB* were performed in MDFA medium with (3 g/liter) and without DL-methionine. Results showed that in medium without methionine (Fig. 6A to C), the growth behavior of the inactivated strains (T6 and T24) and their specific levels of cephalosporin production were similar to those of the control strain, *A. chrysogenum* C10. The lack of cystathionine-γ-lyase activity (Fig. 6C) did not affect growth in MDFA medium.

However, in cultures supplemented with methionine, the behavior was different (Fig. 6D to F). Mutants T6 and T24 exhibited a smaller growth extent than the parental strain *A. chrysogenum* C10, i.e., the lack of cystathionine-γ-lyase results in less growth than in the parental strain C10, probably because methionine reduces the synthesis of cysteine through the autotrophic pathway, which is the only remaining route for cysteine formation in the *mecB* mutants. The specific production of cephalosporin was decreased by 40 to 50% in the mutants T6 and T24 compared with that in the control strain, *A. chrysogenum* C10. The differences in cephalosporin biosynthesis correlate with the lack of cystathionine-γ-lyase activity observed in these mutants (Fig. 6C and F). In *A. chrysogenum* C10, the cystathionine-γ-lyase activity was slightly higher in cultures grown with methionine than in those without methionine, and the same happened with cephalosporin biosynthesis (see below). However, the T6 and T24 mutants showed no significant cystathionine-γ-lyase activity with or without methionine, thus excluding the possibility of the existence of a second silent *mecB* gene that might be triggered by DL-methionine in *mecB* mutants. Both *mecB* mutants behaved identically.

DL-Methionine still induces cephalosporin gene expression in *mecB*-disrupted mutants. It is well known that exogenous DL-methionine induces the transcription of cephalosporin C biosynthetic genes (35). It was unclear, however, whether the methionine induction effect could be mediated by α-ketobutyrate (a product of cystathionine splitting by cystathionine-γ-lyase) or by the cystathionine-γ-lyase protein itself acting as a regulatory protein. To determine if the methionine induction effect was still present in *mecB*-disrupted mutants lacking cystathionine-γ-lyase activity, Northern blot analysis was performed using RNAs extracted from mycelia of *A. chrysogenum* C10 and mutant T6, which were grown for 48 h in MDFA without and with methionine (3 g/liter). Results showed (Fig. 7) that the induction effect on expression of the *A. chrysogenum pcbC* gene was still present in the strain lacking cystathionine-γ-lyase activity. These results indicate that cystathionine-γ-lyase is not involved in cephalosporin gene expression. The involvement of α-ketobutyrate in cephalosporin induction cannot be ruled out since α-ketobutyrate may still be formed in the cell by other catabolic pathways.

DISCUSSION

Gene disruption is a technique difficult to apply to filamentous fungi (9). However, there are significant differences among filamentous fungi. Some of them are able to efficiently integrate homologous DNA into the genome, as is the case with *Aspergillus nidulans* (33, 37), *Ascobolus immersus* (11), and *Cochiobolus heterostrophus* (15). On the other hand, with other filamentous fungi such as *A. chrysogenum* (14, 36), *Podospora anserina* (2), and *Penicillium chrysogenum* (5), the frequency of homologous recombination is very low (usually lower than 1 in 100 transformants).

Only two cases of targeted gene disruption have been described for *A. chrysogenum*. Hoskins et al. (14) inactivated the *pcbAB* gene by the single-crossover integration approach. However, this technique generates direct repeats in the genome and it has been shown to be highly unstable in the genomes of filamentous fungi (4). Waltz and Kück (36) studied the frequency of gene disruption by double crossover in *A. chrysogenum* and established that only in 0.5% of the transformants was the targeted gene inactive.

In this work, we have adapted the gene disruption enrichment method developed for mouse embryo-derived stem cells (18) based on the use of two selection markers. This technique resulted in obtaining 5% gene disruption (one event in every 20 transformants); thus, the expected gene disruption efficiency (0.5%) was increased 10-fold. The use of this enrichment method in our laboratory has resulted in very high efficiencies (30% of gene disruption events) for the inactivation of
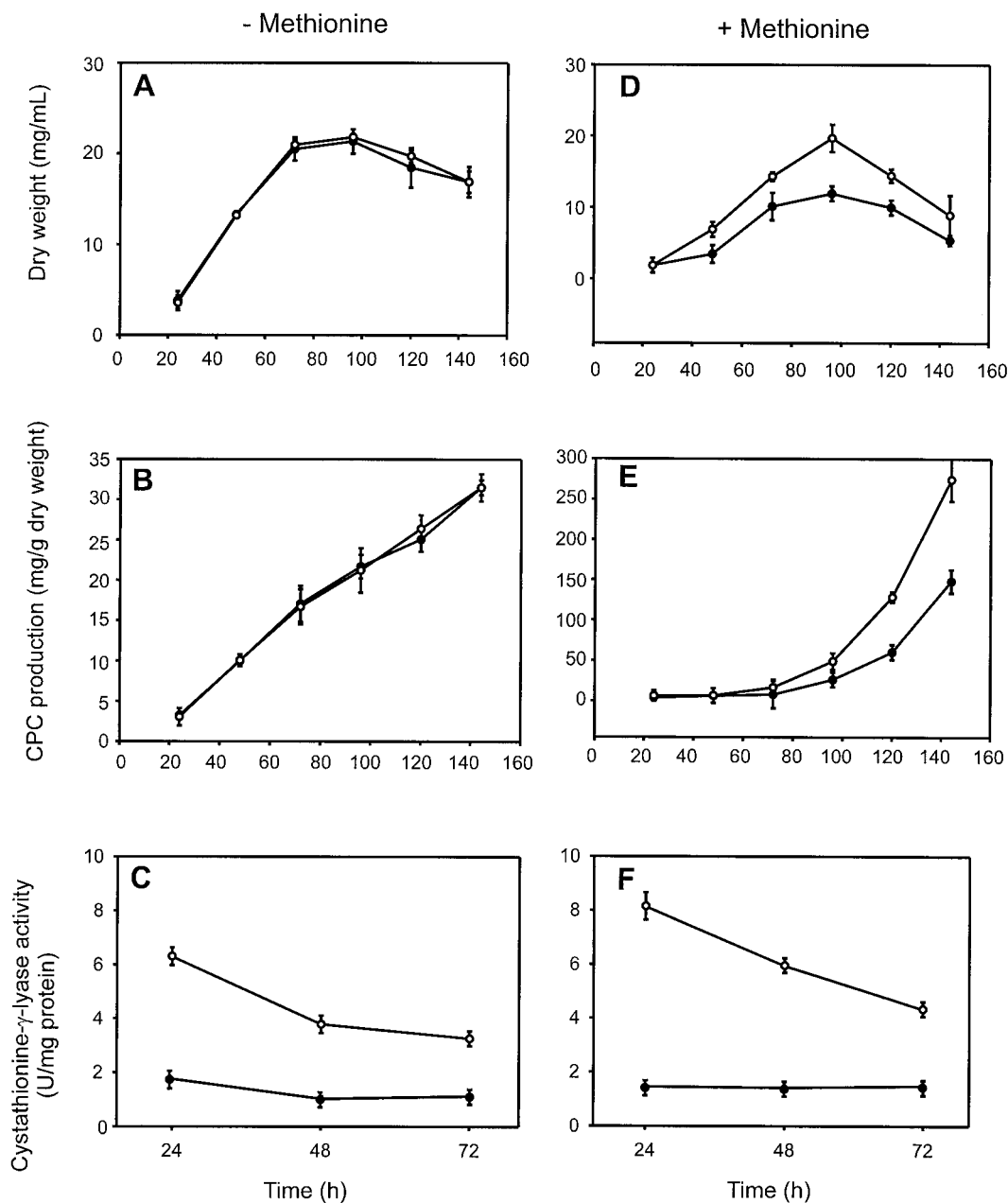
FIG. 6. Growth kinetics (A and D), cephalosporin production (B and E), and cystathionine-γ-lyase activity (C and F) in *A. chrysogenum* C10 (○) and mutant T6 or T24 (●) in MDFA medium without methionine (left) and in MDFA medium supplemented with 3 g of DL-methionine per liter (right). Note the different scales in panels B and E. The increase in cephalosporin C (CPC) production (E) is due to the induction of cephalosporin biosynthesis by methionine. Results are the averages of three determinations from triplicate flasks. Vertical bars indicate the standard deviations from the averages using nine determinations.

other *A. chrysogenum* genes (G. Liu, J. Casqueiro, and J. F. Martín, unpublished results).

In *Saccharomyces cerevisiae* and filamentous fungi, cysteine is synthesized by two pathways (25, 26) (Fig. 1). The L-cysteine molecule required for the biosynthesis of the ACV tripeptide in cephalosporin-producing *A. chrysogenum* strains was proposed to be synthesized mainly from methionine via the reverse transsulfuration pathway through homocysteine and cystathionine (Fig. 1) (23). Treichler et al. (34) at CIBA-Geigy suggested that cystathionine-γ-lyase, the enzyme that splits

cystathionine into cysteine and α-ketobutyrate, is a key enzyme for cephalosporin biosynthesis. An *A. chrysogenum* mutant isolated by random mutagenesis and defective in cystathionine-γ-lyase activity showed a drastic reduction in cephalosporin biosynthesis (34). However, this mutant might have contained a second mutation affecting cephalosporin production and, unfortunately, the CIBA-Geigy mutant is not available for further studies (J. Heim, personal communication). In this article, we have inactivated the *mecB* gene, which encodes cystathionine-γ-lyase activity in *A. chrysogenum* (19). The in-
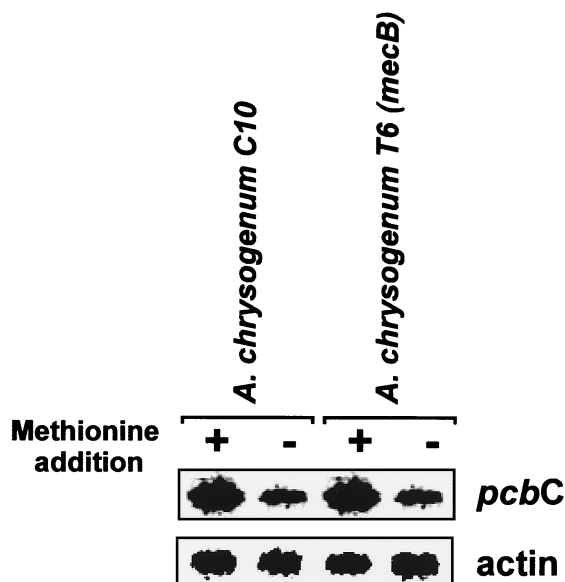
FIG. 7. Methionine stimulation of transcription of the *pcbC* gene in the parental strain *A. chrysogenum* C10 and the *A. chrysogenum* T6 mutant, disrupted in *mecB*. Northern hybridizations were performed with a 0.8-kb *Nco*I/*Kpn*I fragment containing the actin gene of *Aspergillus nidulans* and a 1.8-kb *Sal*I/*Bam*HI fragment that contains the *pcbC* gene of *A. chrysogenum*.

activation of the *mecB* gene does not affect cephalosporin biosynthesis when the sulfur source is sulfate, i.e., cysteine for cephalosporin biosynthesis may be synthesized from either sulfate or methionine. Addition of methionine stimulates cephalosporin biosynthesis in the parental strain *A. chrysogenum* C10. But when *mecB* is inactivated (mutants T6 and T24), there is a clearly lower production of cephalosporin, indicating that the supply of cysteine from methionine via the reverse transsulfuration pathway is required for high-level production of cephalosporin. A correlation between the positive effect of methionine and cystathionine-γ-lyase activity in *A. chrysogenum* C10 was observed (Fig. 6). The cystathionine-γ-lyase activity is higher in the presence of methionine (as previously described by Zanca [38]), in agreement with a direct supply of cysteine from the reverse transsulfuration pathway. The growth of mutants T6 and T24 is retarded in methionine-supplemented medium, probably because in the presence of methionine the utilization of sulfate is depressed (17). Sulfate utilization is needed for cysteine biosynthesis in these *mecB* mutants because they cannot synthesize cysteine through the reverse transsulfuration pathway. Complementation tests of the *mecB* mutants would be desirable to confirm this hypothesis.

DL-Methionine greatly stimulates cephalosporin biosynthesis (16, 20) by inducing expression of the cephalosporin biosynthesis genes *pcbAB*, *pcbC*, and *cefEF* (30, 35). Disruption of the *mecB* gene did not affect the induction of *pcbC* by methionine, i.e., induction of the cephalosporin genes is not mediated by a putative regulatory mechanism exerted by the cystathionine-γ-lyase protein. The induction mechanism may be triggered by methionine itself or by a catabolite derived from methionine, e.g., α-ketobutyrate. α-Ketobutyrate (a product of cystathionine splitting) cannot be formed in *mecB* mutants. However,

the involvement of α-ketobutyrate as an inducer in *A. chrysogenum* cannot be ruled out since it may still be formed by methionine-deaminases similar to those that occur in *Aspergillus nidulans* (R. E. Cardoza and J. F. Martín, unpublished results), although their presence in *A. chrysogenum* has not yet been demonstrated.

In conclusion, targeted inactivation of the *mecB* gene shows that methionine has two positive effects on cephalosporin biosynthesis; one of them, the supply of cysteine from the reverse transsulfuration pathway, required for high-level cephalosporin biosynthesis, is catalyzed by cystathionine-γ-lyase.

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