Mol Genet Genomics (2002) 268: 352–361
DOI 10.1007/s00438-002-0755-5

ORIGINAL PAPER

Y. Abe · C. Ono · M. Hosobuchi · H. Yoshikawa

Functional analysis of *mlcR*, a regulatory gene for ML-236B (compactin) biosynthesis in *Penicillium citrinum*

Received: 30 July 2002 / Accepted: 4 September 2002 / Published online: 25 October 2002

Abstract The *mlcR* gene encodes a putative 50.2-kDa protein with a $Zn(II)_2Cys_6$ DNA-binding domain, which may be involved in the regulation of ML-236B biosynthesis in *Penicillium citrinum*. The induction of ML-236B production appears to correlate with the expression of *mlcR*, and the ML-236B biosynthetic genes *mlcA–mlcH*, and occurs mostly during the stationary phase. The present study was designed to examine the effects of alterations in *mlcR* expression on ML-236B biosynthesis. We first set out to increase the *mlcR* copy number in the chromosome of *P. citrinum*. Transformants with additional copies of native *mlcR* showed increased transcription of *mlcR* and produced larger amounts of ML-236B than the parent strain. Altered *mlcR* expression was also achieved by introducing a construct, designated *pgkA*(P)::*mlcR*, that contained the *mlcR* coding region fused to the (constitutively active) promoter and terminator sequences of the *Aspergillus nidulans* 3-phosphoglycerate kinase (*pgkA*) gene. Transformants carrying the *pgkA*(P)::*mlcR* construct expressed *mlcR* constitutively, and produced ML-236B during the exponential growth phase, suggesting that the *pgkA*(P)::*mlcR* construct does affect the regulation of ML-236B biosynthesis. Comparative expression analysis by RT-PCR showed that altering the expression profile of *mlcR* influenced the expression of some of the ML-236B biosynthetic genes. The evidence suggests that *mlcR* may indeed be involved in the transcriptional activation of some of the pathway-specific genes required for ML-236B biosynthesis.

Keywords *Penicillium citrinum* · ML-236B biosynthesis · Gene regulation

Communicated by C. A. M. J. J. van den Hondel

Y. Abe (✉)
Lead Discovery Research Laboratories,
Sankyo Co. Ltd., 2-58 Hiromachi 1-Chome,
Shinagawa-ku, Tokyo 140-8710, Japan
E-mail: abyuuk@shina.sankyo.co.jp
Tel.: +81-3-34923131
Fax: +81-3-54368565

C. Ono · M. Hosobuchi
Lead Discovery Research Laboratories,
Sankyo Co. Ltd., 389-4 Aza-Ohtsurugi, Shimokawa,
Izumi-machi, Iwaki-shi, Fukushima 971-8183, Japan

H. Yoshikawa
Biomedical Resarch Laboratories,
Sankyo Co. Ltd., 2-58, Hiromachi 1-Chome,
Shinagawa-ku, Tokyo 140-8710, Japan

Introduction

ML-236B (compactin), a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, is produced by *Penicillium citrinum*, and can be used as a substrate for microbial conversion to pravastatin sodium (Brown et al. 1976; Endo et al. 1976; Serizawa et al. 1983). Pravastatin sodium has been widely used as a pharmaceutical agent for the therapy of hypercholesterolemia.

The gene cluster for ML-236B biosynthesis in *P. citrinum* has been cloned and characterized (Abe et al. 2002a). Nine genes, *mlcA–mlcH* and *mlcR*, are presumed to encode polypeptides required for ML-236B biosynthesis. Northern analysis showed that these nine genes are transcribed during the stationary phase. Functional inactivation of the genes in the cluster provided evidence for their direct involvement in ML-236B biosynthesis. Reintroduction of the gene cluster into *P. citrinum* led to overproduction of ML-236B (Abe et al. 2002b).

Clarification of the transcriptional regulation of the ML-236B biosynthetic genes in *P. citrinum* is important for the characterization of ML-236B biosynthesis at the molecular level, and could also help to improve ML-236B productivity. However, our knowledge of the molecular biology of the regulation of ML-236B biosynthesis is currently inadequate.

As a first step towards characterization of the transcriptional regulation of ML-236B biosynthesis, we examined the function of *mlcR*, which encodes a polypeptide with a GAL4-type zinc-finger cluster DNA-binding domain (Johnston and Dover 1988).

DNA-binding proteins containing the $Zn(II)_2Cys_6$ structure represent an important class of positively acting regulatory factors in filamentous fungi and yeasts, and are necessary for the expression of multiple genes within clusters (Lockington et al. 1985; Beri et al. 1987; Witte and Dickson 1988; Yuan et al 1991; Schjerling and Holmberg 1996; Todd and Andrianopoulos 1997; Todd et al. 1997; Masloff et al. 1999). One such gene product, AlfR, is involved in the transcriptional activation of the aflatoxin biosynthetic genes in *Aspergillus parasiticus* and *A. flavus* (Woloshuk et al. 1994, 1995; Chang et al. 1995; Flaherty and Payne 1997; Ehrlich et al. 1999; Cary et al. 2000).

In the present study, we examined the effects of altering the expression of *mlcR* on the transcriptional regulation of the genes for ML-236B biosynthesis and on ML-236B production. Similar experiments were previously used to determine the function of *aflR* in *A. flavus* (Chang et al. 1995; Flaherty and Payne 1997). We demonstrate that altering the level and pattern of *mlcR* expression influences both the expression of genes responsible for ML-236B biosynthesis, and ML-236B productivity. We now consider that *mlcR* encodes the positively acting transcription factor that regulates ML-236B biosynthesis.

Materials and methods

Fungal strains and culture conditions

Penicillium citrinum No. 41520 (Abe et al. 2002a) was used as the recipient strain for fungal transformation. For conidia formation, *P. citrinum* was incubated on PGA plates at 26°C for 10 days. YPL-20 liquid medium and VGS plates were used for fungal transformation as described previously (Nara et al. 1993b). MBG3-8 liquid medium was used for nucleic acid isolation and ML-236B production (Hosobuchi et al. 1993). About 10^7 spores were transferred to a 100-ml flask containing 10 ml of MBG3-8 medium, and incubated on a rotary shaker at 24°C.

Bacterial strains used

E. coli JM109 was used for the construction, propagation and amplification of plasmids. *E. coli* JM109 was purchased from Takara Shuzo (Kyoto, Japan).

Nucleic acid manipulations

Fungal chromosomal DNA was isolated, in principle, according to the method developed by Yelton et al. (1984). Total RNA of *P. citrinum* was extracted and isolated using the RNeasy Total Plant kit (Qiagen K.K., Tokyo, Japan) according to the supplier's instructions. Southern and Northern hybridizations were performed using the DIG system (Nippon Roche K.K., Tokyo, Japan). All other nucleic acid manipulations were carried out by standard methods (Sambrook et al. 1989).

Amplification of the *mlcR* cDNA by reverse transcription (RT)-PCR

First-strand cDNA was synthesized with the LA PCR kit ver1.1 (Takara) and used as the template for PCR. Cloning of the full-length cDNA was carried out with a pair of oligonucleotide primers containing the *Bam*HI recognition site: FR (5′-GGATCCATGTCCCTGCCGCATGCAAC-3′) and RR (5′-GGATCCCTAAGCAATATTGTGTTTCT-3′). Reaction conditions for RT-PCR were as follows: 94°C for 30 s; 60°C for 30 s; and 72°C for 1 min for 30 cycles. The 1.4-kb amplified fragment was recovered after electrophoresis, and ligated into pCR2.1 (Invitrogen BV, Groningen, The Netherlands). Cycle sequencing reactions were carried out using the BigDye primer/terminator cycle sequencing kit and sequencing was carried out with the ABI Prism 377 sequencer (Applied Biosystems, Calif.).

RT-PCR analysis

Rapid amplification of transcripts was carried out with the One-step RNA PCR kit (Takara) according to the instructions provided. Specific pairs of primers which could detect cDNA products derived from transcripts of *mlcR* expressed from the native promoter or the *A. nidulans pgkA* promoter (Clements and Roberts 1985) are listed in Table 1. The primer combinations used for amplification of cDNAs corresponding to individual *mlcA–mlcH*, *orf1*, *orf11–orf17*, *orf19* and *P. citrinum pgk* transcripts (Nara et al. 1993a) are also listed in Table 1. Aliquots (50 ng) of total RNA were used as templates for RT-PCR. Reaction conditions were: 94°C for 30 s; 60°C for 30 s; and 72°C for 1 min, for 20–25 cycles. The amplified cDNAs were analyzed by electrophoresis, stained with ethidium bromide and visualized by densitometry (Atto, Tokyo, Japan).

Construction of integrating vectors

To increase *mlcR* copy number in the chromosome of *P. citrinum*, a 3.9-kb *Eco*RI-*Not*I fragment containing the native *mlcR* locus was recovered from pML51, blunted with T4 DNA polymerase, ligated into the *Pvu*II site of pDF333 (Nara et al. 1993b) and transformed into *E. coli* JM109. The resulting plasmid, which retained the *Eco*RI recognition site but lacked the *Not*I recognition site of the insert, was named pSAKincR. An expression vector, pSAKexpR, designed for expression of the *mlcR* cDNA under the control of the constitutively active *A. nidulans pgkA* promoter, was constructed as follows. pDF333 was first digested with *Bam*HI, blunted with T4 DNA polymerase, religated and transformed into *E. coli* JM109. The resulting plasmid was named pSAK360. A second sample of pDF333 was digested with *Hin*dIII and *Bam*HI, blunted with T4 DNA polymerase, ligated to an *Eco*RI-*Not*I-*Bam*HI adaptor and transformed into *E. coli* JM109. The resulting plasmid was named pSAK410. Then, pSAK410 was digested with *Pvu*II and dephosphorylated with bacterial alkaline phosphatase. The fragment containing the hygromycin B phosphotransferase gene (*hph*; Gritz and Davies 1983), with the *A. nudulans pgkA* promoter and terminator cassette was recovered from SAK360 and introduced to the *Pvu*II site of pSAK410. The resulting plasmid was named pSAK700, a fungal expression vector with the *pgkA* promoter/terminator cassette. A 1.4-kb *Bam*HI fragment encoding the *mlcR* cDNA was isolated from pCR2.1, and introduced into the *Bam*HI site of pSAK410. The resulting plasmid with the expression construct, designated *pgkA*(P)::*mlcR*, was named pSAKexpR.

Transformation of *P. citrinum*

Protoplast preparation and DNA transformation were performed as described previously (Nara et al. 1993b). Briefly, a sample (0.5 g) of germinating conidia was treated with an enzyme solution containing chitinase (Sigma, Deisenhofen, Germany) and Zymolyase 20-T (Seikagaku, Tokyo, Japan). After incubating at 30°C for 0.5–1 h, protoplasts were filtered through a sterile glass filter (3G-2) and washed in 550 mM $MgCl_2$. Transformations were carried out in a solution containing 20% (w/v) PEG8000, 50 mM $CaCl_2$, 10 mM MOPS and 10 mM EDTA (pH 8.0), and 120-μg aliquots of the transforming vectors (pSAKincR and pSAKexpR) were used. Hygromycin B-resistant transformants were selected on agar plates

Table 1 Oligonucleotides used for RT-PCR

Gene/primer	Sequence (5′→3′)
mlcA	GCAAGCTCTGCTACCAGCAC and CTAGGCCAACTTCAGAGCCG
mlcB	AGTCATGCAGGATCTGGGTC and GCAGACACATCGGTGAAGTC
mlcC	AAACCGCACCTGTCTATTCC and CTTTGTGGTTGGATGCATAC
mlcD	CGCTCTATCATTTCGAGGAC and TCAATAGACGGCATGGAGAC
mlcE	ATGTCAGAACCTCTACCCCC and TCAAGCATCAGTCTCAGGCA
mlcF	ATGAGTCCAGCGAGGATTAC and CTAGACAAATGAACCTGACA
mlcG	TATGAAGCTGCTCGGTATGC and GACCTATTGCTTTGTAGCAC
mlcH	TGAGAATGTCAACAGGCTTC and GATTTGCACCATAGATCCAC
mlcR	TCACCTCACATTATTTGATC and GAGCCAGACCAGTACCAATC
pgk	ATGTCTCTCTCCAACAAGCTC and TTACTTACTGGACAGAGCAGC
orf1	GGGTTTCTTGTGACTACTCC and GAACGGAAAGCCCATTGTCC
orf11	GCTATGTGAGTGGAGTCGGC and CATCCGGGATGTTGTTTTGCC
orf12	TTACTCTGGCGGTCATCTCC and TCCAGCGCTGAGGATTGATC
orf13	GAAACACAGTGCGATGCGTC and TGGAGCTTCCTGGTAATCTC
orf14	ATCGGACACGTCGACATCGC and TCACCCAGAGTAAGAATTAC
orf15	TTCATCGAAGCTGGTTTTTC and TATGCTTGGCGCAGTTCTGC
orf16	GATTGGAGGTCACGATGATC and GAAAGCAACTGGCTAGTAGC
orf17	CATTGACCGGAGGAGTTGTC and TGACTATGATCCTCCTGATC
orf19	TCGACAAATCCAAGTCTTGC and CGCAGTCTGCTCAATATCGC
mlcR/NR1	CCTCACATTATTTGATCTTAATCC
mlcR/NR2	TAAGCAATATTGTGTTTCTTCGC
mlcR/NR3	ATGTCCCTGCCGCATGCAACGATTC
mlcR/pgk-R	CGTCCATCTCTCTCCGTACAGC

containing the antibiotic at 200 μg/ml, after incubation at 26°C for 14 days.

Measurement of ML-236B and related compounds in culture broth

ML-236B and related compounds were analyzed as follows. The fermentation broth was diluted sixfold with 0.2 N NaOH. After shaking for 1 h on a rotary shaker, the alkaline broth was diluted tenfold with 50% methanol. After centrifugation, the supernatant was analyzed for ML-236B sodium and its intermediates by high-performance liquid chromatography (HPLC) on an SSC-ODS-262 column (Sensyu Chemicals), using 75% methanol-0.1% acetic acid-0.1% triethylamine-24.8% water as the mobile phase at a flow rate of 1–2 ml/min. Eluted compounds were detected by scanning at 236 nm. The amounts of ML-236B produced are expressed in arbitrary units.

Sequence accession number

The nucleotide sequence of the gene cluster in *P. citrinum*, spanning a 72-kb region, has been deposited in the DDBJ database under the Accession No. AB072893.

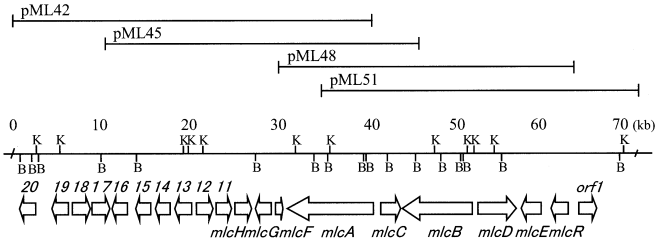


Fig. 1 A 72-kb gene cluster in *P. citrinum*. The restriction map, locations and transcriptional orientation of the ML-236B biosynthetic genes are shown, based on sequence analysis and homology searches. *mlcA* and *mlcB* are predicted to encode polyketide synthase; *mlcC*, *orf12-13*, *orf16*, *orf18*, cytochromes P450; *mlcD*, HMG-CoA reductase; *mlcE* and *orf17*, efflux pumps; *mlcF*, an oxidoreductase; *mlcG* and *orf14*, dehydrogenases; *mlcH*, a transesterase; *mlcR* and *orf1*, transcription factors; *orf11*, an acetyltransferase; *orf15*, aristolochene synthase; *orf19*, HMG-CoA synthase; and ORF20, fructosyl amine oxygen oxidoreductase. Abbreviations: K, *Kpn*I; B, *Bam*HI; *orf*, open reading frame

Results

Identification of the 72-kb gene cluster

In the present study, the clone pML42, which overlaps with several sequenced previously (Fig. 1), was subjected to sequence analysis, and the nucleotide sequence of the entire gene cluster, spanning a 72-kb region, was determined. BLAST searches revealed the existence of twenty open reading frames (*orfs*) as shown in Fig. 1. Nine genes, *mlcA–mlcH* and *mlcR*, are assumed to be required for ML-236B biosynthesis (Abe et al. 2002a). Some of the other 11 ORFs appear to encode polypeptides required for aristolochene-related sesquiterpenoid biosynthesis.

orf1 encodes a polypeptide with a GAL4-type zinc-finger cluster motif like *mlcR*. ORF11 shows weak similarity to trichothecene O-acetyltransferase from *Fusarium graminearum* and *Gibberella zeae* (Kimura et al. 1998). ORF12, ORF13, ORF16 and ORF18 have strong similarity to P450 monooxygenases (Noshiro and Okuda 1990; Cohen and Feyereisen 1995; Alexander et al. 1998). ORF14 shows similarity to dehydrogenases (Lin et al. 1999). ORF15 displays strong similarity only to aristolochene synthases from *Penicillium roqueforti* and *A. terreus* (Proctor and Hohn 1993; Chang et al. 1995). ORF17 shows strong similarity to efflux pumps. ORF 19 displays strong similarity to HMG-CoA synthases (Katayama et al. 1995). ORF20 has similarity to fructosylamine:oxygen oxidoreductase (Takahashi et al. 1997).

Structure of *mlcR*

Both the 5′ and 3′ ends of the *mlcR* transcripts were determined previously by the RACE method (Abe et al. 2002a), and the 5′ RACE products were found to exhibit heterogeneity, as shown in Fig. 2. A comparison of the genomic DNA and cDNAs showed that the gene contained two introns, each of which has the consensus intron splicing sequences GT..AG. *mlcR* encodes an ORF for a putative polypeptide of 459 amino acids, whose molecular weight was calculated to be 50.2-kDa. A BLAST search revealed that the putative polypeptide (MlcR) had a GAL4-type zinc-finger cluster motif in the N-terminal region (Fig. 3) and exhibited 34% identity to LovE, a putative transcription factor required for lovastatin biosynthesis in *A. terreus* (Kennedy et al. 1999).

Transcriptional regulation of the ML-236B biosynthetic genes in *P. citrinum*

To analyze the expression profiles of *mlcR* and the other predicted ML-236B biosynthetic genes in *P. citrinum*, RT-PCR was performed. The expression profile of the *P. citrinum pgk* gene (Nara et al. 1993a) was also examined as a control. RT-PCR products of the ML-236B biosynthetic genes were mostly detectable on the third day of cultivation and remained detectable throughout the examined period as shown

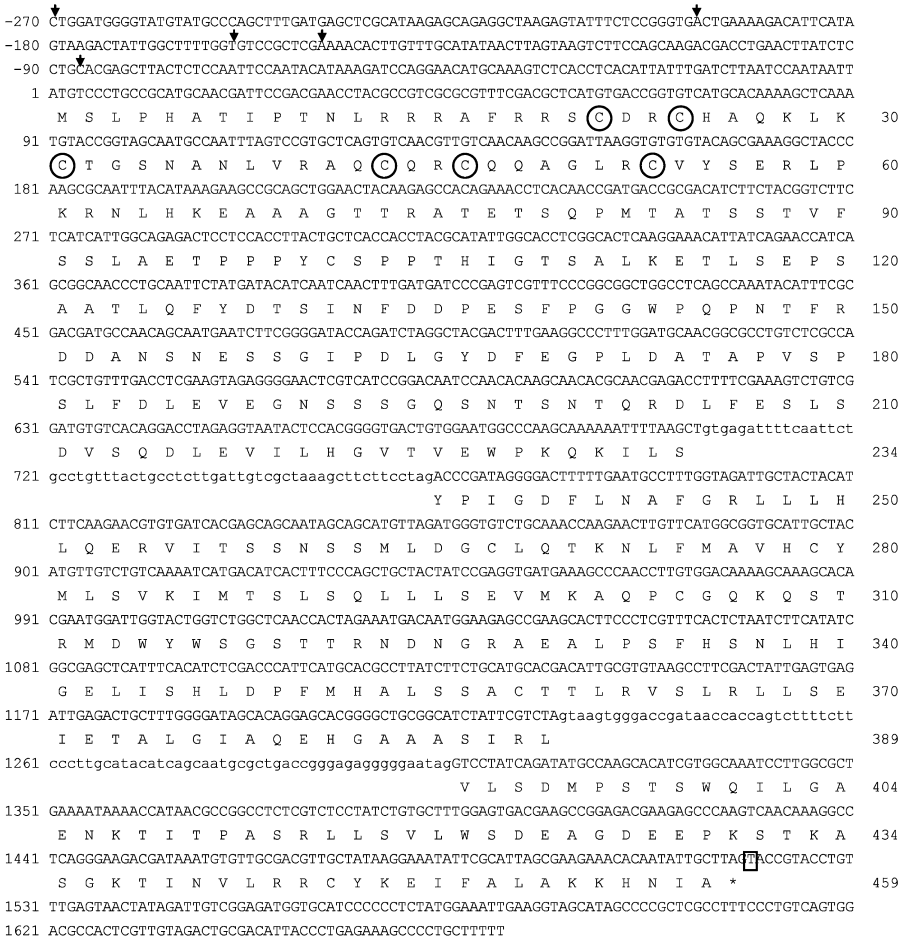


Fig. 2 Nucleotide and deduced amino acid sequences of the *mlcR* gene and its product. Putative active cysteine residues in the zinc-finger cluster motifs are *circled*. The transcriptional start sites determined by the 5′RACE method are indicated by the *arrowheads*. The transcription termination site determined by 3′RACE is *boxed*. Intron sequences are written in *lower case letters*

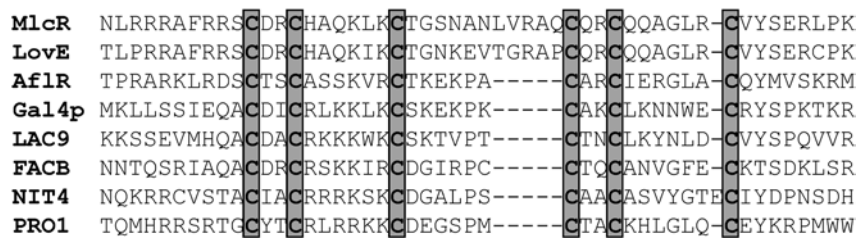


Fig. 3 Multiple alignment of Zn_2Cys_6 zinc-cluster motifs. Conserved cysteine residues are indicated by the *vertical boxes*. The dashes represent gaps introduced into the sequence to optimize the alignment

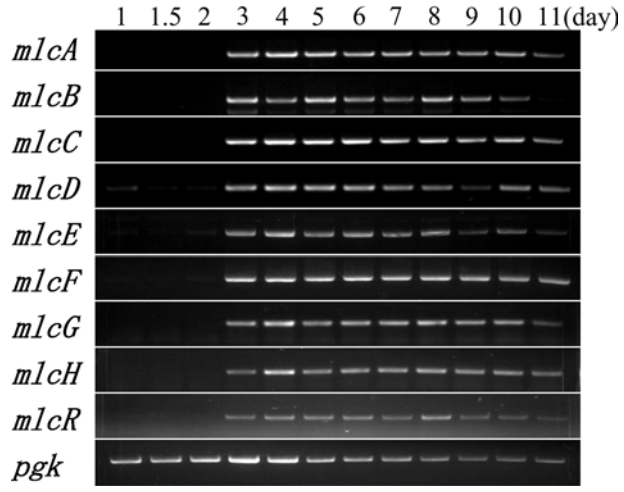


Fig. 4 Transcriptional profile of the *mlc* genes determined by RT-PCR in *P. citrinum*. Samples were obtained at the times indicated (days after inoculation)

in Fig. 4. This coincides with the timing of ML-236B production.

Increased *mlcR* copy numbers lead to overproduction of ML-236B

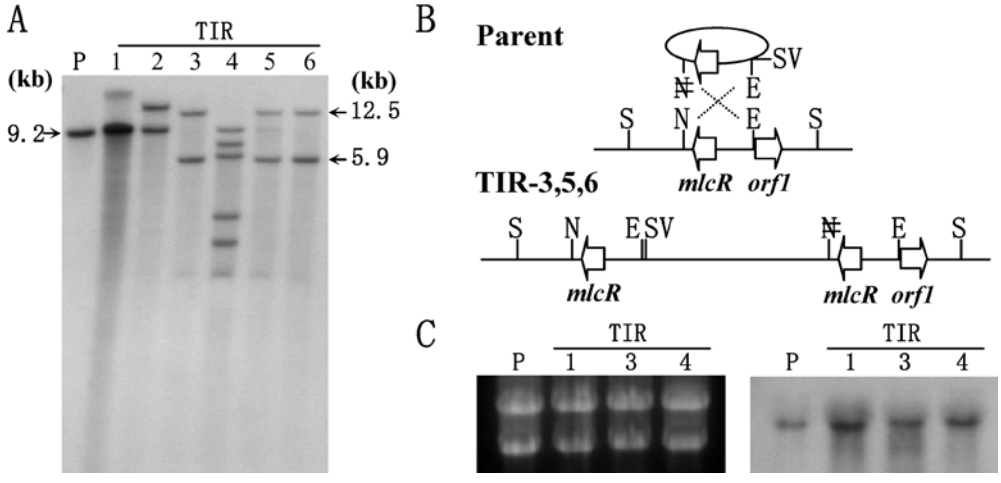
To examine the effects of an increase in the *mlcR* copy number on ML-236B productivity, pSAKincR containing a 3.9-kb genomic fragment of the native *mlcR* locus was transformed into *P. citrinum*. The insert fragment consists of the 1.7-kb intergenic region between the putative translational initiation codons of *mlcR* and *orf1*, the 1.6-kb *mlcR* coding region with two introns and 0.6 kb of 3′ uncoding sequences. Thirty-six hygromycin B-resistant transformants, referred to as the TIR series, were obtained and the ML-236B productivity of each transformant was measured and compared to that of the parent strain. Most of the transformants produced 20–30% more ML-236B in comparison to the parent strain (data not shown).

As shown in Fig. 5A, Southern analysis of DNAs extracted from six of the high-producing transformants (TIR-1 to TIR-6) had additional copies of *mlcR*. In three of the transformants (TIR-3, TIR-5 and TIR-6) the disappearance of the native 9.2-kb *Sal*I fragment and the appearance of two new bands, of 12.5 kb and 5.9 kb, suggested that pSAKincR had been introduced by a homologous recombination event as shown in Fig. 5B. In the other three transformants (TIR-1, TIR-2 and TIR-4) the native *Sal*I fragment was observed, suggesting that pSAKincR had been integrated in a non-homologous manner. Northern analysis of RNAs extracted on day 3 of culture demonstrated that the expression level of *mlcR* in the transformants appeared to be higher than that in the parent strain. These data suggest that the increase in ML-236B production in the transformants may result from the increased transcription of *mlcR* (Fig. 5C).

Fig. 5A–C Characterization of TIR strains. **A** Genomic DNAs were digested with *Sal*I and subjected to Southern hybridization analysis. **B** The predicted mode of integration of the vector pSAKincR is depicted, and the corresponding restriction patterns of the genomic DNAs of the parent strain (*above*) and some of the TIR strains, TIR-3, -5 and –6, (*below*) are illustrated. The blunt-ended *Eco*RI recognition site of the fragment was ligated in *Pvu*II site of pSAK333, which restores the *Eco*RI recognition site. However, the *Not*I recognition site of the ligated 3.9-kb fragment in pSAK333 was truncated with T4 DNA polymerase. The *Sal*I recognition site of the vector was indicated as SV. **C** Northern analysis of *mlcR* expression in the indicated transformants (TIR-1, -3 and -4) and the parent strain (P)

Construction of transformants with altered expression of *mlcR*

In order to examine further the effects of altering the expression profile of *mlcR* on transcriptional regulation of the ML-236B biosynthetic genes, plasmid-borne *mlcR* was placed under the control of the *A. nidulans pgkA* promoter in *P. citrinum*. The full-length *mlcR* ORF was cloned by RT-PCR and inserted between the *A. nidulans pgkA* promoter and terminator sequences in the fungal



expression vector pSAK700. The resulting vector pSAKexpR with the inserted construct, designated *pgkA*(P)::*mlcR*, was then transformed into *P. citrinum*.

Three transformants, named TR-1, TR-2 and TR-4, were selected and characterized by Southern hybridization as shown in Fig. 6A. In TR-1 and TR-2, additional copies of the 1.4-kb *Bam*HI fragment encoding the full-length *mlcR* ORF, and the 13.3-kb *Bam*HI frangment containing the native *mlcR* locus, were detected, suggesting that pSAKexpR had integrated outside the cluster in a non-homologous manner. In TR-4, the 1.4-kb *Bam*HI fragment encoding the introduced *mlcR* was detected. However, the native 13.3-kb *Bam*HI fragment was absent, which suggests that pSAKexpR had integrated within the cluster region by homologous recombination, as shown in Fig. 5B.

ML-236B titers produced by the parent strain and the transformants are also shown in Fig. 6B. TR-1 and TR-2 produced slightly increased titers of ML-236B that were 10–15% higher than the titer of the parent strain in shake flasks. In contrast, TR-4 produced decreased titers of ML-236B. But the transformants could produce ML-236B constitutively, while the parent strain produced it only after the third day of cultivation (data not shown).

Altered *mlcR* expression affects the expression of the ML-236B biosynthetic genes

Primer sets for RT-PCR were constructed to detect specifically transcription of *mlcR* driven either by the *pgkA* promoter or by the native *mlcR* promoter (Fig. 7A). RT-PCR showed that *mlcR* transcription driven by the *pgkA* promoter occurred on the first, second and third day of cultivation in the transformants TR-1, TR-2 and TR-4, which suggested that the introduced *pgkA*(P)::*mlcR* construct was functional (Fig. 7B). On the other hand, the expression profile of the native *mlcR* in the transformants TR-1 and TR-2 was quite similar to that in the parent strain, suggesting that altering the expression of the *pgkA*(p)::*mlcR* construct does not lead to the transcriptional activation of the native *mlcR* gene. As for TR-4, RT-PCR products derived from the native *mlcR* decreased, which is compatible with the assumption that the native *mlcR* locus had been disrupted by the homologous recombination of the *pgkA*(p)::*mlcR* construct.

In order to examine the effects of altering the expression of *mlcR* in the transformants TR-1, TR-2 and TR-4, the expression profiles of the other eight ML-236B biosynthetic genes (*mlcA–mlcH*) were analyzed and compared with those in the parent strain. As shown in Fig. 8, the timing of the expression of most of the biosynthetic genes (*mlc*A–*mlcD* and *mlcF–mlcH*, but not *mlcE*) appeared to be altered, in accordance with the constitutive expression of the *pgkA*(P)::*mlcR* construct.

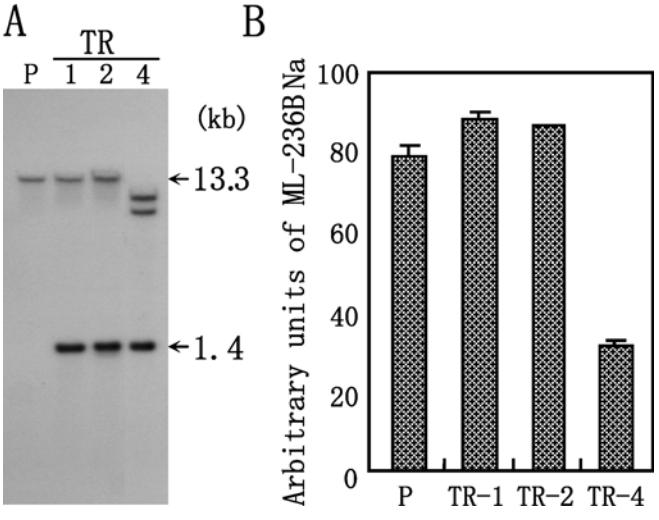


Fig. 6A, B Characterization of TR strains. Southern analysis (**A**) and ML-236B productivity of the parent strain and transformants (**B**) are shown. **A** Integration of pSAKexpR into the genome of the transformants gives rise to a 1.4-kb *Bam*HI band, which includes the full-length *mlcR* coding region. A 13.3-kb *Bam*HI band observed in the parent, TR-1 and -2, corresponding to the endogenous *mlcR* locus was not detected in the genome of TR-4. **B** Each value represents the mean of quadruplicate samples and the *error bars* indicate standard deviations. P, parent strain

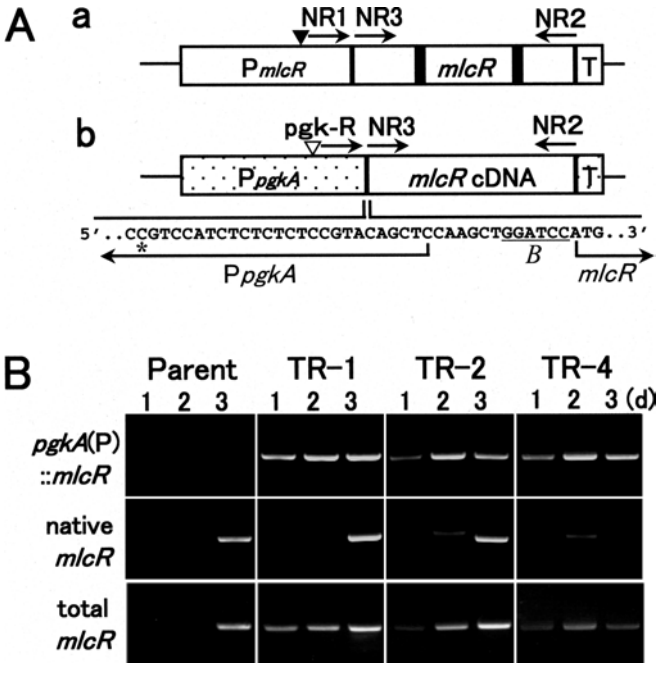


Fig. 7A, B RT-PCR analysis of *mlcR* expression in the parent strain and transformants TR-1, -2 and -4. **A** The structures of the endogenous *mlcR* locus (a) and the *pgkA*(P):*mlcR* gene are depicted. The sites of the PCR primers NR1, NR2, NR3 and pgk-R are indicated by the *arrows*. NR1, NR2 and NR3 were derived from the native *mlcR* locus. Primer pgk-R was derived from the sequence of the *A. nidulans pgkA* promoter. The *filled* and *open arrowheads* represent the transcription start sites in the *P. citrinum mlcR* and the *A. nidulans pgkA* sequences, respectively. The *filled bars* in the native *mlcR* locus represent intron sequences. **B** Expression profiles of the *pgkA* promoter-driven *mlcR*, the endogenous *mlcR* and total *mlcR*. P, promoter. T, terminator. B, *Bam*HI

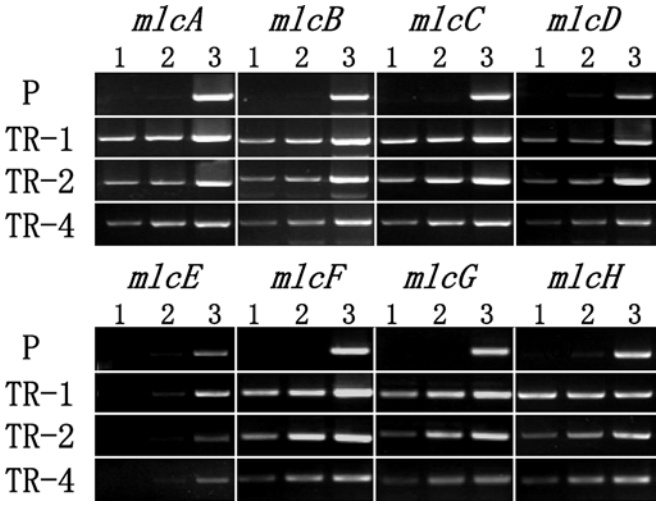


Fig. 8 Comparative expression analysis by RT-PCR of the ML-236B biosynthetic genes in the parent strain and transformants TR-1, -2 and -4. The primer combinations used are listed in Table 1. RNA samples were obtained at the times indicated (days after inoculation)

The expression profile of *mlcE* in TR-1, TR-2 and TR-4 was the same as that in the parent strain.

These data suggest that altered expression of *mlcR* affected the expression of several genes, *mlcA–mlcD* and *mlcF–mlcH*, required for ML-236B biosynthesis, but did not affect the expression profiles of *mlcE* or *mlcR* itself.

Altered *mlcR* expression does not affect the expression of genes outside the ML-236B gene cluster

The effects of altering the expression of *mlcR* in TR-1 and TR-2 were also examined for several of the *orfs* identified outside the ML-236B gene cluster. Oligonucleotides designed to examine the expression profiles of ten *orfs* (*orf1* and *orf11–orf19*) were constructed, and RT-PCR products were successfully amplified for *orf1*, *orf11–orf17* and *orf19*. Several sets of oligonucleotides failed to detect an RT-PCR product of *orf18* (data not shown). As shown in Fig. 9, ML-236B biosynthetic genes and some of the other *orfs* appeared to be co-transcribed on the third day of cultivation in the parent strain. However, comparative analysis by RT-PCR showed that the expression profiles of the genes *orf1*, *orf11–orf17* and *orf19* in TR-1 and TR-2 were quite similar to those in the parent strain, suggesting that the altered *mlcR* expression does not affect the expression of genes outside the ML-236B gene cluster (Fig. 9).

Fig. 9 Comparative expression analysis by RT-PCR of genes outside the ML-236B gene cluster in the parent strain and transformants TR-1 and TR-2. The primer combinations used are listed in Table 1. RNA samples were obtained at the times indicated (days after inoculation)

Discussion

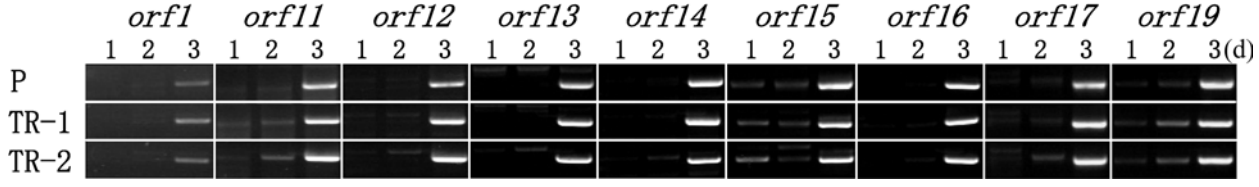
The *mlcR* gene encodes a polypeptide with a $Zn(II)_2Cys_6$ zinc-finger cluster DNA-binding motif typical of the GAL4-type family of positive regulatory proteins in yeasts and filamentous fungi (Lockington et al. 1985; Beri et al. 1987; Johnston and Dover 1988; Witte and Dickson 1988; Yuan et al. 1991; Schjerling and Holmberg 1996; Todd and Andrianopoulos 1997; Todd et al. 1997; Masloff et al. 1999). The zinc binuclear cluster motif has been identified in more than 80 fungal regulatory proteins (Schjerling and Holmberg 1996). Many of them are reported to be involved in catabolic processes, such as ethanol, quinic acid, nitrate, acetate and amide utilization (Lockington et al. 1985; Beri et al. 1987; Yuan et al. 1991; Todd and Andrianopoulos 1997). Some of them are involved in cell differentiation and the regulation of biosynthetic pathways (Woloshuk et al. 1994; Masloff et al. 1999)

AflR, one of the GAL4-type transcription factors, is reported to be required for the biosynthesis of the polyketide derivative aflatoxin, acting as a transcriptional activator in *A. parasiticus* and *A. flavus* (Woloshuk et al. 1994, 1995; Chang et al. 1995; Flaherty and Payne 1997; Ehrlich et al. 1999; Cary et al. 2000). Overexpression of *aflR* enhances the expression of the aflatoxin biosynthetic genes (Chang et al. 1995; Flaherty and Payne 1997). Disruption of *aflR* results in the abolition of aflatoxin production (Cary et al. 2000). A DNA binding assay also provided evidence for the binding of AflR to *cis*-regulatory elements in the intergenic regions of the aflatoxin biosynthetic genes (Ehrlich et al. 1999).

Recently, another transcription factor, LovE, has been described in *A. terreus*, which is thought to be involved in lovastatin biosynthesis (Kennedy et al. 1999).

The regulation of ML-236B biosynthesis in *P. citrinum* is an interesting and important aspect of ML-236B biosynthesis, and further knowledge of the mechanisms involved should help to improve productivity. In order to characterize the transcriptional regulation of ML-236B biosynthesis, we first examined the role of *mlcR* in *P. citrinum*.

In a previous report, a slight increase in ML-236B production was observed when the gene cluster required for ML-236B biosynthesis was transformed into



P. citrinum (Abe et al. 2001b). In the present study, we first examined the effect of introducing *mlcR* alone on ML-236B productivity. Transformants with additional copies of the native *mlcR* locus were found to produce increased titers of ML-236B. Southern analysis and comparative expression analysis by Northern hybridization showed that increased copy numbers of *mlcR* led to increased transcription of *mlcR*, concomitantly with ML-236B overproduction (Fig. 5). But effects on the expression of the other ML-236B biosynthetic genes could not be examined in detail by Northern blot analysis (data not shown).

In order to clarify further the transcriptional regulation of *mlcR*, the expression profile of each ML-236B biosynthetic gene was determined by RT-PCR. As shown in Fig. 4, the expression of most of the ML-236B biosynthetic genes was induced on the third day of cultivation, when cells were already in the stationary phase. The timing of ML-236B production was coincident with that of the expression of all the ML-236B biosynthetic genes (data not shown).

The relationship between the time-course of ML-236B production and the expression profiles of the biosynthetic genes is very interesting, and raises the following question. If *mlcR* is constitutively expressed in *P. citrinum* from the early period of cultivation on, i.e., even in the exponential growth phase, does the timing of ML-236B production and the expression pattern of the ML-236B biosynthetic genes change? Flaherty and Payne (1997) reported that constitutive overexpression of *aflR* using the *gpdA* promoter led to higher accumulations of the transcripts of pathway genes and increased aflatoxin production, but the initiation of aflatoxin biosynthesis was not altered in *A. flavus* (Flaherty and Payne 1997). In the present study, the promoter and terminator cassette of the *A. nidulans pgkA* gene (Clements and Roberts 1985) was used to alter the expression profile of *mlcR*.

All the hygromycin B-resistant transformants produced ML-236B during the exponential growth, while the parent strain produces ML-236B only at the stationary phase, suggesting that the transformed *pgkA*(P)::*mlcR* construct indeed affected the regulation of ML-236B biosynthesis. Southern hybridization showed that the *pgkA*(P)::*mlcR* construct was integrated into a locus outside the ML-236B gene cluster in TR-1 and TR-2, and into the *mlcR* locus itself in TR-4.

Primer sets for RT-PCR were constructed to examine the effects of introducing the *pgkA*(P)::*mlcR* construct. The primers pgk-R and NR1 (Table 1) were derived from the 5′ non-coding region between the putative transcription start point and the translation initiation codon of the *A. nidulans pgkA* and native *mlcR*, respectively as illustrated in Fig. 6A. The transcription start point of *mlcR* was localized in our previous study and that of *A. nidulans pgkA* has been mapped 32 bp upstream from the translation initiation codon (Clements and Roberts 1986). Therefore, we believe that transcripts derived from the *pgkA*(P)::*mlcR* construct and native *mlcR* should be distinguishable. As shown in Fig. 6B, constitutive expression of *mlcR* was indeed achieved by introducing the *pgkA*(P)::*mlcR* construct into TR-1 and TR-2, but this did not appear to affect the expression profile of native *mlcR*. On the other hand, in TR-4, although constitutive expression was achieved, inducible expression of the native *mlcR* gene was abolished. Altered expression of *mlcR* in TR-1 and TR-2 appeared to enhance ML-236B production, while decreased expression of native *mlcR* appeared to decrease the titer in TR-4 (Fig. 6B). This can be explained if the native *mlcR* locus was disrupted by the homologous recombination of the *pgkA*(p)::*mlcR* construct. These data also suggest that the expression profile and level of *mlcR* influence ML-236B production.

Exactly how *mlcR* regulates ML-236B biosynthesis in *P. citrinum* remains unclear. RT-PCR analysis of the ML-236B biosynthetic genes in the TR series, even in TR-4, provided evidence for the altered expression of several genes, *mlcA–mlcD* and *mlcF–mlcH*, in the biosynthetic gene cluster (Fig. 8). RT-PCR analysis also suggests that the expression profiles of *mlcE* and other genes (*orf1*, *orf11* to *orf17* and *orf19*) around the ML-236B gene cluster did not appear to be influenced by the constitutive expression of the *pgkA*(P)::*mlcR* construct (Figs. 8 and 9). Taken together, these data also suggest that *mlcR* is involved in transcriptional activation of at least some of the pathway-specific genes responsible for ML-236B biosynthesis.

Most GAL4-type transcription factors bind to the sites with terminal CG-rich, triad inverted repeats such as 5′-CGGN$_n$CCG-3′ or 5′-CCGN$_n$CGG-3′ (Reece and Ptashne 1993). AflR binds to the related motif 5′-TCGN$_5$CGA-3′ in the promoter regions of some of the aflatoxin biosynthetic genes in *A. parasiticus* (Ehrlich et al. 1999). MlcR may bind to DNA targets in a similar manner, but the Zn_2Cys_6 domain of MlcR has a characteristic feature with respect to the numbers of amino acids between the third and fourth cysteines (Fig. 3). Structural difference in the zinc-cluster DNA-binding domains between MlcR and the other Zn_2Cys_6 proteins may alter their recognition of DNA targets. On the other hand, the strong resemblance between the DNA-binding domains of MlcR and LovE suggests that they may recognize similar DNA targets. Comparative analysis of the promoter regions of the pathway-specific genes, e.g. *mlcA–mlcD* and *mlcF–mlcH*, and those of their counterparts in the lovastatin biosynthetic cluster may provide information on the *cis* acting elements required for the binding of MlcR.

The functional analysis in the present study suggests that expression of *mlcR* is not autoregulated. This implies that other factors that regulate ML-236B biosynthesis must exist in *P. citrinum* No.41520. *orf1*, which is next to *mlcR*, also encodes a putative polypeptide with a $Zn(II)_2Cys_6$ DNA-binding domain. The function of *orf1* will also be determined in the near future. Further analyses using gene disruptions, DNA-binding assays and DNase I footprinting will be needed to confirm the

direct role of *mlcR* in the transcriptional activation of the ML-236B biosynthetic genes. We are now concentrating on studies to characterize the regulation system for the transcriptional activation of *mlcR* in *P. citrinum*.

Acknowledgements The authors wish to thank Drs. M. Inukai, N. Nakamura and N. Serizawa for their encouragement and suggestions, and Drs. F. Nara and I. Watanabe for their technical advice.

References

Abe Y, Suzuki T, Ono C, Iwamoto K, Hosobuchi M, Yoshikawa H (2002a) Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in *Penicillium citrinum*. Mol Genet Genomics 267:636–646

Abe Y, Suzuki T, Mizuno T, Ono C, Iwamoto K, Yoshikawa H (2002b) Effect of increased dosage of the ML-236B (compactin) biosynthetic gene cluster on ML-236B production in *Penicillium citrinum*. Mol Genet Genomics 268:130–137

Alexander NJ, Hohn TM, McCormick SP (1998) The *TRI11* gene of *Fusarium sporotrichioides* encodes a cytochrome P-450 monooxygenase required for C-15 hydroxylation in trichothecene biosynthesis. Appl Environ Microbiol 64:21–225

Brown AG, Smale TC, King TJ, Hasenkamp R, Thompson RH (1976) Crystal and molecular structure of compactin, a new antifungal metabolite from *Penicillium brevicompactum*. J Chem Soc Perkin Trans 1:1165–1170

Beri RK, Whittington H, Roberts CF, Hawkins AR (1987) Isolation and characterization of the positively acting regulatory gene *QUTA* from *Aspergillus nidulans*. Nucleic Acids Res 15:7991–8001

Cary JW, Ehrlich KC, Wright M, Chang PK, Bhatnagar D (2000) Generation of *aflR* disruption mutants of *Aspergillus parasiticus*. Appl Microbiol Biotechnol 53:680–684

Chang PK, Ehrlich KC, Yu J, Bhatnagar D, Cleveland TE (1995) Increased expression of *Aspergillus parasiticus aflR*, encoding a sequence-specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. Appl Environ Microbiol 61:2372–2377

Clements JM, Roberts CF (1985) Molecular cloning of the 3-phosphoglycerate kinase (PGK) gene from *Aspergillus nidulans*. Curr Genet 9:293–298

Clements JM, Roberts CF (1986) Transcription and processing signals in the 3-phosphoglycerate kinase (PGK) gene from *Aspergillus nidulans*. Gene 44:97–105

Cohen MB, Feyereisen R (1995) A cluster of cytochrome P450 genes of the *CYP6* family in the house fly. DNA Cell Biol 14:73–82

Endo A, Kuroda M, Tsujita M (1976) ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by *Penicillium citrinum*. J Antibiot 29:1346–1348

Ehrlich KC, Montalbano BG, Cary JW (1999) Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. Gene 230:249–57

Flaherty JE, Payne GA (1997) Overexpression of *aflR* leads to upregulation of pathway gene transcription and increased aflatoxin production in *Aspergillus flavus*. Appl Environ Microbiol 63:3995–4000

Gritz L, Davies J (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25:179–188

Hosobuchi M, Shioiri T, Ohyama J, Arai M, Iwado S, Yoshikawa H (1993) Production of ML-236B, an inhibitor of 3-hydroxy -3-methylglutaryl coenzyme A reductase, by *Penicillium citrinum*: improvements of strain and culture conditions. Biosci Biotech Biochem 57:1414–1419

Johnston M, Dover J (1988) Mutational analysis of the *GAL4*-encoded transcriptional activator protein of *Saccharomyces cerevisiae*. Genetics 120:63–74

Katayama S, Adachi N, Takao K, Nakagawa T, Matsuda H, Kawamukai M (1995) Molecular cloning and sequencing of the *hcs* gene, which encodes 3-hydroxy-3-methylglutaryl coenzyme A synthase of *Schizosaccharomyces pombe*. Yeast 11:1533–1537

Kennedy J, Auclair K, Kendrew SG, Park C, Vederas JC, Hutchinson CR (1999) Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. Science 284:1368–1372

Kimura M, Kaneko I, Komiyama M, Takatsuki A, Koshino H, Yoneyama K, Yamaguchi I (1998) Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of *Tri101*. J Biol Chem 273:1654–1661

Lin X, et al (1999) Sequence and analysis of chromosome 2 of the plant *Arabidopsis thaliana*. Nature 402:761–768

Lockington RA, Sealy-Lewis HM, Scazzocchio C, Davies RW (1985) Cloning and characterization of the ethanol utilization regulon in *Aspergillus nidulans*. Gene 33:137–149

Masloff S, Pöggeler S, Kück U (1999) The *pro1*(+) gene from *Sordaria macrospora* encodes a C_6 zinc finger transcription factor required for fruiting body development. Genetics 152:191–199

Nara F, Watanabe I, Serizawa N (1993a) Cloning and sequencing of the 3-phosphoglycerate kinase (PGK) gene from *Penicillium citrinum* and its application to heterologous gene expression. Curr Genet 23:134–140

Nara F, Watanabe I, Serizawa N (1993b) Development of a transformation system for the filamentous ML-236B (compactin)-producing fungus *Penicillium citrinum*. Curr Genet 23:28–32

Noshiro M, Okuda K (1990) Molecular cloning and sequence analysis of cDNA encoding human cholesterol 7 alpha-hydroxylase. FEBS Lett 268:137–140

Proctor RH, Hohn TM (1993) Aristolochene synthase. Isolation, characterization, and bacterial expression of a sesquiterpenoid biosynthetic gene (*Ari1*) from *Penicillium roqueforti*. J Biol Chem 268:4543–4548

Reece RJ, Ptashne M (1993) Determinants of binding-site specificity among yeast C6 zinc cluster proteins. Science 261:909–911

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schjerling P, Holmberg S (1996) Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. Nucleic Acids Res 24:4599–4607

Serizawa N, Serizawa S, Nakagawa K, Furuya K, Okazaki T, Terahara A (1983) Microbial hydroxylation of ML-236B (compactin). Studies on microorganisms capable of 3 beta-hydroxylation of ML-236B. J Antibiot 36:887–891

Takahashi M, Pischetsrieder M, Monnier VM (1997) Molecular cloning and expression of amadoriase isoenzyme (fructosyl amine:oxygen oxidoreductase, EC 1.5.3) from *Aspergillus fumigatus*. J Biol Chem 272:12505–12507

Todd RB, Andrianopoulos A (1997) Evolution of a fungal regulatory gene family: the $Zn(II)_2Cys_6$ binuclear cluster DNA binding motif. Fungal Genet Biol 21:388–405

Todd RB, Murphy RL, Martin HM, Sharp JA, Davis MA, Katz ME, Hynes MJ (1997) The acetate regulatory gene *facB* of *Aspergillus nidulans* encodes a $Zn(II)_2Cys_6$ transcriptional activator. Mol Gen Genet 254:495–504

Witte MM, Dickson RC (1988) Cysteine residues in the zinc finger and amino acids adjacent to the finger are necessary for DNA binding by the LAC9 regulatory protein of *Kluyveromyces lactis*. Mol Cell Biol 8:3726–3733

Woloshuk CP, Foutz KR, Brewer JF, Bhatnagar D, Cleveland TE, Payne GA (1994) Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. Appl Environ Microbiol 60:2408–2414

Woloshuk CP, Yousibova GL, Rollins JA, Bhatnagar D, Payne GA (1995) Molecular characterization of the *afl-1* locus *in Aspergillus flavus*. Appl Environ Microbiol 61:3019–3023

Yelton MM, Hamer JE, Timberlake WE (1984) Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc Natl Acad Sci USA 81:1470–1474

Yuan GF, Fu YH, Marzluf GA (1991) *nit-4*, a pathway-specific regulatory gene of *Neurospora crassa*, encodes a protein with a putative binuclear zinc DNA-binding domain. Mol Cell Biol 11:5735–5745