APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 753–756 Vol. 63, No. 2
0099-2240/97/$04.00+0

Regulated System for Heterologous Gene Expression in *Penicillium chrysogenum*

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Received 6 August 1996/Accepted 27 November 1996

A system for regulated heterologous gene expression in the filamentous fungus *Penicillium chrysogenum* was established. This is the first heterologous expression system to be developed for this organism. Expression of a recombinant fungal xylanase gene (*xylP*) and the cDNA for the human tear lipocalin (*LCN1*) was achieved by placing the encoding sequences under the control of the repressible acid phosphatase gene (*phoA*) promoter of *P. chrysogenum*. Secreted recombinant proteins were detected in the growth media of transformed *P. chrysogenum* cells by means of bioassays, zymogramography, and Western blotting. Levels of transcription and amounts of recombinant proteins secreted varied among transformants, mainly due to the copy number and the integration site of the expression vector on the fungal chromosome.

Filamentous fungi are saprophytic organisms secreting a wide array and high level of proteins involved in the breakdown and recycling of complex polymers from both plants and animal tissues (13). These characteristics and the existence of a well-established technology for large-scale fermentation of these organisms advanced their industrial application in secretion of heterologous proteins. Species of *Aspergillus* and *Trichoderma* have been extensively used as model organisms for diverse transformation and expression systems (20). Although *Penicillium chrysogenum* is of significant industrial importance and has the "generally recognized as safe" status of the U.S. Food and Drug Administration, only preliminary attempts have been made to utilize this fungus as a host for homologous and heterologous protein production and secretion. One of the major reasons might be that in contrast to *Aspergillus* and *Trichoderma*, no suitable promoter system for *P. chrysogenum* has been available so far.

We have recently cloned the gene of *P. chrysogenum* encoding a secreted phosphate-repressible acid phosphatase (*phoA*) (9), which holds several features required for an efficient expression system. The expression of *phoA* seems to be similar to that of phosphatases from *Saccharomyces cerevisiae* (1) and *Aspergillus niger* (15); thus, it can be turned on by the simple procedure of simultaneously lowering the P_i concentration and pH (14).

To demonstrate the feasibility of this system for recombinant protein production in *P. chrysogenum*, a fungal xylanase gene (10) and the cDNA encoding the human tear lipocalin (16) were fused as model genes to the promoter region of *phoA* in order to achieve expression of the recombinant genes by derepressing the *phoA* promoter.

Construction of expression vectors and transformation of *P. chrysogenum*. Vectors employed for the construction of expression plasmids were derived from pUC (21), Bluescript (19), and GEM (Promega). For propagation of plasmids, *Escherichia coli* TG2 (5) and JM 109 (21) were used. The *phoA* promoter sequence (9), the gene for the fungal xylanase (10), and the cDNA encoding the human tear lipocalin (16) were amplified by PCR from genomic fungal DNA (*phoA* promoter, *xylP*) and cDNA obtained from the human lacrimal gland (*LCN1*). At the fusion sides of the fragments, PCR primers with 5′ add-on sequences were used as adapters for PCR-mediated ligation (11). The overlapping PCR products were gel purified, and equimolar amounts were used as the templates in a second PCR with the left- and the right-most primers of the hypothetical full-length product. The PCR fusion product was inserted into a GEM-T vector, resulting in pPLgXB 5Z(+) (xylanase expression vector), and into a Bluescript vector, resulting in pPLLCN1 KS(−) (tear lipocalin expression vector). In both constructs (Fig. 1), the original signal peptides encoding sequences and termination sites of the model genes were retained. The junction regions of the fusion fragments were verified by sequencing.

For the transformation procedure, the nitrate reductase (*niaD*)-deficient *P. chrysogenum* strain CRB 47, which is a derivative of the high-efficiency penicillin producer strain P-2 (12), was employed. A plasmid carrying the *niaD* gene of *P. chrysogenum* (6) was used as a selection marker for cotransformation. Protoplasts were transformed by the method of Cantoral et al. (3), obtaining a transformation efficiency of two to five transformants/μg of selection vector. DNA was isolated from transformants growing on minimal medium (4), and screening of positive clones was performed by PCR, with one primer based on the *phoA* promoter sequence and the other deduced from the coding sequences of the genes analyzed. About 30% of cells transformed with the selectable marker gene were also found to carry the specific expression constructs. In order to obtain homokaryotic transformants, colonies from single homokaryotic spores were picked and genomic integration of the expression constructs was verified again by PCR and Southern analysis.

Determination of integration site and copy number. To confirm the integration sites of the expression constructs in the genome of *P. chrysogenum* and to determine the number of copies integrated, Southern blot analyses were performed (not shown). Genomic DNA from *P. chrysogenum* transformants was isolated (17), digested, separated on a 0.7% agarose gel, and blotted onto nylon membranes. The membranes were hybridized with ^{32}P-labeled PCR fragments of the two model

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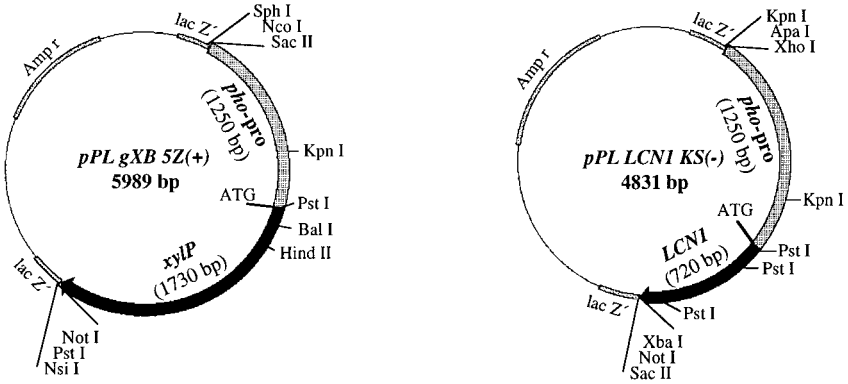

FIG. 1. Schematic representations of the *xylP* expression vector pPLgXB 5Z(+) and the *LCN1* expression vector pPLLCN1 KS(−). The *phoA* promoter sequence (shaded area) was fused to the model genes (filled area) by recombinant PCR. Arrows indicate the orientations of the coding sequences.

genes. Recombinant sequences were detected in all transformants selected. Sites of genomic integration were random in all cases, in spite of the presence of the homologous *phoA* promoter region (1,250 bp) and the coding region of the endogenous *xylP* gene (1,730 bp). Furthermore, the number of plasmid copies integrated into the genome ranged from 1 to >12 per strain (Table 1). Multicopy transformants predominantly carried oligomers of the *xylP* expression vector.

The *LCN1* transformant strain selected held a single copy of the corresponding expression vector integrated ectopically.

Secretion of the recombinant proteins in *P. chrysogenum*. All liquid cultures were inoculated with 10^8 conidiospores. Transformed *P. chrysogenum* strains were grown aerobically at 25°C in 500-ml Erlenmeyer flasks on a rotary shaker (250 rpm) in 125 ml of medium containing 3.0 g of $NaNO_3$, 0.5 g of $MgSO_4 \cdot 7\ H_2O$, 0.1 g of $FeSO_4 \cdot 7\ H_2O$ and 20.0 g of sucrose in 1 liter of 25 mM potassium phosphate buffer (pH 6.0). The *phoA* promoter was derepressed by shifting the mycelia into P^- medium (0.25 mM potassium phosphate buffer, 25 mM citrate buffer [pH 4.8]). For measuring induction of the recombinant *phoA* promoter, the activity of endogenous PhoA was determined by a phosphatase assay with *p*-nitrophenyl phosphate (Sigma) as the substrate (7). Cultures were grown until the activity of endogenous PhoA reached 0.1 U/ml of growth medium.

TABLE 1. Yields, levels of transcription, and activities of recombinant xylanases of transformants carrying various copy numbers of the expression construct pPLgXB 5Z(+) growing under *phoA*-derepressing conditions

Strain[a]	Copy no.	XylP activity			Yield (mg/liter)	*xylP* mRNA level (%)
		U/g of mycelia	U/liter	U (%)		
1311	1	91	1,900	39	1.8	41
2811	1	94	1,900	39	1.8	45
2911	6	331	6,600	135	6.2	142
2431	9	211	4,200	86	4.0	92
2531	9	213	4,400	90	4.2	97
1300	>12	380	8,200	167	7.8	175
1000		ND[b]	0	0	0	0
1000*		ND	4,900	100	4.6	100

[a] Strain 1000 is mutant strain CRB 47 transformed only with the *niaD* selection vector (negative control). Results were compared with the transcription level and the activity of the endogenous xylanase (100%) of the control strain grown with oat spelt xylan as the sole carbon source (1000*).

[b] Not determined.

In order to determine the amount of recombinant proteins secreted by the transformed cells, the supernatant was concentrated 1:20 (Ultrafree-CL filter 1000; Millipore) and proteins were analyzed by native polyacrylamide gel electrophoresis (PAGE), zymogramography, sodium dodecyl sulfate (SDS)-PAGE, or Western blot analysis. Activity of the recombinant xylanase in native polyacrylamide gels was localized with agarose replicas containing 0.5% remazol brilliant blue–xylan as the substrate (2). Proteins in the medium were subjected to electrophoresis in gels containing 10% acrylamide with a basic buffer system. From Fig. 2, it is evident that cells harboring the recombinant plasmid secreted an active xylanase detectable as a white zone within a dark agarose gel (lane 5). No xylanase activity could be observed after derepression of the endogenous *phoA* gene in a control strain harboring only the selection plasmid (lane 6). A faint band on the activity gel (lane 4) indicates that the *phoA* promoter is not completely inactive under the repressing conditions used.

To quantify the amount of recombinant XylP, the prominent 36-kDa xylanase bands on SDS polyacrylamide gels (Fig. 3) were scanned with a densitometer, model PDSI, equipped with Image-Quant software (Molecular Dynamics, Sunnyvale, Cal-

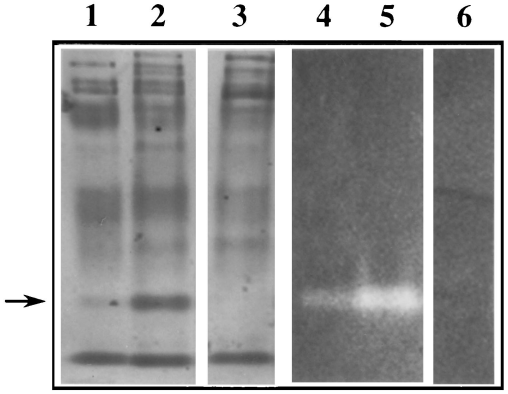

FIG. 2. PAGE of proteins in the growth medium of an *xylP* transformant strain, under nondenaturing conditions. Lanes contain 20 μl of culture medium (20-fold concentrated) and were stained with Coomassie brilliant blue (lanes 1 to 3) or developed as zymograms with remazol brilliant blue-xylan agar gels (lanes 4 to 6). Active recombinant XylP (indicated with an arrow) appears as a white zone within the dark agar gel. Lanes 1 and 4, strain 2811 with its *phoA* promoter repressed; lanes 2 and 5, strain 2811 with its *phoA* promoter derepressed; lanes 3 and 6, control strain 1000 (transformed only with the selection vector) with its *phoA* promoter derepressed.

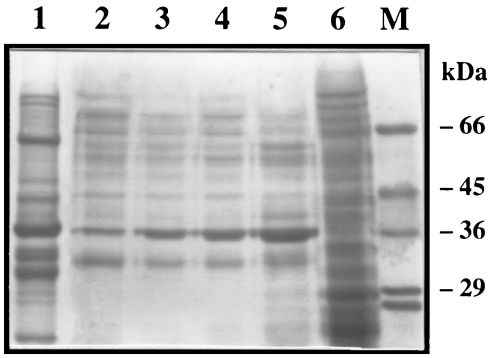

FIG. 3. SDS-PAGE analyses of secreted proteins of *P. chrysogenum* strains transformed with the expression vector pPLgXB 5Z(+). Lanes contain 20-μl aliquots of growth medium (20-fold concentrated). Lane 1, control strain 1000 (transformed only with the selection vector) with induced endogenous *xylP*; lane 2 to 5, strains 2811, 2431, 2531, and 2911, respectively, with their *phoA* promoters derepressed; lane 6, strain 2911 with its *phoA* promoter repressed; lane M, molecular size markers. The prominent 36-kDa bands in lanes 2 to 5 represent various levels of expression of the recombinant xylanase in four different transformants.

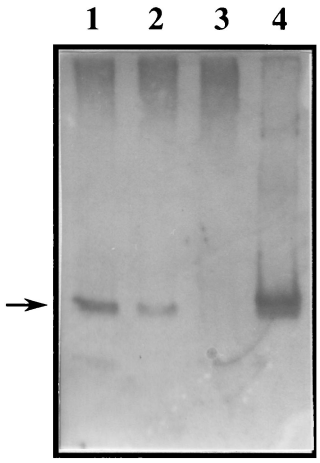

FIG. 4. Western blot of proteins in the growth medium of the *LCN1* transformant strain incubated with an LCN1-specific antiserum. The position of the recombinant human protein is indicated with an arrow. Lanes 1 and 2, 20 μl of culture medium (20-fold concentrated) with the *phoA* promoter derepressed and repressed, respectively; lane 3, control strain 1000 with the *phoA* promoter derepressed; lane 4, 1 μg of purified LCN1 from human tear fluid.

if.) and compared with a protein standard (glycerinaldehyde-3-phosphate dehydrogenase; Sigma). Results corresponded with the xylanase activities in the medium as determined by a spectrophotometric bioassay (8) using oat spelt xylan (Sigma) as the substrate and D-(+)-xylose (Sigma) as the standard (Table 1). It is worth mentioning that yields of secreted recombinant XylP of some strains surpassed that of the endogenous xylanase induced in control strain 1000 grown with oat spelt xylan as the sole carbon source. The amount of recombinant protein produced correlated in most cases with the copy number of constructs integrated in the fungal genome. Despite the strong tendency of high-copy-number transformants to be high producers, no strict relationship was evident from our data; e.g., strain 2911 (six copies integrated) showed xylanase activity in the supernatant about 50% higher than those of strains 2431 and 2531 (both with nine copies integrated). As with other systems, the genomic site of integration also seemed to affect xylanase expression (13).

Secretion of the recombinant human tear lipocalin by the transformant harboring the human cDNA under *phoA* control was investigated by Western blot analysis (Fig. 4) with an LCN1-specific antiserum. The immunoreactive 20-kDa band confirmed the presence of the human protein in the medium of the corresponding transformant. It should be mentioned that this secretion is obviously mediated by the original human signal sequence. The presence of an immunoreactive band under noninducing conditions again demonstrates that the *phoA* promoter is not strictly repressed under the conditions used.

Northern blot analyses. To determine if *xylP* and *LCN1* were efficiently transcribed in *Penicillium* transformants and to give a rough correlation between transcription and protein production, Northern blot analyses were performed. Total RNA was isolated from *P. chrysogenum* by the Trisolv method (Biotecx), size fractionated on a 1.2% agarose-formaldehyde gel, and blotted onto nylon membranes (18). Hybridization with ^{32}P-labeled PCR fragments of the recombinant genes showed specific *xylP* transcripts and specific *LCN1* transcripts in all transformants (but not in the untransformed control strains). Northern blots hybridized with *xylP* were quantified by a Storm PhosphorImager equipped with Image-Quant software (Table 1). Values were corrected for the total amount of RNA by using the amount of actin RNA as an internal control. The yield of protein correlated with the mRNA level, indicating transcription to be the main regulatory determinant of this system.

The blots that hybridized with the human *LCN1* gene (Fig. 5) showed a distinct transcript of 800 bp in the strain transformed with the *LCN1* expression construct under derepressed growing conditions (lane 2), whereas no transcript could be detected in a control strain harboring only the selection vector (lane 4).

In sum, our investigations clearly demonstrate the usefulness of the system described for secretion of recombinant proteins

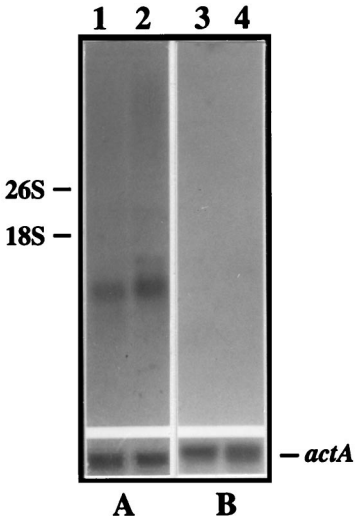

FIG. 5. Northern blot analyses of the *P. chrysogenum* strain transformed with the *LCN1* expression vector (A) and the control strain transformed only with the selection vector (B). Total RNA (5 μg) was hybridized with a radiolabeled *LCN1* fragment. Lanes 1 and 2, RNA of the *LCN1* transformant grown under *phoA* promoter-repressing and -derepressing conditions, respectively; lanes 3 and 4, RNA of the control strain grown under *phoA* promoter-repressing and -derepressing conditions, respectively. Subsequently, blots were stripped and rehybridized with *actA*, which encodes γ-actin from *P. chrysogenum* (GenBank accession number U61733). The positions of 26S and 18S RNAs are indicated.

in *P. chrysogenum*. The production of xylanase mediated by the *phoA* promoter gives an example for the economical relevance of the system, since it is induced by the simple and inexpensive manipulation of lowering the P_i instead of adding an expensive inducer (xylan) to the growth medium. The universal feasibility of the expression system was confirmed by using the human *LCN1* cDNA as a model gene, which was produced and secreted as well.

We thank our colleague Paul Holzfeind for his contribution to this work and helpful discussions.

REFERENCES

1. **Bajwa, W., B. Meyhack, H. Rudolph, A. M. Schweingruber, and A. Hinnen.** 1984. Structural analysis of the two tandemly repeated acid phosphatase genes in yeast. Nucleic Acids Res. **12:**7721–7739.
2. **Biely, B. J., O. Markovic, and D. Mislovicoca.** 1985. Sensitive detection of endo-1,4-β-glucanases and endo-1,4-β-xylanases in gels. Anal. Biochem. **144:** 147–151.
3. **Cantoral, J. M., B. Diez, J. L. Barredo, E. Alvarez, and J. F. Martin.** 1987. High-frequency transformation of *Penicillium chrysogenum*. Bio/Technology **5:**494–497.
4. **Cenis, J. L.** 1992. Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Res. **20:**2380.
5. **Gibson, T. J.** 1984. Studies on the Epstein Barr virus genome. Ph.D. thesis. Cambridge University, Cambridge, United Kingdom.
6. **Gouka, R., J. W. van Hartingsveldt, R. A. L. Bovenberg, C. A. M. J. J. van den Hondel, and R. F. M. van Gorcom.** 1991. Cloning of the nitrate-nitrite reductase gene cluster of *Penicillium chrysogenum* and use of the *niaD* gene as a homologous selection marker. J. Biotechnol. **20:**189–200.
7. **Haas, H., B. Redl, E. Leitner, and G. Stöffler.** 1991. *Penicillium chrysogenum* extracellular acid phosphatase: purification and biochemical characterization. Biochim. Biophys. Acta **1074:**392–397.
8. **Haas, H., E. Herfurth, G. Stöffler, and B. Redl.** 1992. Purification, characterization and partial amino acid sequences of a xylanase produced by *Penicillium chrysogenum*. Biochim. Biophys. Acta **1117:**279–286.
9. **Haas, H., B. Redl, E. Friedlin, and G. Stöffler.** 1992. Isolation and analysis of *Penicillium chrysogenum phoA* gene encoding a secreted phosphate-repressible acid phosphatase. Gene **113:**129–133.
10. **Haas, H., E. Friedlin, G. Stöffler, and B. Redl.** 1993. Cloning and structural organization of a xylanase-encoding gene from *Penicillium chrysogenum*. Gene **129:**237–242.
11. **Higuchi, R.** 1990. Recombinant PCR, p. 177–183. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. Academic Press, Inc., San Diego, Calif.
12. **Lein, L.** 1983. The panlabs penicillin strain improvement program, p. 105–139. *In* Z. Vanek and Z. Hostalek (ed.), Overproduction of microbial metabolites. Butterworths, Boston, Mass.
13. **MacKenzie, D. A., D. J. Jeenes, N. J. Belshaw, and D. B. Archer.** 1993. Regulation of secreted protein production by filamentous fungi: recent developments and perspectives. J. Gen. Microbiol. **139:**2295–2307.
14. **MacRae, W. D., F. P. Buxton, D. I. Gwynne, and R. W. Davies.** 1993. Heterologous protein secretion directed by a repressible acid phosphatase system of *Aspergillus niger*. Gene **132:**193–198.
15. **MacRae, W. D., F. P. Buxton, S. Sibley, S. Garven, D. I. Gwynne, R. W. Davies, and H. N. Arst, Jr.** 1988. A phosphate-repressible acid phosphatase gene from *Aspergillus niger*: its cloning, sequencing and transcriptional analysis. Gene **71:**339–384.
16. **Redl, B., P. Holzfeind, and F. Lottspeich.** 1992. cDNA cloning and sequencing reveals human tear prealbumin to be a member of the lipophilic-ligand carrier protein superfamily. J. Biol. Chem. **267:**20282–20287.
17. **Rozman, D., and R. Komel.** 1994. Isolation of genomic DNA from filamentous fungi with high glucan level. BioTechniques **16:**382–384.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Shortl, J. M., J. M. Fernadez, J. A. Sorge, and W. D. Huse.** 1988. Lambda-ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res. **16:**7583–7600.
20. **van den Hondel, C. A. M. J. J., P. J. Punt, and R. F. M. van Gorcom.** 1991. Heterologous gene expression in filamentous fungi, 396–428. *In* J. W. Bennett and L. L. Lasure (ed.), More gene manipulations in fungi. Academic Press, Inc., San Diego, Calif.
21. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.