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Heterologous transformation of *Agrocybe aegerita* with a bacterial neomycin-resistance gene fused to a fungal promoter-like DNA sequence

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Abstract DNA sequences of the basidiomycete *Agrocybe aegerita* were cloned in *E. coli* based on their ability to drive the expression of the bacterial promoterless tetracycline (Tc)-resistance gene. A 0.48% frequency of the cloned sequences promoted antibiotic-resistance. The sequence conferring the highest Tc resistance (40 µg/ml) was selected to drive the expression in *E. coli* of two other promoterless genes encoding chloramphenicol and neomycin resistance. One of the derivative vectors, pN13-A2, carrying a chimeric neomycin-resistance gene, was used to transform an *A. aegerita* neomycin-sensitive strain by protoplast electroporation. Transformation frequencies ranged from 1 to 2.8 transformants per µg of DNA per 10^3 viable cells, in a relatively high background of spontaneous-resistant colonies (2% of the surviving protoplasts). Molecular analyses showed that transformation had occurred by the integration of pN13-A2 sequences, either ectopically or at the resident locus carrying the *A. aegerita* promoter-like sequence, with probable molecular rearrangements. The nucleotide sequence of the promoter-like fragment revealed the presence of a CT motif that is known to be involved in a promoter function in some highly expressed genes of filamentous fungi.

Key words *Agrocybe aegerita* · Heterologous transformation · Neomycin resistance · Electroporation

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Introduction

Agrocybe aegerita is an edible cultivated Agaricale for which a transformation system, relying upon the complementation of uracil auxotrophy with the homologous *URA1* gene, has recently been developed (Noël and Labarère 1994). This system allowed determination of the conditions for the uptake of DNA and a study of the molecular fate of the vector in transformants, but its use is restricted to a mutant genetic background. A prerequisite for the study and genetic improvement of the industrially cultivated edible basidiomycetes is transformation based on dominant selectable markers such as antibiotic resistance.

Among the heterologous transformation systems developed in basidiomycetes (for a review see Peng et al. 1992), the vector pAN7-1 (Punt et al. 1987), conferring hygromycin resistance under the control of *Aspergillus nidulans* transcriptional signals, has often been used (Barret et al. 1990; Marmeisse et al. 1992; Peng et al. 1992). However, because of a high frequency of spontaneous hygromycin-resistant colonies, this vector did not allow direct selection of *Schizophyllum commune* transformants (Mooibroek et al. 1990). For this reason, the heterologous transformation of *A. aegerita* was based on the resistance to aminoglycosides, whose toxicity has already been reported in *A. aegerita* (Labarère et al. 1989). The aminoglycoside-resistance genes were used as genetic markers to select transformants in several types of cells and organisms, such as mamalian cells (Southern and Berg 1982; Gorman et al. 1983), plants (Herman et al. 1986; Teeri et al. 1986), and fungi (Jimenez and Davies 1980; Hirth et al. 1982; Suarez and Eslava 1988; Wang et al. 1988; Randall et al. 1991).

As an extension of the use in transformation of antibiotic-resistance markers which are subordinate to fungal transcriptional signals, we report here a simple strategy for the characterization of DNA sequences from *A. aegerita* acting as promoters in *E. coli* when cloned upstream from promoterless antibiotic-resistance genes. A heterologous transformation vector, carrying a bacterial neomycin-resistance gene fused to a fungal promoter-like sequence,

was constructed and used to transform a *A. aegerita* neomycin-sensitive strain by protoplast electroporation.

Materials and methods

Strains, growth conditions and plasmids

The *A. aegerita* homokaryotic strain G30 *A3 B3*, derived from the germination of a basidiospore of the wild dikaryotic strain WT 51 (Noël et al. 1991), was used for the cloning of promoter-like sequences and transformation experiments. Mycelial cultures were grown on solid or liquid CYM (Raper and Hoffman 1974) at 27°C in the dark.

Escherichia coli strain HB101 (Maniatis et al. 1982) was used for the cloning experiments and for plasmid propagation. Bacteria were grown in LB medium at 37°C. Unless otherwise stated, ampicillin (Ap), tetracycline (Tc), chloramphenicol (Cm) and neomycin (Nm) were used at concentrations of 50 µg/ml, 15 µg/ml, 25 µg/ml and 15 µg/ml, respectively.

The vector pT13 (Simoneau and Labarère 1990) was used for the cloning of promoter-active DNA sequences. This vector is a derivative of pBR322 in which the *Eco*RI/*Hin*dIII fragment located in the promoter region of the Tc-resistance gene was replaced by the polylinker region of pUC13, leading to the inactivation of the resistance gene. The chloramphenicol- and neomycin-resistance genes were derived from pBR328 (Bolivar et al. 1977) and pRSVneo (Gorman et al. 1983), respectively.

DNA isolation from *A. aegerita* and sonication conditions for shot-gun cloning

DNA was isolated as previously reported (Noël and Labarère 1989). Time points of 5, 10, 15, and 20 s of sonication were performed with constant amounts (500 ng) of high-molecular-weight DNA fragments (160 kb) using a Vibra Cell sonicator (adjusted to position 2), and the size of the DNA fragments was determined by 1.2% agarose-gel electrophoresis. A sonication time of 20 s generated a majority of DNA fragments with an average size of 0.5 kb.

Protoplast electroporation

Electroporations were performed as previously described (Noël and Labarère 1994) using 10^6 protoplasts in suspension in 100 µl of electroporation buffer (10 mM potassium phosphate pH 7.2, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.6 M sorbitol), and 20 µg of plasmid DNA. Electroporations were realized with the Cellject Electroporation System (Eurogentec) in cuvettes having a 0.2-cm interelectrode distance. Electric field strengths of 2.25 and 2.5 kV/cm were applied under a capacitance of 40 µF. After electroporation, protoplasts were diluted in 10 ml of non-selective CYM medium and incubated for 48 h at 27°C. For regeneration, 1-ml aliquots of diluted protoplasts were mixed with 7 ml of CYM containing 1% Sea plaque agarose (FMC BioProducts) kept at 40°C, 0.5 M sucrose, and different concentrations of G418 (Gibco BRL). The protoplast suspension was then overlayed on 0.5-M sucrose CYM plates supplemented with G418. The regenerating mycelial colonies were observed after 8 days incubation at 27°C.

Standard procedures

Bacterial transformation was carried out according to Hanahan (1985). Plasmid DNA was prepared by the alkaline-lysis method and purified by a CsCl density gradient (Maniatis et al. 1982). Endonucleases, modifying enzymes, and bacterial alkaline phosphatase were used as specified by the suppliers (BRL, Boehringer Mannheim and Amersham). For vector construction, digested DNA fragments were separated and excised from Sea plaque agarose-gel electrophoresis, and in-gel ligated with T4 DNA ligase. DNA transfer from 0.8% agarose-gel electrophoresis to a Hybond-N^+ nylon membrane (Amersham) was carried out as recommended by the manufacturer. Probes were labeled with a random primer DNA labeling kit (BRL) using 30 µCi of α-^{32}P-dCTP at 3000 Ci/mmol (Amersham). In order to make a probe from a restriction fragment, the DNA fragment was excised from Sea Plaque agarose (FMC)-gel electrophoresis and in-gel labeled as described above. Hybridizations were performed in standard 5 × SSC buffer (Maniatis et al. 1982) for 16 h at 65°C. The final post-hybridization washes were at 65°C, in 0.1 × SSC, 0.1% (w/v) SDS. DNA sequencing of the promoter-like fragment was performed after re-cloning in plasmid pUC18, according to the method of Sanger et al. (1977), with the Sequenase system (US Biochemical Corp.) and [α-^{32}S]dATP. Four 18-mer oligo primers (Eurogentec) were needed to obtain the full sequence on both strands.

Results

Cloning promoter-like sequences from *A. aegerita* in *E. coli*

For the selection of *A. aegerita* DNA sequences acting as promoters in *E. coli*, end-repaired sonicated DNA fragments were cloned at the *Sma*I site of pT13, upstream of the promoterless *Tc* bacterial gene. The ligation mixture and the control pT13 were separately used to transform *E. coli* to Ap resistance. Over 2500 clones replica-plated from each transformation experiment on Tc-supplemented medium, 0.48% of the recombinant clones derived from the ligation mixture was able to grow on 15 µg/ml of Tc, while all the clones transformed with pT13 could no longer grow on 5 µg/ml of Tc.

The Tc-resistance level of 11 recombinant clones was determined in liquid LB medium supplemented with increasing concentrations of the antibiotic (from 5 µg/ml to 100 µg/ml). The resistance levels ranged from 15 µg/ml, i.e. the minimal concentration used for the selection, to 40

Table 1 Resistance levels conferred on *E. coli* by plasmids carrying the *A. aegerita* promoter-like sequence fused to *Tc*-, *Cm*- and *Nm*-resistance genes and by the control plasmids carrying the genes with or without their own bacterial promoters
Table 1

Plasmid	Structural antibiotic resistance gene	Promoter	Resistance level (µg/ml)[a]
pT13	*Tc*	None	5
pT13-A2	*Tc*	Fungal	40
pT13-A2*id*	*Tc*	Fungal	40
pT13-A2*inv*	*Tc*	Fungal	5
pBR322	*Tc*	Bacterial	75
pC13	*Cm*	None	10
pC13-A2	*Cm*	Fungal	80
pBR328	*Cm*	Bacterial	500
pN13	*Nm*	None	5
pN13-A2	*Nm*	Fungal	100
pNeo	*Nm*	Bacterial	1250

[a] The resistance levels conferred on *E. coli* by the different plasmids was determined by cultivating *E. coli* for 16 h without shaking in liquid LB medium containing increasing concentrations of the relevant antibiotic

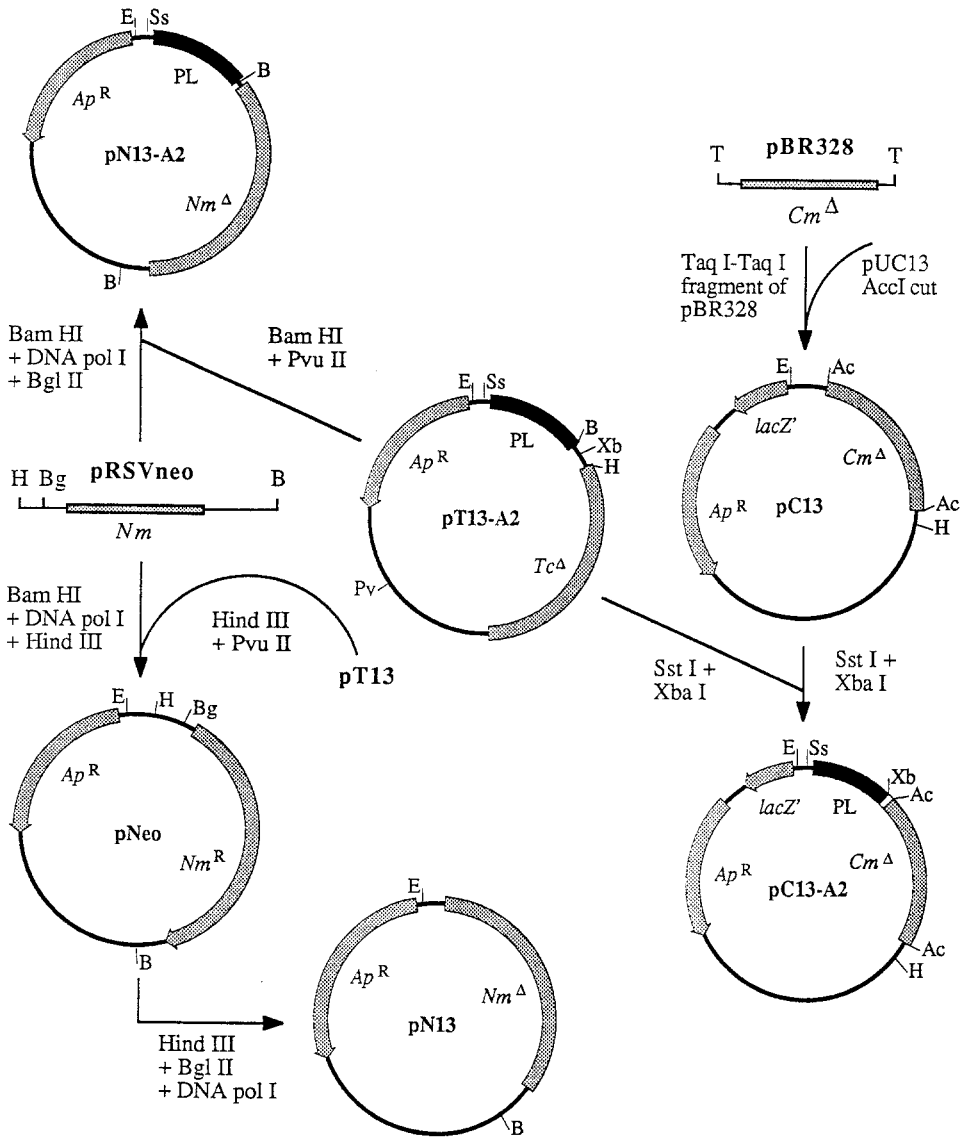


Fig. 1 Construction of pC13-A2 and pN13-A2 from pT13-A2, and control plasmids pC13, pN13 and pNeo. *Black box*: the 0.8-kb promoter-active DNA fragment from *A. aegerita*. When present, the symbol Δ means that the gene is devoid of its endogenous promoter. *Ap* ampicillin, *Cm* chloramphenicol, *Nm* neomycin, *Tc* tetracycline; Ac *Acc*I, B *Bam*HI, Bg *Bgl*II, E *Eco*RI, H *Hin*dIII, Pv *Pvu*II, Ss *Sst*I, T *Taq*I, Xb *Xba*I, *DNA pol I* DNA polymerase I-Klenow fragment. Plasmids are not represented on the same scale

μg/ml. Concurrently, the plasmid content of each clone was extracted and digested with *Sst*I/*Bam*HI (two restriction sites flanking the insert) to determine the size of the cloned inserts promoting expression of the resistance gene. No correlation existed between the resistance level and the size of the cloned fragments, which varied from 0.2 to 1.45 kb. For further experiments, we employed the plasmid pT13-A2 containing an *A. aegerita* insert of 0.9 kb and conferring a Tc-resistance level of 40 μg/ml on *E. coli*.

Characterization of the DNA sequence cloned in pT13-A2 and construction of chimeric genes encoding chloramphenicol and neomycin resistance

To determine whether the *A. aegerita* insert of pT13-A2 could carry a specific fixation site for the *E. coli* RNA polymerase, the polarity of the fungal insert was inverted in pT13-A2. For this, pT13-A2 was digested with *Sst*I/*Bam*HI, the resulting 4.3-kb (pBR322-derived) and 0.9-kb (insert) restriction products were purified, separately end-repaired to blunt-end, ligated, and transformed into *E. coli*. The polarity of the insert was determined by a restriction analysis since re-cloning the insert in the same orientation (pT13-A2*id*) resulted in the creation of a new unique *Cla*I restriction site at the junction of the *Bam*HI-repaired ends of the insert and of the vector, while an insertion in the opposite direction (pT13-A2*inv*) did not.

Two bacterial clones harboring pT13-A2*id* and pT13-A2*inv* respectively, were tested for Tc resistance (Table 1). The Tc-resistance level conferred by pT13-A2*id* was as high as that obtained with the original pT13-A2 (40 μg/ml), whereas the change of insert polarity in pT13-A2*inv* abolished the resistance gene expression. This result strongly suggested that the bacterial RNA polymerase had a specific fixation site on the fungal fragment.

The promoter activity of the *A. aegerita* sequence was then studied through its ability to drive the expression of

*Eco*RI .... TGGCCTGGGA ACGTGGGAAA TTTGCTTGAA GACTCCTTTG GTATGTACTT TGCAACTACC 60

AACCACCGTG TGTATTATGA GTCAGACGTC GACGGTCTCT TCATTCGACA CCCTCGGGCC AGTTCTCTTA 130

TGGTTATTGT CTTCGAACCC GAACACATGA CCGATCGAAT ATTAAGGTTG CACGGACGGC ATTGCCCCAA 200

ATCCCCAGAT TCAACCCTTC TAGCCTTGAT CTCAGGTGAC GCGCTAACGT TCGGAACATC TCCCCCAAGT 270

TTCTCAATCC CAACGTCAGC AACCCCGTCA GAAACATCAA TCATTAGATA ATTGTCTCTT CATTACTAAA 340

TGACTCCATG ACATGTACAA AATGATGTCC ATTGCATCGG ACGCCCAGGT ATTTGACCCG TAGCCTCCCT 410

GGCTACTAGC AGATTTTCCA AAACCCATCT TCTGCATATT ACTAGCAGAG CACTATTCCC TCGTTCAGTG 480

TCCCATCCCG GCTCGGTAGC ATCGCGGCCC AAATATTCCA GCACTGGGTA TTGATGCAGC ATATAACCCG 550

TACACCATGC GCCCGGTCAC CCTCTTCCCT CAACGTTTCC CGTCTTGAAG CTACTGGCCT GAGTTGTCTC 620

ATGGCAAGCC CCCATCTGCT GCAGGCCACA AACATCATTC CCCTATTCTC CGCCTGGACT GCCACTGACT 690

TTCTCTTCTT CTCTTCTATC GGCGTTCCTT TTACCACTTC TGTGTATGTA TTTTTTCAGG ATATCAATAC 760

TCTTCGCCTG GCACCGCATT TCTGGTCCCG AGTGCATTTC TTTCGGCACC ATTGGCTACT ATGCGACTCA 830

CGGAGCTGGC ACTCTGGCAT CCTGCGCACA CCTCCGTTTG TTTCCTTCTT GACAAATTAT GT ...*Bam*HI

Fig. 2 Nucleotide sequence of the 892-bp promoter-like DNA fragment of *A. aegerita*. The *numbers* on the right side indicate nucleotide positions. Putative CAAT and TATA boxes are *underlined*. Positions of the CT motif preceding an ATG initiation codon are both indicated by *wavy lines*

two other promoterless bacterial genes coding for chloramphenicol (Cm) and neomycin (Nm) resistance (Fig. 1). For the construction of the chimeric Cm^R gene, a *Taq*I fragment from pBR328 containing the entire *Cm* coding region without its endogenous promoter was inserted into the *Acc*I site of pUC13 to produce plasmid pC13. The small *Sst*I/*Xba*I fragment of pC13 was then replaced by the *A. aegerita* promoter-like sequence to derive the plasmid pC13-A2. For the construction of the hybrid Nm^R gene, the major part of the *Tc* coding region of pT13-A2 was removed with *Bam*HI/*Pvu*II and replaced by the *Bgl*II/*Bam*HI fragment of pRSVneo, containing the promoterless *Nm* coding region, to give the plasmid pN13-A2. The positive control plasmid, pNeo, was created by replacing the *Hin*dIII/*Pvu*II fragment of pT13 with the *Hin*dIII/*Bam*HI fragment of pRSVneo overlapping the entire *Nm* coding region with its own promoter. The negative control plasmid, pN13, was obtained from pNeo by the deletion of a *Hin*dIII/*Bgl*II fragment located in the promoter of the Nm^R gene.

The resistance levels conferred by the plasmids pC13-A2, pN13-A2, as well as control plasmids pBR328, pC13, pNeo and pN13, were determined in *E. coli* (Table 1). The bacteria transformed with the negative control plasmids pC13 and pN13 were sensitive to 10 µg/ml of Cm and 5 µg/ml of Nm, respectively. These values correspond approximately to the minimal inhibitory concentrations of these antibiotics for *E. coli* HB101. Transformation of *E. coli* with pC13-A2 or pN13-A2 resulted in an increase in resistance from 8-fold (80 µg/ml) for Cm to 20-fold (100 µg/ml) for Nm. Although these resistance levels were always lower than those observed when the resistance genes are under the control of their own promoters, they suggested that an active protein was produced from both chimeric genes.

Sequence of the promoter-like fragment

Sequencing of the promoter-like fragment was performed after subcloning into pUC18 an *Eco*RI/*Bam*HI restriction fragment derived from pN13-A2. The sequence was constituted by 892 bp, containing 49% GC. Typical eukaryotic promoter sequences are not present in this sequence (Fig. 2), although elements resembling TATA and CAAT boxes can be found, notably at positions 541–546 (ATATAA) and 509–514 (CCAAAT). More interesting is the presence of a 19-bp CT block located at position 689–707, 28 bp upstream of an ATG which could constitute an initiation codon, suggesting a possible role as promoter element.

Transformation of an *A. aegerita* neomycin-sensitive strain with pN13-A2

In a previous study (Labarère et al. 1989), we reported that the mycelium of *A. aegerita* was about ten-times more sensitive to antibiotics from the aminoglycoside family than to chloramphenicol. Accordingly, we used the vector pN13-A2 (5.5 kb) in an attempt to transform protoplasts of the homokaryotic strain G30, for which a neomycin concentration of 500 µg/ml inhibits 90% of the vegetative growth when compared to growth on a medium without antibiotic. Transformation was achieved by electroporation with electric parameters (voltage/resistance) known to give either a high transformation efficiency or a high number of stable transformants (Noël and Labarère 1994). After electroporation of 10^6 protoplasts in the presence of 20 µg of pN13-A2 or pBR322 (control), the protoplasts

Table 2 Results of the transformation of the *A. aegerita* strain G30 with pN13-A2 and pBR322 by protoplast electroporation

Experiment (electric parameters)	Vector	Percentage of resistant colonies selected on G418 (g/l)[a]				Transformation efficiency[b]
		0.25	0.5	0.75	1.0	
Exp 1	pBR322	2.08	0	0	0	
(500 V/1320 Ω)	pN13-A2	7.14	0.6	0	0	56 (2.8)
Exp 2	pBR322	0.75	0	0	0	
(450 V/282 Ω)	pN13-A2	2.25	0.25	0.25	0	20 (1.0)

[a] Expressed per 100 viable regenerable protoplasts after electroporation
[b] Expressed per 100 viable regenerable protoplasts before electroporation and substracting the number of spontaneous colonies. The number of transformants per µg of DNA is given in brackets

were incubated for 48 h in liquid CYM without antibiotic, before plating on solid CYM supplemented with increasing concentrations of the neomycin analog G418 (from 250 to 1000 µg/ml).

Despite a high frequency of spontaneous resistant colonies, the number of $G418^R$ colonies obtained with pN13-A2 was about 3-fold higher than that recovered with the control pBR322 (Table 2). Moreover, spontaneous resistant colonies arose only on 250 µg/ml of G418, while some resistant colonies derived from pN13-A2 transformation experiments could be selected at higher antibiotic concentrations (500 and 750 µg/ml). The transformation efficiencies with pN13-A2, obtained by subtracting the number of spontaneous resistant colonies, was in the range of 20–56 putative transformants per 10^3 viable protoplasts before electroporation. When expressed per µg of plasmid DNA, the transformation efficiency varied from 1 to 2.8 transformants per 10^3 viable cells.

When subcultured on 250 µg/ml of G418, the putative transformants exhibited different growth rates. The resistance level was more accurately assessed by the ratio of the radial growth of the putative transformants, and of the untransformed strain G30, on CYM medium supplemented with 250 µg/ml of G418, to their growth on non-selective CYM (Fig. 3). The untransformed strain G30 showed 40% of growth on 250 µg/ml of G418 when compared to its growth on the medium without antibiotic. The results for 11 $G418^R$ colonies, randomly chosen on the 250-µg/ml G418 transformation plates, indicated that some of them had lost the resistant phenotype (colonies 3 and 6), while the resistance level of the other putative transformants ranged from 60 to 100%. As expected, all the colonies selected on 500 and 750 µg/ml of G418 had a resistance level of 100% on 250 µg/ml (data not shown). The molecular analyses were performed with 20 putative transformants (named TG1–TG20) which had retained a resistance level greater or equal to 60% on 250 µg/ml of G418 after three successive subcultures on CYM without antibiotic.

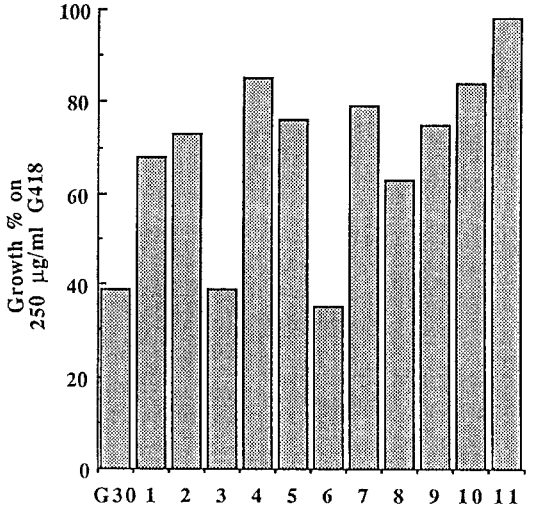
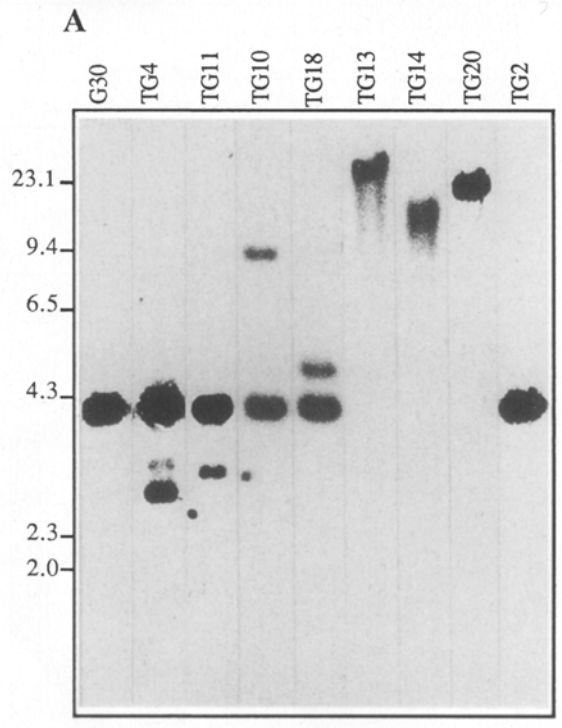


Fig. 3 Resistance levels of 11 colonies derived from pN13-A2 transformation, and of the untransformed control strain G30, on 250 µg/ml of G418. The resistance level is defined by the ratio: (radial growth on G418-supplemented CYM medium/radial growth on CYM) × 100. The standard deviation of the ratio, determined every 2 days during 12-days incubation, was always less than 5% (not represented on the diagram)

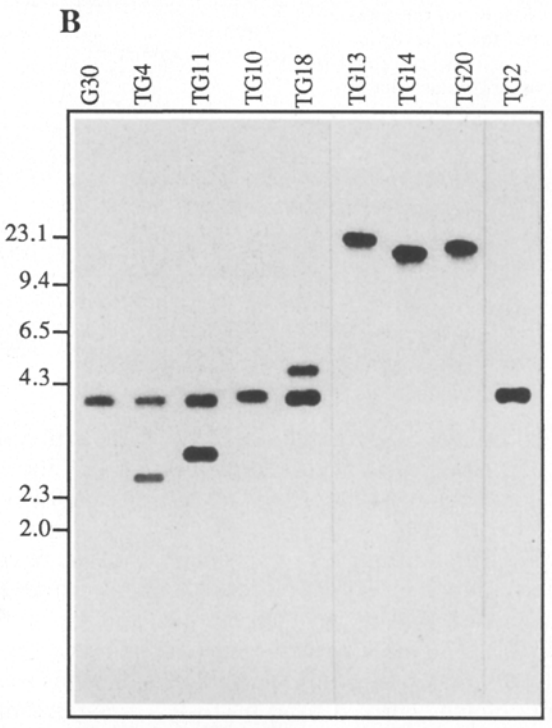
Molecular analyses of the transformants

The DNA from 20 $G418^R$ colonies and from the untransformed strain G30 was digested with *Eco*RI and subjected to a Southern analysis using pN13-A2 as a probe (Fig. 4A). The endogenous locus carrying the *A. aegerita* DNA sequence cloned in pN13-A2 corresponds to a 4.0-kb *Eco*RI fragment, as shown in the control strain G30. Three kinds of molecular events could be characterized in the $G418^R$ colonies: (1) eight transformants possessed additional signals, either smaller or greater in size than the 4.0-kb *Eco*RI fragment (see TG4, 10, 11 and 18); (2) five transformants lost the 4.0-kb signal and acquired a unique high-molecular-weight signal (see TG13, 14 and 20); and (3) seven colonies showed no change in their hybridization profile when compared to the untransformed strain G30 (e.g. TG2).

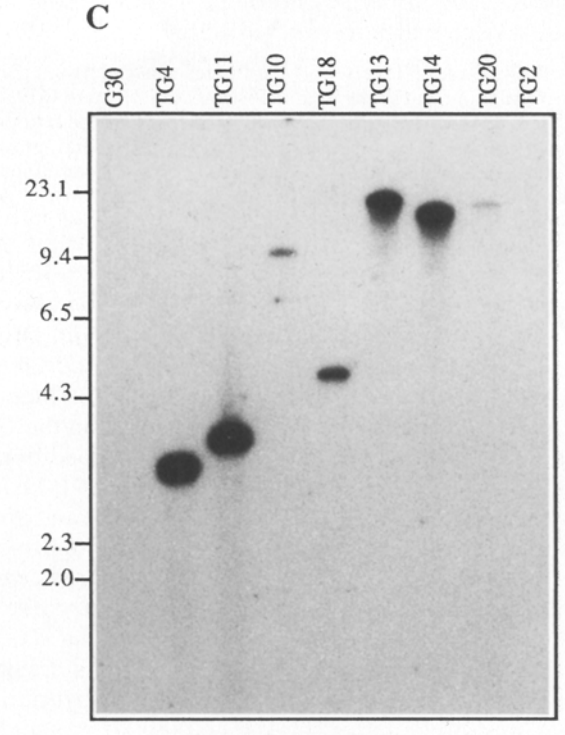
The *Eco*RI-digested DNA of the untransformed and transformed colonies was then subjected to hybridization using as a probe either the isolated 0.9-kb promoter-like fragment (Fig. 4B) or the Nm^R gene (Fig. 4C). These analyses confirmed that most of the additional and high-molecular-weight fragments, previously detected by pN13-A2 in transformants, contained sequences homologous to the 0.9-kb promoter-like fragment and to the Nm^R gene. How-



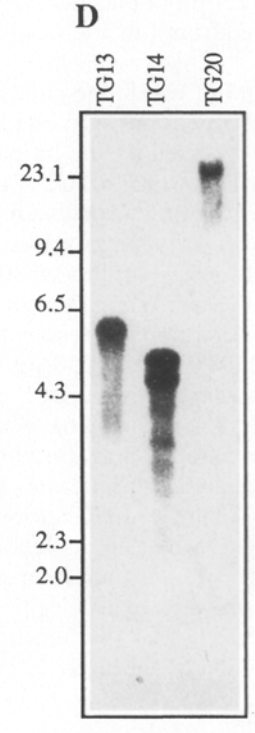
DNA: *Eco*RI-digested
Probe: pN13-A2



DNA: *Eco*RI-digested
Probe: 0.9 kb promoter-like fragment



DNA: *Eco*RI-digested
Probe: Nm^R gene



DNA: *Hin*dIII-digested
Probe: *pN*13-A2

ever, the lack or the weakness of the signals obtained with the Nm^R gene in transformants TG10 and TG20 suggests that the integrated sequences have undergone rearrangements or deletions. Lastly, the DNA from transformants TG13, 14 and 20 was digested with *Hin*dIII (which cuts once in pN13-A2) and hybridized with pN13-A2. The endogenous locus carrying the promoter-like fragment corresponds to a 3.5-kb *Hin*dIII fragment (data not shown). One or several signals were obtained for TG13 (near 6.5 kb) and TG14 (main signals at 4.8 and 5.5 kb), which is of particular interest since the 5.5-kb fragment corresponded in size to the linearized pN13-A2. A high-molecular-weight signal was still revealed for TG20, which could confirm the occurrence of important deletions in the integrated vector sequences.

Overall, these results allow us to arrange the resistant colonies into three groups: (1) the transformants possessing additional signals were derived from the ectopic integration of at least a part of the transforming vector; (2) the transformants characterized by the disappearance of the endogenous 4.0-kb *Eco*RI fragment probably resulted from the integration of several copies of pN13-A2 (with possible molecular rearrangements) at the resident locus carrying the promoter sequence with loss or methylation of the *Eco*RI site; and (3) the colonies having the same hybridization pattern as the untransformed control strain most likely corresponded to spontaneous resistant colonies.

Discussion

Cloning of *A. aegerita* DNA sequences upstream of the inactive Tc-resistance bacterial gene in plasmid pT13 resulted in the recovery of 0.48% of *E. coli* recombinant clones able to grow on 15 μg/ml of Tc, suggesting that the cloned fungal DNA sequences supported the expression of the Tc-resistance gene. The promoter activity of eucaryotic DNA sequences in *E. coli* has been diversely explained either by the cloning of true transcriptional signals (West et al. 1979) or by the fortuitous recognition of a binding site by the RNA polymerase of *E. coli* (Brosius 1984). Nevertheless, some of the properties of the *A. aegerita* 0.9-kb promoter-like sequence cloned in pT13-A2 suggested that it could carry a specific binding site for the bacterial RNA polymerase. This sequence allowed expression of the Tc^R gene to confer a resistance level up to 40 μg/ml, while inverting its polarity in the plasmid abolished the gene expression. Moreover, it was further successfully used to drive the expression of the promoterless chloramphenicol- and neomycin-resistance genes. The nucleotide distance between the *A. aegerita* fragment and each structural gene being different in the three constructions (86 bp in pT13-A2, 45 bp in pC13-A2 and 19 bp in pN13-A2), the fungal DNA sequence is in-frame at +2 in pT13-A2, at 0 in pC13-A2 and at +1 in pN13-A2 with respect to each bacterial ATG initiation codon. The production of a functional protein in each case suggests that translation was started from the procaryotic ATG initiation codon. As frequently reported from the analysis of gene structure in filamentous fungi (Gurr et al. 1987), the nucleotide sequence of the 0.9-kb fragment did not provide evidence for the promoter nature of this DNA fragment. Although putative atypical TATA and CAAT boxes could be localized on the sequence, the more interesting feature is the presence of a CT motif in the 3' region, that is to say close to the structural Nm^R gene, which could be involved in a promoter function. Indeed, the CT motif has previously been observed in genes lacking the TATA and CAAT sequences, and in highly expressed genes (Gurr et al. 1987).

The plasmid pN13-A2, carrying the Nm^R gene under the control of the *A. aegerita* promoter-like sequence, was used to transform protoplasts of a wild-type *A. aegerita* neomycin-sensitive homokaryotic strain. With the homologous transformation vector pUra1-1, we previously found that *A. aegerita* transformants could be selected using electroporation and after incubation of the electroporated protoplasts during 48 h in a non-selective medium before plating (Noël and Labarère 1994). Under these conditions, the transformation efficiencies reached with pN13-A2 (1–2.8 transformants per μg of plasmid DNA per 10^3 viable cells before electroporation) are comparable to the efficiencies obtained in other agaricales with heterologous vectors (Barret et al. 1990; Randall et al. 1991; Marmeisse et al. 1992; Peng et al. 1992), but are 2–20-times lower that those previously obtained in *A. aegerita* with the homologous vector pUra1-1. Differences of the same order of magnitude between heterologous and homologous transformation systems have already been described (Webster and Dickson 1983; Suarez and Eslava 1988; Mooibroek et al. 1990). They may result from the differential methylation of the foreign transforming DNA, which could play a role in the expression of the resistance genes and thus explain the different resistance levels of the transformants (Mooibroek et al. 1990).

Despite a high frequency of spontaneous mutations to neomycin resistance (up to $2–10^{-2}$ of the protoplast fraction surviving to electroporation), the ratio of the number of transformants to the number of spontaneous mutants was 3/1. This ratio was confirmed by the molecular analysis of the fate of the plasmid DNA in the colonies derived from pN13-A2 transformations. Using pN13-A2 as a probe, about one-third of the 20 putative transformants analysed had the same hybridization profile as the untransformed control strain, i.e. a single hybridizing 4.0-kb *Eco*RI fragment carrying the promoter-like sequence, and therefore probably correspond to untransformed spontaneous mutants. The other transformants were shown to derive either

Fig. 4A–D Southern analysis of digested DNA from representative G418R colonies, and from the untransformed control strain G30. **A** DNA digested with *Eco*RI and probed with pN13-A2. **B** DNA digested with *Eco*RI and probed with the isolated 0.9-kb promoter-like fragment. **C** DNA digested with *Eco*RI and probed with the isolated *Nm* structural gene. **D** DNA digested with *Hin*dIII and probed with pN13-A2. DNA fragment sizes are given in kb. Representative patterns of several hybridization experiments carried out with different DNA preparations from independent cultures of each transformant

from the integration of vector sequences at the promoter-like locus or somewhere else in the genome. However, no correlation could be found between the integration site of pN13-A2 and the neomycin-resistance level. Evidence was provided for the occurrence of sequences homologous to the 0.9-kb promoter-like fragment and the structural Nm^R gene in the genomic DNA of most of the transformants. Nevertheless, the hybridization pattern of two transformants indicated that the integrated Nm^R gene may have suffered molecular rearrangements or deletions. In the case of the integration events at the promoter-like locus, the *Eco*RI signals obtained were greater in size (around or above 23 kb) than the 9.5 kb expected from the integration of a single copy of pN13-A2. This suggested the integration of several copies of the vector, as frequently described in fungal transformation (for a review see Fincham 1989), even in basidiomycetes (e.g. Binninger et al. 1987; Marmeisse et al. 1992). The use of *Hin*dIII to digest the DNA from these transformants strongly suggested tandem duplications of the vector in one transformant (recovery of a signal for a 5.5-kb fragment corresponding to the size of the linearized vector). Depending on the probe used, particularly pN13-A2 and the isolated Nm^R gene, some of the newly detected signals in transformants appeared fainter than the endogenous promoter-like locus or else were heterogeneous in intensity. This could be due either to the presence of less than one copy of integrated vector sequences per genome, or to molecular rearrangements which could decrease homology with the probe. Indeed, in *A. aegerita* we previously showed that transformation with the homologous vector pUra1-1 by electroporation could favor the emergence of heterokaryotic mycelia, carrying both untransformed and transformed nuclei. Furthermore, we also reported an instability of the integrated vector sequences which resulted in frequent excision events and, therefore, in important molecular rearrangements (Noël and Labarère 1994).

The fungal DNA sequence cloned in pN13-A2 allows the expression of three different bacterial structural genes in *E. coli* and the selection of Nm-resistant transformants in *A. aegerita*. This result supports the hypothesis that this fungal sequence contains a versatile promoter activity. However, one cannot omit other possibilities to explain the emergence of *A. aegerita* Nm-resistant transformants. Although a molecular study of the DNA sequences at the site of ectopic integration showed that very little or no homology is required between the vector and recipient DNA (Razanamparany and Bègueret 1988), the presence of a DNA fragment of the donor species in the transforming vector, as in pN13-A2, could only have a physical role in favouring and driving integration into the recipient genomic DNA. Thus, expression of the neomycin-resistance gene could result from the random integration of the vector in highly expressed parts of the genome; it is also possible that spontaneous resistant mutants had simultaneously acquired DNA vector sequences, as could be the case for transformants TG10 and TG20 in our experiments.

Our aim was to derive a simple transformation system for *A. aegerita* usable in any wild genetic context. Although there is no direct evidence that the fungal DNA sequence of pN13-A2 is a true promoter in *A. aegerita*, it behaves like a versatile promoter in both *E. coli* and *A. aegerita*. The cloning strategy and the transformation procedure outlined here could apply to other industrial fungi for which the genetics and molecular biology is still poorly developed, allowing the creation of a diversity of chimeric genetic markers that could be further tested in transformation experiments.

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