Journal of Industrial Microbiology & Biotechnology (1998) 21, 311–314

http://www.stockton-press.co.uk/jim

Malachite green treatment of industrial *Penicillium chrysogenum* protoplasts results in increased penicillin-V formation

B Ribár[1,2,3] and A Szentirmai[1]

[1]*Department of Microbiology and Biotechnology;* [2]*Department of Genetics, University of Debrecen, Hungary*

We attempted protoplast fusion in order to generate gene transfer between an industrial strain of *Penicillium chrysogenum* and a fission yeast, *Schizosaccharomyces pombe*. The *Penicillium* strain was treated with malachite green. The *S. pombe* strain was auxotrophic for lysine. The regenerated colonies showed *Penicillium* morphology. The number of *Penicillium* colonies was significantly higher when the inactivated *Penicillium* protoplasts were fused to *S. pombe* protoplasts than in the self-fusion control experiments. We randomly isolated colonies from the regeneration plates and measured beta-lactam formation in cultures from shaken flasks. Antibiotic production was increased in colonies originated from the malachite green-treated protoplasts.

Keywords: beta-lactam production; *P. chrysogenum; S. pombe*; malachite green; protoplasts

Introduction

We examined gene transfer between an industrial strain of *Penicillium chrysogenum* and a fission yeast, *Schizosaccharomyces pombe*, with the aim of constructing a new penicillin producer strain. Genetic recombination, especially when the fusing strains are in different species, can influence metabolite production or may lead to the development of new metabolites if the biochemical properties of the two species are combined into a single genome [8,16]. Protoplast fusion techniques in filamentous fungi were first described by Anne and Peberdy [2] and Ferenczy *et al* [5]. They provide an efficient method for genetic manipulation and strain improvement [13]. This technique is extensively used in penicillia to improve antibiotic titre [3]. In our experiments two methods were used to select the fusion products. First, we used recessive auxotrophic mutants of both species. Selection is based upon complementation of auxotrophic strains. This approach failed to generate fusion products. However, the introduction of auxotrophic mutations into industrial strains is not a desirable practice because of the likely deterioration in productivity of the strain. In the second approach, *Penicillium* protoplasts were inactivated with malachite green and these biochemically inactive protoplasts were fused to lysine auxotrophic *S. pombe* protoplasts. Malachite green is an antifungal material which irreversibly inactivates some important enzymes, and may cause cell death. Cell reactivation may occur after transfer of functioning enzymes via protoplast fusion (enzyme-transfusion) [6].

[3]Correspondence to B Ribár at his present address: H-MED Diagnostic Ltd, PCR Laboratory, Military Hospital, Building H, H-1134 Budapest, Robert Károly krt 44, Hungary
Received 2 June 1998; accepted 30 November 1998

Materials and methods

Strains and media

A high penicillin-producing prototrophic and a low penicillin-producing leucine-auxotrophic industrial strain of *Penicillium chrysogenum* were used. These strains were kindly provided by Biogal Pharmaceutical Co, Debrecen, Hungary. *Schizosaccharomyces pombe lys1–131* was kindly provided by U Leupold (Bern, Switzerland).

YEL (liquid growth medium) contained 0.5% yeast extract and 1% glucose. SM (seed medium) contained: peptone 0.4%, yeast extract 0.4%, KH_2PO_4 0.2%, $MgSO_4 \cdot H_2O$ 0.05% and glucose 1%. MMAK (synthetic minimal medium) contained: glucose 1%, agar 2%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.01%, $MgSO_4$ 0.005%, KCl 0.7 M and vitamins (biotin 10 μg L^{-1}, Ca-pantothenate 1 mg L^{-1}, nicotinic acid 10 mg L^{-1} and meso-inositol 10 mg L^{-1}). PPM (penicillium producing medium) contained: lactose 4%, whey powder 8%, corn extract 0.5%, soya flour 1%, $(NH_4)_2SO_4$ 0.6%, KH_2PO_4 0.2%, $CaCO_3$ 0.66%. Na-phenoxy acetic acid 0.65%, Tween-20 0.005%, pH 5.8, sunflower oil 0.3% [7,14,15].

Measuring beta-lactam formation

SM medium was inoculated with 10^6 spores per 100 ml, in a 500-ml Erlenmeyer-flask. The culture was grown on a rotary shaker at 300 rpm, at 25°C for 48 h. Beta-lactam production medium (PPM) was inoculated by 10% (v/v) from the preceding SM culture and incubated on the rotary shaker at 300 rpm, at 25°C for 48 h. Beta-lactam formation was measured in the fermentation medium by hydroxylamin reagent in an automated system [7,10].

Preparation of protoplasts from P. chrysogenum

10^6 spores of *P. chrysogenum* were inoculated into 100 ml seed medium and were incubated for 48 h. Mycelium was harvested on a glass filter and washed with an aqueous solution of 0.7 M KCl. The suspension was mixed with

enzyme solution (10 mg ml^{-1} Lysing enzyme, Sigma L2265; it contains cellulase, protease and chitinase activities. This mixture was incubated with gentle shaking at 32°C for 2 h. After the incubation all mycelium was completely digested, the average protoplast number was 4–7 $\times 10^7$ ml^{-1}. The protoplasts liberated were separated from mycelial debris by filtration through a sintered glass filter and washed three times with 0.7 M KCl.

Inactivation of P. chrysogenum protoplasts

The *P. chrysogenum* protoplast pellet was suspended in 1% (w/v) malachite green solution containing 0.7 M KCl. The mixtures were incubated for 1 h at room temperature. The inactivated protoplasts were washed in 1 M mannitol until the supernatant became colourless.

Preparation of protoplasts from S. pombe

S. pombe was grown in yeast extract medium (YEL) to mid-log phase. The cells were harvested, washed in 0.7 M KCl, then they were digested in enzyme solution (5 mg ml^{-1} Lysing enzyme, Sigma). The protoplasts liberated were washed with 1 M mannitol [15,17].

Protoplast fusion

Fusion was induced using 30% (w/v) polyethylene glycol (PEG, molecular weight 4000) solution containing 10 mM $CaCl_2$. Protoplast suspensions were mixed and the mixture was centrifuged for 10 min at 2500 $\times$ *g*. After centrifugation, the supernatant fluid was carefully removed, and 2 ml 30% PEG was layered onto the protoplast pellet and mixed very gently with a sterile pipette tip. The fusion mixture was incubated at room temperature for 1 h, diluted in soft-agar (MMAK with 1% agar) and spread onto minimal plates (MMAK) and incubated at room temperature for 1 week [15].

Results

Fusion of S. pombe lys1–131 and P. chrysogenum leucine-auxotrophic mutants

The lysine biosynthesis pathway is conserved among fungi, therefore we used a *lys1–131* mutant of *S. pombe*. Assuming that interspecific complementation of these mutations was possible, fusion products were selected on minimal medium (MMAK). However, no prototroph colony was formed in these experiments, although the protoplasts regenerated efficiently: *S. pombe lys1–131* was 15% and *P. chrysogenum* was 22% on MMAK with lysine.

Fusion of inactivated prototrophic P. chrysogenum and S. pombe lys1–131 auxotroph mutants

Penicillium protoplasts were inactivated with malachite green and mixed under fusion conditions with *S. pombe* protoplasts. The level of inactivation was 99% relative to the total number of protoplasts. The inactivated protoplasts are able to regenerate only if the donor protoplast provides intact cell particulums and enzyme-systems. Table 1 shows the results of these fusion experiments. In all cases the number of colonies was two to four times higher when the inactivated *Penicillium* protoplasts were fused to *S. pombe* protoplasts than in the self-fusion control experiments. Equal numbers of *Penicillium* protoplasts were used in both the interspecific fusion experiments and in the regeneration control experiments.

Morphology of the regenerated colonies

We examined the morphology of the regenerated colonies by stereo microscopy. The *P. chrysogenum* colonies showed a wrinkled and dull surface, the *S. pombe* colonies showed a smooth and bright surface. All of them showed *P. chrysogenum* morphology.

Penicillin-V formation of the regenerated colonies

We isolated 30 random regenerated colonies from each of the fusion experiments: MG-treated *P. chrysogenum* $\times$ *S. pombe lys1–131* (series A), the first control experiment: MG-treated *P. chrysogenum* $\times$ MG-treated *P. chrysogenum* (series B) and the second control experiment: *P. chrysogenum* $\times$ *P. chrysogenum* (series C). Figure 1 shows histograms of the production of penicillin-V in these colonies. The beta-lactam formation of the series A and B increased compared with series C. The colonies isolated from series B (MG-treated *P. chrysogenum* protoplasts) produced 988 U more penicillin on average than the C series (*P*-value: 0.0001). The colonies isolated from series A produced 473 U more penicillin on average than series C (*P*-value: 0.0468) (Figure 2). The average penicillin titer of the series: A: 17333 U; B: 17848 U; C: 16860 U.

Discussion

Intergeneric fusion experiments with malachite green-inactivated *Penicillium chrysogenum* protoplasts showed two kinds of results.

Intergeneric protoplast fusion has an influence on the regeneration ability of MG-treated *P. chrysogenum* protoplasts: the number of colonies was two to four times higher when inactivated *Penicillium* protoplasts were fused to *S. pombe* protoplasts than in self-fusion control experiments (equal numbers of *Penicillium* protoplasts were used in both experiments). The efficiency of colony formation was dependent on the ratio of the *S.* pombe protoplasts to the *Penicillium* protoplasts: that the higher this proportion, the more colonies appeared (Table 1). These results suggest that *S. pombe* protoplasts can complement the MG-treated *P. chrysogenum* protoplasts. The viability of the transitory intergeneric fusion products is highly dependent on a number of factors that arise from the genetic and physiological incompatibilities of the fusion partners. These differences can be lethal either at the cytoplasmatic level or at the nuclear level [1]. In our experiments the cytoplasm of *S. pombe* did not prove lethal for MG-treated *P. chrysogenum* protoplasts because their fusion did not reduce. On the contrary, it increased the regeneration ability of MG-treated *P. chrysogenum*.

The malachite-green (MG) treatment has an effect on penicillin-V production. The average penicillin-V production of series B (colonies from only MG-treated protoplasts), is significantly larger than that of control C series (an average 988 U more penicillin-V than the series C; *P*-value: 0.0001). The value of the A series is slightly lower than that of the B series because the effect of MG

Table 1 The regeneration of malachite green-inactivated *P. chrysogenum* protoplasts without intergeneric fusion (a) and fusion with *S. pombe lys1–131* (b) in three independent experiments

Experiment number	*Penicillium* protoplast number in fusions	Regenerated *Penicillium* colonies	Regeneration %	*S. pombe* protoplast number in fusions	Ratio of *S. pombe* protoplasts to *Penicillium* protoplasts
I(a)	0.94×10^6	240	2.5×10^{-2}	0	0
I(b)	2.3×10^6	1303	5.5×10^{-2}	5.2×10^8	226
II(a)	1.14×10^8	125	1.2×10^{-4}	0	0
II(b)	1.14×10^8	413	3.9×10^{-4}	2.36×10^8	2
III(a)	1.36×10^7	170	0.12×10^{-4}	0	0
III(b)	1.36×10^7	677	0.49×10^{-4}	9.48×10^8	70

(a) Inactivated *Penicillium chrysogenum* × inactivated *Penicillium chrysogenum*.
(b) Inactivated *Penicillium chrysogenum* × *Schizosaccharomyces pombe lys1–131*.

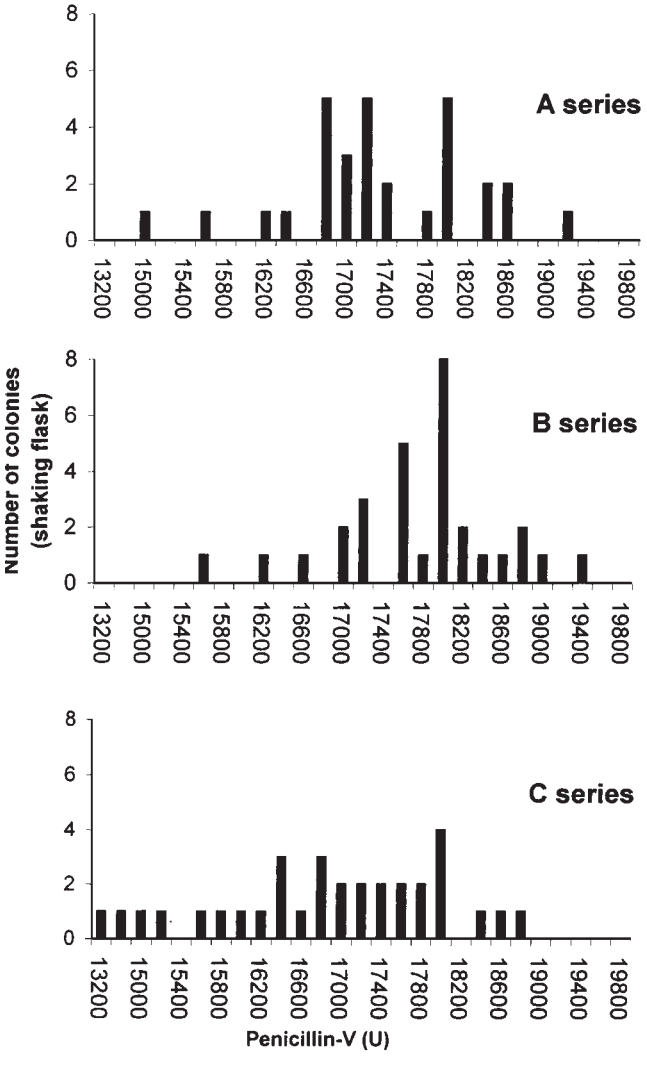

Figure 1 Production of penicillin-V of regenerated *Penicillium* colonies: series A: inactivated *P. chrysogenum* × *S. pombe lys1–131*; series B: inactivated *P. chrysogenum* × inactivated *P. chrysogenum*; series C: *P. chrysogenum* × *P. chrysogenum*.

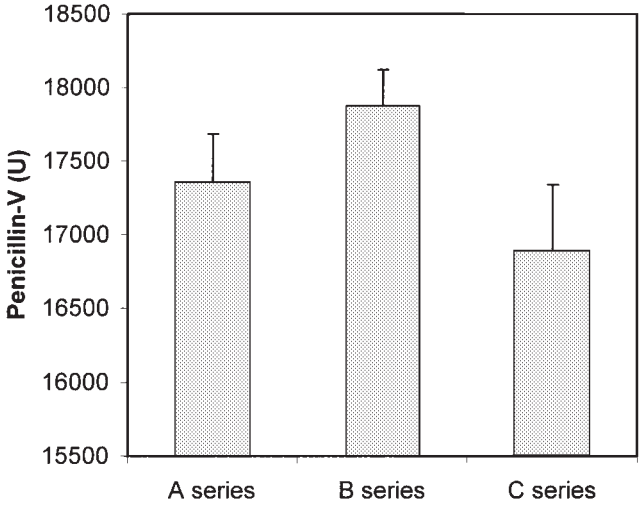

Figure 2 The average penicillin-V formation of the series.

was complemented via *S. pombe* cytoplasm. However the value of the A series is significantly higher than that of series C at the 0.05 level (an average 473 U more penicillin than series C; *P* value: 0.0468). Penicillins are peptide antibiotics synthesized by condensation of l-alpha-aminoadipic acid, l-cysteine and l-valine to form the tripeptide delta (l-alpha aminoadipyl)-l-cysteinyl-d-valine by a non-ribosomal peptide synthetase [9]. The pathway is catalyzed by three enzymes encoded by pcbAB, pcbC and penDE. The genes are organized in a cluster. The beta-lactam biosynthesis genes are controlled by a complex regulatory network, eg, by the ambient pH, carbon source and amino acids [4,7].

In series B the increase of the penicillin-V titer might cause a mutagenic effect of malachite green. The MG is a triaryl-methane dye. The dye is not intercalated into the DNA but is bound to exposed hydrophobic regions in the major groove. The ligand is in part, (the charged amino groups), in contact with the phosphoribose chain but its main surface lies against the hydrophobic base-pair stack [11]. The exposure of mammalian cells to MG demonstrated a dose-dependent increase in the generation of free radicals, lipid perodixation and DNA damage. The accumulation of free radicals caused DNA damage [12]. Our

observations suggest that increasing the beta-lactam formation is caused by a mutagenic effect of MG.

Acknowledgements

We thank U Leupold and A Oláh for providing strains. We also thank B Grallert (ICRF, London), M Sipiczki (University of Debrecen, Hungary) and Z Szallasi (Department of Pharmacology, USUHS, Bethesda, MD, USA) for critical reading and correction of the manuscript.

References

1 Anne J and JF Peberdy. 1985. Protoplast fusion and interspecies hybridization in *Penicillium*. In: Fungal Protoplasts (Peberdy JF and L Frerenczy, eds), pp 259–277, Marcel Dekker, New York.
2 Anne J and JF Peberdy. 1975. Conditions for induced fusion of fungal protoplasts in polyethylene glycol solutions. Arch Microbiol 105: 201–205.
3 Anne J. 1982. Comparison of penicillins produced by inter-species hybrids from *Penicillium chrysogenum*. Eur J Appl Microbiol Biotechnol 15: 41–46.
4 Brakhage AA. 1998. Molecular regulation of beta-lactam biosynthesis in filamentous fungi. Appl Microbiol Biotechnol 50: 1–15.
5 Ferenczy L, F Kevei and M Szegedi. 1975. Increased fusion frequency of *Aspergillus nidulans* protoplasts. Experientia 31: 1028–1029.
6 Ferenczy L, RE Bradshaw, F Kevei and JF Peberdy. 1987. Biochemical alternative to mutagenesis: model experiments with auxotrophic strains of *Aspergillus nidulans*. Eur Congr Biotechnol 1: 505.
7 Kozma J, G Bartók and A Szentirmai. 1993. Fructose-2,6-bisphosphate level and β-lactam formation in *Penicillium chrysogenum*. J Basic Microbiol 33: 27–34.
8 Javadekar VS, H SivaRaman and DV Gokhale. 1995. Industrial yeast strain improvement construction of highly flocculent yeast with a killer character by protoplast fusion. J Ind Microbiol 15: 94–102.
9 Martin JF. 1998. New aspects of genes and enzymes for beta-lactam antibiotic biosynthesis. Microbiol Mol Biol Rev 62: 547–585.
10 Niedermayer AO, FM Russo-Alesi, CA Lendzian and JM Kelly. 1960. Automated system for continuous determination of penicillin in fermentation media using hydroxilamine reagent. Anal Chem 32: 664–666.
11 Norden B, F Tjerneld and E Palm. 1978. Linear dichroism studies of binding site structures in solution. Complexes between DNA and basic arylmethane dyes. Biophys Chem 8: 1–15.
12 Panandiker A, GB Maru and KV Rao. 1994. Dose-response effects of malachite green on free radical formation, lipid perodixation and DNA damage in Syrian hamster embryo cells and their modulation by antioxidant. Carcinogenesis 15: 2445–2448.
13 Peberdy JF. 1980. Protoplast fusion — a tool for genetic manipulation and breeding in industrial microorganisms. Enzyme Microbiol Technol 2: 23–29.
14 Queener SW and RW Schwartz. 1978. Penicillins: biosynthetic and semisynthetic. In: Economic Microbiology (Rose AH, ed), pp 35–121, Vol 3, Academic Press, London.
15 Sipiczki M and L Ferenczy. 1977. Protoplast fusion of *Schizosaccharomyces pombe* auxotrophic mutants of identical mating-type. Mol Gen Genet 151: 77–81.
16 Tahoun MK. 1993. Gene manipulation by protoplast fusion and penicillin production by *Penicillium chrysogenum*. Appl Biochem Biotechnol 39–40: 445–453.
17 Zemanova Z, I Pavlicek and V Vondrejs. 1991. Formation and reversion of *Schizosaccharomyces pombe* cells with apical protoplast protuberances. Folia-Microbiol Praha 36: 153–157.