Protoplast production and transformation of morphological mutants of the Quorn® myco-protein fungus, *Fusarium graminearum* A3/5, using the hygromycin B resistance plasmid pAN7-1

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A protocol for the generation of high yields of viable protoplasts has been developed for several highly branched (colonial) strains of the Quorn® myco-protein fungus, *Fusarium graminearum* A3/5. Driselase was found to produce higher protoplast yields (*ca* 10^9 g^{-1} wet weight) than the other lytic enzymes tested (Glucanex, Novozyme, β-glucuronidase, Sigma lytic enzyme, or ICN lytic enzyme), although yields differed for the various strains. Protoplast regeneration frequencies of 25–50 % were observed when glucose (1·0 M) or sucrose (1·0 M) was used as the osmotic stabilizer. A highly branched strain of *F. graminearum* CC1-5, which grows better in submerged culture than the more sparsely branched wild-type strain (A3/5) was transformed using the hygromycin B resistance plasmid pAN7-1.

In the past decade, transformation of filamentous fungi has progressed from being a novel experimental technique to a routine procedure used in the study of gene expression (Fincham, 1989; van den Hondel & Punt, 1991) and in the production of homologous and heterologous proteins (Fowler & Berka, 1991; Jeenes *et al.*, 1991; van den Hondel, Punt & van Gorcom, 1991). However, it remains necessary to optimize the general transformation procedure for each individual fungal species to be studied (Fincham, 1989). In particular, protoplast production varies greatly from species to species (Deed & Seviour, 1990; Lynch, Collin & Isaac, 1985; Tschen & Li, 1994) and without efficient protoplast production, efficient transformation is difficult to achieve. In addition, an appropriate marker to select transformants must be available. Homologous transformations have been shown to yield higher transformation frequencies than heterologous systems (Ridder & Osiewacz, 1992; Diolez *et al.*, 1993; Rikkerink *et al.*, 1994). However, heterologous transformations systems based on resistance to hygromycin B using plasmid pAN7-1 have also been successfully used for a wide range of fungi (Punt & van den Hondel, 1992). Using resistance to an antibiotic as the selectable marker has the advantage of not requiring the isolation of specific auxotrophic mutants which is often difficult and time consuming. In addition, lower background growth occurs on antibiotic-containing plates that on nitrate (nitrate reductase, *niaD*, as the selectable marker) or acetamide (acetamidase, *amdS*, as the selectable marker) media.

Fusarium graminearum Schwabe strain A3/5 is used in the production of Quorn® myco-protein and can be readily cultured in submerged culture. As it is sold for human consumption, it has been through extensive toxicity testing and is known to be a safe organism (Trinci, 1992). Therefore, *F. graminearum* A3/5 is a good potential host for the production of heterologous proteins, particularly as the technology exists to grow it in 155 m^3 continuous flow culture at high dilution rates (Trinci, 1994). Royer *et al.* (1995) have recently demonstrated that strain A3/5 can be used to produce heterologous proteins. However, even though strain A3/5 itself grows well in submerged culture, it has been shown that in long-term (600 h +) continuous flow cultures A3/5 is supplanted by highly branched mutants of this strain which are even more adapted to growth in submerged culture (Wiebe *et al.*, 1991). Therefore, a highly branched mutant of *F. graminearum* A3/5, strain CC1-5, was chosen to evaluate its potential for producing heterologous proteins. An efficient protoplasting protocol has been developed together with a transformation system based on resistance to hygromycin B. Protoplast production of other highly branched and sparse strains of *F. graminearum* was also studied.

MATERIALS AND METHODS

Strains, media and plasmid

Fusarium graminearum strains MC3-8, CC1-5, CC1-2, CC2-1 (Wiebe *et al.*, 1991) and A27-1 (Wiebe *et al.*, 1994)

were isolated from glucose-limited chemostat cultures of *F. graminearum* A3/5 (provided by Mr T. W. Naylor, Marlow Foods, Billingham). Strain C106 (provided by Mr T. W. Naylor, Marlow Foods) was isolated from a myco-protein production glucostat.

The plasmid pAN7-1, containing the gene for resistance to hygromycin B with the glyceraldehyde phosphate dehydrogenase promoter from *Aspergillus nidulans* and the *trpC* terminator from *A. nidulans* (Punt *et al.*, 1987), was kindly provided by Professor G. Turner (University of Sheffield). Cosmid pAB6-10, which contains four copies of the *A. niger* glucoamylase gene, was provided by Dr P. J. Punt.

The fungus was grown on the defined medium of Vogel (1956) with glucose substituted for sucrose as the carbon source. In some media, glutamine was substituted for NH_4NO_3 as the nitrogen source. Some media also contained hygromycin B (50–200 µg ml^{-1}). Medium was solidified with 1–1·5% (w/v) agar (Lucas Meyer). Osmotically stabilized medium included NH_4Cl (1·0 M) glucose (1·0 M), sucrose (1·0 M), or sorbitol (1·0 M).

Culture conditions

Shake flask (50 ml medium in 250 ml Erlenmeyer flasks) cultures were inoculated with *ca* 1×10^7 conidia (*ca* 2×10^5 conidia ml^{-1}, final concentration) or with 1–1·5 ml of mycelial suspension from a pre-inoculated shake flask (typically in mid- or late-exponential phase growth when subcultured). Flasks were incubated at 25° C on a rotary shaker at 200 rpm (throw = 2·5 cm).

Petri dish (9 cm diam.) cultures were inoculated with spore suspensions spread across the surface of the plate or by mycelial transfer. All cultures were incubated at 25°.

Protoplast production

Mycelia were harvested from shake flask cultures which were in the exponential phase of growth (*ca* 21 h after inoculation) by vacuum filtration through Whatman nitrocellulose filters (3 µm pore size). The mycelium was washed with 5–20 ml NH_4Cl (1 M) and transferred to a sterile container. Lysing enzyme, dissolved in NH_4Cl (1 M), was added to give approximately 10–30 mg wet weight of biomass ml^{-1} lysing solution. A variety of lysing enzymes were tested to determine the most efficient: Driselase (0·1–5·0% w/v; source, basidiomycetes; enzymes, laminarinase, xylanase and cellulase; activity, unknown; Sigma), Glucanex (5% w/v; source, *Trichoderma* sp.; enzyme, β-glucanase; activity, 300 units g^{-1}; Novo Nordisk), Novozyme (0·5% w/v; source, *Trichoderma harzianum*; enzymes, cellulase, protease and chitinase; activity, unknown; Novo Nordisk), β-glucuronidase (4% w/v; source, *Helix pomatia*; activity, 89 400 units ml^{-1}; Sigma), Sigma lysing enzyme (1% w/v; source, *Rhizoctonia solani*; enzymes, glucanase and protease; activity, unknown), ICN Yeast lytic enzyme (5% w/v; source, *Arthrobacter luteus*; enzymes, unknown; activity, 5500 units g^{-1}; ICN). The mycelial suspension was dispensed into 100 ml Erlenmeyer flasks (10–15 ml flask^{-1}) and incubated at 25–30° on an orbital shaker (100 rpm, throw, 2·0 cm) for up to 3·5 h. Protoplasts

were harvested by filtration through two layers of lens tissue (Whatman 105).

Protoplast regeneration

Harvested protoplasts were concentrated by centrifugation (MSE Mistral 3000i, 3600 rpm, 10 min) and washed twice in osmoticum (1·0 M, NH_4Cl, glucose, sucrose or sorbitol). For transformation procedures, the osmoticum contained Tris (pH 8, 10 mM) and $CaCl_2$ (50 mM). The protoplasts were resuspended in the same osmoticum which was used to wash them. Cooled molten agar (*ca* 45°), with or without osmoticum (1 M), was added and the protoplasts were rapidly dispensed into Petri dishes (9 cm diam., 10 ml medium plate^{-1}).

Transformation

For transformation procedures, protoplasts which had been washed twice in 1 M GTC osmoticum (1 M glucose, 50 mM $CaCl_2$, 10 mM Tris pH 8) were concentrated to 5×10^8–2×10^9 protoplasts ml^{-1} and 200 µl aliquots were dispensed into sterile plastic universals to which 10–20 µg plasmid DNA was added (for co-transformations 10 µg of both pAN7-1 and pAB6-10 were added). Protoplasts were incubated 30 min on ice before addition of 1·2 ml (in three stages, 0·2 ml + 0·2 ml + 0·8 ml) PEG 4000 (60%, w/v, containing 50 mM $CaCl_2$ and 10 mM Tris pH 8, or 1 M GTC) and incubation on ice for a further 30 min. The PEG solution was diluted by adding 24 ml 1 M GTC with gentle mixing and then removed following centrifugation (3600 rpm, 12 min). Protoplasts were suspended in 1·2–2·4 ml^{-1} osmoticum and dispensed with agar into Petri dishes, as described above. Hygromycin B (100–200 µg ml^{-1}) overlays (8 ml molten agar) were dispensed over the protoplasts either 3 or 18 h after initial plating of the protoplasts.

Extraction of fungal DNA

DNA was extracted from small colonies growing on agar solidified medium or from mycelia harvested from shake flask cultures (10–20 ml in 100 ml flasks). The mycelium was ground (after filtration for shake flask cultures) in liquid nitrogen to disrupt the cell walls and suspended in 600 µl Qiagen (Dorking, U.K.) genomic G2 buffer (300 mM guanidine HCl, 30 mM EDTA, 30 mM Tris/HCl, 5% w/v Tween 20, 0·5% w/v Triton X-100, 200 µg RNase A ml^{-1}, 400 µg proteinase ml^{-1}; pH 8·0). Samples were incubated at 50–55° for 45–60 min. Proteins were extracted with phenol: chloroform:pentanol (25:24:1, 500 µl) and then chloroform:pentanol (24:1, 500 µl). The aqueous phase was removed and the DNA precipitated with 0·1 vol. 3 M Na acetate (pH 5·2) and 0·6 vol. isopropanol. The precipitate was washed with cold 70% (v/v) ethanol, before being resuspended in 25–50 µl Tris (10 mM, pH 8) EDTA (1 mM) buffer (TE).

Southern blot analysis

DNA was digested with *Eco*R I (Boehringer Mannheim) for at least 1 h at 37° and DNA fragments were separated by gel

electrophoresis on 0·8% (w/v) agarose. Gels were washed twice in denaturing solution (1·5 M NaCl, 0·5 M NaOH; 15 and 30 min) and then twice in neutralizing solution (1·5 M NaCl, 0·5 M Tris–HCl, pH 7·2, 0·001 M Na$_2$EDTA; 15 and 30 min). The DNA was blotted onto a nylon membrane (ICN Biotrans Nylon Membrane) by capillary blotting overnight, using 20 × SSC (3·0 M NaCl, 0·3 M Na$_3$ citrate). DNA was fixed to the membrane by 2 min exposure to uv light.

Membranes were probed using digoxigenin labelled pAN7-1. Hybridization and detection were carried out following the Boehringer Mannheim DIG chemiluminescent protocol. For some membranes, a low stringency wash of 1% SSC, 0·1% SDS at 55° was used. The low stringency wash resulted in clearer bands being observed on the Southern blots, although some non-specific binding was also observed.

RESULTS

Protoplast production

Lynch *et al.* (1985) found that 1·4 M NH$_4$Cl was the optimal osmoticum for protoplast release from *Fusarium culmorum* and that 1·2 M NH$_4$Cl was optimal for protoplast release from *F. tricinctum* (using Novozyme as the lytic enzyme). However, very poor yields of protoplasts were obtained when *F. graminearum* MC3-8 mycelia were incubated in 1·4 M NH$_4$Cl (Novozyme, 0·5% w/v). Protoplast release was improved in 1·2 and 1·0 M NH$_4$Cl. When Glucanex (5%, w/v) was used as the lytic enzyme, protoplast yields were also enhanced with either 1·0 or 1·2 M NH$_4$Cl in comparison with 1·4 M NH$_4$Cl. Therefore, 1·0 M NH$_4$Cl was used as the osmoticum for all further experiments.

Fig. 1 compares protoplast yields of strain MC3-8 in a variety of lysing solutions and shows that Protoplast yields were highest in Driselase (5%, w/v). Different batches of Driselase consistently produced high protoplast yields. The effectiveness of different Glucanex batches, however, was variable. Protoplast production was not enhanced by addition
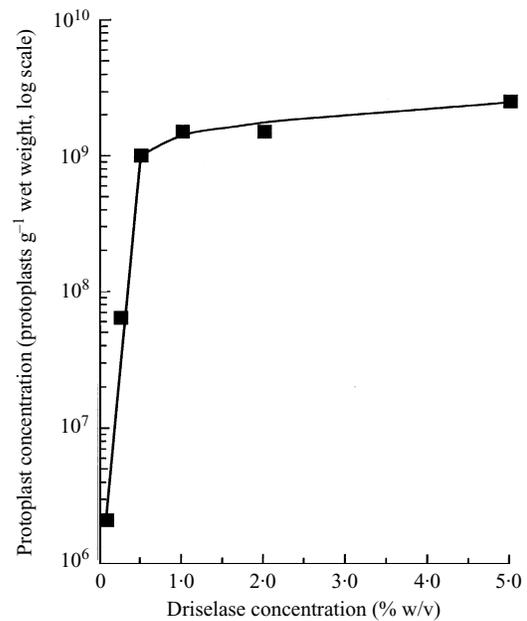
Fig. 2. Protoplast yield (Protoplasts [g wet weight]$^{-1}$) from exponential phase *Fusarium graminearum* MC3-8 mycelia after 3 h incubation at 25° in various concentrations (0·1–5·0% w/v) of Driselase. NH$_4$Cl (1·0 M) was used as the osmotic stabilizer.

of β-glucuronidase or Novozyme to Driselase solutions, nor was there a significant reduction in cellular debris following these treatments. Good protoplast release also occurred in Glucanex (5% w/v) and ICN lytic enzyme (5% w/v) solutions, although the yields were 4–5 times lower than in Driselase (5% w/v). Novozyme (0·5% w/v) was relatively inefficient for protoplast production from MC3-8. However, addition of β-glucuronidase (4% v/v) to Novozyme (0·5% w/v) solutions resulted in increased protoplast yield, with a reduction in cellular debris. Adams *et al.* (1987) also found protoplast release from *Gibberella zeae* was higher and left less debris in Novozyme + β-glucuronidase solution than in
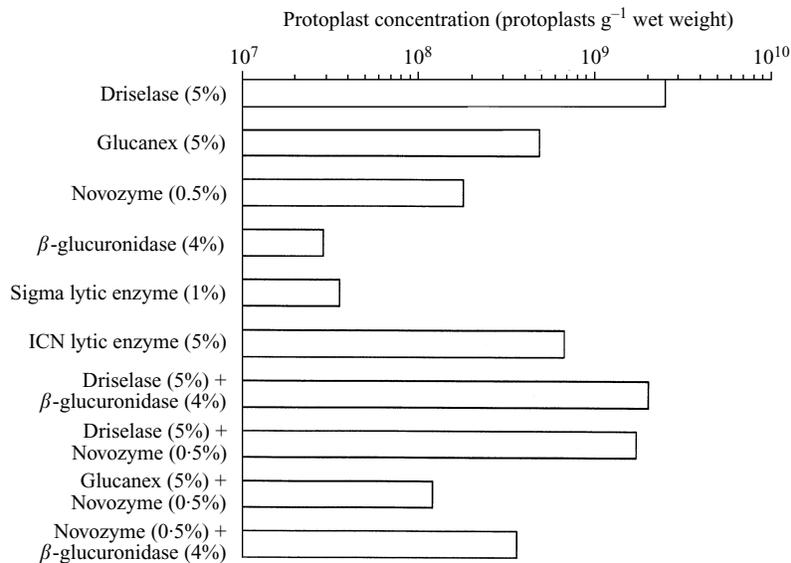
Fig. 1. Protoplast yield (Protoplasts [g wet weight]$^{-1}$) from exponential phase *Fusarium graminearum* MC3-8 mycelia after 3 h incubation at 25° in various lytic enzyme solutions. NH$_4$Cl (1·0 M) was used as the osmotic stabilizer.
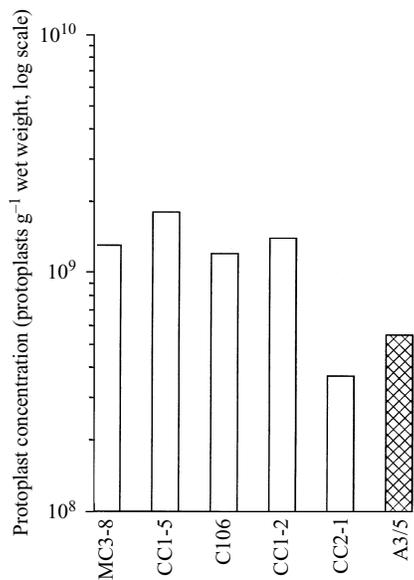
Fig. 3. Protoplast yield (Protoplasts [g wet weight]$^{-1}$) from exponential phase mycelia of five highly branched strains (MC3-8, CC1-5, C106, CC1-2 and CC2-1, □), and one sparsely branched strain (A3/5, ⊠) of *Fusarium graminearum* after 3 h incubation at 25° in Driselase (2 % w/v) solution. NH_4Cl (1·0 M) was used as the osmotic stabilizer.

Novozyme alone. β-Glucuronidase (4 % v/v) and Sigma lytic enzyme (1 % w/v) on their own produced poor yields of protoplasts. The amount of cellular debris remaining in protoplast suspensions was similar for protoplasts produced in Driselase, Novozyme or Glucanex.

Although Driselase is not completely soluble in 1 M NH_4Cl, even at 0·1 % (w/v), the active component is soluble. Protoplast concentration increased exponentially with enzyme concentration up to 0·5 % (w/v) (Fig. 2). Above 0·5 % (w/v) Driselase, a more gradual increase in protoplast release was observed. To ensure high yields of protoplasts, 2 % Driselase (w/v) was used for all subsequent experiments.

Fig. 3 compares protoplast yields (in Driselase, 2 % w/v and 1·0 M NH_4Cl) from five highly branched strains of *F. graminearum*, as well as from A3/5. The hyphal growth unit lengths (a measure of hyphal branching) of the highly branched strains ranged from 75 ± 2 μm for CC1-2 to 105 ± 2 μm for MC3-8 compared with 232 ± 11 μm for A3/5 (Wiebe *et al.*, 1992). MC3-8, CC1-5, C106 and CC1-2 produced similar yields of protoplasts, but CC2-1 produced only a quarter of that obtained from the other highly branched strains, while the yield from the sparsely branched A3/5 was approximately one third of that from CC1-5.

Protoplast yields from A3/5 and C106 were also determined in Glucanex (5 % w/v), Novozyme (0·5 % w/v) and Novozyme (0·5 % w/v) + β-glucuronidase (4 % v/v) lytic solutions. As with MC3-8, protoplast yields were lowest in Novozyme solutions (2–3 % of yields obtained in Driselase). Addition of β-glucuronidase to the Novozyme again improved protoplast yields (28–50 % of yields in Driselase). Yields in Glucanex were lower than in the combined Novozyme + β-glucuronidase solution (10–15 % of yields in Driselase).

Protoplast regeneration

Protoplasts did not regenerate well when NH_4Cl (1·0 M) was used as the osmoticum. Initially, regenerating protoplasts formed chains of budding cells, similar to those formed by *Aspergillus flavus* protoplasts regenerating in medium containing NH_4Cl (0·4 M; Moore & Peberdy, 1976) and by *Beauveria bassiana* protoplasts regenerating in medium containing $(NH_4)_2SO_4$ (0·6 M; Pfeifer & Khachatourians, 1992). A germ-tube then emerged from the original protoplast. However, regeneration frequencies were too low to be determined accurately. Protoplast regeneration was also poor when sorbitol (1 M) was used as the osmoticum (0·02–0·7 % for strain MC3-8 and 2–9 % for strain CC1-5), although protoplasts did regenerate by forming a germ-tube directly from the original protoplast, with no budding cells. Regeneration frequencies were higher in medium containing either glucose (1 M, 6–10 % for strain MC3-8 and 15–50 % for strain CC1-5) or sucrose (1 M, 36–52 % for strain MC3-8) as osmoticum than in medium containing sorbitol (1 M).

Transformation

Strain CC1-5 was selected for transformation because it produced high yields of protoplasts and was known to have a selective advantage in glucose-limited chemostat culture (selection coefficient = 0·010 h^{-1} at a dilution rate of 0·18 h^{-1}; M. G. Wiebe, unpublished results) and therefore was a good potential host for production of heterologous proteins. Strain A27-1 was also used for transformation and co-transformation procedures; it is the most highly adapted colonial mutant isolated in our laboratory.

When hygromycin B overlays were poured 18 h after incubation of protoplasts with DNA and PEG, a significant amount of growth occurred even on the control plates, although it was possible to isolate transformants because of their more vigorous growth. Approximately 0·5 transformants (μg DNA)$^{-1}$ were observed. These transformants (e.g. T1–T5 in Table 1) grew well on hygromycin B. When the overlays were poured after only 3 h of incubation, background growth was reduced appreciably, but transformants tended to stop growing if not subcultured onto non-selective medium soon after they appeared. Using this method, transformation efficiency was improved approximately 10-fold to *ca* 8 transformants (μg DNA)$^{-1}$. These experiments included osmoticum with the PEG (Crowhurst *et al.*, 1992) and the increased transformation frequency may be a result of this, as well as reflecting the improved ability to see the transformants with the low background growth. Not all the putative transformants which were subcultured onto selective medium were able to grow on hygromycin B when transferred back onto medium containing the antibiotic, although others (e.g. T6, Table 1, and transformants of A27-1) were able to do so.

Several of the putative transformants were grown on medium containing hygromycin B (at final concentrations of 25, 50, 75, 100, 125 and 150 μg ml^{-1}) to determine the extent of their growth in comparison to the non-transformed CC1-5, which was unable to grow at hygromycin B concentrations above 50 μg ml^{-1} (Table 1). The putative transformants differed in their ability to grow in the presence of

Table 1. A comparison of the growth of CC1-5 and several putative transformants on agar-solidified Vogel's medium containing increasing concentrations of hygromycin B (25–150 µg ml⁻¹)

| Strain | Hygromycin B concentration (µg ml⁻¹) | | | | | | Number of bands present in Southern blot analysis |
	25	50	75	100	125	150	
CC1-5	+ +	+	−	−	−	−	0
T1	+ + + +	+ + +	+ + +	+ + +	+ + +	+ +	4
T2	+ + + +	+ + + +	+ + +	+ +	+ +	+ +	2
T3	+ + +	+ + +	+ + +	+ +	+ +	+	1
T4	+ + +	+ +	+ +	+	+	−	1
T5	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	3
T6	+ + +	nd	+ +	+	+	−	1
T7	+ +	+	−	−	−	−	0

Growth is indicated by +, the number of plus signs indicating the extent or vigour of growth; − indicates the lack of growth; nd, no data. The presence or absence of bands detected with digoxigenin-labelled pAN7-1 is also indicated.
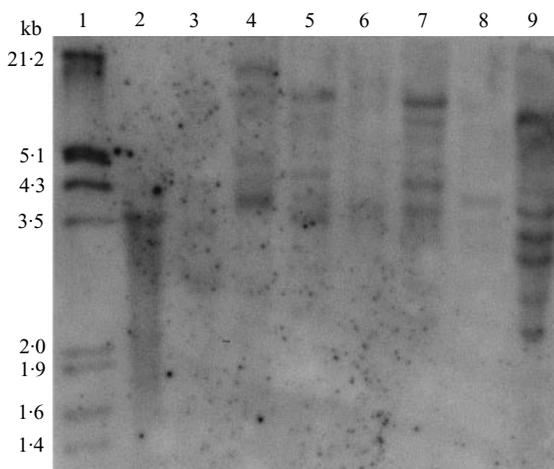
Fig. 4. Southern blot of *F. graminearum* A27-1 and several putative transformants of this strain, probed with DIG-labelled pAN7-1 following the Boehringer Mannheim DIG chemiluminescent protocol. Lanes 1 = λ ladder, 2 = A27-1, 3 = 48, 4 = 16, 5 = 10, 6 = 2, 7 = 46, 8 = 36, 9 = 8.

Fig. 5. Southern blot of *F. graminearum* A27-1 and several strains co-transformed with plasmids pAN7-1 and pAB6-10, probed with DIG-labelled pAB6-10 following the Boehringer Mannheim DIG colour detection protocol. Lanes 1 = λ ladder, 2 = A27-1, 3 = 24, 4 = 60, 5 = pAB6-10, *Pst* I restriction, 6 = CT7/46, 7 = CT7/31, 8 = CT7/31. Lanes 1 to 7 were *Pst* I restrictions, lane 8 was restricted with *Eco*R I. Arrowheads mark location of bands in lane 6; arrow marks location of band in lanes 7 and 8.

hygromycin B. However, all except one isolate (T7), which resembled the untransformed CC1-5 in its resistance to hygromycin B, were capable of at least some growth even at 125 µg ml⁻¹ hygromycin B. Only one isolate (T5) grew vigorously on 150 µg ml⁻¹ hygromycin B.

DNA was extracted from 11 putative transformants (T1 to T7, Table 1, and four other strains, T8–T11) and restricted with *Eco*R I. Southern blot analysis using digoxigenin-labelled pAN7-1 confirmed that 10 of the isolates contained pAN7-1 DNA. No label bound to CC1-5 or T7 (Table 1), but from 1 to 4 bands were labelled in transformants T1–T6 (Table 1) and T8–T11 (1, 3, 2 and 1 band respectively). Several strains had a band of approximately 7·4 kb (varying from *ca* 6·5 to 8·0 kb, T1, T3, T4, T5, T6, T8); other bands varied from 0·8 to 3·0 kb.

DNA was also extracted from several putative transformants of strain A27-1, another strain which is well adapted for growth in chemostat culture. Southern blot analysis again demonstrated the incorporation of pAN7-1 DNA into the fungal genome (Fig. 4). Using a lower stringency wash resulted in the appearance of one band at *ca* 3·6 kDa in the non-transformed A27-1. This band was also present in each of

the putative transformed strains, although its size appeared to vary slightly. Strains 16, 10, 2, 46 and 8 each showed larger bands (between 4 and 18 kb in size) which were not present in A27-1 or in strain 48. Again it appeared that at least one of the *Eco*R I restriction sites had been lost in the incorporation of pAN7-1 into the genome. Strains 2 and 8 were shown to grow slowly on concentrations of up to 180 and 240 µg hygromycin ml⁻¹ respectively.

The co-transformation of A27-1 with pAN7-1 and pAB6-10 resulted in two transformants with incorporated pAB6-10 DNA (Fig. 5, lanes 6, 7 and 8). These strains were subcultured from hygromycin B containing agar onto non-selective medium shortly after the transformation procedure and did not retain the ability to grow on hygromycin B. Expression of glucoamylase was not increased in these strains.

DISCUSSION

An efficient protocol for protoplast production has been developed for several strains of *F. graminearum* using Driselase as the lytic enzyme and NH_4Cl (1·0 M) as the osmoticum. Four of the highly branched strains produced protoplast yields of more than $10^9 g^{-1}$ wet weight (Fig. 4). However, as another highly branched strain yielded fewer protoplasts than the sparse (A3/5) strain, it seems unlikely that mycelia with more hyphal tips release more protoplasts than mycelia with fewer tips. Rather, differences in protoplasting efficiency between strains are more likely to reflect differences in cell wall composition. Deed & Seviour (1990) note that the location of protoplast release, whether from the entire hypha or primarily at hyphal tips, appears to be dependent on the lytic enzyme used, the osmoticum and also the composition of the fungal wall.

Protoplast regeneration in medium containing 1·0 M NH_4Cl as the osmotic stabilizer was poor. Although sorbitol is the most commonly used osmotic stabilizer in fungal transformation protocols (Fincham, 1989), regeneration frequencies were below 1% for strain MC3-8 and less than 10% for strain CC1-5 in sorbitol (1·0 M)-containing medium. In contrast, regeneration frequencies of 10–50% were observed on glucose (1·0 M) and sucrose (1·0 M) containing media. Glucose was chosen as the regeneration osmoticum for transformation procedures because of the difficulty of centrifuging protoplasts in a 1·0 M sucrose solution. Deed & Seviour (1990) note that protoplast regeneration is often higher in organic osmotica than in inorganic osmotica, but the reason for the differential response is not understood.

CC1-5 and A27-1 were transformed using the hygromycin B plasmid, pAN7-1. When restricted with *Eco*R I, two fragments of *ca* 2·7 and 4·8 kb are produced from pAN7-1. One of the *Eco*R I sites occurs within the hygromycin B phosphotransferase gene, the other occurs in front of the promoter (Punt *et al.*, 1987). It appears that, in incorporating pAN7-1 DNA into the *F. graminearum* genome, the *Eco*R I restriction site located in front of the promoter may have been lost, otherwise bands at either 2·7 or 4·8 kb would be expected in all the transformants. The presence of several bands in some transformants may suggest insertion of several copies of the hygromycin B gene into the genome. Although many of the initially observed colonies subsequently stop growing in medium containing 100 μg ml^{-1} hygromycin B, Southern blot analysis demonstrated that plasmid DNA had been incorporated into the fungal genome. The occurrence of aborted hygromycin B transformants has been reported for several other fungi including *Fusarium oxysporum* (Kistler & Benny, 1988), *Penicillium roquefortii* (Durand, Reymond & Fevre, 1991), *Aspergillus terreus* (Ventura & Ramon, 1991), *Botrytis cinerea* (Hamada *et al.*, 1994), *Glomerella cingulata* (Rikkerink *et al.*, 1994), *Penicillium paxilli* (Itoh, Johnson & Scott, 1994) and *Pyricularia oryzae* (Kimura *et al.*, 1995). However, fast-growing transformants of *F. graminearum* CC1-5 were not observed, possibly reflecting poor expression of the *A. nidulans* promoter sequence. *A. niger* hygromycin B transformants grew poorly when the fungal promoter and terminator sequences were absent (Punt *et al.*, 1987). Use of a

homologous promoter sequence in *Glomerella cingulata* (Rikkerink *et al.*, 1994) resulted in improved transformation frequency, compared to the use of the heterologous promoter sequence in pAN7-1. Similarly, use of an homologous promoter in *F. graminearum* might be expected to improve the growth of transformants on hygromycin B-containing medium. However, co-transformations using the heterologous plasmid pAN7-1 for the selectable marker could be carried out as described in this paper, provided that the transformed colonies were transferred to non-selective medium for subsequent screening for the second product. Two transformants of A27-1 with DNA from cosmid pAB6-10 (containing four copies of the *A. niger* glucoamylase gene) were isolated in this manner.

Royer *et al.* (1995) isolated transformants of *F. graminearum* A3/5 using the *A. nidulans* acetamidase gene as a selectable marker and demonstrated the potential of using this strain in the production of heterologous proteins. As this strain has been used to produce Quorn® myco-protein for 10 years it is known as a safe organism and as an organism which can be grown in large-scale submerged culture. Further, productivity of growth-correlated heterologous proteins would be higher in continuous flow culture (high dilution rate chemostat or turbidostat culture) than in batch or fed-batch culture. Thus, *F. graminearum* A3/5 is an attractive host for the production of heterologous proteins. CC1-5 is a mutant of strain A3/5 which has been adapted for growth in glucose-limited chemostat culture at a high dilution rate (D = 0·19 h^{-1}). Because it is more highly branched than A3/5, the viscosity of the fermentation broth would be reduced and hence aeration of the culture would be enhanced (Metz, 1976). Thus, CC1-5 is even more attractive than A3/5 as a host for the production of heterologous proteins. The protocols for protoplasting and transforming CC1-5 (and other strains of *F. graminearum*) described in this paper will facilitate the use of this strain for heterologous protein production.

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