How do highly branched (colonial) mutants of *Fusarium graminearum* A3/5 arise during Quorn® myco-protein fermentations?

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Chlorate-resistant, highly branched (colonial) mutants and auxotrophic mutants were used to study the nuclear distribution, morphology and growth of heterokaryons of the Quorn® myco-protein fungus, *Fusarium graminearum* A3/5. The results showed that for several complementary homokaryons, even a strong selective pressure was insufficient to maintain heterokaryons in a 'balanced' condition (i.e. exhibiting a wild-type or near wild-type phenotype). Furthermore, the margins of heterokaryotic colonies generally contained nuclei from only one of the parental homokaryons, indicating imperfect nuclear mixing within the mycelium. These observations suggest that recessive, colonial mutants may appear during Quorn® myco-protein production following shear-induced separation of hyphal fragments which contain a sufficiently high ratio of colonial:wild-type nuclei for the colonial phenotype to be expressed.

Keywords: *Fusarium graminearum*, chlorate resistance, heterokaryons, morphological mutants, nuclear ratios

INTRODUCTION

Fusarium graminearum A3/5 is grown in continuous flow culture by Marlow Foods Ltd to produce Quorn® myco-protein for human consumption. The appearance of highly branched (colonial) mutants of *F. graminearum* during the production of Quorn® myco-protein results in premature termination of the fermentation and consequently reduces the cost-effectiveness of the process (Trinci, 1992). Mutations resulting in highly branched phenotypes in *F. graminearum* are recessive (Wiebe *et al.*, 1992) and therefore mycelia with this phenotype will only appear in the fermentation following separation of mutant from parental nuclei. This separation may occur during sporulation (phialides of *F. graminearum* are uninucleate and macroconidia are produced when this fungus is grown in continuous flow culture; Wiebe & Trinci, 1991) or during shear-induced fragmentation of mycelia resulting in the release of hyphae which contain a sufficiently high ratio of mutant:parental nuclei for the colonial phenotype to be expressed.

The ratio of parental nuclei in a heterokaryotic mycelium may influence both its morphology and growth. For

example, Pittenger & Atwood (1954) found that reduced hyphal extension rates were observed when heterokaryons of *Neurospora crassa* contained certain ratios of wild-type: mutant nuclei, whilst Barratt & Garnjobst (1949) observed that hyphae of a *N. crassa* heterokaryon formed between colonial (i.e. highly branched) and non-colonial (sparsely branched) mutants extended at the wild-type extension rate when the parental nuclei were present in a ratio of 1:1, but reduced hyphal extension rates were observed when more colonial than non-colonial nuclei were present in the heterokaryon. Inadequate nuclear mixing resulted in a heterogeneous distribution of nuclei in heterokaryons of *F. oxysporum* (Puhalla, 1984) and consequently heterokaryotic mycelia did not grow vigorously and were unstable. Thus, morphology, growth and stability of a heterokaryon are influenced by the ratio of parental nuclei present, the degree of nuclear mixing (Puhalla, 1984; Bowden & Leslie, 1992) and the presence of selection pressures which favour heterokaryon maintenance (Jinks, 1952).

The present work was carried out for two reasons. Firstly, to study nuclear distribution in heterokaryons of *F. graminearum* and to assess whether or not mycelia with a colonial mutant phenotype might arise in Quorn® myco-protein fermentations following fragmentation of hetero-

Abbreviation: K_r , colony radial growth rate.

karyotic mycelia. Secondly, to assess the extent to which selective pressures might ensure the maintenance of 'balanced' heterokaryons of *F. graminearum* (and hence form the basis of a strategy to prevent or delay the appearance of colonial mutants).

METHODS

Organisms and media. *F. graminearum* Schwabe strains A3/5 (wild-type strain) and C106 (a highly branched, colonial mutant) were obtained from Mr T. W. Naylor, Marlow Foods, Billingham, UK. *F. graminearum* strains MC3-6, CC1-2 and CC2-1 were isolated from glucose-limited chemostat cultures of *F. graminearum* A3/5 (Wiebe *et al.*, 1991) and MC1-1 was isolated from a magnesium-limited chemostat culture (Wiebe *et al.*, 1992); all are highly branched, colonial mutants.

Spontaneous chlorate-resistant mutants of strains A3/5, C106, MC3-6, CC1-2 and CC2-1 were generated by spreading about 5×10^4 macroconidia over the surface of modified Vogel's medium containing 300 mM potassium chlorate. Other auxotrophic mutants of strain A3/5 were isolated after UV irradiation of macroconidia.

The defined medium of Vogel (1956) was used with 10 g glucose l^{-1} as the carbon source instead of sucrose. Vogel's mineral salts solution was prepared at $50 \times$ final concentration, sterilized by membrane filtration (0.2 μm pore diam.) and added to the sterile glucose solution (autoclaved at 121 °C for 15 min). Semi-solid medium was prepared by adding agar (Lucas Meyer; 15 g l^{-1} , final concentration) to the glucose solution before autoclaving. For some media 2 g $NaNO_3$ l^{-1} , 0.4 g $NaNO_2$ l^{-1} , 2 g hypoxanthine l^{-1} or 2 g glutamine l^{-1} were substituted for 2 g NH_4NO_3 l^{-1} as the nitrogen source in the medium.

Classification of chlorate-resistant strains. Chlorate-resistant mutants were designated (Cove, 1976) as *nirA* (unable to grow on nitrate), *nirA/nirA* (unable to grow on nitrate or nitrite) or *cnx* (unable to grow on nitrate and hypoxanthine). To characterize the ability of the strains to grow on various nitrogen sources, plates were inoculated with a small drop of macroconidial suspension, with up to five strains inoculated onto the same plate. At least 10 isolates were characterized for each strain; the isolate number is given at the end of the strain number (e.g. for strain MC3-6[1], MC3-6 is the designation of the colonial mutant and [1] is the chlorate-resistant isolate number).

Culture conditions. Colonies were grown in 9 cm diameter Petri dishes containing 20 ml agar-solidified medium. For colony radial growth rate (K_r) measurements, plates were inoculated centrally with a loop of mycelial suspension. To characterize the morphology of heterokaryotic mycelia, agar-solidified medium was first overlaid with sterile Cellophane (boiled 10 min in two changes of distilled water to remove plasticizers) which was then inoculated with 0.1 ml of a dilute mycelial suspension. Mycelial suspensions of heterokaryons were prepared by scraping (with a sterile metal or plastic rod, in the presence of sterile water) the surface of colonies growing on agar-solidified medium. The suspension was then ground using a sterile mortar and pestle and filtered (about 0.5 mm pore diam) to obtain a suspension of mycelial fragments.

Stationary liquid cultures were grown in 0.3 ml or 0.5 ml medium in 1.5 ml sterile Eppendorf tubes. Cultures were agitated after inoculating and again after about 24 h. Agitated liquid cultures were grown in 20 ml volumes of medium in 250 ml Nephlos flasks (Trinci, 1972). The flasks were inoculated with 2 ml of a suspension of homokaryotic (A3/5 or auxotrophic mutants) or heterokaryotic mycelia, and incubated on a

Fig. 1. Diagram of a fungal colony showing where measurements of mean light transmittance were made (areas 1-7) and where plugs were collected to sample for macroconidia (areas 1-4). Measurements of mean light transmittance were made in the approximate centre of each area, except at the colony margins, where the measurements were made at the margin edge. Colonies were inoculated centrally with a mycelial suspension and incubated for 144 h at 25 °C before measurements were made.

rotary shaker (throw = 2.5 cm) at 200 r.p.m. All cultures were incubated at 25 °C.

Measurements of fungal growth and morphology. For K_r determinations, colony diameters were measured with a rule at $10 \times$ magnification using a Shadowmaster as described by Trinci (1969). Absorbance measurements of shake flask cultures were made using a colorimeter (Evans Electroselenium) with a green (540-560 nm) filter.

The percentage transmittance of light through colonies growing on agar-solidified medium was measured using the Quantimet 570 system (Leica Cambridge Ltd). Images were relayed to the computer monitor using a Sanyo VC-2512 video camera mounted on a Leitz Medilux microscope. The colony was viewed using a $\times 2.5$ objective, and transmittance was measured at up to seven locations (1.6 $\times$ 1.6 mm) along a single transect across each colony, so that measurements were made at the colony margin, the centre of the colony and the opposite colony margin, and sometimes at two sites between each margin and the middle (Fig. 1), depending on the diameter of the colony. The intensity of the light from the microscope lamp was set to allow measurement of very sparse mycelial growth (gain = 52.3, offset = 62.1, lamp = 8.7 V), although this made it impossible to distinguish differences in the denser parts of the colony.

Hyphal growth unit length measurements (a measure of mycelial branching; Trinci, 1974) and mycelial tracings were made using a MeasureMouse graphics system (Analytical Measuring Systems) as described by Wiebe & Trinci (1991).

Heterokaryon formation. Heterokaryons were formed using a method modified from Wiebe *et al.* (1992). Macroconidia (0.05 or 0.10 ml of a 1×10^8 macroconidia ml^{-1} suspension) of the two parental strains were suspended in 0.2-0.4 ml Vogel's medium containing glutamine as the nitrogen source (all strains could grow on this nitrogen source) in sterile Eppendorf tubes. After 48-72 h incubation, mycelium was harvested from the surface of the liquid and from the suspension and inoculated onto agar-

solidified Vogel's medium containing NaNO_3 as the sole nitrogen source.

Determination of nuclear ratios in heterokaryons. Multi-nucleate macroconidia of *F. graminearum* are formed from uninucleate phialides and are therefore homokaryotic (Miller, 1946). For the present analysis it was assumed that every nucleus in a heterokaryon had an equal opportunity of being incorporated into a phialide and hence into macroconidia. Consequently, the ratio of parental nuclei in a heterokaryon formed between two colonial mutants could be estimated by harvesting macroconidia from the heterokaryon, allowing them to germinate and identifying the parental colonial mutants from their distinctive mycelial morphology (Wiebe *et al.*, 1992). In this study, 5 mm diameter plugs were removed from up to four locations in a colony (Fig. 1). The plugs were incubated for 24 h in Vogel's medium containing 0.2 ml glutamine. The medium was then drained off and the plugs were allowed to sporulate for 72 h before harvesting the macroconidia in 5 ml distilled water. The filtered (two layers of Whatman 105 lens tissue) macroconidial suspension was diluted and used to inoculate glutamine-containing agar-solidified Vogel's plates (five replicates for each agar plug); 0.1 ml macroconidial suspension, harvested from the heterokaryon, was spread across the plate with a sterile metal rod. The plates were incubated for 5 d and the colonies of the two parental strains were counted. Nuclear ratio determinations were calculated from the appearance of 176 ± 21 (mean $\pm$ SE) colonies (range 10–813) derived from macroconidia harvested from a particular heterokaryon.

RESULTS

Formation of heterokaryons between *niaD*, *nirA/niiA* and *cnx* mutants of *F. graminearum*

To identify complementing chlorate-resistant mutants of *F. graminearum* A 3/5, paired crosses were set up between each of four *niaD*, *nirA/niiA* or *cnx* mutants. Each strain

Table 2. K_t of individual heterokaryons of nitrate-non-utilizing, colonial mutants of *F. graminearum* grown at 25 °C for 144 h on agar-solidified Vogel's medium containing sodium nitrate as the sole nitrogen source

Heterokaryon	K_t ($\mu\text{m h}^{-1}$)	
	MC3-6[1] (<i>cnx</i>)	C106[3] (<i>niaD</i>)
C106[6] (<i>nirA/niiA</i>)	189 336 280	
MC3-4[1] (<i>nirA/niiA</i>)	95 71 131	191 121 202
CC1-2[1] (<i>nirA/niiA</i>)	147 191 172	330 174 167
CC2-1[4] (<i>nirA/niiA</i>)	204 186 203	168 335 145
C106[3] (<i>niaD</i>)	327 280 322	

was crossed with each other strain and also with itself and two replicates were made of each cross. In all except one cross (involving two *cnx* strains), duplicate crosses produced identical results. None of the crosses involving either two *niaD* or two *nirA/niiA* parents produced heterokaryons capable of utilizing nitrate. However, some complementation (indicated by an ability to grown on nitrate) was observed between the *cnx* mutants, although

Table 1. K_t and biomass density (expressed as a percentage of light transmittance) of colonies of *F. graminearum* A3/5 and nitrate-non-utilizing colonial mutants grown at 25 °C for 144 h on agar-solidified Vogel's medium containing sodium nitrate as the nitrogen source

Mean transmittance was measured in various areas (1.6×1.6 mm) across a diameter of each colony; the lower the level of light transmitted, the higher the biomass density. Measurements from three (*six radii) colonies have been combined and are expressed as mean $\pm$ SE. For the colonial mutants, measurements of biomass were only made at the margins and the middle of colonies, as these colonies were smaller in diameter and showed little variation in biomass density across the colony. ND, not determined.

Strains	K_t ($\mu\text{m h}^{-1}$)	Intensity of light transmitted by colony expressed as percentage of incident light						
		1 Margin	2	3	4 Middle	5	6	7 Margin
A3/5	241 $\pm$ 4*	50 $\pm$ 5	0	0	0	0	0	55 $\pm$ 2
MC3-6[1]	63 $\pm$ 5	86 $\pm$ 3	ND	ND	72 $\pm$ 4	ND	ND	89 $\pm$ 1
C106[6]	59 $\pm$ 3	83 $\pm$ 1	ND	ND	83 $\pm$ 3	ND	ND	82 $\pm$ 1
C106[3]	59 $\pm$ 1	80 $\pm$ 1	ND	ND	72 $\pm$ 8	ND	ND	83 $\pm$ 1
MC3-4[1]	84 $\pm$ 2	84 $\pm$ 2	ND	ND	82 $\pm$ 1	ND	ND	88 $\pm$ 1
CC1-2[1]	72 $\pm$ 4	76 $\pm$ 11	ND	ND	72 $\pm$ 8	ND	ND	83 $\pm$ 1
CC2-1[4]	107 $\pm$ 6	67 $\pm$ 1	ND	ND	76 $\pm$ 4	ND	ND	73 $\pm$ 7

Table 3. K_p , biomass density (expressed as a percentage of light transmittance) and ratio of parental macroconidia (nuclei) in heterokaryons between nitrate-non-utilizing, colonial mutants of *F. graminearum* grown at 25 °C for 144 h on agar-solidified Vogel's medium containing sodium nitrate as the sole nitrogen source

Mean transmittance was measured in seven areas (1.6×1.6 mm) across a diameter of each colony (for simplicity areas 5–7 are not shown as they were almost identical to areas 1–3) and the ratio of parental macroconidia (nuclei) was determined for samples taken from four areas along a radius of each colony. Data from only one heterokaryon of each pair is shown, as the other heterokaryons showed similar trends. The biomass densities of C106[3] + CC1-2[1] heterokaryons were not determined, but were similar to the biomass densities of C106[3] + CC2-1[4] heterokaryons. Measurements were made only for the one colony of a C106[3] + CC2-1[4] heterokaryon shown. ND, not determined.

Pairs of colonial mutants used to produce heterokaryons	K_p , ($\mu\text{m h}^{-1}$)*	Intensity of light transmitted by colony expressed as a percentage of radiant light				Number of MC3-6[1] (a and b) or C106[3] (c) macroconidia (nuclei) in sample expressed as a percentage of all macroconidia (nuclei)			
		1†	2	3	4	1	2	3	4
		Margin			Middle	Margin			Middle
(a) Heterokaryons formed between a <i>cnx</i> colonial mutant (MC3-6[1]) and four <i>nirA/niiA</i> colonial mutants (C106[6], MC3-4[1], CC1-2[1], CC2-1[4])									
MC3-6[1] + C106[6]	336	66	8	0.3	0	0	0	95	100
MC3-6[1] + MC3-4[1]	131	89	0	ND	0	74	19	ND	42
MC3-6[1] + CC1-2[1]	147	42	0	0	0	86	62	43	52
MC3-6[1] + CC2-1[4]	203	80	0	0	0	0	94	~ 50	51
(b) Heterokaryons formed between a <i>cnx</i> colonial mutant (MC3-6[1]) and a <i>niaD</i> colonial mutant									
MC3-6[1] + C106[3]	280	85	73	0	0	0	26	98	100
(c) Heterokaryons formed between a <i>niaD</i> colonial mutant (C106[3]) and two <i>nirA/niiA</i> colonial mutants (MC3-4[1], CC1-2[1], CC2-1[4])									
C106[3] + MC3-4[1]	202	78	75	51	4	100	7	7	7
C106[3] + CC2-1[4]	335	79	65	83	13	100	100	100	3

* The colonial growth rates of homokaryotic colonies of the mutants are shown in Table 1.

† See Fig. 1. for location of zones.

only one complementation group was observed. Although all *niaD* + *cnx* and *nirA/niiA* + *cnx* heterokaryons were able to grow on nitrate, most of the *nirA/niiA* + *cnx* heterokaryons grew more vigorously (81 % of *nirA/niiA* + *cnx* heterokaryons grew vigorously, compared to only 31 % of *niaD* + *cnx* heterokaryons). Only 25 % of heterokaryons formed between *niaD* and *nirA/niiA* strains were able to utilize nitrate, and these all showed only poor growth. Correll *et al.* (1987), Klittich & Leslie (1988), Toth & Lacy (1991) and Bowden & Leslie (1992) also found that, for *Fusarium* spp., *niaD* mutants did not form vigorous heterokaryons with *nirA/niiA* mutants.

Measurement of the biomass density of colonies

It was possible to measure the relative density of biomass present in colonies using the Quantimet 570 image analysis system. The mean transmittance of low intensity light through homokaryotic colonies of *cnx*, *nirA/niiA* and *niaD* colonial mutants growing on medium with nitrate as the nitrogen source varied between 54 % and 90 % of the incident light (Table 1). In contrast, no light was transmitted through nitrate-utilizing, A3/5 colonies

growing on the same medium, except at the colony margin where mean light transmittance varied between 43 % and 59 % of the incident light (Table 1). Unfortunately, the relatively low light intensity used at the colony margin made it impossible to make meaningful biomass measurements in the denser parts of A3/5 colonies. Thus, for heterokaryons formed between different chlorate-resistant mutants (see below), mean light transmittance values below 50 % indicate higher biomass densities than those present in the parental homokaryotic colonies (Table 1). Values of 0 % represent substantial, but not necessarily identical, biomass densities.

Heterokaryons formed between colonial, chlorate-resistant mutants

To study heterokaryon formation between the nitrate-non-utilizing, colonial mutants, a *cnx* colonial mutant (MC3-6[1]) was selected and crossed with four *nirA/niiA* colonial mutants (C106[6], MC3-4[1], CC1-2[1] and CC2-1[4]) and with one *niaD* colonial mutant (C106[3]) on Vogel's medium containing nitrate as the sole nitrogen source. All heterokaryotic colonies, except those formed

Fig. 2. Tracings of mycelia of *F. graminearum* growing on agar-solidified Vogel's medium containing glutamine as a nitrogen source. (a) A3/5, $G = 334 \mu\text{m}$; (b) MC1-1, $G = 56 \mu\text{m}$; (c) heterokaryotic fragment of A3/5+MC1-1, $G = 554 \mu\text{m}$; (d) heterokaryotic fragment of A3/5+MC1-1, $G = 101 \mu\text{m}$. The more highly branched ends of fragment (d) have G values of 77 and $95 \mu\text{m}$, whereas the central portion has a G value of $308 \mu\text{m}$.

between MC3-6[1] and MC3-4[1], exhibited K_r values similar to those of the A3/5 wild-type (Tables 1 and 2), confirming their heterokaryotic nature and complementation of the colonial mutations. Dense mycelial biomass was present in the centre of all heterokaryons which had MC3-6[1] as one of the homokaryotic parents (Table 3 presents data from representative heterokaryons and Table 2 shows the variation in K_r observed). In heterokaryons which had either CC1-2[1] or CC2-1[4] as one of the homokaryotic parents, this dense mycelial biomass extended to the colony margin. Dense mycelial biomass was also formed by one MC3-6[1]+MC3-4[1] colony (data not shown), confirming that a heterokaryon had been formed, but the K_r of this colony was only $131 \mu\text{m h}^{-1}$ (54% of the A3/5 K_r , but faster than that of either homokaryon; Table 1). The other two heterokaryons formed between MC3-6[1] and MC3-4[1] did not form dense mycelial biomass.

MC3-6[1] macroconidia were not detected in samples of macroconidia taken from the margins of heterokaryotic colonies formed between MC3-6[1] and the other colonial mutants tested, except for one heterokaryon formed between MC3-6[1] and CC1-2[1] and the MC3-6[1]+MC3-4[1] heterokaryon described above (Table 3). Of the three heterokaryons formed between MC3-6[1] and CC1-2[1], macroconidia of MC3-6[1] were absent

from the margin of one colony (data not shown), present in small numbers at the margin of a second colony and present in large numbers at the margin of a third colony (Table 3). However, MC3-6[1] macroconidia were found in all samples taken from the centres of these colonies and were present in all other samples except in some taken more than 2 cm from the centre of heterokaryotic colonies formed between MC3-6[1] and C106[6] or C106[3]. C106[3] and C106[6] macroconidia were found predominantly towards the colony margins. In some heterokaryons involving MC3-6[1], macroconidial ratios of about 1:1 were observed in samples taken within an 8–13 cm radius of the colony centre. High nuclear ratios generally occurred in heterokaryons with less dense mycelial growth.

Heterokaryons were also made between the *niaD* colonial mutant C106[3] and the *nirA/niiA* colonial mutants MC3-4[1], CC1-2[1] and CC2-1[4]. Growth remained relatively sparse throughout these heterokaryotic colonies, but their centres were denser than those of parental homokaryotic colonies. However, K_r values of these heterokaryotic colonies were much faster than the parental homokaryotic colonies (indicating complementation between the colonial mutants) and, for some, actually exceeded that of the A3/5 wild-type (Tables 1 and 2). Macroconidia from both parents were found in each heterokaryotic colony

Table 4. Specific growth rates of *F. graminearum* A3/5, adenine- and histidine-requiring auxotrophs of A3/5, and various heterokaryons of auxotrophic mutants of A3/5 on supplemented and unsupplemented Vogel's medium

Cultures were grown in 20 ml medium in 250 ml Nephlos flasks on a rotary shaker (200 r.p.m.) at 25 °C. *ade*⁻, adenine requiring; *his*⁻, histidine requiring; *cys*⁻, cysteine requiring; *met*⁻, methionine requiring; *arg*⁻, arginine requiring; *leu*⁻, leucine requiring.

Strain type	Strain	Medium	Specific growth rate (h ⁻¹)	Mean doubling time (h)
Parent	A3/5	Vogel's	0.21 ± 0.01	3.3
Auxotrophs of A3/5	<i>ade</i> ⁻	Vogel's + 1 g adenine l ⁻¹	0.19 ± 0.01	3.6
	<i>his</i> ⁻	Vogel's + 1 g histidine l ⁻¹	0.22 ± 0.01	3.2
Heterokaryons formed between various auxotrophs of A3/5	<i>his</i> ⁻ + <i>ade</i> ⁻	Vogel's	0.05 ± < 0.01	13.9
	<i>cys</i> ⁻ + <i>met</i> ⁻	Vogel's	0.03 ± < 0.01	23.1
	<i>his</i> ⁻ <i>arg</i> ⁻ + <i>leu</i> ⁻ <i>ade</i> ⁻	Vogel's	0.013 ± < 0.01	53.3

sampled, but only C106 macroconidia were found in samples taken from the margins of colonies and few, if any, C106 macroconidia were found at the centre of colonies (Table 3).

Breakdown of heterokaryons

Fig. 2 shows mycelia of A3/5 (a) ($G = 334 \mu\text{m}$), the colonial mutant MC1-1 (b) ($G = 56 \mu\text{m}$) and of a heterokaryon (c) formed between chlorate-resistant mutants of the two strains. The MC1-1 + A3/5 heterokaryotic fragment (d) had an overall hyphal growth unit length of $101 \mu\text{m}$, but the two ends of the fragment were much more highly branched ($G = 77 \mu\text{m}$ and $95 \mu\text{m}$) than the centre ($G = 308 \mu\text{m}$).

Growth of heterokaryons in liquid culture

Specific growth rates of heterokaryons formed between auxotrophic mutants (*ad*⁻ *his*⁻; *cys*⁻ *met*⁻; *his*⁻ *arg*⁻ and *leu*⁻ *ade*⁻ mutants) of *F. graminearum* were measured on Vogel's medium in shake flask culture. Table 4 shows that the heterokaryons grew at significantly slower specific growth rates on Vogel's medium ($0.013\text{--}0.05 \text{ h}^{-1}$) than did the auxotrophs on Vogel's supplemented medium (about 0.19 and 0.22 h^{-1}) or the wild-type A3/5 on Vogel's medium ($0.21 \pm 0.01 \text{ h}^{-1}$).

DISCUSSION

Puhalla & Spieth (1983) suggested that heterokaryons of *F. moniliforme* could only be sustained by repeated anastomosis of adjacent hyphae, because nuclear migration (mixing) did not occur in this species. Similarly, anastomosis appeared necessary to sustain heterokaryons

between coloured variants of *F. oxysporum* (Puhalla, 1984) and auxotrophic mutants of *Gibberella zeae* (= *F. graminearum*) (Adams *et al.*, 1987). Lack of nuclear migration (mixing) in heterokaryons will eventually result in the formation of homokaryotic sectors, and, if such sectors occurred in heterokaryons formed between auxotrophic mutants, they would not be capable of sustained growth on minimal medium. The phenotype of the A3/5 + MC1-1 mycelium shown in Fig. 2 suggests that nuclear segregation occurred in this heterokaryon. Sanchez *et al.* (1976) observed that heterokaryons formed between auxotrophic mutants of *F. oxysporum* generally did not grow (measured as colony diameter) as rapidly as the wild-type but found that colonies which contained a 1:1 ratio of auxotrophic nuclei most closely resembled wild-type colonies. Although 1:1 nuclear ratios were not essential for the attainment of fast hyphal extension rates (K_r) in the *F. graminearum* heterokaryons examined here, they were important for nitrogen utilization. In general, those colonies (MC3-6[1] + CC1-2[1], MC3-6[1] + CC2-1[4] and one colony of MC3-6[1] + MC3-4[1]) which contained nuclear ratios close to 1:1 throughout most of the colony also produced dense biomass (Table 3). However, as noted by Wiebe *et al.* (1992), complementation for the colonial mutations (as judged by the K_r values observed) occurred even in mycelia in which the mutant nuclei had become localized in different parts of the mycelium (Table 3; MC3-6[1] + C106[6]). These observations suggest that translocation or diffusion of some gene product(s) (those involved in hyphal extension) occurs in some heterokaryons, while other gene product(s) (such as enzymes for nitrate utilization) remain localized.

Our results indicate that at least for some pairs of

complementary homokaryons of *F. graminearum*, even a strong selective pressure was insufficient to maintain heterokaryons in a 'balanced' condition exhibiting a wild-type phenotype. This conclusion is supported by the poor biomass production of heterokaryons formed between some complementary pairs of chlorate-resistant, colonial mutants grown on nitrate medium (Table 3), by the poor growth of heterokaryons formed between complementary pairs of auxotrophic mutants grown on minimal medium (Table 4), by the variation in hyphal branching within a heterokaryon formed between complementary chlorate-resistant, colonial mutants grown on medium containing glutamine (Fig. 2), and particularly, by the fact that it is not possible to isolate the nuclei of both parental homokaryons from some parts of heterokaryotic colonies formed between complementary chlorate-resistant, colonial mutants grown on nitrate medium (Table 3). Presumably, in the absence of a selective pressure favouring their maintenance, breakdown of heterokaryons of *F. graminearum* would occur even more rapidly.

Homokaryotic hyphal tips have been observed in heterokaryons of other *Fusarium* spp., however, the occurrence of nuclei of only one parental homokaryon in the middle of a heterokaryotic colony, as observed here in some heterokaryons involving strain C106 (Table 3), has not previously been reported. Puhalla & Spieth (1983) found only one nuclear type in hyphae taken 1–2 mm from the margins of the colonies of *F. moniliforme* heterokaryons, although nuclei of both parents were found at distances greater than 3 mm from the colony margin; this corresponded to the distance behind the colony margin at which hyphal anastomoses were observed. Homokaryotic hyphal tips were also observed in heterokaryons of *F. oxysporum* (Puhalla, 1984) and *G. zeae* (Adams *et al.*, 1987). These results emphasize the importance of taking samples across a colony when determining nuclear ratios. In contrast to the situation observed in *F. graminearum* (Table 3) and other *Fusarium* spp. (Puhalla & Spieth, 1983; Puhalla, 1984), hyphal tips of heterokaryons of *N. crassa* contain both types of parental nuclei (Beadle & Coonradt, 1944).

Our observations suggest that the appearance of highly branched colonial mutants (which are recessive) in long-term continuous cultures of *F. graminearum* A3/5 (Trinci, 1992; 1994) occurs following: firstly, spontaneous colonial mutations; secondly, the formation of homokaryotic or relatively homokaryotic hyphal tips containing colonial mutant nuclei (as apparently occurred in the A3/5 + MC1-1 heterokaryon shown in Fig. 2); and, thirdly, the isolation (by shear-induced fragmentation of mycelia in the fermenter) of these hyphal tips from the rest of the mycelium (which contains wild-type nuclei). Furthermore, Table 4 shows that heterokaryons formed between auxotrophic mutants do not grow sufficiently rapidly to be used in Quorn® myco-protein fermentations. Thus, it is not possible to develop a strategy to prevent the appearance of colonial mutants in Quorn® myco-protein fermentations by using a heterokaryon formed between auxotrophic mutants (to prevent segregation of colonial mutant and wild-type nuclei).

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