Mutants with general growth rate advantages are the predominant morphological mutants to be isolated from the Quorn® production plant

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Sixteen highly branched (colonial) mutants of *Fusarium graminearum* A3/5 were isolated at the end of 11 Quorn® myco-protein production fermentations. These ranged from the highly branched C134-3 to the sparsely branched C145, which was similar to A3/5 in liquid culture. Although allelic complementation was observed to occur between some of the mutants, heterokaryon analysis revealed that all the highly branched mutants belonged to a single complementation group. Mixed cultures of A3/5 and six of the colonial mutants were grown in glucose-, ammonium-, magnesium- and sulphate-limited chemostat culture. One mutant (C153) had a selective advantage over A3/5 in all nutrient limitations tested; four mutants (C134-1, C134-3, C137-1, and C135) had a selective advantage over A3/5 in all except one nutrient limitation; and one mutant (C139) had a selective advantage over A3/5 in magnesium-limited chemostat culture only. Four mutants (C134-3, C139-1, C153 and C135) had a selective advantage over A3/5 when the dilution rate was increased above the critical dilution rate (0·22 h^{-1}) and nutrients were present in excess. These results suggest that the growth conditions in the Quorn® production plant (which is operated as a glucose-stat) favour mutations that confer general growth rate advantages, but these mutations may result in growth disadvantages when nutrient limitations are imposed. As all of the mutations appear to occur in one gene or gene cluster, the differing patterns of selective advantage/disadvantage for the six mutants studied suggests that regulatory genes may also be involved or that different sites of mutation can lead to a variety of pleiotropic effects.

Quorn® myco-protein, a protein substitute for human consumption, was introduced into the U.K. in 1985. It is produced from the biomass of a filamentous fungus, *Fusarium graminearum* Schwabe A3/5, the hyphae of which give the product a fibrous, meat-like texture. A continuous flow process was chosen as the most economical means of producing the high biomass output required for Quorn® production (Pirt, 1975; Trinci, 1992). The appearance, however, of highly branched (colonial) mutants after 4–8 weeks of production (Pirt, 1975; Trinci, 1992) results in termination of the fermentation and consequently reduces the cost-effectiveness of the process; the presence of colonial mutants affects both the filtration properties and overall texture of the biomass.

Wiebe *et al.* (1992*a*, *b*, 1996*b*) have studied a variety of colonial mutants isolated from glucose-limited chemostat cultures. All of these colonial mutations were recessive to the wild type morphology and most were found to belong to the same heterokaryon complementation group. This suggested that although numerous genes are involved in determining mycelial morphology (Garnjobst & Tatum, 1967) mutations in only a small proportion of these genes were selectively advantageous in glucose-limited chemostat culture (Wiebe *et al.*, 1992*a*). Furthermore, the mutations involved appear to be specific to the glucose metabolic pathway and confer a selective advantage only when glucose (or certain related sugars) is limiting in the culture (Wiebe *et al.*, 1992*b*). As the strains were isolated from glucose-limited chemostats, this was not surprising. The Quorn® production plant is not operated as a nutrient-limited chemostat at a dilution rate below the maximum specific growth rate (μ_{max}, i.e. the maximum rate of biomass production per unit biomass for the given cultural conditions), but as a nutrient-unlimited glucose-stat (i.e. a continuous flow culture in which the residual glucose in the vessel is maintained at a constant concentration) at μ_{max}. In a glucose-stat, mutations which result in an improvement in μ_{max} should be the most frequently occurring mutations that confer a selective advantage. Wiebe *et al.* (1993, 1994*a*, *b*, 1995) have demonstrated that other mutations, which apparently confer no selective advantage relative to the original strain, also accumulate during long term chemostat cultures of *F. graminearum* A3/5 and that non-morphological mutants with selective advantages relative to the original strain may also appear. The colonial mutants, however, are the most readily identified.

It has not been established whether the size of the production plant (40 m^3) prevents thorough mixing thereby resulting in local limitations which induce unexpected selection pressures. In order to understand the nature of the selection pressures in such a large continuous flow system and to assess

whether an optimal strategy for controlling the occurrence of colonial mutations in the production plant can be devised, 16 more colonial mutants have been isolated from Quorn® production campaigns terminated due to the appearance of colonial mutants. Complementation analysis was used to determine the relationship between these mutants and six were further analysed in competition with A3/5 in chemostat cultures to determine the general nature of their selective advantage.

MATERIALS AND METHODS

Organisms and medium

F. graminearum strains A3/5 and C106 were obtained from Mr T. W. Naylor, Marlow Foods, Stokesley, U.K. All other strains were isolated at the University of Manchester from samples from the Quorn® myco-protein production plant, Billingham, U.K. Isolates were named according to the campaign (i.e. production run) number (e.g. C106 was isolated from campaign 106, C134-1 was isolated from campaign 134, etc.), with a second number (−1, −2, −3, etc.) being added when more than one mutant was obtained from the same campaign sample. Stock cultures of all strains were maintained as macroconidia at −70 °C in 20% (v/v) glycerol and as mycelia at 4° on sterile soil.

Spontaneous chlorate- and selenate-resistant mutants were generated by spreading about 5×10^4 macroconidia over the surface of modified Vogel's medium containing 300 mM potassium chlorate or 50 mM selenate. Resistant colonies were isolated after 14–20 d.

The defined medium of Vogel (1956) was used with glucose as the carbon source instead of sucrose. For submerged cultures, $(NH_4)_2SO_4$ was substituted for NH_4NO_3 as the nitrogen source. For some media $NaNO_3$ 2 g l^{-1}, $NaNO_2$ 0·4 g l^{-1}, hypoxanthine 2 g l^{-1}, or glutamine 2 g l^{-1} were used as the nitrogen source. The final concentrations of glucose, ammonium, magnesium sulphate and phosphate in nutrient-limited media used in chemostat cultures are given in Table 1. NH_4Cl (1·34 g l^{-1}) replaced the $(NH_4)_2SO_4$ in sulphate-limited medium, which was supplemented with $MgCl_2.6H_2O$ (0·2 g l^{-1} final concentration). Vogel's mineral salts solution was prepared at ×50 final concentration, sterilized by membrane (0·2 μm diam. pore size) filtration and added to the sterile glucose solution (autoclaved at 121°). Semi-solid medium was prepared by adding agar (Lucas Meyer; 15 g l^{-1} final concentration) to the glucose solution before autoclaving.

Table 1. Concentrations of glucose, ammonium, magnesium and sulphate in glucose-, ammonium-, magnesium- and sulphate-limited, modified Vogel's media used for chemostat cultivation of *F. graminearum* A3/5 and colonial mutants

	Nutrient concentrations (l^{-1})			
Limiting nutrient	Glucose (g)	NH_4^+ (g)	Mg^{2+} (mg)	Sulphate (g)
Glucose	3*	0·45	9·9	1·25
NH_4^+	6	0·2*	9·9	0·6
Mg^{2+}	6	0·45	1·5*	1·25
Sulphate	6	0·45	24·0	0·0135*

* Indicates the limiting nutrient in the medium.

Classification of chlorate-resistant strains

Chlorate-resistant mutants were designated (Cove, 1976) as *niaD* (unable to grow on nitrate), *nirA/niiA* (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 per plate.

Culture conditions

Colonies were grown in 9 cm diam. Petri dishes containing 20 ml agar-solidified medium. For colony radial growth rate measurements, plates were inoculated centrally with a loop of macroconidial or mycelial suspension.

Stationary liquid cultures were grown in 0·5 ml medium in 1·7 ml sterile Eppendorf tubes. Agitated liquid cultures were grown in 50 ml volumes of medium in 250 ml flasks which had been inoculated with *ca* 10^6 macroconidia (*ca* 2×10^4 macroconidia ml^{-1}, final concentration). Flasks were incubated on a rotary shaker (throw = 2·5 cm) at 200 rpm.

Continuous flow cultures were grown at pH 5·8 (±0·1) in a Braun Biostat M fermenter (2 l working volume) as described by Wiebe & Trinci (1991) aerated with 0·8 l air l culture^{-1} min^{-1} and stirred at 1000 rpm. Biomass retention in the fermenter vessel was monitored by dry weight measurements of culture samples taken from inside the fermenter vessel and from the overflow.

All cultures were incubated at 25°.

Measurements of fungal growth and morphology

To determine colony radial growth rates (K_r), colony diameters were measured at ×10 magnification using a Shadowmaster as described by Trinci (1969). Measurements were made of colony growth rates and hyphal growth unit length (G, a measure of mycelial branching; Trinci, 1974) using a MeasureMouse graphics system (Analytical Measuring Systems) as described by Wiebe & Trinci (1991). Maximum specific growth rates (μ_{max}) in submerged culture were calculated from the rate of biomass washout at a dilution rate above μ_{max} (Esener *et al.*, 1981).

Viable counts

Samples (containing both macroconidia and mycelial fragments) from continuous flow cultures were diluted serially with sterile distilled water to yield a suspension containing *ca* 4×10^2 colony-forming units (cfu) ml^{-1}, and 0·1 ml volumes of these suspensions were spread evenly over the surface of 9-cm diam. Petri dishes. The plates (10 per culture sample) were incubated for 3 d at 25° and the cfu counted to determine the proportion of mutant (small, densely-branched colonies) to wild-type (A3/5) for each sample. Selection

coefficients were calculated using the equation derived by Dykhuizen & Hartl (1981):

$$s = \frac{\ln\left(\frac{p_{(t)}}{q_{(t)}}\right) - \ln\left(\frac{p_{(0)}}{q_{(0)}}\right)}{t}, \quad (1)$$

where $p_{(t)}$ = concentration of one strain at time t, $q_{(t)}$ = concentration of the second strain at time t, $p_{(0)}$ and $q_{(0)}$ = the initial concentrations of each strain, and s = the selection coefficient.

Heterokaryon formation

Heterokaryons were formed using the method described by Wiebe *et al*. (1996*a*). Macroconidia of two chlorate-resistant strains (or one chlorate- and one selenate-resistant strain) were suspended in 0·4 ml Vogel's medium containing glutamine as the nitrogen source (and methionine for selenate-resistant strains) and incubated in sterile Eppendorf tubes. After 48 h, mycelium was harvested from the surface of the liquid and inoculated onto agar-solidified Vogel's medium containing $NaNO_3$ as the sole nitrogen source. Colony radial growth rate measurements were made as described above.

RESULTS

Morphological characteristics of 16 colonial mutants

The colony radial growth rates and hyphal growth unit lengths were measured for 16 colonial mutants isolated from 11 Quorn® production plant campaigns (Table 2). The K_r of the mutants varied from 34 ± 2 to 116 ± 3 μm h^{-1} and the fastest extending colonial mutant had a K_r of 86% of that of A3/5 (135 ± 5 μm h^{-1}). Hyphal growth unit lengths of the colonial mutant varied between 29 ± 9 and 227 ± 10 μm compared with 195 ± 9 μm for A3/5. A high correlation was observed between K_r and G.

Table 2. Hyphal growth unit length and colony radial growth rate (measured in shake flask cultures) of *F. graminearum* A3/5 and colonial mutants of A3/5 which were isolated from Quorn® myco-protein production fermentations. Cultures were grown at 25° on modified Vogel's medium containing 1·65 g NH_4^+ l^{-1}.

	Hyphal growth unit length (G, μm)	Colony radial growth rate (K_r, μm h^{-1})
A3/5	$195 \pm 9^{a*}$†	135 ± 5^{a}
C106	65 ± 0	55 ± 1^{b}
C134-1	n.d.	29 ± 2^{b}
C134-2	41 ± 7^{b}	66 ± 1^{bc}
C134-3	29 ± 9^{bc}	34 ± 11^{b}
C135	91 ± 4^{d}	80 ± 3^{bcd}
C137-1	81 ± 3^{d}	79 ± 3^{bcd}
C137-3	n.d.	34 ± 2^{b}
C138	139 ± 4^{f}	116 ± 3^{ac}
C139	87 ± 4^{d}	88 ± 1^{cde}
C145	227 ± 10	94 ± 10^{cde}
C150	88 ± 3^{d}	103 ± 2^{def}
C151	132 ± 5^{f}	82 ± 1^{bcdf}
C152	n.d.	127 ± 2^{aef}
C153	99 ± 3^{d}	69 ± 1^{bcd}
C154-1	91 ± 2^{d}	78 ± 3^{bcdf}
C154-2	92 ± 7^{d}	51 ± 3^{bcd}
C154-3	100 ± 4^{e}	90 ± 2^{cdef}

* Mean (±S.E.) of six and 25 replicates for K_r and G, respectively.

† Values in the same column with the same superscript (a–f) are not significantly different ($P < 0{\cdot}05$, Bonferroni multiple range test).

n.d. no data.

Heterokaryon analysis

Wiebe *et al*. (1996*a*) found that the most vigorous heterokaryons produced between chlorate-resistant colonial mutants of *F. graminearum* A3/5 were formed between *nirA*/*niiA* and *cnx* mutants. Only these mutants were, therefore, used in the heterokaryon analysis of the colonial mutants isolated from the Quorn® production plant. For some colonial mutants (C145 and C150), no *cnx* mutant was isolated, so selenate-resistant mutants were generated instead. Heterokaryons were formed between a *nirA*/*niiA* isolate of each colonial mutant and a *cnx* mutant of the wild type strain (A3/5). In all cases the heterokaryon was able to grow on minimal medium (with nitrate as the sole nitrogen source). The heterokaryons formed with the wild type strain all had colony radial growth rates which were 85–107% of the wild type colony radial growth rate (313 ± 4 μm h^{-1}).

Table 3 shows the maximum K_r values observed for heterokaryons formed between different Quorn® production plant colonial mutants. A complementation map derived from the data is shown in Fig. 1. Two colonial mutants were considered to complement each other if the heterokaryon formed between them had a K_r at least 50% higher than that of the faster extending homokaryotic parent and at least 50% of the wild type (A3/5) K_r. If the heterokaryon grew vigorously on modified Vogel's medium with nitrate as the sole nitrogen source, but did not achieve the K_r values indicated above, the two colonial mutants were considered not to complement each other. With the exception of strain C106 (isolated from a much earlier campaign than the remainder) all the colonial mutants mapped into one complex complementation group that included all other colonial mutants (Fig. 1).

Competitive ability of colonial mutants relative to A3/5

The competitive ability, relative to A3/5, of 6 colonial mutants (C134-1, C134-3, C137-1, C135, C139, and C153) was determined in glucose-, ammonium-, sulphate-, and magnesium-limited chemostat cultures grown at a dilution rate (D, dilution rate is calculated as flow rate/volume and determines the specific growth rate in chemostat culture) of $0{\cdot}18 \pm 0{\cdot}01$ h^{-1} and at a dilution rate of $0{\cdot}30 \pm 0{\cdot}01$ h^{-1} (i.e. above the critical dilution rate, D_{crit}, at which biomass is washed out of the fermenter vessel at a faster rate than it can be replaced by growth within the vessel, so that all nutrients were present in excess). The mutants chosen for these experiments were readily distinguishable from the wild type, had relatively low K_r values (Table 2), and showed different complementation patterns (Fig. 1). For each competition experiment, a mixture of wild type and mutant spores were inoculated into the fermenter to give an initial population

Table 3. Complementation matrix showing the maximum colony radial growth rate (K_r) of heterokaryons formed by crossing auxotrophic colonial mutants of *F. graminearum* A3/5. The colony radial growth rate for each strain grown as a homokaryon, is given at the foot of each column as the mean ± S.E. of six replicates. Colonies were grown on modified Vogel's medium with sodium nitrate as the sole nitrogen source. The colony radial growth rate for A3/5 parental strain was 313 ± 4

Strain	C154-3	C154-2	C154-1	C153	C152	C151	C150	C145	C139	C138	C137-3	C137-1	C135	C134-3	C134-2	C134-1
C134-1	102	84	70	101	141	110	75	170	86	200	166	107	111	111	107	68 ± 2
C134-2	121	131	120	110	156	122	120	269	267	198	319	141	134	139	103 + 4	
C134-3	110	122	110	124	167	91	155	200	230	177	293	91	112	87 ± 2		
C135	110	115	110	139	n.d.	121	114	n.d.	151	247	n.d.	140	111 ± 1			
C137-1	122	75	50	95	136	180	99	215	169	182	265	36 ± 1				
C137-3	151	144	201	209	n.d.	175	314	n.d.	197	313	109 ± 6					
C138	246	222	226	220	181	211	227	348	286	160 ± 10						
C139	165	150	248	227	152	207	196	163	108 ± 6							
C145	141	170	163	278	n.d.	227	163	163 ± 6								
C150	117	141	118	300	152	168	101 ± 9									
C151	131	141	125	130	144	100 ± 2										
C152	168	188	161	134	159 ± 7											
C153	117	127	126	107 ± 4												
C154-1	100	64	46 ± 1													
C154-2	89	47 ± 4														
C154-3	87 ± 4															

n.d. = no data.

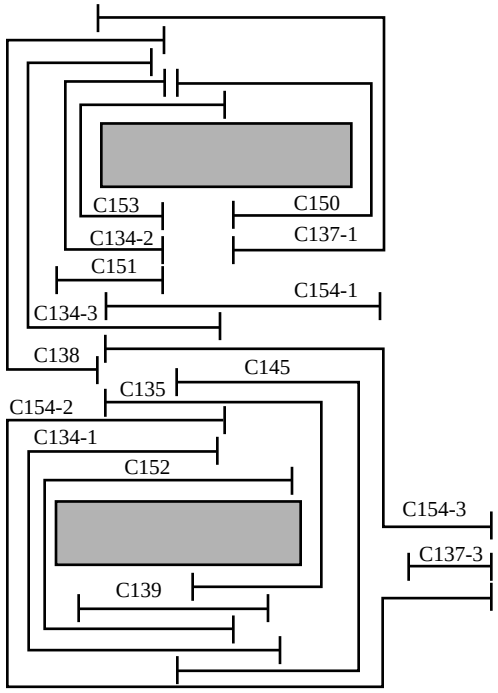

Fig. 1. Complementation map, based on Table 3, for 16 colonial mutants of *F. graminearum* A3/5. Each strain is represented by a continuous line. Complementation occurs between mutants whose lines do not overlap. Lines on opposite side of the shaded boxes do not overlap (i.e. the mutants represented by the lines did not complement each other unless indicated by lines overlapping on the same side of a shaded box).

containing 10–50% colonial mutants. Selection coefficients (s) were calculated from the relative proportions of colonial and wild type fragments present during each fermentation and the outcome of the competitions are presented in Table 4. Table 4 also includes selection coefficients for strains C106, CC1-1 (a glucose-limitation specific μ_{max} mutant isolated from a glucose-limited chemostat) MC1-1 (a general μ_{max} mutant isolated from a magnesium-limited chemostat; Wiebe *et al.*, 1992*a*) and C301 (isolated prior to 1985 from a Quorn® production run by Rank Hovis McDougal; M. G. Wiebe, unpublished data).

Table 4. Selection coefficients (*s*, h^{-1}) measured when *F. graminearum* A3/5 and colonial mutants were grown as mixed cultures in a nutrient-limited chemostat or at a dilution rate above D_{crit} (washout cultures). Chemostat cultures were grown on modified Vogel's medium with four separate nutrient-limitations (Table 1). Dilution rates were 0·17 ± 0·1 h^{-1} in chemostat cultures and 0·30 ± 0·1 h^{-1} in washout cultures. Culture conditions were 25°, pH 5·8, 1200 rpm, and an aeration rate of 0·8 l min^{-1} l^{-1}. Values of the selection coefficients presented are for the colonial mutant relative to A3/5

	Limiting-nutrient in chemostat				Washout
Strain	Glucose	Ammonium	Sulphate	Magnesium	culture
C134-1	+0·041	+0·008	+0·018	**−0·017**	−0·001
C134-3	+0·017	+0·022	+0·009	0·000	+0·028
C137-1	+0·008	+0·004	+0·015	−0·017	−0·034
C153	+0·021	+0·019	+0·013	+0·030	+0·012
C135	0·000	+0·011	+0·009	+0·008	+0·017
C139	−0·003	0·000	0·001	+0·012	+0·021
C106	−0·006	−0·007	0·000	+0·038	+0·010
C301	+0·010	0·000	n.d.	+0·010	n.d.
CC1-1	+0·033	0·000	−0·008	−0·016	−0·022
MC1-1	+0·018	+0·017	+0·016	+0·026	+0·024

n.d. = not determined.

The colonial mutants tested showed differing patterns of selective ability (relative to A3/5), but could be arranged into broad groups showing similarities. Mutants C134-1, C134-3 and C137-1 each had a selective advantage over A3/5 in glucose, ammonium and sulphate-limited chemostat cultures, but were disadvantaged (or neutral in the case of C134-3) in magnesium-limited chemostat culture. Of these three strains, only C134-3 had a selective advantage over A3/5 during culture washout. The data for C134-1 were inconclusive, however, as very few C134-1 propagules were present in the population at the onset of the experiment. Mutants C153, C135, and MC1-1 generally had a selective advantage over A3/5 in all nutrient-limited conditions tested (with the exception of C135, which behaved as a neutral mutant in glucose-limited culture) and also had a selective advantage over A3/5 during culture washout. C301 may also belong to this group, although the data are insufficient. C139 and C106 had a selective advantage over A3/5 only in magnesium-

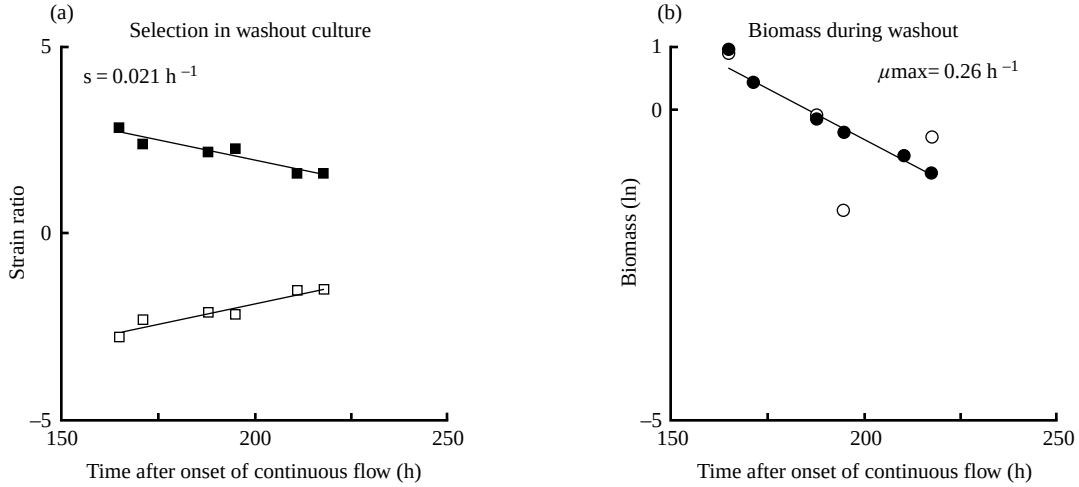

Fig. 2. *F. graminearum* A3/5 (■) and colonial mutant C139 (□) in mixed washout culture, $D = 0{\cdot}29\ h^{-1}$. Cultures were grown in modified Vogel's medium with 6 g glucose l^{-1} at 25°, pH 5·8, 1200 rpm and aeration rate $0{\cdot}8\ l\ min^{-1}\ l^{-1}$. A. Relative proportions of A3/5 and C139, expressed as the natural log of the ratio of the two strains. The slope of the line is the selection coefficient (s). B. Biomass concentration (expressed as natural log) taken from the fermenter sample port (●) and the culture overflow (○).

Table 5. Calculated maximum specific growth rates ($\mu_{max}\ h^{-1}$ of individual strains and/or of populations) for mutants C134-3, C137-1, C135, and C139 growing in mixed culture with A3/5 in a chemostat operated above D_{crit} (i.e. during washout)

	For mutant	For A3/5	For population
C134-3	0·31	0·29	0·30
C137-1	0·27	0·30	0·28
C135	0·25	0·24	0·24
C139	0·27	0·26	0·26

limited and in washout cultures. In glucose-, ammonium-, and sulphate-limited cultures they behaved as neutral or disadvantageous mutations. None of the Quorn® production plant derived mutants showed patterns of selective ability which were similar to CC1-1, which had been isolated from a glucose-limited chemostat.

Estimation of μ_{max} from washout kinetics

During mixed culture experiments in chemostats with a dilution rate of 0·30 ($\pm$0·01) h^{-1} (above the μ_{max}, 0·22 h^{-1}, for *F. graminearum* A3/5 in chemostat culture; Wiebe *et al.*, 1994*b*) biomass was washed out of the culture vessel. Fig. 2 shows the biomass concentration and the proportions of A3/5 and C139 strains during washout from the fermenter. For cultures above D_{crit}, the maximum specific growth rate of the culture can be calculated from the equation

$$\ln x = (\mu_{max} - D)\,t, \qquad (2)$$

where x is the biomass concentration, D the dilution rate and t the time (Cove, 1976). Assuming that the proportion of cfu of each strain in the population reflected the proportion of biomass contributed to the population by each strain, and that adhesion or detachment of biomass from fermenter surfaces did not occur during the washout experiment, it is possible to estimate the μ_{max} for individual strains, as well as μ_{max} for the total population. Specific growth rates determined from washout experiments and differences between specific growth rates for various strains are presented in Table 5. In the competition between A3/5 and C134-1, there were insufficient cfu of C134-1 in the population to make independent measurements of μ_{max} values for the two strains. In the competition involving A3/5 and C153, detachment of a significant amount of biomass from fermenter surfaces during the experiment meant that estimations of specific growth rates were inaccurate. For A3/5, the estimated value of μ_{max} varied from 0·24 h^{-1} to 0·30 h^{-1}. For the mutants, the estimated value of μ_{max} varied from 0·25 h^{-1} to 0·31 h^{-1}.

DISCUSSION

Wiebe *et al.* (1996*a*) demonstrated that vigorous growth only occurred between *nirA/niiA* and *cnx* mutants in heterokaryons in which the nuclear ratio of the two strains was close to 1:1. The high K_r values of the heterokaryons formed between colonial mutants and A3/5 demonstrate, therefore, the recessive nature of the mutations and shows that the phenotype was not due to an imbalanced nuclear ratio. The recessive nature of the mutations was also indicated by the results of complementation analysis of heterokaryons formed between complementing colonial mutants, as wild type or near wild type K_r values were again observed. In most heterokaryons, however, no complementation was observed and thus all mutants could be arranged into a single complementation group (Fig. 1), suggesting that the mutations were within the same gene or cluster of genes. Partial and inter-allelic complementation has often been observed between mutants of filamentous fungi (Woodward, Partridge & Giles, 1958; Ishikawa, 1962; Fincham & Coddington, 1963; Ratner & Rodin, 1976; Fincham, Day & Radford, 1979). If inter-allelic complementation does occur, the complementation maps are generally complex (Ishikawa, 1962; Wiebe *et al.*, 1992*a*) as was observed here and by Wiebe *et al.* (1992*a*) for colonial mutants isolated from glucose-limited chemostat cultures. That all of the colonial mutants mapped to a single complementation group indicates that mutations in only a small proportion of morphology-determining genes are selectively advantageous in the Quorn® myco-protein pro-

duction plant. Thus, altering the fermentation conditions to eliminate the selective advantage of mutants in this gene group(s) would provide a strategy for prolonging the length of production plant campaigns.

The competition between A3/5 and mutants C134-1, C134-3, C135, C137-1, C139, and C153 showed that although all of these mutants mapped to the same complementation group, they did not all share the same selective advantage in nutrient-limited chemostat cultures. Only C153 had a selective advantage over A3/5 in all of the conditions tested, suggesting that it is a general μ_{max} mutant, similar to MC1-1 (a strain isolated from a magnesium-limited fermenter; Wiebe *et al.*, 1992*b*). As the Quorn® myco-protein production plant is run as a glucose-stat, with theoretically no nutrient limitation, general μ_{max} mutants were expected to be the most frequent type of selectively advantageous mutation. C135 and C301 (isolated from a very early production campaign) may also be general growth rate mutants, although the data are inconclusive. C135 and C153 showed no complementation, suggesting that they may be related (Fig. 1). Of the remaining mutants, C134-1, C134-3 and C137-1 also had selective advantages over A3/5 in several different cultural conditions, although only C134-3 had a selective advantage over A3/5 during washout culture. In order to displace A3/5 in different nutrient conditions, these mutants must also have some general growth rate advantage over A3/5, although this advantage was not expressed in magnesium-limited culture. None of these mutants complemented C135 or C153, although C137-1 did complement C134-1 (Fig. 1). C139 and C106 were the only strains which had selective advantages in only one nutrient limitation (magnesium). However, both of these strains did have selective advantage over A3/5 in washout culture. Thus, it appears that they also are growth rate (μ_{max}) mutants.

As mutations with pleiotropic effects have previously been observed in chemostat adapted mutants of *Escherichia coli* with improved growth rates (Kurlandzka *et al.*, 1991), it seems probable that the genes involved in improved growth rate in *F. graminearum* also have pleiotropic effects. For example, a mutation which confers a general increase in growth rate in nutrient excess may also affect some metabolic pathways in such a way that the strain is disadvantaged or neutral in nutrient limited growth, even at high growth rates, particularly if the gene affects pathways involved in cell signalling or is a regulatory gene. Significantly, Kurlandzka, Rosenzwerg & Adams (1991) found that although expression of some proteins was increased in their mutants, expression of other proteins was decreased or absent. These are described as maladaptive pleiotropic effects by Lenski (1988) and are also known to occur in other organisms. Thus, most of the mutants isolated from the Quorn® production plant have improved growth rates (μ_{max}) when all nutrients are in excess (i.e. during washout culture), but do not necessarily have a selective advantage in all nutrient-limited chemostat cultures. Further, all of the mutants except C139 and C106 also had a selective advantage in at least three of the four nutrient-limited conditions. C106 was isolated from an early Quorn® production campaign, in which the operating conditions differed somewhat from later campaigns and these differences may account for its belonging to a separate complementation group.

Washout experiments were useful in determining selective advantages in conditions of nutrient excess, but were less useful for measurement of μ_{max} values. Table 5 shows that the estimated μ_{max} for A3/5 (0·26–0·30 h^{-1}) was found to be consistently above the dilution rate at which A3/5 has previously been observed to wash out of chemostat fermentations (0·22 h^{-1}) (Wiebe *et al.*, 1994*a*; Simpson *et al.*, 1995). However, the calculated values were comparable to the μ_{max} for A3/5 in batch culture ($0{\cdot}28 \pm 0{\cdot}01\ h^{-1}$, Wiebe, Robson & Trinci, 1989). It is unclear why μ_{max} is consistently lower in chemostat than in batch culture or why the washout experiments apparently had maximum specific growth rates which were more similar to batch growth than to growth in continuous flow culture.

Wiebe *et al.* (1992*b*, 1996*b*) suggested that periodic variations in nutrient-limitation or pH could be used to delay the appearance of colonial mutants in *F. graminearum* populations. Operating the production fermenter with oscillations between nutrient-excess (glucose-stat) and nutrient-limitation (chemostat) would not, however, be a viable option because, although this combination would continue to provide a high output of biomass, the present results show that many of the colonial mutants which have a selective advantage in conditions of nutrient excess also have a selective advantage in several different nutrient limitations.

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