Evolution of *Fusarium graminearum* A3/5 grown in a series of glucose-limited chemostat cultures at a high dilution rate

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The evolution of *Fusarium graminearum* A3/5 was studied in a series of glucose-limited chemostat cultures grown at a dilution rate of 0·18 h^{-1}: the second chemostat was inoculated with macroconidia harvested from a sub-culture derived from the first chemostat, and the third chemostat was inoculated with macroconidia harvested from a sub-culture derived from the second chemostat. Using this method it was possible to follow the evolution of *F. graminearum* for *ca* 3 months (531 generations). Periodic selection of advantageous mutants was detected in the population by monitoring increases and decreases in the proportion of cycloheximide-resistant macroconidia; 15 peaks of cycloheximide resistance were observed in the three cultures representing 14 adaptive changes with an adaptive change occurring in the population on average once every 135 ± 10 h (mean ± SE.). After 648 h of cultivation, a highly branched (colonial) mutant was detected in the population and this mutant increased in concentration (with a selection coefficient of 0·016 h^{-1} compared with the parental strain) and eventually formed 82–92% of the population but never completely supplanted the sparsely-branched population. A second mutant (A21–XS), more sparsely branched than A3/5, was isolated at 1277 h.

Few studies have been made of the evolution of filamentous fungi in chemostat culture, partly because of the technical difficulty of maintaining steady states of these organisms for prolonged periods. One problem is the tendency of moulds to adhere to vessel and probe surfaces, especially at the air/culture interface, resulting in biomass retention in the fermenter vessel. For this reason, chemostat cultures of filamentous fungi are usually grown in fermenters filled so that the culture broth continuously washes the top plate of the vessel (Rowley & Bull, 1973). However, even this precaution does not completely eliminate the accumulation of biomass on fermenter surfaces and, in addition, the continual washing of the top plate causes wear on the bearings and oil seals of top-driven vessels, necessitating frequent maintenance. Thus, steady states of filamentous fungal cultures can only be maintained for limited periods. Further, even for bacteria and yeasts, the selection of 'adherent' mutants can be avoided only if the culture is periodically transferred to a new vessel (Chao & Ramsdell, 1985; Helling *et al.*, 1987).

During chemostat culture, neutral mutants (mutants which have neither a selective advantage nor disadvantage when grown in competition with the parental strain) initially accumulate in the population at linear rates (Kubitschek, 1970). However, this linear accumulation is followed by oscillations in the concentrations of neutral mutants (Paquin & Adams, 1983; Trinci, 1992) with a decrease occurring when a mutant arises which has a selective advantage over the rest of the population but does not carry the neutral mutations being monitored. Thus, oscillations in the proportion of neutral mutants in a chemostat have been taken as indicative of 'periodic selection' (Dykhuizen & Hartl, 1983), i.e. the appearance of selectively advantageous mutants of unknown phenotype. It is possible to study periodic selection in chemostat cultures of *F. graminearum* because this organism produces macroconidia (Wiebe & Trinci, 1991) which, although multinucleate, are homokaryotic (Miller, 1946). Since macroconidia are formed from uninucleate phialides, the nuclei in a population of macroconidia harvested from a culture provide a sample of the nuclei present in the mycelial biomass.

If chemostat cultures of *F. graminearum* are maintained at a high dilution rate for more than *ca* 400 h, highly branched (colonial) mutants invariably appear and supplant (i.e. they have a selective advantage) the sparsely-branched 'parental' population (Wiebe *et al.*, 1991, 1993; Trinci, 1992). In a chemostat, the selective advantage of a mutant strain (e.g. a colonial mutant) compared with the parental strain, can be quantified by determining the selection coefficient(s) (Dykhuizen & Hartl, 1981).

$$s = \frac{\ln\left[\frac{p_{(t)}}{q_{(t)}}\right] - \ln\left[\frac{p_{(o)}}{q_{(o)}}\right]}{t}, \qquad (1)$$

where, s = the selection coefficient; $p_{(t)}$ = concentration of

the mutant strain at time t; $q_{(t)}$ = concentration of the parental strain at time t; $p_{(o)}$ and $q_{(o)}$ = the initial concentrations of each strain.

Previously, we have described the appearance of morphological mutants of the Quorn® myco-protein fungus, *F. graminearum* A3/5 grown in chemostat cultures for up to 744 h (Wiebe *et al.*, 1991, 1993). We demonstrated that several changes had occurred in the population before the appearance of morphological mutants and that the morphological mutants did not usually completely displace the parental strain (Wiebe *et al.*, 1991, 1993). Additionally, we have grown some of these morphological mutants in mixed culture with the parental strain to determine the biochemical basis of their selective advantage (Wiebe *et al.*, 1992*a*). However, the subsequent evolution of morphological mutants has not been studied. In this paper we demonstrate that the evolution of *F. graminearum* can be studied using a series of chemostat cultures, each inoculated with a suspension of macroconidia derived from a sub-culture of the population from the previous chemostat. This paper also reports the isolation of a mutant (A21-XS) of *F. graminearum* which is more sparsely branched than A3/5.

MATERIALS AND METHODS

Organisms and media

Fusarium graminearum Schwabe strain A3/5 was obtained from Mr T. W. Naylor, Marlow Foods, Billingham, U.K. Stock cultures were maintained as macroconidia at −70 °C in 20% (v/v) glycerol. Inocula were prepared as described previously (Wiebe *et al.*, 1991).

The defined medium of Vogel (1956) was used with glucose as the carbon source instead of sucrose, and 1·65 g $(NH_4)_2SO_4$ l^{-1} was substituted for 2·0 g NH_4NO_3 l^{-1} as the nitrogen source. 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. Glucose solutions (final concentration of 3·0 g glucose l^{-1} medium) for chemostat cultures were prepared in 10 l volumes and autoclaved for 60 min at 121°. No caramelization and no significant loss of glucose was observed. For plate cultures, modified Vogel's medium containing 10 g glucose l^{-1} and 3·3 g $(NH_4)_2SO_4$ l^{-1}/2 g NH_4NO_3 l^{-1} was solidified with agar (Davis Gelatine; 15 g l^{-1}, final concentration). For media to detect resistant strains, cycloheximide (0·25 mM final concentration) was added to modified Vogel's medium. All cultures were incubated at 25°.

Chemostat cultures

Chemostat cultures were grown at a dilution rate of 0·18 h^{-1} (under the prevailing conditions, and in the absence of glucose limitation, A3/5 has a maximum specific growth rate of *ca* 0·23 h^{-1}) in a Braun (B. Braun Medical Ltd, Aylesbury, Bucks) Biostat M (2 l) fermenter as described by Wiebe & Trinci (1991). Biomass retention in the fermenter vessel was monitored by comparing dry weight measurements of culture samples both from inside the fermenter vessel and from the overflow; no retention of biomass in the vessel was observed. Biomass concentrations remained approximately constant throughout the fermentation.

Detection of cycloheximide-resistant mutants and morphological mutants

Samples (15–25 ml) were removed daily from the fermenter vessel. Macroconidia in the sample were separated from the mycelial biomass by filtering the suspension through two layers of sterile lens tissue, and the percentage of cycloheximide-resistant macroconidia was determined. Viable counts (5 or 10 replicate plates per sample) of macroconidia were made using modified Vogel's medium as described by Wiebe *et al.* (1991). In addition, *ca* 3×10^4 macroconidia per plate were used to inoculate modified Vogel's medium containing 0·25 mM cycloheximide (5 or 10 replicates per sample). The plates were incubated for 3 d (for viable counts) or 6–8 d (to detect cycloheximide resistance).

Highly branched morphological mutants were identified from their colonial morphologies (Trinci, 1992) and the proportion of these mutants to parental strain in a sample was determined for the total culture population (i.e. macroconidia and mycelial fragments) as described by Wiebe *et al.* (1991). Mutants which were more sparsely branched than the parental strain were identified as colonies with longer colony diameters than parental colonies of the same age. As the colonies plated out for total counts were of different ages (macroconidial germination occurring later than fragment growth), it was not possible to determine the proportion of the more sparsely branched mutants in the total population.

Inoculation protocols

Chemostat AC20 was inoculated with macroconidia harvested from spread plates of *F. graminearum* A3/5 following the procedure of Wiebe & Trinci (1991); after the onset of continuous flow, the fermenter population was not disturbed for 48–72 h (9–14 retention times) to allow a steady state to develop. At 720 h (196 generations) after the onset of continuous flow, samples (0·1 ml containing *ca* 10^5 propagules) containing mycelia and macroconidia were taken from the AC20 culture and used to inoculate spread plates of modified Vogel's medium which were incubated for *ca* 10 d at 25° before being stored at 4°. Macroconidia harvested from these plates were used to inoculate a second chemostat culture (designated AC21) 2 d later. Similarly, 647 h (175 generations) after the onset of continuous flow in culture AC21, a sample was taken from the culture vessel to inoculate spread plates, and macroconidia from these plates were used after a 16 month period of storage at 4° to inoculate a third chemostat culture (designated AC25).

Measurements of colony radial growth rate and mycelial morphology

For colony radial growth rate (K_r) measurements, Petri dishes (9 cm diam.) containing 20 ml agar-solidified modified Vogel's medium were inoculated centrally with a small volume of a suspension of macroconidia in water. Colony diameter was

measured at × 10 magnification using a Shadowmaster (Baty & Co., Burgess Hill, Sussex), as described by Trinci (1969).

Measurements of hyphal growth unit length (*G*) were made on mycelia which had been grown for *ca* 24 h either on Cellophane overlaid agar-solidified medium or in liquid medium. The submerged cultures were grown in 50 ml volumes of medium in 250 ml Ehrlenmeyer flasks and were incubated on a rotary shaker (with a throw of 2·5 cm) at 200 r.p.m. *G* is a measure of hyphal branching (Trinci, 1974). Hyphal growth unit lengths of mycelia with at least six tips were measured using a MeasureMouse system (Analytical Measuring System) and a Nikon Microscope linked to a videocamera and an Amstrad 1512PC as described by Wiebe & Trinci (1991).

RESULTS

Appearance of colonial mutants in the three chemostat cultures

A highly branched (colonial) mutant was observed in the first chemostat (AC20) at 648 h (177 generations) after the onset of continuous flow (Fig. 1). Only one colonial mutant (designated A20-1) was observed in this chemostat. When the AC20 chemostat was terminated 864 h (235 generations) after the onset of continuous flow, the colonial mutant made up 69·6 ± 1·6% (mean ± S.E.) of the population; the selection coefficient of A20-1 over A3/5 was 0·023 h^{-1}.

At 720 h (196 generations) after the onset of continuous flow, samples from culture AC20 (at this time the colonial mutant formed 9·8 ± 1·1% of the AC20 population) were taken and used to inoculate spread plates. Macroconidia from these plates were used to inoculate the second chemostat (AC21) but, to allow the new culture to attain steady state, the population was not sampled until 48 h (14 generations) after the onset of continuous flow. At this time, a colonial mutant made up 5·5 ± 0·8% of the AC21 population, increasing to 13·2 ± 3·3% at 96 h (28 generations) after the onset of continuous flow. Thus, *ca* 90 h after the onset of continuous flow, the concentration of the colonial mutant in the second chemostat (AC21) was approximately the same as that in the first chemostat (AC20) at the time when the sample was taken to inoculate spread plates. At this point, the AC21 culture was considered to be equivalent to the AC20 culture at 720 h. The selection coefficient (0·018 h^{-1}) of the colonial mutant isolated from the AC21 culture was slightly lower than that (0·023 h^{-1}) isolated from the AC20 population. However, the colonial mutant present in the AC21 fermentation was morphologically indistinguishable from the colonial mutant from AC20 and the two mutants did not complement each other (Wiebe *et al.* 1992*b*). The colonial mutant increased in concentration in the AC21 culture until, 336 h (93 generations) after the onset of continuous flow (representing 966 h of evolution), it formed 90·6 ± 1·4% of the total population. This chemostat was maintained for a further 311 h (82 generations), during which period the concentration of colonial mutant fluctuated between 81·0 ± 2·5 and 92·2 ± 1·5% of the total population. At this time (647 h, or 175 generations after the onset of continuous flow; representing 1277 h of evolution), a sample was taken from the population to inoculate plates which after storage at 4° for 16 months were used to provide a suspension of macroconidia to inoculate the third chemostat (AC25).

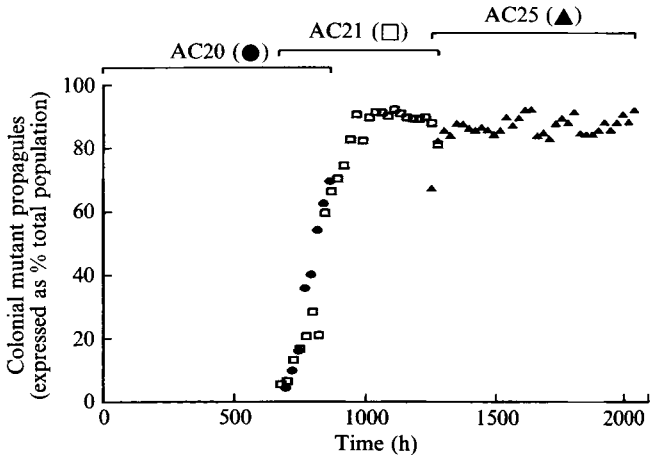

Fig. 1. Colonial mutant population (expressed as a percentage of the total population) in the series of three chemostat cultures initially inoculated with *F. graminearum* A3/5. The population was monitored throughout the three chemostat cultures (AC20, AC21 and AC25) as described in the methods. The period over which each chemostat culture was monitored is shown in the bars above the figure. The percentage of colonial mutants present in the population was used to determine the extent of overlap between experiments, as described in the text.

At 48 h after the onset of continuous flow, the colonial mutant in the third chemostat (AC25) made up 67 ± 1·8% of the population. This increased to 81·9 ± 2·4% at 72 h after the onset of continuous flow, a concentration comparable with that present in the AC21 chemostat (81·0 ± 2·5%) at the time when the sample was taken to inoculate spread plates. Throughout the remainder of the AC25 chemostat (total time = 840 h, or 204 generations; representing 2045 h of evolution), the percentage of the colonial mutant in the population varied between 81·9 ± 2·4% and 91·7 ± 2·0% (Fig. 1; and compare with the data above for the AC21 chemostat). Colonial mutants which were phenotypically different from A20–1 were occasionally observed in the third chemostat (AC25) but never contributed more than 1·1 ± 0·8% of the total population.

Thus, 90 h after the onset of continuous flow, the population in the second chemostat (AC21) resembled (in terms of the percentage of colonial mutant) the population in the first chemostat (AC20) from which it was derived, and, similarly 72 h after the onset of continuous flow, the population in the third chemostat (AC25) resembled the population in the second chemostat (AC21) from which it was derived (Fig. 1).

Periodic selection in the chemostat cultures

The concentration of cycloheximide-resistant macroconidia in chemostat populations were determined to monitor periodic selection (Wiebe *et al.*, 1993). For the combined data from the three chemostats, and, after appropriate allowance had been made for the periods of overlap between one chemostat and the next in the series, 15 cycloheximide-resistant macroconidia

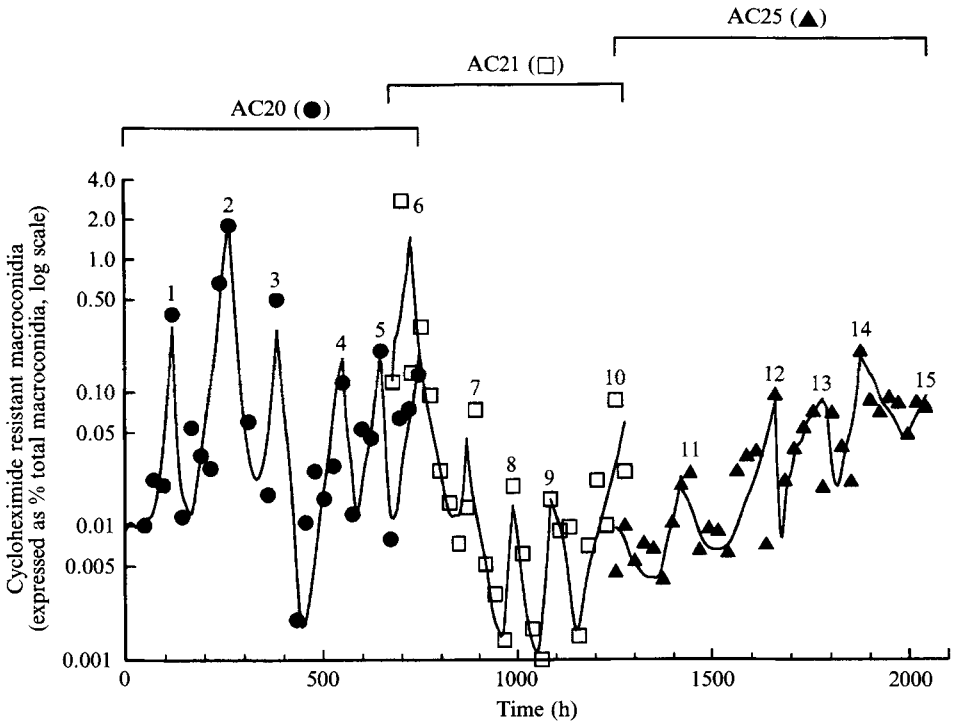

Fig. 2. Variation in the concentrations of cycloheximide-resistant macroconidia in three chemostat cultures [AC20 (●); AC21 (□) and AC25 (▲)]. The period over which each chemostat culture was monitored is shown in the bars above the figure. The percentage of colonial mutants present in the population was used to determine the extent of overlap between experiments, as described in the text and illustrated in Fig. 1. The line indicates the increases and decreases in the cycloheximide resistant population, following the principle that three or more consecutively increasing or decreasing data points indicate the trends in the population. Standard errors varied between ±0·0005 and ±0·29 (for values above 1·5%). The average standard error, for all data points, was ±0·015.

Table 1. Periods between peaks in the concentration of cycloheximide-resistant macroconidia present in a population *of F. graminearum**

	Time between peak maxima (h)														
Population	Peak 0–1	Peak 1–2	Peak 2–3	Peak 3–4	Peak 4–5	Peak 5–6	Peak 6–7	Peak 7–8	Peak 8–9	Peak 9–10	Peak 10–11	Peak 11–12	Peak 12–13	Peak 13–14	Peak 14–15
Total population	120	144	120	168	96	102	144	96	96	168	191	216	96	120	144
Sparsely branched mycelial sub-population	120	144	120	168	96	102	144	96	120	144	191	—	312	120	168
Highly branched mycelial sub-population	—	—	—	—	—	—	72	96	97	168	191	216	—	216	144

* *F. graminearum* A3/5 was grown in three glucose-limited (3 g glucose l^{-1} medium) chemostat cultures on modified Vogel's medium (25°, pH 5·8±0·1) at a dilution rate of 0·18 h^{-1}. The concentration of the colonial mutant in the population was used to normalize the data obtained from the three chemostats (see Figs 1, 2). Samples were removed from the vessel at intervals and the proportion of cycloheximide (0·25 mM) resistant macroconidia in the total macroconidial population in these samples was determined. The proportion of cycloheximide-resistance (macroconidia) in the sparsely branched sub-population mycelial and the highly branched (colonial) sub-population were also determined.

peaks were observed, suggesting that there had been 14 adaptive changes in the populations in the three chemostats (Fig. 2). The interval between the peaks did not increase or decrease in a regular manner and on average an interval of 135±10 h (mean±S.E.) was observed between peaks. No cycloheximide-resistant colonial mutants were observed in the first six peaks (Table 1) but after 822 h (245 generations), cycloheximide-resistant colonial mutants and A3/5-like cycloheximide-resistant mutants were both observed. In addition, fluctuations (indicative of periodic selection) in the concentrations of cycloheximide-resistant A3/5-like strains and cycloheximide-resistant colonial strains were observed. Oscillations in cycloheximide-resistant A3/5-like strains were observed throughout the experiment, even when the colonial mutant made up 90% of the total population (Table 1). Cycloheximide-resistant strains which were more sparsely branched than A3/5 were included with the A3/5-like strains, because of the difficulty in distinguishing the sparse strains without subculturing each resistant colony.

Isolation of a sparsely branched mutant

A sparsely branched mutant (designated A21-XS) was isolated from the AC21 chemostat after *ca* 1277 h (345 generations) of

Table 2. Comparison of hyphal growth unit length and colony radial growth rate of *F. graminearum* A3/5 and A21-XS*

Strain	Hyphal growth unit length† in liquid medium (G, μm)	Hyphal growth unit length† on semi-solid medium (G, μm)	Colony radial growth rate‡ (K_r μm h^{-1})
Parental strain (A3/5)	224 ± 10a	333 ± 16a	101 ± 3a
A21-XS	274 ± 10b	874 ± 37b	119 ± 3b
	(22%)	(162%)	(18%)

* *F. graminearum* A3/5 and A21-XS (a sparsely-branched strain isolated from the third chemostat culture) were grown in batch culture at 25° on modified Vogel's medium containing ammonium sulphate as the nitrogen source.

The results are the means (± S.E.) of 25† and 6‡ replicates, respectively.

The percentage increases, in K_r or G, for A21-XS relative to A3/5 are indicated in parentheses.

Figures in the same vertical column with the same letter are not significantly different ($P > 0.05$).

evolution. When grown in submerged batch culture on modified Vogel's medium, A21-XS was significantly ($P < 0.05$) more sparsely branched (longer G value) than A3/5 (Table 2). The difference in morphology between these strains was observed on both semi-solid and liquid media but was more marked on the former (Table 2). In addition, on semi-solid media, A21-XS had a significantly ($P < 0.05$) faster colony radial growth rate than A3/5 (Table 2).

DISCUSSION

Helling *et al.* (1987) used a small subculture from one chemostat culture to study the continued evolution of *E. coli* in the next culture in a series of chemostats. We have used this procedure to study the evolution *F. graminearum* A3/5 in three chemostats, each lasting *ca* 700 h (*ca* 190 generations), but with an interval of more than 1 yr between the end of the second fermentation and the start of the third. Using this method it was possible to follow the evolution of *F. graminearum* for *ca* 3 months (*ca* 531 generations). However, an interval of unpredictable duration (here, 72–90 h) was observed between inoculation of a chemostat and the time when its population apparently attained the same composition as the chemostat from which the macroconidial inoculum was derived.

The initial A3/5 population was supplanted by a highly branched (colonial) mutant (A20-1) which was first observed at 648 h (177 generations) and eventually formed *ca* 90% of the population. The colonial mutant never formed more than *ca* 92% of the total population, and was not replaced by any other colonial mutant. The results (Fig. 1) showed that the colonial mutant present in one chemostat appeared in the next chemostat in the series, and that in both chemostats the mutant behaved in a similar manner, i.e. they had similar selection coefficients. Further, the colonial mutants from the various chemostats failed to complement one another, suggesting that they are related. Where, as here, a morphological mutant is used as an indicator of the stage of evolution of a culture, differential growth/sporulation of the sub-populations in the sample plated out to provide inoculum for the next chemostat may affect the duration of this interval. In this instance, the mutant colonies expanded at only *ca* 15% of the rate of the parental colonies. Thus, the parental strain would colonise a greater proportion of the surface area of the plate than the colonial strain and therefore might contribute a higher proportion of the macroconidia harvested from this plate than it had in the sample used to inoculate the Petri dish. This problem might be avoided by storing the chemostat sample in glycerol at −70°. However, this technique is only suitable for the storage of macroconidia and would therefore result in the loss of the variation present in the coenocytic mycelial fragments in the population. For whatever reason, the results suggest that there was a difference between the proportions of the sub-populations present in the fermenter sample and in the suspension of macroconidia harvested from plates inoculated with this sample.

A sparsely branched mutant (A21-XS) was isolated at 1277 h (346 generations). This mutant never contributed more than 19% of the total population and was at a selective disadvantage relative to the highly branched colonial mutant (A20-1). Mutants more sparsely branched than A3/5 have not been isolated previously, although a mutant more sparsely branched than the parental strain has been observed in chemostat cultures of *Aspergillus oryzae* (Withers *et al.*, 1994). The co-existence of *F. graminearum* strains A21–XS and A20-1 in the population from 1277 h onwards resembles the co-existence of four strains of *E. coli* grown in a series of chemostats (Helling *et al.*, 1987): although one strain of *E. coli* was at a selective disadvantage relative to the others, it persisted in the population because of its ability to use certain carbon sources.

By monitoring changes in the concentration of cycloheximide-resistant macroconidia in the *F. graminearum* population (Table 1), it was possible to deduce that mutations occurred in the population. Fifteen peaks in cycloheximide resistance were observed, implying that the predominant population in the fermenter changed 14 times. The highly branched colonial mutant (A20-1) was responsible for the sixth population change. The interval between peaks did not increase or decrease in a regular pattern and, on average, an interval of 135 ± 10 h (mean ± SE) was observed. However, the presence of at least two sub-populations within the population may have masked variations in the duration of the interval between peaks. Intervals between peaks in the cycloheximide-resistant, colonial mutant population increased towards the end of experiment AC25 and a similar trend was observed in the cycloheximide-resistant, sparsely-branched population.

Data for the both sparsely strain and the highly branched mutant suggested that periodic selection was occurring (i.e. fluctuations were observed in the proportion of cycloheximide-resistant propagules in the population) and that the two strains evolved at approximately the same rate. The sparsely branched strain continued to evolve (i.e. periodic selection continued) even after it had been almost completely supplanted by the highly branched colonial strain. This co-evolution may explain why the sparsely branched strain was never completely eliminated from the population.

These experiments demonstrate that a series of chemostats can be used to follow the evolution of a fungus grown under constant environmental conditions and there is no technical reason why this period of evolution could not be extended beyond the 3 months reported here.

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