Evolution of *Aspergillus niger* and *A. nidulans* in glucose-limited chemostat cultures, as indicated by oscillations in the frequency of cycloheximide resistant and morphological mutants

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Aspergillus niger and *A. nidulans* were grown separately in glucose-limited chemostat cultures on modified Vogel's medium. Periodic selection (the appearance of new mutant populations) in *A. niger* was determined by monitoring oscillations in the concentration of cycloheximide-resistant mycelial fragments in samples from the fermenter vessel. Using these data, the interval between the periodic selection of each new mutant population was 32 ± 6 generations (mean $\pm$ S.E.M.). Periodic selection in cycloheximide resistance in three morphologically distinct sub-populations of the *A. niger* culture averaged 36 ± 2, 31 ± 11 and 28 ± 5 generations and these values were not significantly different from that of the whole population (32 ± 6 generations). Also, it was possible to estimate the rate of evolution taking place in these cultures by monitoring oscillations in the frequency of morphological mutants. The intervals between the periodic selection of morphological mutant populations in *A. niger* and *A. nidulans* were 28 ± 3 and 22 ± 2 generations respectively.

The rate of evolution of a continuous flow culture can be monitored by measuring the frequency of a neutral marker in the population (Novick & Szilard, 1950). The neutral marker accumulates in the culture at a linear rate until the resident population starts to become displaced by a new mutant which has a selective advantage over the previous strain but lacks the neutral marker (Kubitschek, 1970). The oscillations in neutral marker frequency form a jagged curve and the process is termed periodic selection (Atwood, Lillian & Ryan, 1951). Periodic selection has been observed in bacteria (Novick & Szilard, 1950; Helling, Vargas & Adams, 1987), haploid and diploid yeast (Paquin & Adams, 1983) and filamentous fungi (Wiebe *et al*., 1993, 1994*a*, *b*, 1995).

It is possible to monitor evolution in *Fusarium venenatum* (formerly called *F. graminearum*; Yoder & Christianson, 1998 and O'Donnell, Cigelnik & Casper, 1998) because it produces macroconidia in submerged culture (Wiebe & Trinci, 1991); periodic selection in this fungus can be monitored by harvesting macroconidia from the culture, plating them on selective medium and testing for the appearance of cycloheximide or chlorate resistant mutants. Unfortunately, industrially important fungi such as *Aspergillus niger* and *Penicillium chrysogenum* do not normally sporulate in submerged culture and consequently periodic selection in these fungi is difficult to measure. Further, use of second generation conidia (conidia harvested from plates inoculated with a sample from the fermenter; Wiebe *et al*., 1993) may yield non-representative data because of the presence of non or poorly sporulating morphological mutants in the population (Withers *et al*., 1994, 1995).

Using a strain of *A. niger* which forms morphological mutants in chemostat culture, we demonstrate that periodic selection in non-sporulating fungi can be monitored by plating mycelial fragments from the fermenter onto selective media. Resistance to cycloheximide was chosen for this work because it was known to be a neutral marker in glucose-limited populations of *Saccharomyces cerevisiae* (Paquin & Adams, 1983) and was nearly neutral in glucose-limited populations of *F. venenatum* (Wiebe *et al*., 1994b, 1995) and *A. niger* B1 was sensitive to it. Further, oscillations in the frequency of morphological mutants in the population occur at approximately the same interval as periodic selection.

MATERIALS AND METHODS

Strains and culture media

Aspergillus niger Tiegh. strain N402[pAB6-10] B1 (subsequently referred to as B1) is a transformant of *A. niger* N402 containing an additional 20 copies of the *A. niger gla*A (glucoamylase) gene (Verdoes *et al*., 1993). The transformation was carried out at the TNO Medical Biological Laboratory, Rijswijk, The Netherlands, and the strain was kindly provided by Dr P. J. Punt. *A. nidulans* (Eidam) G. Winter 70 is a haploid strain auxotrophic for pyridoxine and was kindly donated by Professor G. Turner (University of Sheffield, UK).

Stock cultures were maintained as conidial suspensions at −80 °C in 20% (w/v) glycerol. For use as inocula, conidia were harvested with (2 × 5 ml) sterile distilled water from 10 d old cultures grown at 30° on agar-solidified modified Vogel's medium (Vogel, 1956) in 9 cm Petri dishes; modified

Vogel's medium contained 5 (shake flask and fermenter cultures) or 10 g (Petri dish cultures) glucose l^{-1} as the carbon source instead of 10 g sucrose l^{-1}. For liquid media 1.65 g $(NH_4)_2SO_4$ l^{-1} was substituted for 2 g NH_4NO_3 l^{-1} as the nitrogen source. Vogel's stock solution was prepared at $50\times$ concentration and diluted 1:100 before use in liquid cultures. Stock Vogel's solution was filter sterilized through a 0.22 µm pore size filter (Millipore) and added to the sterile carbohydrate solution. Solutions containing glucose were sterilized by autoclaving at 121° for up to 60 min depending upon the volume used. Semi-solid medium was prepared by the addition of agar (1.5 g l^{-1} final concentration, Lucas Meyer). Pyridoxine was prepared as a 5 g l^{-1} stock solution and used at a final concentration of 500 µg l^{-1}.

Continuous culture conditions

Cultures were grown in either an Applikon (FT Applikon Ltd, Tewkesbury, UK) fermenter (working volume 2.3 l) or a 2 l Braun Biostat M fermenter (Braun, Reading, UK), according to the methods of Wiebe & Trinci (1991). Unless otherwise stated, cultures were maintained at $30\pm1°$ (mean $\pm$ S.E.M.) and pH 5.4 ± 0.1, agitated at 1000 rpm (using 3 six-bladed 48 mm diam. Rushton turbine impellers) and aerated with *ca* 0.7 l air (l culture)$^{-1}$ min^{-1}. Foaming was controlled by the addition of polypropylene glycol (mixed mol. wt; Foamaster, Henkel Performance Chemicals Ltd, Leeds, UK) to give a final concentration of *ca* 0.1% (v/v). Biomass accumulation on internal surfaces (head plate and probes) was minimized by periodically (generally once a day) increasing the stirrer speed to *ca* 1800 rpm for 5–10 min. Biomass accumulation was generally lower than, and did not exceed, approx. 1% v/v for *A. niger* or 10% v/v for *A. nidulans*.

Batch culture conditions

Shake flask cultures used 50 ml medium in 250 ml Erlenmeyer flasks. A final concentration of 0.16% (w/v) Junlon 110; (Honeywill and Stein Ltd, Wallington, Surrey, UK) was included in the medium to enhance filamentous growth (Trinci, 1983) and 0.05 M 2-(*N*-morpholino) ethane sulphonic acid (MES) was used to buffer the cultures at pH 5.4. Unless stated otherwise, all cultures were grown at 30° with rotary shaking (throw of 2.5 cm) at 200 rpm.

Measurement of fungal biomass and glucose concentration

Biomass concentration in the fermenter was monitored in 2×10 ml samples from both the vessel and the overflow. These were filtered through pre-weighed dried Whatman No. 1 filter papers and dried to constant weight at 70°; each sample was washed with *ca* 50 ml distilled water prior to drying. Glucose concentration in culture filtrates was estimated using a Reflolux glucose analyser (Boehringer–Mannheim).

Monitoring of cycloheximide-resistant mutants and viable counts

Samples (about 5 ml) were removed daily from the fermenter vessel. Ten replicate 100 µl aliquots were plated on to Vogel's medium containing 100 µM cycloheximide and incubated for 10 d at 30°. For viable counts, each sample was diluted 1:100 in sterile distilled water and ten replicate 100 µl aliquots plated on Vogel's medium. Plates were incubated at 30° for 4 d.

Changes in the population were assessed from the cycloheximide resistance data following the principle that three or more consecutively increasing or decreasing data points indicated the trends in the population (Wiebe *et al.*, 1995).

RESULTS

Evolution of Aspergillus niger *in chemostat culture as indicated by periodic selection in the entire population*

A. niger B1 is sensitive to cycloheximide (100 µM). When conidia (harvested from Petri dish culture) or mycelial fragments from the fermenter vessel were plated onto Vogel's agar medium containing 100 µM cycloheximide, however, spontaneous resistant mutants were observed at a frequency of 7.1×10^{-3} conidium^{-1} or 1.6×10^{-2} mycelial fragment^{-1}.

A glucose-limited chemostat culture of *A. niger* B1 was grown for 644 h (111 generations) at a dilution rate of 0.12 ± 0.002 h^{-1} (doubling time of 5.8 h). At this time the culture was terminated because of technical problems with the Applikon fermenter, but a sample (0.2 ml) removed from the chemostat after 628 h and plated on modified Vogel's medium was allowed to grow for 10 d, after which conidia were harvested and used to inoculate a second chemostat which was operated for a further 413 h under the same conditions as the first (Helling *et al.*, 1987; Wiebe *et al.*, 1995). Thus, the total evolutionary time of the combined chemostat cultures was 1041 h or about 180 generations. The decrease in cycloheximide resistance at the start of the culture (Fig. 1*a*) was attributed to wash-out of the B1 parental strain from the fermenter and the selection of a less densely sporulating light brown mutant. For the combined data from the two chemostats, and after appropriate allowance had been made for the period of overlap between both chemostats (Wiebe *et al.*, 1995), six peaks (including the initial decline) in the concentration of cycloheximide-resistant mycelial fragments were observed (Fig. 1*a*), representing six evolutionary changes in the population with an average interval between peaks of 186 ± 34 h (32 ± 6 generation).

Fig. 2 shows the relative proportions of the morphological sub-populations (i.e. the parental phenotype, light brown mutants and dark brown mutants) in a glucose-limited chemostat culture of *A. niger* B1. Samples removed from the start of the second chemostat revealed that the dark brown mutant predominated over the light brown mutant which had previously been dominant in the population towards the end of the first chemostat. In addition, the parental-like (B1-like) strain could not always be detected and had either been washed out of the culture after 800 h or was present in numbers too low to be detected by the present methods. The average interval between peaks for the morphological mutants was 161 ± 17 h (or 28 ± 3 generations; $n=10$).

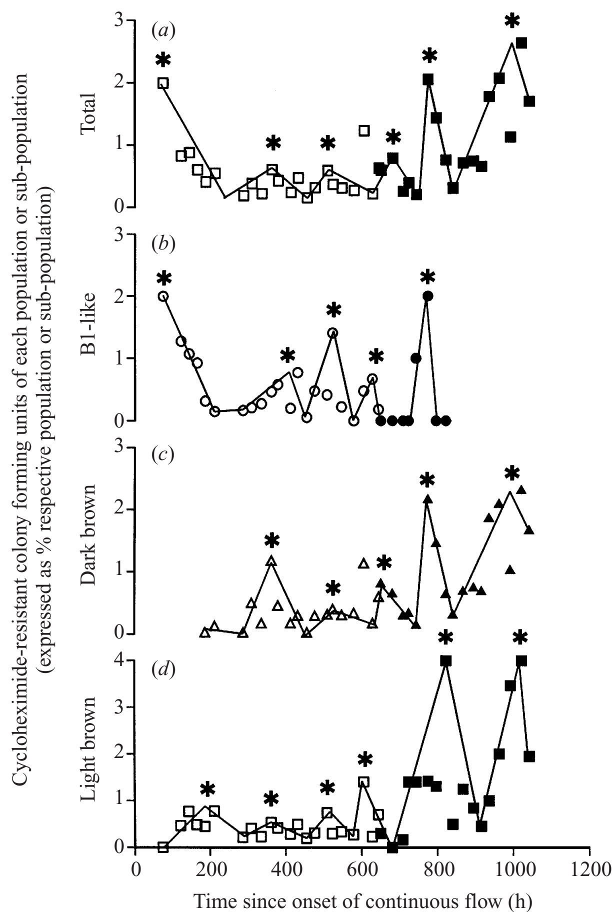

Fig. 1. Cycloheximide-resistant colony-forming units (cfu) in (*a*) a population initially inoculated with *A. niger* B1 and in (*b*, *c* and *d*) morphologically distinct sub-populations of B1 (*b*, B1-like, ○ and ●; *c*, dark brown, △ and ▲; *d*, light brown, □ and ■), grown in a glucose-limited chemostat on modified Vogel's medium, with glucose as the carbon source ($D = 0.12 \pm 0.002\ h^{-1}$ pH 5.4, 30°, 1000 rpm, 0.7 l air (l culture)$^{-1}$ min^{-1}. The open and closed symbols represent data taken from the first and second chemostat, respectively. Compounded S.E.M. was determined for each data point (based on 10 replicates for both total and cycloheximide-resistant counts) and varied from 1 to 100% of the mean, with an average of 38% error (1 to 40% of mean for total population; average error = 22%). Asterisks (*) mark peaks representing the selection of each new mutant population (sub-population). For each population (sub-population) the percentage of cycloheximide-resistant cfu is expressed relative to the total number of that population (sub-population) in the culture, i.e. number of B1-like cycloheximide-resistant cfu relative to the total number of B1-like cfu, etc.

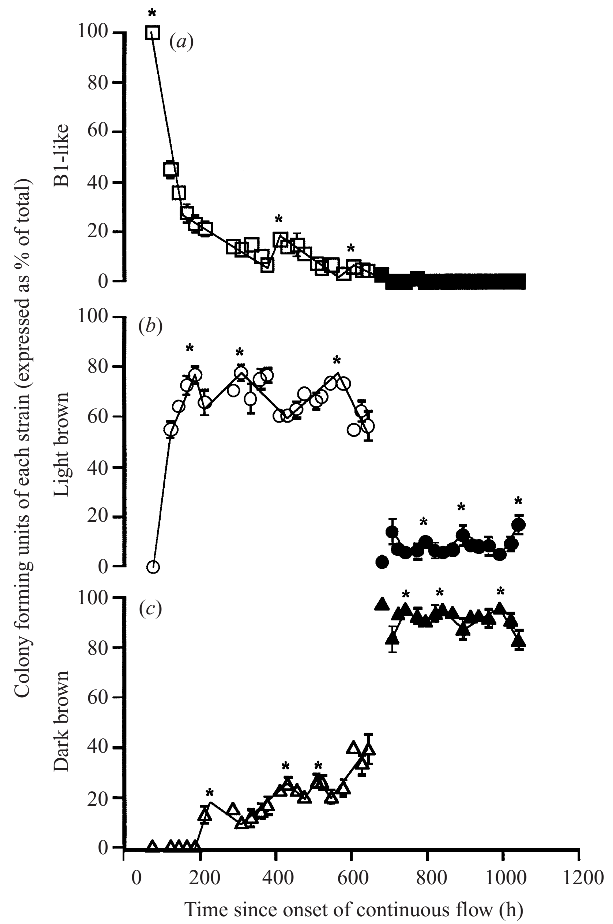

Fig. 2. The population composition of *A. niger* B1 grown in a glucose-limited chemostat on modified Vogel's medium ($D = 0.12 \pm 0.002\ h^{-1}$ pH 5.4, 30°, 1000 rpm, 0.7 l air (l culture)$^{-1}$ min^{-1}. The morphologically distinct sub-populations (*a*, B1-like, □ and ■; *b*, light brown, ○ and ●; and *c*, dark brown, △ and ▲) are expressed as a percentage of the total population. The open and closed symbols represent data taken from the first and second chemostat, respectively. Error bars denote the S.E.M. for 10 replicates; where no error bars are visible, the S.E.M. was less than the size of the symbol. Asterisks (*) mark peaks representing the new mutant sub-populations.

Evolution of Aspergillus niger *in chemostat culture as indicated by periodic selection in sub-populations*

The frequency of cycloheximide resistance in each of the three sub-populations of morphological mutants is shown in Fig. 1*b*–*d*. Five peaks (including the initial decline) could be observed in the parental-like (B1-like) sub-population, with an average interval between peaks of 180 ± 61 h (31 ± 11 generations; $n = 4$; Fig. 2*b*). Five peaks could be seen in the dark brown mutant sub-population also with an average interval between peaks of 164 ± 28 h or 28 ± 5 generations ($n = 4$; Fig. 1*c*). Finally, five peaks were observed in the light brown mutant sub-population with an average interval between peaks of 206 ± 14 h or 36 ± 2 generations ($n = 4$; Fig. 1*d*). The intervals between the peaks in each sub-population did not increase or decrease in a regular manner and, importantly, the maxima of the peaks in one sub-population did not necessarily coincide with the maxima in the other two sub-populations, implying that each mutant sub-population might have evolved independently of the others. No significant difference (Fisher's multiple range test; $P > 0.05$) existed, however, between the average intervals calculated for each sub-population.

Evolution of Aspergillus nidulans *in chemostat culture*

When *A. nidulans* 70 was grown for *ca* 1000 h at a dilution rate of 0.09 h^{-1} in a glucose-limited chemostat culture at pH 5.6, four morphological sub-populations (groups I–IV) were distinguished on the basis of their colonial morphology. Group I colonies (which resembled the parental strain, *A. nidulans* 70) produced abundant yellow conidia and no aerial

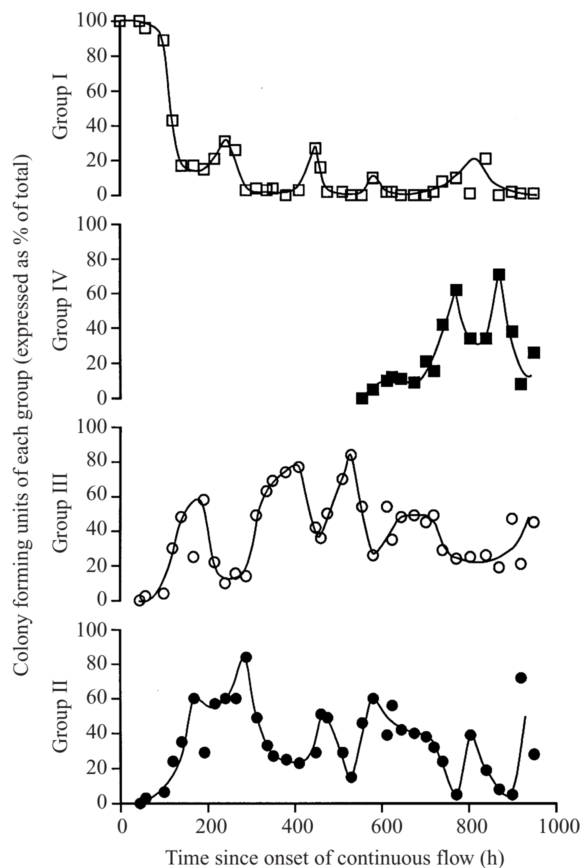

Fig. 3. The population composition of *A. nidulans* haploid 70 grown in a glucose-limited chemostat on modified Vogel's medium, with glucose as the carbon source ($25 \pm 0.1°$; pH 5.6 ± 0.2, $D \sim 0.09\ h^{-1}$, 1.0–1.4 air (l culture)$^{-1}$ min^{-1}; □, group I; ●, group II; ○, group III; ■, group IV).

hyphae. Group II colonies were cream coloured and produced fewer conidia than the parental strain. Group III colonies were cream, had 'fluffy' aerial hyphae but produced no conidia. Group IV colonies were cream and did not produce conidia but had 'spiky' aerial hyphae. When representatives of each colony type were sub-cultured onto agar medium further differences in morphology of the progeny were identified. These minor differences, however, could not be distinguished on the viable count plates, so only four different morphological sub-populations were scored.

Fig. 3 records the colony-forming units of each group as a percentage of the total population in the chemostat. After the initial substantial displacement of the group I (parental like) population from the fermenter (reduced from 100% at 73 h to *ca* 15% at 143 h after onset of continuous flow), groups II and III constituted approximately 80% of the total population. Between 143 h and 652 h after the onset of continuous flow, frequencies of these mutants in the fermenter varied between *ca* 15 and 85% for group II and between *ca* 10 and 85% for group III, as each group dominated the population and was then partially displaced by another strain. Towards the end of the culture (*ca* 718 h after the onset of continuous flow), the group IV population increased in the population and subsequently contributed to the oscillations in the percentage of groups II and III in the population. Periodic oscillations in mutant frequency were observed throughout the duration of the fermentation (Fig. 3), and the average interval between peaks for the morphological mutants was 172 ± 14 h (22 ± 2 generations).

DISCUSSION

Strain evolution is important in industrial fermentations where loss of productivity may occur with time due to the selection of less productive populations (Dunn-Coleman *et al.*, 1994). In carbon-limited chemostat cultures at dilution rates near to the maximum specific growth rate of the organism in a chemostat, as have been studied here, mutations which result in increased specific growth rates are likely to occur (Wiebe *et al.*, 1992). Small differences in specific growth rate may confer a substantial advantage to the mutant, although the growth rate difference may not be measurable directly, and if the mutation is not accompanied by a change in morphology these mutants may be difficult to identify.

Plating mycelial fragments directly from the fermenter vessel onto selective medium instead of using conidia (Wiebe *et al.*, 1993) offers a method to monitor evolution in continuous flow cultures of filamentous fungi that do not sporulate in submerged culture. It should be noted, however, that cycloheximide resistance has been shown to be a dominant mutation (Hsu, 1963; Paquin & Adams, 1983) and, therefore, the concentration of cycloheximide resistance may be over-estimated in a population when coenocytic mycelial fragments (as compared to haploid conidia) are used to determine cycloheximide resistance. Conversely, the use of a recessive marker could lead to underestimating neutral marker frequency.

Morphology mutants have been observed in chemostat cultures of *A. oryzae* (Withers *et al.*, 1994), *A. niger* (Swift *et al.*, 1998) and *A. nidulans* (Craig, 1997). In the present study, the average interval (161 ± 17 h or 28 ± 3 generations) between peaks in the proportion of morphological mutants present in the *A. niger* culture was not significantly different ($P > 0.05$) to the average interval (186 ± 34 h or about 32 ± 6 generations) estimated using cycloheximide resistance data obtained by plating out mycelial fragments. This suggests that oscillations in the morphological populations fairly accurately represented the number of selective events occurring in the chemostat culture, i.e. all selectively advantageous mutations could be distinguished morphologically, in contrast to evolution in populations of *F. venenatum* in which a significant number of advantageous mutations occur with no alteration of phenotype (Wiebe *et al.*, 1993). A similar (172 ± 14 h or 22 ± 2 generations) number of selective events was observed in the *A. nidulans* population and similar rates of evolutions have been observed in *Fusarium venenatum* (148 ± 22 h or 11 ± 2 generations, Wiebe *et al.*, 1994*b*; and 135 ± 10 h or 40 ± 3 generations, Wiebe *et al.*, 1995) and *Saccharomyces cerevisiae* (about 145 h or 41 generations, Paquin & Adams, 1983). In bacterial populations, however, intervals of about 300 h or 86 generations between selective events have been observed (Helling *et al.*, 1987).

The evolution of *A. nidulans* and *A. niger* into easily distinguishable groups on the basis of their morphological

appearance on viable count plates demonstrates that morphologically different sub-populations can co-exist in a chemostat for prolonged periods and that the sub-populations evolve at similar rates to the total population. The average interval (32 ± 6 generations) between the selection of each new mutant observed in the entire *A. niger* population was not significantly different ($P > 0.05$) to those observed for the mutant sub-populations (31 ± 11 generations for the B1-like population; 36 ± 2 generations for the light brown mutant; 28 ± 5 generations for the dark brown mutant). In some instances, however, co-evolution of several sub-populations may make it difficult to interpret data based only on the whole population.

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