Reducing fitness costs associated with antibiotic resistance

Experimental evolution in the filamentous fungus *Aspergillus nidulans*

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Sijmen Ecco Schoustra

Reducing fitness costs associated with antibiotic resistance

Experimental evolution in the filamentous fungus *Aspergillus nidulans*

Het verminderen van fitnesskosten verbonden aan antibioticumresistentie

Experimentele evolutie in de filamenteuze schimmel *Aspergillus nidulans*

Proefschrift

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Thesis Abstract

The use of antibiotics may result in a persistent resistance in the organism targeted by the antibiotic. Since in most cases resistance comes with fitness costs in the absence of the antibiotic, fitness restoration by compensatory adaptation is essential for the resistance to persist in a population. We have studied the process of compensatory evolution using a fludioxonil resistant mutant of the filamentous fungus *Aspergillus nidulans*. In an evolution experiment lasting for 27 weeks (about 3000 cell cycles) 40 parallel strains of this mutant evolved under three different environmental conditions.

Our results show that there is a severe cost of resistance (around 50%) in the absence of fludioxonil and that in all conditions used the mutant strain was able to restore fitness. Resistance was caused by a single mutation. Fitness compensation occurred in one, two or three discrete steps. Genetic analysis of crosses between different evolved strains and between evolved and ancestral strains revealed interaction between compensatory mutations and provided information on the number of loci involved in fitness compensation.

Since the results show that resistance and compensatory evolution to eliminate associated fitness costs require only a limited number of mutations, resistance management strategies only focusing on prevention of these steps without altering the use of fungicide are unlikely to be successful.

We have investigated the relative effectiveness of artificial selection versus natural selection on the rate of compensatory evolution. Using mycelial growth rate as a fitness measure, artificial selection involved the weekly transfer of the fastest growing sector onto a fresh plate. Transferring random samples of all the spores produced by the mycelium approximated natural selection. Results show that the transfer of a random sample of all spores leads to more rapid adaptation than the transfer of the visually 'fittest' sector.

Many fungi have a 'parasexual cycle' in which haploid nuclei may fuse to form relatively stable diploid nuclei. Diploid nuclei can spontaneously produce haploid recombinants by repeated loss of chromosomes, and/or diploid recombinants by mitotic crossing-over. In an experimental study of adaptation over 3000 cell cycles with isogenic haploid and diploid strains of *A. nidulans,* we demonstrate that evolved populations that started as diploid but reverted to haploidy have a higher rate of adaptation than populations that began and remained haploid or diploid. We propose that the parasexual cycle enables fungi to exploit an extended vegetative diploid phase to accumulate recessive mutations and subsequent haploidization to generate high-fitness recombinants, thus enhancing adaptation to novel environments.

The velvet allele (*ve*A1) is known to prevent the formation of banding patterns in spore formation in *A. nidulans*. We observed a light inducible rhythm in the formation of conidiospores and ascospores in a velvet- *A. nidulans* mutant. Further characterization showed that the rhythm is light entrainable, however needs to be induced by a cycle of light and darkness and therefore is non-circadian.

We present a model and experiments on the population dynamics of lysogeny. The results of competition experiments with sensitive and lysogenic bacteria show that lysogens can successfully prevent invasion of sensitive bacteria and are able to invade in and recruit from a population of sensitive bacteria. This is consistent with the model we developed. The ecological role of lysogeny is discussed as similar to the ecological role of bacteriocins: temperate phage can act as allelopathic agents.

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1 Introduction

Antibiotics were discovered by Alexander Flemming in 1928. He realized that the fungus infecting his bacterial culture was excreting a substance that killed bacteria. This substance is now known as penicillin. His important finding started the era of antibiotics. In the 1940s, antibiotics were used for the first time in human medicine. Since then they have proven to be very powerful to treat microbial infections, not only in human medicine, but also in agriculture. The success of antibiotics led to an abundant use.

In the last decades, it has become evident that this abundant use of antibiotics has caused the wide-spread development of resistance. Figure 1 illustrates the worldwide prevalence of penicillin resistant Streptococcus pneumoniae, a human pathogen (Baquero and Blazquez 1997). Multiple resistant Staphylococcus aureus (MRSA) is another well-known example of a human pathogen that is increasingly difficult to cure with antibiotics and recently, the emergence of a novel type of vancomycin resistant S. aureus (VRSA) has been reported (Kacica and McDonald 2004). Resistance genes can spread into nature, resistance to clinical antibiotics is found in bacteria in wild rodents who themselves have never been exposed to the antibiotic (Gilliver et al. 1999).

In agriculture, the main pest microorganisms are fungi. Farmers growing crops have to contend with approximately 80 000 mostly fungal plant diseases, 30 000 species of weeds, 10 000 species of insects and 1 000 species of nematodes (IFIC 2004). Since the 1950s, fungicides (antibiotics directed against fungi) have been applied. At first, broad-spectrum fungicides such as sulfur and copper

compounds were used; resistance was not a problem then. In the 1960s, synthetic chemical research led to the introduction of new narrow spectrum fungicides such as phenylamides, aminopyrimidines and azoles (Russell 1999). In the first years, the new fungicides revolutionized crop protection. However, in the late 1970s resistance became a problem. Since then, for example, 50% of all *Phytophthora infestans* isolates in The Netherlands had become fungicide resistant (Staub 1991).

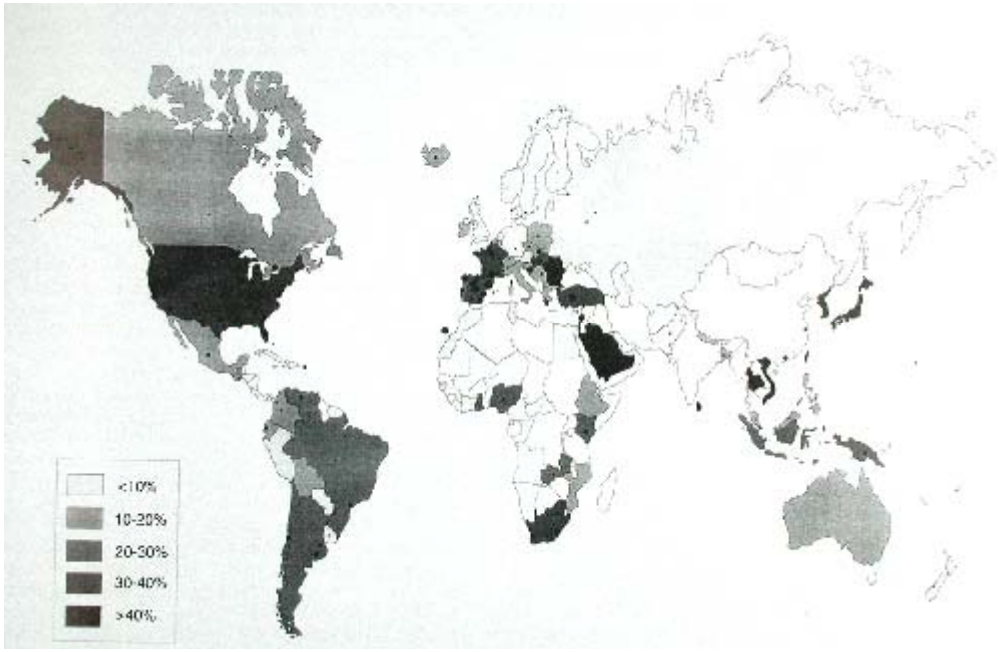

Figure 1. Prevalence of penicillin resistant *Streptococcus pneumoniae* as percentage of the total population. *S. pneumoniae* is a human bacterial pathogen causing pneumonia, meningitis and other diseases. In the 1970s this organism was considered as constantly susceptible to penicillin, at present, resistance rates of up to 70 % are reported among clinical isolates. This figure was taken from Baquero and Blazquez (1997).

By the 1990s programs and policies were implemented to reduce resistance development, the formation of the European Fungicide Resistance Action Committee (FRAC) being one of these initiatives (Christ and May 2003; Follet 2000; Locke et al. 2001). Measures have been suggested to reduce the risk of

resistance development; these include non-chemical ways of pest control, fragmentation of the area of use, the use of mixtures of multiple fungicides and close monitoring of resistance development (Brendt and Hollomon 1998; Christ and May 2003; Lossbroek 1996; Russell 1999; Staub 1991).

In the Netherlands, fungicides are primarily used for pest control in the cultivation of flower bulbs and potatoes. Several agencies of government and farmers (LTO) are working together in fungicide resistance management and, in 1990, initiated the so-called Meerjarenplan Gewasbescherming. An evaluation published in 2001 reports that the fungicide use in the Netherlands had increased to 4,000,000 kg active compound per year in the years 1990-2000 (Ekkes et al. 2001). The goal formulated by farmers' organization LTO was a decrease of 35% of fungicide use in this time period.

Microorganisms such as bacteria and fungi can develop resistance by a single mutation in the genome and resistance can spread when conditions are favorable. However, in most if not all cases, there is a cost for the resistant organism associated to the resistance mutation; i.e. the newly resistant organism has a reduced fitness when growing in the absence of the antibiotic (Cohan et al. 1994; Schrag and Perrot 1996). The persistence of resistance will (in part) depend on the rate and degree in which natural selection will be able to ameliorate these costs (Andersson and Levin 1999; Maisnier-Patin and Andersson 2004). The process whereby these costs are alleviated by mutation and natural selection is referred to as compensatory evolution. Experimental data on genetic details of compensatory evolution are mainly from prokaryotic systems (Bouma and Lenski 1988; Levin et al. 2000; Maisnier-Patin and Andersson 2004; Reynolds 2000; Schrag et al. 1997).

Antibiotic resistance and adaptive landscapes

Antibiotic resistance and associated costs of resistance can be seen in the context of the adaptive landscapes, as first proposed by Wright in the 1930's (Wright 1982) and as illustrated by the cartoon in Figure 2. In a stable situation without antibiotics in the environment, a population will be on or around a fitness peak. Introducing antibiotics changes the environment and thus the landscape, now a sensitive population may suddenly be in an adaptive valley. By acquiring a mutation that renders resistance, the population will adapt to the novel environment, climb an adaptive peak and thus change position in the landscape. When the antibiotic is no longer present in the environment, i.e. placing the newly resistant population back into the original landscape, this resistant population may now no longer be on an adaptive peak but rather in a valley. This can explain the phenomenon of cost of resistance (Cowen et al. 2001; Moore et al. 2000b; Uyenoyama 1986). From the adaptive valley, the population can again improve itself by acquisition of one or more adaptive mutations. Different adaptive peaks could be approached causing the final fitness of different parallel resistant populations to differ from one another. In principle, the final fitness could be even higher than the original sensitive wild type.

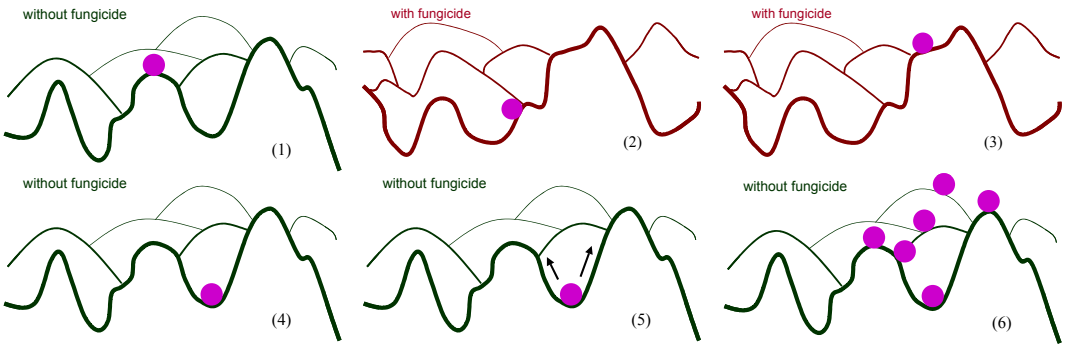

Figure 2. Development of resistance and subsequent compensatory evolution in the context of adaptive landscapes. Panel (1) shows a stable situation, the population is on a fitness peak. Introduction of fungicide changes the landscape and a mutation leading to higher fitness can occur, panels (2) and (3). Removing the fungicide places the population back in the original landscape, panel (4); the population is no longer on a peak and compensatory mutations will enhance fitness, panel (5). Different fitness peaks can be approached by different populations, panel (6).

Based on the above, three predictions can be made regarding the fitness development of a newly formed resistant mutant. First, we expect that a newly formed resistant mutant bears costs associated with the resistance in an antibiotic free environment. Second, the resistant mutant can improve its fitness by acquiring one or more adaptive mutations. There are different adaptive trajectories; parallel populations of the same resistant mutants may evolve to a different peak. Additionally, adaptive evolution may lead to higher fitness than the original sensitive ancestor. Third, when during the process of adaptation the environment keeps changing, the final fitness of the evolved resistant mutant may be even higher because the population has moved into a larger part of the adaptive landscape.

Principles of experimental evolution

Resistance development and subsequent compensatory adaptation to ameliorate fitness costs is an evolutionary process. Darwin started the study of evolutionary processes, mainly by studying organisms that had evolved in a certain way and by trying to reconstruct the trajectory using fossils. Since the 1990s, the study of evolution has become experimental by introducing microorganisms as model organism. This has enabled the testing of evolutionary hypotheses on the dynamics of adaptation and diversification since in contrast to mammals and popular model organisms such as *Drosophila,* microorganisms can go through thousands of generations within the time of a research project and samples of different time points can be stored for later analysis. Additionally, microorganisms have shown to be sufficiently genetically complex to address general evolutionary questions that go beyond just microorganisms (Elena and Lenski 2003; Lenski and Travisano 1994).

Advantages of microorganisms as model system in experimental evolution include the following: they are easy to grow; they reproduce quickly; they allow large populations in small spaces; (ancestral) samples can be stored and revived for later analysis and competition; environmental variables can be easily manipulated and the genetics is well known (Elena and Lenski 2003). After determining the evolutionary question, a typical evolution experiment is set up in the following way. From a single ancestor, several identical independent populations are established. These populations are grown in a well-controlled and reproducible environment; the environmental conditions are set by the evolutionary question. To keep the populations growing for many generations, a sample of the population is propagated to fresh medium at appropriate time points. Samples of the ancestor and the evolving populations are taken at certain time points and stored in a non-evolving state, making it possible to analyze the ancestor together with evolved populations in one experiment.

Experimental studies in antibiotic resistance development and compensatory evolution

The evolutionary question of development of resistance to antibiotics and subsequent compensatory evolution has been studied experimentally using bacteria. Lenski (1997) has argued that the experimental results found in laboratory showing fitness costs associated to resistance and compensatory evolution are very likely to occur similarly in nature. In one of the first studies, *E. coli* was transformed with a resistance encoding non-conjugative plasmid bearing fitness costs and propagated for 500 generations. By mutations on the bacterial genome, the costs of carriage of the plasmid were eliminated and turned into a beneficial bacterium-plasmid association, even in the absence of antibiotic (Bouma and Lenski 1988). A similar study with another non-conjugative plasmid confirmed these results (Modi and Adams 1991). Dahlberg

and Chao (2003) used a conjugative low-copy plasmid coding for resistance bearing fitness costs and obtained similar results when growing the bacterium with the plasmid in the absence of antibiotic. Rather than loss of the plasmid the fitness costs are compensated for by second site mutations on the bacterial genome. Cohan et al. (1994) studied the development of rifampin resistance in *Bacillus subtilis* and discussed the potential for compensatory evolution after observing different mutants with different costs.

In *E. coli*, ribosomal mutations (*rpsL*) can result in streptomycin resistance with a substantial fitness cost. Schrag and Perrot (1996) studied compensatory evolution in *rpsL* mutants in the absence of antibiotic and found that the fitness was restored to nearly wild-type levels without loss of resistance. A follow up study (Schrag et al. 1997) showed the second site mutations compensating for fitness costs create a genetic background in which strains with the $rpsL^+$ allele have a lowered fitness; i.e. the process of resistance development and compensation is an irreversible one. These results were confirmed in a study with rifampin resistant *E. coli* (Reynolds 2000). The ascent of intermediate-fitness compensatory mutants, rather than high-fitness revertants can be attributed to higher rates of compensatory mutations relative to that of reversions. This effect is further enhanced by the numerical bottlenecks associated by serial passage (Levin et al. 2000).
Bjorkman et al. (1998) have studied the virulence of antibiotic-resistant *Salmonella typhimurium* in mice. They found a cost of resistance resulting in a decreased virulence, however, the virulence could be increased by compensatory mutations without loss of resistance. Different types of compensatory mutations where found in bacteria evolving through serial passage in mice than in bacteria evolving in a laboratory medium (Bjorkman et al. 2000).

In eukaryotes, the development of antibiotic resistance has been studied experimentally. In *Candida albicans*, development of fluconazole resistance

shows divergence in parallel populations in level and mechanism of resistance (Cowen et al. 2000; Cowen et al. 2001). Different modes of selection to which populations are exposed influence the evolution of resistance in experimental populations as was shown with *Saccharomyces cerevisiae* growing with increasing and permanently high fluconazole concentrations (Anderson et al. 2003).

The genetics of *Aspergillus nidulans*

In this study, the filamentous fungus *Aspergillus nidulans* is used as model organism. *A. nidulans* has a well studied genetics that was first described by Pontecorvo et al. in 1953. This fungus grows to make a mycelium consisting of a web of hyphae that are divided into compartments by septa. Septa are believed to stabilize the hyphae and protect the mycelium by localizing damage to a limited area and to enable the mycelium to undergo differentiation (Gull 1978).

A. nidulans is homothallic (i.e. there are no mating types) and has an asexual and a sexual reproductive cycle. The lifecycle is drawn schematically in Figure 3. The formation of mycelium usually starts from a single spore; this spore can be a sexually derived ascospore or an asexually derived conidiospore. A small part of mycelium containing at least one nucleus can also be used to initiate the formation of a new fungal colony. The mycelium will consist of nuclei isogenic to the founding spore and will thus be homokaryotic. However, mycelium of different *A. nidulans* genotypes can fuse to form heterokaryons with two or more different types of nuclei. Heterokaryons are unstable but can be maintained in permissive conditions, such as on a medium where the two genotypes need to complement each other in order to grow (e.g. in the case of having different auxotrophic markers; Pontecorvo et al. 1953).

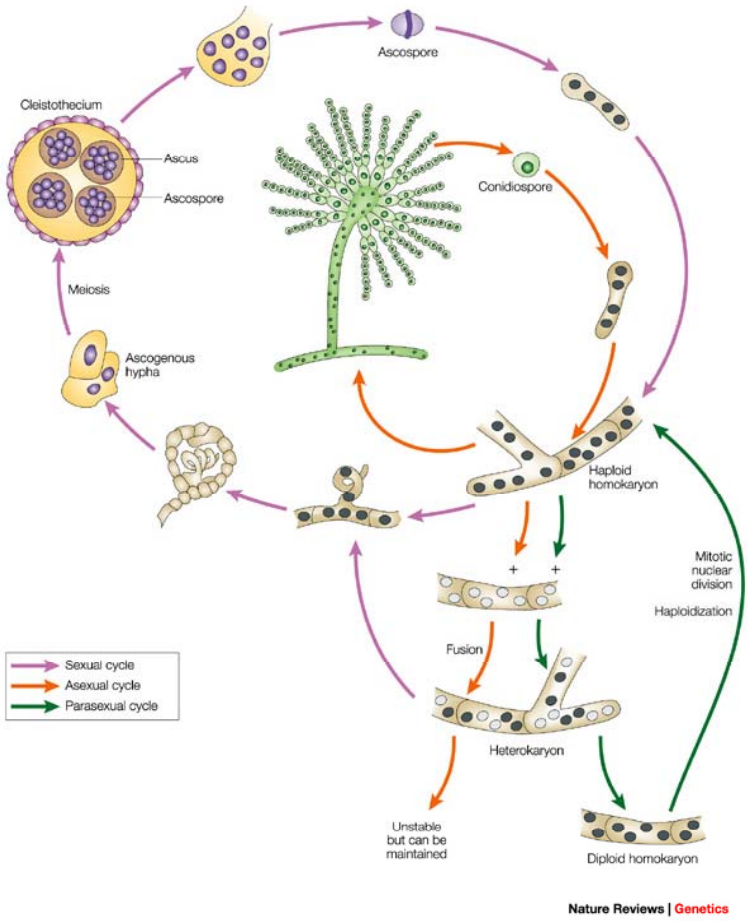

Figure 3. Lifecycle of *A. nidulans*.
The asexual cycle starts when mycelium, which develops from a single haploid spore, differentiates into a foot cell and a spore head. Many identical asexual spores known as conidia or conidiospores are formed (see the asexual cycle in the figure).

The sexual cycle starts when a pair of nuclei divides in synchrony to form the ascogenous hypha. In the tip of the hypha, two nuclei fuse and go through meiosis and then through one mitosis, resulting in eight haploid spores that are held together in an ascus. Thousands of asci may be formed within one fruiting body (cleitothetium) that thus can contain up to 100 000 ascospores.

The parasexual cycle starts with the formation of a heterokaryon of two possibly genetically different nuclei. These heterokaryons are not stable but can be maintained in permissive conditions. Two haploid nuclei can fuse to form a (heterozygous) diploid that can form mycelium and can grow vegetatively in a stable way. Mitotic crossing-over may occur between homologous chromosomes. Non-disjunction can cause random chromosome loss; eventually repeated non-disjunction will restore the haploid chromosome number (which is eight) and this haploid will have a recombined set of mostly non-recombined chromosomes. These events can be used to map gene orders and assign new genes to the eight linkage groups. The parasexual cycle and its possibilities for genetic analysis were developed studying *A. nidulans*. Related organisms without sexual cycle are now being analyzed and exploited using the same techniques. Examples are *A. niger*, *A. fumigatus*, *Fusarium oxysporum* and *Penicillium chrysogenum*. This figure was taken from Casselton and Zolan (2002).

Approximately 16 hours after initiation of a new mycelium, the first specialization of hypal cells becomes evident (Adams et al. 1998). Aerial branches will develop differentiating in conidiophores, structures carrying the asexual spores; the formation of these asexual spores commences after 6 to 8 hours (Adams et al. 1998).

Twenty-four hours after initiation if the asexual cycle, the sexual cycle is initiated. The formation of a sexual fruiting body, or cleistothetium, starts with a closed ascocarp in which a dikaryotic hypha is present (Casselton and Zolan 2002; Swart and Debets 2004). The two nuclei in this hypha divide synchronously, the dikaryotic hypha will become branched and subsequently, fusion of nuclei will occur giving rise to a diploid nucleus that can undergo meiosis. This process of fusion and meiosis will be repeated several times in the hypha; the end products of many independent meioses originating from the same parental nuclei will be present in one sexual fruiting body, which will consist of up to 100,000 sexually derived spores (Swart and Debets 2004). In most cases, the two nuclei fusing to subsequently undergo meiosis will be identical, leading to a self-fertilization. However, if the mycelium has two genetically different nuclei like in the case of a heterokaryon, out crossed progeny can be obtained. After the start of a mycelium, it takes around 6 days for the sexual cycle to complete.

By spontaneous fusion of haploid nuclei in the mycelium, diploid nuclei can be formed giving rise to a vegetatively growing diploid mycelium and diploid asexual spores and initiate the parasexual cycle. The parasexual cycle is of use for genetic analysis. In nature, 0.1 % of the nuclei of *Aspergillus nidulans* may be diploid (Pontecorvo 1958). Most diploid nuclei will be completely homozygous, but if emerged from a (haploid) heterokaryon, heterozygous diploids can be formed. By mitotic non-disjunction, the parasexual cycle is initiated. Random loss of chromosomes can lead to aneuploid nuclei and

continued loss of chromosomes will eventually lead to haploid nuclei, the reverted haploid will have a recombined set of non-recombined chromosomes (Swart and Debets 2004).

In somatic diploid cells recombinants may arise by mitotic crossing-over; crossing-over between nonsister chromatids of homologous chromosomes resulting in homozygosity for markers distal to the point of crossing-over (Pontecorvo 1958; Swart and Debets 2004). Together with meiotic mapping, mitotic linkage analysis with heterozygous diploids has proven to be very powerful tool in genetic analysis and has led to a linkage map of *A. nidulans* (Käfer 1958); many genetical markers are known (Clutterbuck 1974). Recently, the full genome has been sequenced (www.genome.wi.mit.edu/annotation/fungi/aspergillus/).

Culturing techniques have been described by Pontecorvo et al. (1953) and Clutterbuck (1974). *A. nidulans* can grow in liquid culture but is usually cultured on (semi-)solid media. In liquid media no sexual or asexual spores are formed (Adams et al. 1998). Strains can be stored as conidia on silica gel at room temperature for many years (Clutterbuck 1974); alternatively, samples of spores and mycelium can be stored in glycerol-peptone at –80 ^{o}C. From heterokaryons, heterozygous diploids can be obtained by plating conidiospores on selective media with a top-layer, haploidisation and thus parasexual recombination and analysis can be enhanced by adding *p*-fluorophenylalanine or benomyl to the medium (Käfer 1958; Swart and Debets 2004). Sexual analysis is done by analyzing the progeny of a crossed cleistothetium (Casselton and Zolan 2002; Swart and Debets 2004).

The fungicide fludioxonil

In this study, the fungicide fludioxonil (4-(2,3-difluoro-1,3-bezodioxol-4-yl)pyrrol-3-carbonitril) is used. It is a derivate of pyrrolnitrin, an fungicide naturally produced by various *Pseudomonas spp* and has a broad spectrum activity against fungi amongst the Ascomycetes, Basiomycetes and Deuteromycetes (Hilber et al. 1995). As mode of action interference of transport related phosphorylation of glucose (Hilber et al. 1995; Jespers et al. 1993). It has been shown that in *A. nidulans,* over expression of ABC transporter B decreases sensitivity to fludioxonil (Andrade et al. 2000).

Fludioxonil is applied in vineyards to control Grey mold caused by *Botrytis cenerea.* On grapes, it usually decays to half its activity in around 24 days, in wine made out of the grapes up to 70 % residue of fludioxonil can be found (Cabras and Angioni 2000; Cabras et al. 1997; Verdisson et al. 2001).

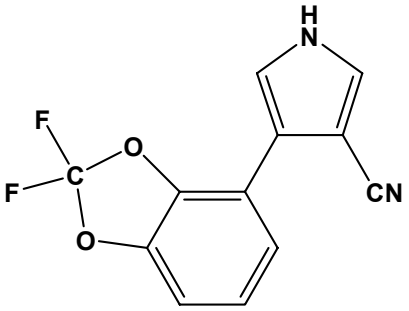

Figure 4. Chemical structure of fludioxonil, 4-(2,3-difluoro-1,3-bezodioxol-4-yl)pyrrol-3-carbonitril.

Aims and outline of the thesis

This thesis aims to unravel the initial phases of the evolution of fungicide resistance and compensatory changes that alleviate fitness costs of resistance mutations in the filamentous fungus *Aspergillus nidulans*. Fungicide resistance not only forms a useful model for the experimental study of adaptation and gene interaction, but also is of great practical importance in it's own right. Especially compensatory evolution may contribute to the longtime persistence of resistant strains, even after the application of fungicide is terminated. The model organism *Aspergillus nidulans* has well known genetics. To study the process of adaptation, the use of a filamentous fungus has several advantages, which are explored in this thesis.

We anticipate the following scenario of the evolution within a fungal population when exposed to fungicide. Resistance mutations will arise in the population. When grown in a fungicide free environment, resistance mutations will most likely have negative pleiotropic effects. Selection will tend to reduce any negative pleiotropic side effects of the genetic resistance by compensatory mutations at other loci affecting the cellular functions that have been negatively affected by the resistance. This will lead to epistatic fitness interactions between loci.

This thesis will provide experimental information on the dynamics of the evolution of resistance in the genetic model system *Aspergillus nidulans* and will have implications for the anticipated dynamics of resistance development and compensatory evolution in natural systems.

Chapter 2 describes the characteristics of *A. nidulans* as a model organism in experimental evolution. We show that resistance to fludioxonil bears a cost. Compensation under various environmental conditions is studied. The number

of loci involved in resistance and fitness compensation is assessed using classical genetical tools.

Chapter 3 reports a study with resistant haploid and diploid strains of *A. nidulans* in compensatory evolution. Apart from the process of compensation, we evaluate the role of parasexual recombination in filamentous fungi.

Chapter 4 compares two approaches in the experimental evolution procedure, resembling artificial selection and natural selection. We show that the natural selection approach leads to faster adaptation than the artificial selection approach.

Apart from the study of fungicide resistance, this thesis describes two side projects.
Chapter 5 describes the presence of rhythms in spore formation in an *Aspergillus nidulans* strain, which was observed phenotypically in several evolved strains obtained from the study described in Chapter 2. Circadian and other rhythms are believed to be present in many if not all living organisms but were never observed phenotypically in *A. nidulans.*

Chapter 6 describes a project on the possible allelopathic properties of temperate phage that was carried out in the laboratory of and in collaboration with professor Bruce Levin in Atlanta (USA).

Chapter 7 is a summarizing discussion of the thesis work including practical implications of the results found in this thesis and a guideline for future research.

2 Reducing the cost of resistance; experimental evolution in the filamentous fungus *Aspergillus nidulans*

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Abstract

The use of antibiotics may result in a persistent resistance in the organism targeted by the antibiotic. Since in most cases resistance comes with a cost in the absence of the antibiotic, fitness restoration by compensatory adaptation is essential for the resistance to persist in a population. We have studied the process of compensatory evolution using a fludioxonil resistant mutant of the filamentous fungus *Aspergillus nidulans*. In an evolution experiment lasting for 27 weeks (about 3000 cell cycles) 40 parallel strains of this mutant evolved under three different environmental conditions. Our results show that there is a severe cost of resistance (56%) in the absence of fludioxonil and that in all conditions used the mutant strain was able to restore fitness. In 7 cases, the evolved strain reached a higher fitness than the original sensitive ancestor. Fitness compensation occurred in one, two or three discrete steps. Genetic analysis of crosses between different evolved strains and between evolved and ancestral strains revealed interaction between compensatory mutations and provided information on the number of loci involved in fitness compensation. Finally we discuss the opportunities for the experimental study of evolutionary processes provided by the filamentous fungus *Aspergillus nidulans*.

Introduction

The use of antibiotics has resulted in persistent resistance in microbial populations (Bjorkman et al. 2000; Schrag and Perrot 1996). By a single mutation, an organism can become resistant to an antibiotic it encounters. It is common that these resistance mutations come with a cost: in the absence of the antibiotic, the resistant type often has a lower fitness than its sensitive relatives (Andersson and Levin 1999; Cohan et al. 1994; Cowen et al. 2000; Gilliver et al. 1999; Reynolds 2000). Thus, when a resistant population is placed back in an antibiotic-free environment, loss of resistance might be expected. However, instead of reverting to the sensitive wild type, a resistant genotype may gain fitness by accumulating so-called compensatory mutations that take away (part of) the costs while resistance remains unaffected (Cohan et al. 1994; Levin et al. 2000; Lipsitch 2001; Maisnier-Patin and Andersson 2004; Schrag et al. 1997). Clearly, the phenomenon of compensatory evolution in antibiotic-resistant strains is highly relevant for understanding the persistence of resistance in natural populations.

Several experimental studies have investigated compensatory evolution in resistant micro-organisms (see also (Lenski 1997)). Bouma and Lenski (1988) showed that fitness costs associated with a plasmid encoded resistance to chloramphenicol in *E. coli* decreased over 500 generations. Cohan et al. (1994) discussed the amelioration of fitness costs in rifampicin resistant *Bacillus subtilis* and have suggested that the potential for compensatory evolution depends on the magnitude of the cost of resistance, Schrag and Perrot (1996) report compensatory adaptation in streptomycin resistant *E. coli* populations. (Moore et al. 2000b) indeed observed greater compensatory adaptation for deleterious mutations of large effect than for those of small effect. Levin et al. (2000) studied compensatory evolution in streptomycin resistant *Escherichia coli* and concluded that mainly intermediate fitness compensatory mutations

arise, rather than high fitness reversion to the sensitive type. Reynolds (2000) reached similar conclusions in rifampin resistant *E. coli.* Bjorkman et al. (2000) found different compensatory mutations in streptomycin resistant *Salmonella typhimurium* depending on whether bacteria evolved in mice or laboratory medium.

The beneficial effect of a compensatory mutation may depend on the presence of the deleterious resistance mutation. Schrag et al. (1997) observed negative fitness effects of compensatory alleles from a streptomycin resistant *E. coli* strain when transferred into a sensitive background.

We have studied antibiotic resistance and compensatory evolution in a multicellular eukaryote system, the filamentous fungus *Aspergillus nidulans*. In order to do this, we have developed an experimental evolution approach in *A. nidulans*, studying evolution over 3000 cell cycles. Also in fungi like *Aspergillus*, the emerging antibiotic resistance is a problem (Cowen et al. 2000; Cowen et al. 2001; Moore et al. 2000a).

Apart from sharing the well-known advantages of unicellular micro-organisms in experimental evolution (Elena and Lenski 2003), *A. nidulans* has the additional advantages of multicellularity and a eukaryotic sexual cycle that can facilitate genetic analysis (Clutterbuck 1974; Pontecorvo and Käfer 1958; Pontecorvo et al. 1953; Swart and Debets 2004; Timberlake 1990). Filamentous fungi grow on the surface of a substrate and form aerial asexual and sexual spores. The way the fungus expands during growth on solid medium is drawn schematically in Figure 1. From a mononucleate spore, the fungus eventually forms mycelium consisting of a network of hyphae with the nuclei in the mycelium occupying spatially defined positions. Twenty-four hours after their formation the hyphal cells will differentiate into foot cells, giving rise to

conidiophores, each carrying up to 10,000 asexual spores. The production of sexual spores commences after 4 days (Adams et al. 1998).

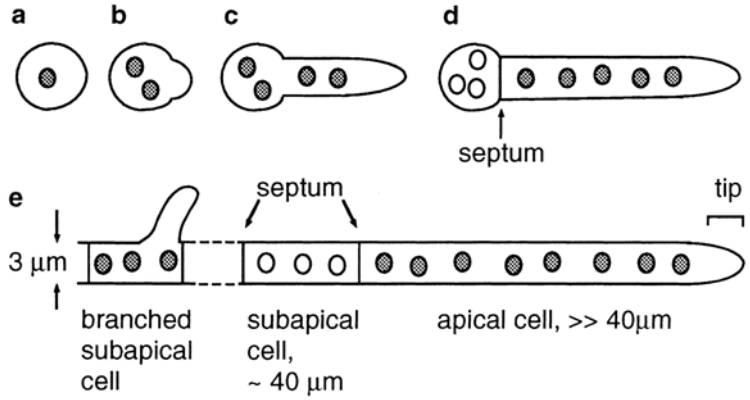

Figure 1. Schematical drawing of a growing *Aspergillus nidulans* colony. From a spore with one single nucleus, the fungus starts by a few cycles of nuclear division. When there are more then eight nuclei, hyphae start to develop (Figure 1. a-c). After cell stretching, septa are formed in the hyphae (Figure 1.d), eventually leading to the situation shown in Fig 1.e: The tip of the mycelium has one large apical cell containing 20 to 40 nuclei. During a cell cycle, all nuclei in this apical cell divide simultaneously. After the nuclear division, septa are formed. Half of the nuclei are then located in the subapical cells formed by the septa, the other half remain in the apical cell to enter the next cycle. Each subapical cell will contain three to four nuclei and will have a length of approximately 40 μm, or one nucleus per 13 μm mycelium. The nuclei in the subapical cells do not divide anymore; they are only metabolically active. (From Kaminskyj & Hamer (1998), with permission.)

The nuclei at the growing front of the colony went through the largest number of mitoses; sampling from the growing front makes the effective number of cell cycles in the context of experimental evolution remarkably high. Based on the time needed for a cell cycle as well as on the distribution of the fixed nuclei in the mycelium, it can be estimated that daily the fungus can go through about 19 cell cycles, see Methods (Bergen and Morris 1983; Kaminskyj and Hamer 1998). Here, the time needed for a cell cycle is defined as the time needed for a full replication of the genome. Each nucleus is omnipotent and potentially can

give rise to spore formation. Therefore, the haploid nucleus in a mycelium is the evolutionarily relevant unit, as is the haploid cell in a bacterial culture.

A special feature of the spatially structured mycelium is the possibility to directly recognize the part of the mycelium containing a (beneficial) mutation that enhances the rate of hyphal growth. All nuclei in a mycelium are mitotically derived from a single ancestral nucleus, resulting in a genetically homogeneous colony. The only source of genetic variability in a fungal colony is mutation resulting in segments of mycelium with different genotypes. The effect of a beneficial mutation that arises in the mitotically active hyphal tips may be seen (phenotypically) at the growing front of the colony as a sector with accelerated growth (Figure 2).

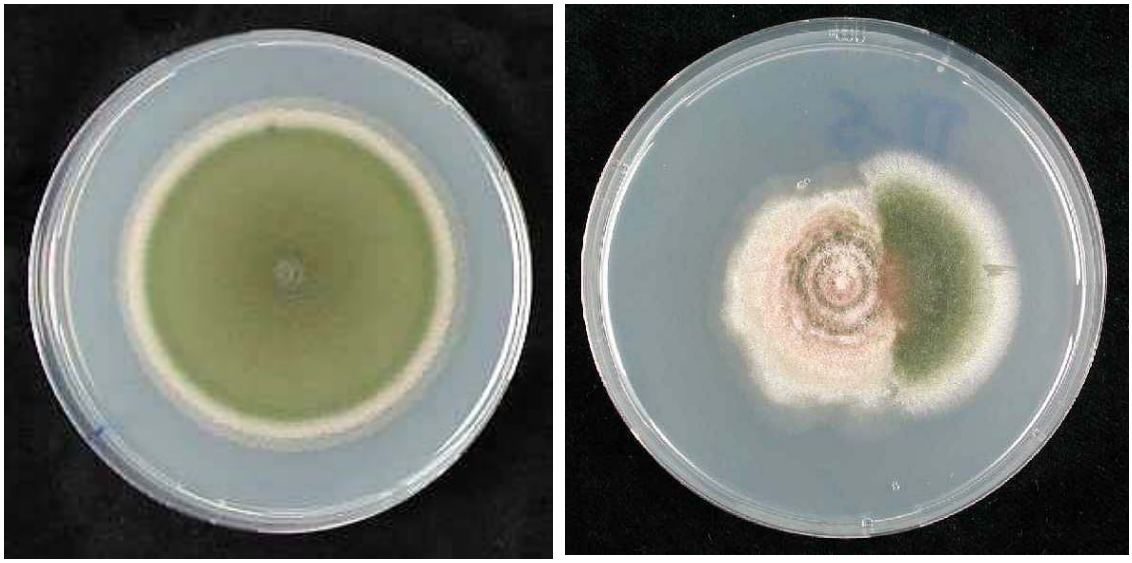

Figure 2. (left panel) A fungal colony (WG562) after 6 days of incubation on MM. The colony has a perfectly circular shape; the surface is covered by asexual conidiospores. At the edge, the colony has a lighter color; here the asexual spores have not yet fully developed, this takes around 24 hours (Adams et al. 1998). (right panel) A fungicide sensitive strain of A. nidulans (WG562), growing on medium with fungicide (fludioxonil), showing a spontaneous beneficial mutation. From the point in the mycelium where the mutation occurred, a sector with higher growth rate develops.

The main aim of our study is to elucidate fundamental aspects regarding adaptation. However, this study has practical implications as well, since the fungicide used, fludioxonil (4-(2,3-difluoro-1,3-bezodioxol-4-yl)pyrrol-3-carbonitril), is widely used in agriculture to control emerging fungal infections on plants, grape vines in particular (Cabras and Angioni 2000; Rosslenbroich and Stuebler 2000; Verdisson et al. 2001). It is thought to act by interfering with sugar transport and sugar phosphorylation and by disordering membrane functions (Hilber et al. 1995; Ziogas and Kalamarakis 2001). Different environmental conditions for compensatory evolution were chosen such that they could mimic aspects of agricultural use of fludioxonil.

Here, we demonstrate the presence of costs associated to fludioxonil resistance in Aspergillus nidulans. Secondly, we show that compensatory evolution occurs over 3000 cell cycles in the following environmental conditions: in the absence of fungicide, with diminishing concentrations of fungicide and with alternating high and low fungicide concentrations. Thirdly, we demonstrate that discrete steps are involved in the process of compensation. Fourthly, we study the presence of epistasic interaction between the resistance mutation and compensatory mutations. And finally, we discuss consequences and implications for the persistence of resistance.

Materials and Methods

Strains. The *Aspergillus nidulans* strains used in this study were derived from the original Glasgow strains (Clutterbuck 1974), see Table 1. WG562 is the wild-type lab strain with a lysine marker to facilitate genetic analysis. WG561 is a spontaneous mutant of WG562 that is resistant to 0.2 ppm of the fungicide fludioxonil (Novartis). We crossed WG562 with WG145, which has a pyridoxin deficiency and white asexual spores, and determined that fludioxonil resistance

is due to a single locus (*fld*A1). From this cross, we recovered WG615, which is fludioxonil resistant, pyrodoxin deficient and has white asexual spores. Compensatory mutations to alleviate the costs of fludioxonil resistance were named *flc*.

Table 1. Strains used.

Strain	Genetic markers per chromosome								
	I	II	III	IV	V	VI	VII	VIII	unknown
WG562					*lys*B5				
WG145		*w*A3		*pyro*A4					
WG561			*fld*A1		*lys*B5				
WG615		*w*A3	*fld*A1	*pyro*A4					
WG619			*fld*A1		*lys*B5	*flc*A4			
WG621		*w*A3	*fld*A1	*pyro*A4					*flc*5, *flc*6, *flc*7
WG525	*pyr*G89	*acr*A1	*act*A1	*paba*B22	*nic*A2	*s*B3	*cho*A1	*cha*A1	
AN116	*y*A2	*w*A3	*s*C0	*pyro*A4					
WG492	*pyr*G89		*phen*A2			*s*B3	*nic*B8	*ribo*B2	
WG626		*w*A3	*fld*A1	*pyro*A4		*s*B3	*nic*B8	*ribo*B2	

fld: resistance to fludioxonil; *flc*: mutation compensating for costs associated with fludioxonil resistance; other markers as described by Clutterbuck (1974).

Para-sexual analysis. Para-sexual analysis was performed essentially as described by Käfer (1958). We determined the chromosomal location of the resistance locus and compensatory mutations by parasexual analysis. In this analysis, a strain with an unlocalised allele is allowed to anastomose and form diploid nuclei with a marker strain that has a scoreable marker on each chromosome. The diploid nuclei are induced to haploidize with medium containing benomyl (1.7 ppm) to give nuclei with new combinations of parental chromosomes in the near absence of intra-chromosomal recombination.

Culture media and growth conditions. We cultured the strains in Petri dishes with Minimal Medium (MM) or Complete Medium (CM). MM consists of 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 10 mg of $FeSO_4$, $ZnSO_4$,

$MnCl_2$ and $CuSO_4$ and agar 15 g + 1000 ml H_2O (pH 5.8). For CM, 2.0 g neopeptone, 1.0 g vitamin assay casamino acids, 1.0 g yeast extract and 0.3 g ribonucleic acids for yeast was added to MM. After sterilization of the media, sucrose was added to a final concentration of 25 mmol/l to both media. CM was supplemented with a vitamin solution after sterilization (2 ml solution/1000 ml medium) containing thiamin 100 mg/l, riboflavin-Na 1.25 g/l, para-aminobenzoicacid 100 mg/l, nicotinamide 1.0 g/l, pyridoxin-HCl 500 mg/l, D-pantothenic acid 100 mg/l and biotin 2.0 mg/l. MM always was supplemented with lysine to a final concentration of 2.0 mmol/l in the medium and with pyrodoxin to a final concentration of 0.1 mg/l. In the assay used to determine reaction norms and in two of the conditions of the evolution experiment MM with the fungicide fludioxonil was used. Fludioxonil was dissolved in methanol to a concentration of 100 ppm. This stock solution was used to add fludioxonil to the medium to a concentration of 0.2 ppm; the methanol concentration in the medium never exceeded 0.8 % and methanol evaporates rapidly since it was added to the medium when warm (>40 ^{o}C). Spore suspensions were made in saline (distilled water with NaCl 0.8 g/l) supplemented with Tween80 (0.05%) by washing off all spores from the surface of a plate with 5 ml saline-tween. Inoculation of agar plates was done either with an inoculation needle or with 5 µl of spore suspension. Plates were incubated at 37 ^{o}C.

Mycelial growth rate. Mutations can lead to isogenic sectors in the mycelium; a mutation enhancing mycelial growth rate has the highest chance to form a large isogenic sector. Figure 2. - right panel shows the emergence of a sector with increased mycelial growth in an *Aspergillus nidulans* colony grown on an unfavorable medium. We transferred a small fraction from this sector of the colony onto fresh medium, resulting in a perfectly circular colony with higher mycelial growth rate, suggesting that the beneficial mutation had been fixed in the mutant sector.

In our selection experiments, we selected for sectors in the mycelium with a higher growth rate, in this way discriminating between genotypes in the process of adaptation. So, we implicitly use *mycelial growth rate* (MGR) as sole fitness measure (Pringle and Taylor 2002). After six days of growth, we determined the MGR by averaging the colony diameters as measured in two randomly chosen perpendicular directions. The MGR of WG562 (fludioxonil sensitive) grown on media without fludioxonil was always used as the reference and set equal to one. The MGR of other strains is given in relation to the reference.

MGR is highly reproducible and independent of inoculum size. Three suspensions with decreasing numbers of spores (100,000, 100 or 10 spores per inoculum of 5 μl) were plated in 5-fold in Petri dishes with Minimal Medium and incubated at 37 ^{o}C. After six days, we measured the colony diameter. For the inoculum sizes of 100,000 and 100 spores no difference in colony diameter was observed (t-test; $t_{11} = 1.01$, $p = 0.34$); we found a MGR of 69.1 (standard error of the mean – SEM = 0.31) and 69.5 (SEM = 0.50) respectively. Only in the case of the extremely small inoculum size of 10 spores the MGR was somewhat reduced (t-test; $t_{11} = 24$, $p < 0.001$); here we found a colony diameter of 65.0 (SEM = 0.10). We conclude that MGR measurements are highly reproducible and that inoculum size in our evolution experiment (transferring between 10,000 and 50,000 nuclei, see below) did not influence the MGR.

Experimental evolution procedure. In the evolution experiment three different environments were used, see Table 2. To facilitate genetic analysis and sexual crosses between evolved strains, we used both resistant strains WG561 and WG615 for each condition in the evolution experiment, see Tables 1 and 2. We measured the mycelial growth rate (MGR) of WG561 and WG615 on media with and without fungicide, showing that the genetic markers are neutral in terms of MGR (t-test; t_8=0.367, p=0.72). WG562 (the fungicide sensitive strain) was used as an evolving control.

At the start of the experiment, we inoculated approximately 1000 spores at the center of a Petri dish. After six days of incubation at 37 ^{o}C, all plates were put at 4 ^{o}C for at least 24 hours (but no longer than 60h). The (mean) MGR of the colony was measured and the part of the colony with the highest MGR was identified by visual inspection (see Figure 2 for an example). Part of the mycelium of this sector was taken off with a needle and transferred to the middle of a Petri dish with fresh medium; in this way between 10,000 and 50,000 nuclei were transferred. This cycle was repeated 27 times. Samples of all replicates were stored weekly at –80 ^{o}C in a solution consisting for 0.7 % neo-peptone in water and 30 % glycerol in water. Each week we measured the MGR of the reference strain (WG562) by starting five replicate cultures from the stock stored at –80 ^{o}C. The plates with the reference strain were incubated together with those with evolving strains.

Table 2. Conditions and strains used in the evolution experiment.

	Condition	Strains used (# of replicates)	Fludioxonil resistant (R) or sensitive (S)
A	MM without fludioxonil	WG 561 (8); WG 615 (7)	R
B	MM with decreasing fludioxonil concentrations, at the start 0.2 ppm, after the fourth week starting to decrease with weekly 0.02 ppm	WG 561 (5); WG 615 (5)	R
C	MM with alternating three weeks no and one week 0.2 ppm fludioxonil	WG 561 (5); WG 615 (5)	R
W	MM without fludioxonil	WG 562 (5)	S

Twenty-seven weekly transfers is equivalent to at least 3000 cell cycles. The number of cell cycles can be estimated in two ways. The first estimate is based on the time the fungus needs for one cell cycle. A cell cycle takes 90 minutes growing at 37 °C (Bergen and Morris 1983; Rosenberger and Kessel 1967). Therefore, in one week the fungus can complete about 112 cell cycles. The second way to estimate the number of cell cycles is by considering the position

of the nuclei in the mycelium combined with the growth characteristics of the fungus, shown in Figure 1. After six days of incubation at 37 ^{o}C, an *Aspergillus nidulans* colony (WG652) inoculated with a needle has a diameter of approximately 60 mm (Suelmann et al. 1997; Wolkow 1996). Therefore, from the starting point of the colony the mycelium has stretched for 30 mm in one week. Since there is on average one nucleus per 13 μm mycelium (Kaminskyj and Hamer 1998), a hypothetical hypha extending radially from the colony center along a straight line would contain around 2300 nuclei after one week of incubation at 37 ^{o}C. How many mitoses are required to obtain 2300 nuclei? Formation of novel nuclei occurs at the growing front of the colony in the apical cells; nuclei "behind" the growing front are only metabolically active. After every mitotic cycle in the apical cells, half of the newly formed nuclei remain in the apical cells and half are directed to the subapical cells. The number of nuclei in the apical cell varies and can reach upto 50 nuclei per apical cell (Kaminskyj and Hamer 1998; Wolkow 1996); 20 seems to be a reasonable conservative estimate just before mitosis. Assuming 20 nuclei in the apical cell, each mitotic cycle will add 20 nuclei to the total present in the growing hypha. To reach the total number of 2300 nuclei, 115 cycles are required. This second estimate for the number of cell cycles is consistent with the first estimate based on the time needed for one cell cycle.

Like in other experimental evolutionary studies, we cannot exclude that the dynamics of the cell cycle is affected by unfavorable growth conditions. Our microscopical observations (unpublished) suggest a shortening distance between septa in unfavorable conditions rather than a decreasing number of nuclei (see Figure 1).

Results

We tested 30 spontaneous resistant mutants of WG562 isolated from minimal medium with fludioxonil present in concentrations ranging from 0.03 ppm up to 0.8 ppm. All mutants found suffered a considerable cost of resistance in terms of linear growth: on average the MGR on standard fungicide-free medium was reduced by 56.0 % (SEM = 0.76 %) as compared to WG562 (sensitive strain).

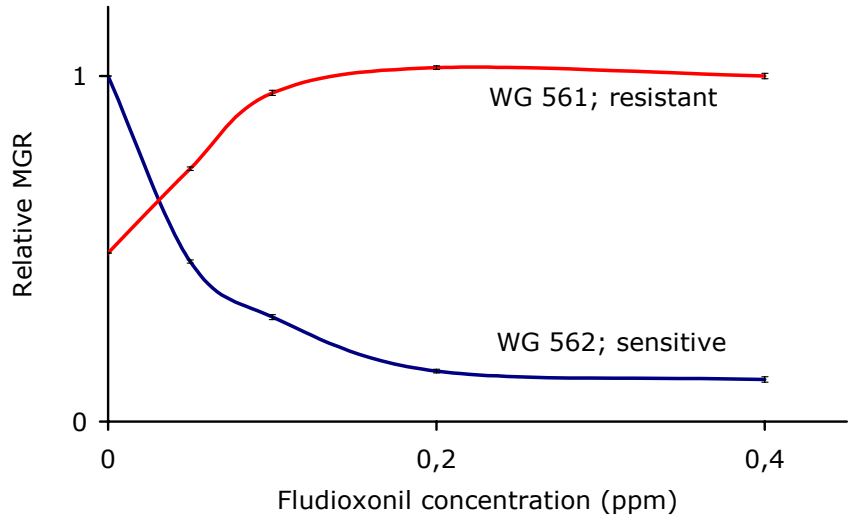

Figure 3. Reaction norms of the sensitive (WG562) and resistant strain (WG561) used in the evolution experiment. Mycelial growth rate (MGR) is expressed relative to the MGR of the sensitive strain growing on minimal medium without fungicide. The reaction norm shows that the presence of fungicide enhances growth of the resistant strain and inhibits the growth of the sensitive strain.

For evolution experiments we selected WG561, which we further analyzed. This mutant was isolated from medium containing 0.2 ppm of fludioxonil. An initial physiological characterization with radioactively labeled fludioxonil showed that the mutant strain accumulates the fungicide within the cells. Figure 3 shows the MGR of WG562 and WG561 on medium with different fungicide concentrations, i.e. the reaction norms. Growth of the resistant mutant (WG561) is enhanced by the presence of fludioxonil; at high concentrations the MGR is

higher than at low concentrations and even higher than the MGR of the fungicide sensitive strain (WG562) on fungicide free medium. Under fungicide-free conditions strain WG561 suffers a fitness cost of approximately 50%. The growth of the wild type strain WG562 is strongly reduced by the fungicide, confirming results found in other studies (Ziogas and Kalamarakis 2001).

By genetic analysis involving a sexual cross and para-sexual analysis, we determined the number of loci involved in the resistance and their chromosomal location. A sexual cross of the resistant mutant (WG561) with a sensitive strain (WG145) showed a clear cut 1:1 segregation of the resistance phenotype (Chi square test; n = 67; χ^2 = 0.134; p > 0.05), indicating that there is one locus involved in the resistance phenotype. We constructed a diploid strain of the resistant strain and the linkage group tester strain WG525. Based on the phenotype given by the reaction norm of this heterozygous- diploid, the resistance allele together with its associated fitness cost appeared to be recessive.

Following benomyl-induced haploidization of the diploid, haploid segregants were isolated and tested, see Table 3. This assay indicated that the resistance locus was located on chromosome III.

Table 3. Linkage data of parasexual analysis of diploids. Diploids were formed of the strain with an unlocalized allele and a tester strain. Segregation was induced with benomyl and haploid segregants were analyzed, see methods. Table 1 shows the strains and their markers.

Strain (with un-localized allele)	Linkage group tester strain	Linked markers*	Number of segregants analyzed	Recombinant frequency (number of recombinants)	Linkage group
WG651 (*fld*A1)	WG525	*fld*A1-*act*A1	133	1.5 % (2)	III
WG651 (*fld*A1)	AN116	*fld*A1-*s*C0	224	0 % (0)	III
WG619 (*flc*A4)	WG626	*flc*A4-*s*B3	120	0.8 % (1)	VI

* Only the linked marker of the tester strain is shown. The other markers of the tester strains were unlinked to the allele of interest.

Evolution experiment over 3000 cell cycles. We set up an evolution experiment over 27 weeks (corresponding to some 3000 cell cycles), weekly selecting for the part of the colony with the highest MGR in three different environments; see Methods, Table 2 and Figure 2.
Figure 4 shows the mean MGR (± standard error of the mean) of all strains for the four conditions and the control.

For all conditions (A, B, C and W), evolved strains after 27 weeks had a higher mean MGR than at the start (t-tests; $p < 0.01$). The sensitive control strains of strain WG562 (condition W), increase in average MGR from 1.00 (SEM = 0.0027) to 1.03 (SEM = 0.0055). This increase is statistically significant (t-test; $t_8 = 5.91$, $p < 0.001$). In condition A, the average MGR of the 15 evolving strains increased from 0.50 (SEM = 0.0034) to 0.82 (SEM = 0.052). In condition B, the resistant mutant showed a higher MGR during the first weeks when growing in the presence of the fungicide (1.08; SEM = 0.0014) than the populations of condition A that grew in the absence of fungicide (MGR = 0.50; SEM = 0.0034); the mean MGR diminished with decreasing fungicide concentration and recovered during the complete absence of the fungicide to a MGR of 0.97 (SEM = 0.058). In condition C, the presence of fungicide leads to a highly increased MGR as shown by the peaks in the curve. By week 27 the evolving strains were adapted to both situations, on fungicide-free medium the mean MGR increased from an initial value of 0.50 (SEM = 0.0034) to 0.90 (SEM = 0.092) on week 27; on fungicide-containing medium the MGR was 1.02 (SEM = 0.049) on week 4 when the strains first encountered fungicide and 1.01 (SEM = 0.049) on week 24 (the difference on medium with fungicide is non-significant; t-test; $t_{16} = 0.18$, $p = 0.86$).

Figure 4 shows the change in MGR in the experimental populations. The mean MGR at week 27 is not different between the conditions A, B and C (one way ANOVA, $F_{2,26} = 1.42$; $p = 0.256$) The rate of adaptation (given by the slope) in

conditions A, B and C is higher than in condition W (multiple t-tests; $p < 0.001$), showing that adaptation in the fungicide resistant strains was indeed compensation for fitness costs and not only adaptation to our laboratory conditions.

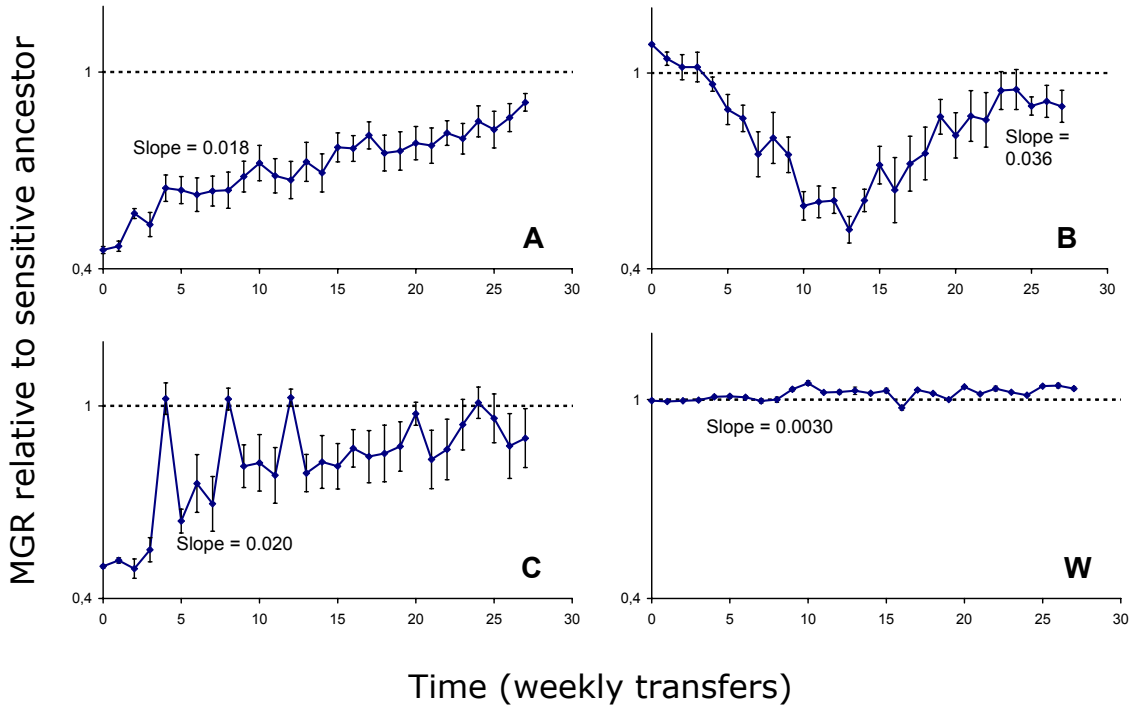

Figure 4. Results of the evolution experiment (27 weeks, i.e. >3000 mitotic cell cycles). W, A, B and C refer to the experimental conditions used (see Table 2). The data points in the graphs represent the weekly mean MGR of all strains per condition, errorbars represent the standard error of the mean (SEM). MGR was made relative to the MGR of the (non-evolved) fungicide sensitive ancestor (WG562) growing without fungicide. The resistant mutant when growing in the presence of high fungicide concentrations has a higher MGR than the sensitive ancestor when growing in the absence of fungicide (see Fig 3). In conditions B and partially C this results in the relative fitness to be greater than 1. Slopes (the mean slope over all strains per condition) are given over de first 13 weeks without fungicide in the environment. For A this was computed over the first 13 weeks, for B over week 15 to 27 and for C over week 1 to 17 (for C, we omitted the weeks that fungicide was present in the medium). The slope in condition B is higher than the slope in A and C, however, this is statistically inconclusive (one way ANOVA; $F_{2,29} = 3.23$; $p = 0.054$).

Changes in level of resistance. Condition C with the alternating presence and absence of fungicide shows that the resistance to fungicide is maintained, while the fitness in the absence of fungicide increases. In addition to the measurements of weekly linear growth, we tested the strains after 27 weeks of evolution for their resistance to different concentrations of the fungicide. In the evolved strains of the other conditions, we similarly observed that all strains maintained their resistance to the fungicide. This indicates that the compensatory changes did not involve back mutation of the resistance allele to the wild type but mutations at other loci.

Stepwise fitness improvement. The appearance of clearly recognizable sectors displaying enhanced growth rate suggests that fitness improves in discrete steps. When weekly measuring the MGR in the course of the evolution experiment, the (mean) MGR not always increased as expected; the occasional drop in MGR could be an artifact due to fluctuations in uncontrolled factors during the experiment (such as exact control of incubation temperature). We therefore selected several strains from each condition and remeasured their MGR for five of the 27 week points in a single well controlled assay, expecting a constant MGR with occasional fitness increase in distinct steps. MGR measurements were done in six-fold; two reconstructions per condition is shown in Figure 5. For one evolving strain of condition A (WG621), we measured the MGR of all 27 week points in one assay, see Figure 6.

Results show that when doing all measurements in a single assay, the MGR of an evolving strain does not decrease during the course of the evolution experiment (suggesting that this indeed was an artifact in the previous dataset). MGR improvement occurred in one to three major steps, suggesting the occurrence of one to three (major) compensatory mutations during the experiment. The differences in MGR before and after a step were highly significant in all cases (t-test; $n = 12$, $p < 0.001$). The evolved strains studied in

this detail all have a different MGR after the 27 weeks of evolution, suggesting they followed different evolutionary trajectories. In the strains studied here, the first step in fitness improvement was always larger than subsequent steps.

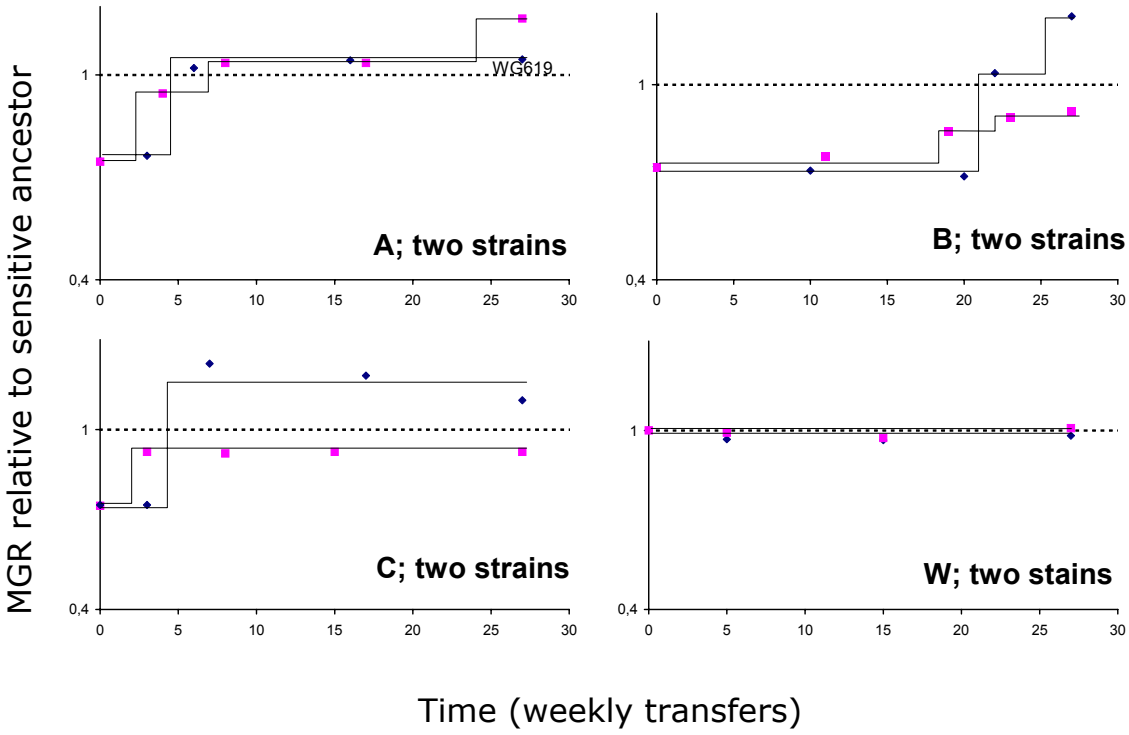

Figure 5. Adaptive trajectories of several independently evolving strains. W, A, B and C refer to the experimental conditions used (see Table 2). Here, two evolving strains per condition are shown (derived from either WG561 or WG615). We measured the MGR at five time points in 3-fold in a single assay, error bars representing the SEM are smaller than the symbols. MGR was made relative to the MGR of the (non-evolved) fungicide sensitive ancestor (WG562) growing without fungicide. On the basis of the MGR, steps in adaptation were identified, the difference in MGR between the steps is highly significant (t-tests; $n = 6$, $p < 0.001$).

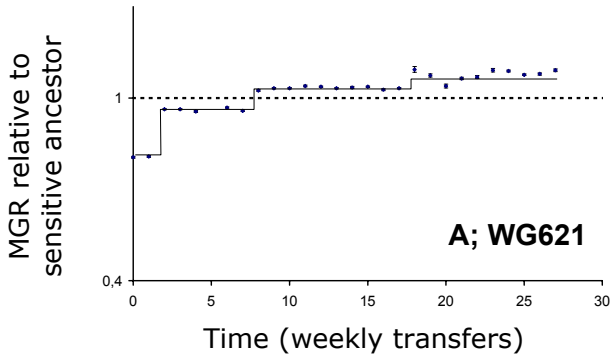

Figure 6. Adaptive trajectory of strain WG621 (which was derived from WG615 and evolved in condition A). Stored samples of all weeks were retrieved from –80 °C and the MGR was measured in 3-fold in a single assay. MGR was made relative to the MGR of the (non-evolved) fungicide sensitive ancestor (WG562) growing without fungicide. Error bars represent the SEM. On the basis of MGR, three steps in adaptation could be identified. (The MGR before and after a step is different; t-test; n = 6, $p < 0.001$).

Sexual crosses to assess the number of mutational steps in fitness improvement. In case of major mutational phenotypic effects, the number of phenotypic classes in the progeny of a sexual cross reveals the number of major loci involved. In our case, a cross between an evolved strain and the ancestor can reveal the number of major compensatory mutations. Here, the phenotype is given by the reaction norm on different concentrations of fungicide (Figure 3).

Firstly, we crossed a strain of condition A, WG619 (which was derived from WG561) with a fungicide sensitive strain (WG145). Based on MGR measurements, WG619 is expected to carry one compensatory mutation. Analyzing 40 progeny, we found four phenotypic classes when growing the progeny on a series of plates with MM with increasing fungicide concentrations, see Table 4. One recombinant has the same phenotype as WG561 (the non-evolved fungicide resistant). The other recombinant is a “novel” type and has a higher MGR than the ancestor WG619, see Table 4, suggesting that the compensatory mutation also has a beneficial effect in the absence of the resistance allele. Parasexual analysis using strain WG626 showed that the compensatory allele is located on chromosome IV (for data see Table 3).

Table 4. Sexual cross between a strain of condition A after 27 weeks of adaptation (WG619; derived from WG651) and a fungicide sensitive strain (WG145) showed four classes in the progeny when measuring their MGR on MM without fungicide (no significant deviation from a 1:1:1:1 segregation; Chi-square test; $\chi^2 = 7.6$; df = 3; $p > 0.05$). The MGR was made relative to the standard fungicide sensitive reference (WG562).

Sexual cross:

WG619	+	*fld*A1	+	*lys*B5	*flc*A4
WG145	*w*A3	+	*pyro*A4	+	+

Progeny genotype*		# of progeny	MGR (*SEM*)	Phenotype description
*fld*A1	*flc*A4	9	1.18 (*0.005*)	Evolved fungicide resistant
+	+	4	1.00 (*0.005*)	Fungicide sensitive ancestor
*fld*A1	+	16	0.78 (*0.005*)	Non-evolved fungicide resistant
+	*flc*A4	11	1.12 (*0.005*)	"New" fungicide sensitive type

* Relevant markers are shown; *fld*A1: resistance allele; *flc*A4: compensatory mutation.

Secondly, we crossed another evolved strain of condition A, WG621 (which was derived from WG615) with WG562, a fungicide sensitive strain. Based on MGR combined with a partial f-test (Elena et al. 1996), three steps in fitness improvement were identified in the evolutionary trajectory of WG621, see Figure 6. By analyzing 40 progeny of the cross, it was clear that there were many phenotypic classes. Therefore, we decided to do a cross with the non-evolved resistant (WG561), eliminating one segregating locus. Out of this cross, again analyzing 40 progeny, we found four distinct phenotypic classes, indicating the presence of two segregating major compensatory mutations. (The ratio of the classes did not deviate from 1:1:1:1; χ^2-test; $p = 0.70$). The mean MGR (± the standard error of the mean) of each of the classes was measured for different fludioxonil concentrations, which gives the reaction norm; the results are in Figure 7.

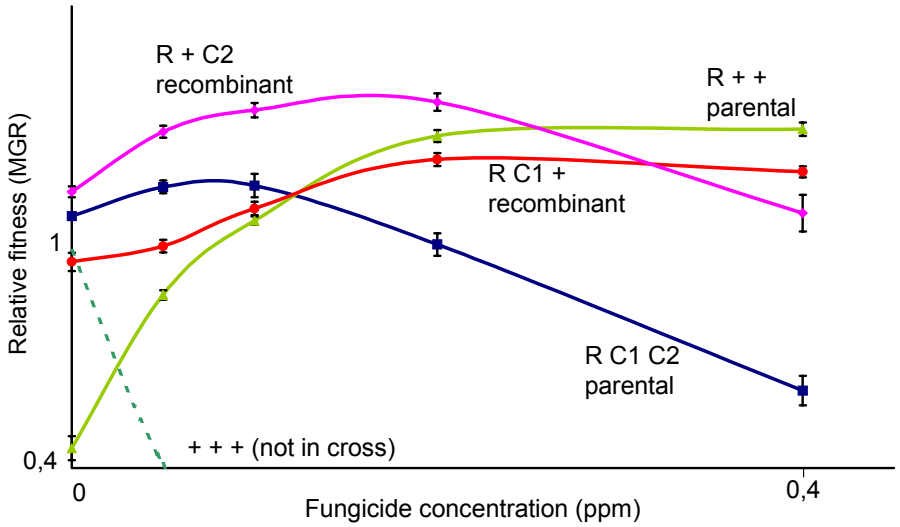

Figure 7. Reaction norms on MM with increasing fungicide concentrations of progeny from a cross between evolved strain WG621 and the non-evolved resistant ancestor (WG561). For comparison, part of the reaction norm of a fungicide sensitive strain (WG562) is also shown. The wild-type allele is represented by +, the resistance allele by R and the compensatory mutations by C1 and C2. The mean MGR (± standard error of the mean) of the four classes in the progeny (n=40) is shown, indicating that two main loci are involved in the fitness compensation.

The result of the cross indicates that two major compensatory mutations are present in the evolved strain, not three as the MGR measurements (see Figure 6) combined with the partial f-test suggested. However, there could be a third compensatory mutation present since the variation in MGR between the progeny within the classes is relatively high (4.5 times higher than in the other MGR assays); this could indicate that a third segregating locus has an effect on all four phenotypic classes. One of the recombinant classes has a higher fitness than both parental types; this must be the progeny with the second compensatory mutation only. The fact that the second compensatory mutation alone has a greater effect than in a background containing the first compensatory mutation is a clear indication of interaction between both compensatory mutations. By chance, the second compensatory mutation having the largest effect on fitness occurred after fixation of the first one.

Finally, we performed test crosses to verify the reconstruction we made. A recombinant suspected of only having the second compensatory mutation without the first was crossed with the non-evolved fungicide resistant (WG561). Here we found two classes among 40 progeny (data not shown), thus confirming the presence of just one major mutation. We also did a cross between the non-evolved resistant (WG561) and the evolved strain (WG621) after 6 weeks. As Figure 6 shows, by this stage only the first compensatory mutation is present; analysis of 34 progeny confirmed this (data not shown).

Sexual crosses between evolved strains. A sexual cross of two strains from the same condition was used to elucidate whether compensatory mutations that have arisen independently in replicate strains lead to even higher fitness when combined. For each condition (A, B and C) two strains were selected on the basis of their increased fitness compared to the ancestor.

For condition A, WG619 and WG621 were crossed (see Figures 5 and 6), for condition B and C the strains shown in Figure 5. From each cross, 40 random progeny were isolated; the MGR of all progeny and the parents of the cross was measured in six-fold on fungicide free MM, results are in Figure 8. We compared the MGR of the progeny with the MGR of the highest parent. In cross A no progeny had a higher MGR than the highest parent (t-test; $t_{10} = 1.00$, $p = 0.34$). In cross B, 7 progeny had a higher MGR than the highest parent (t-tests; $df = 10$, $p < 0.05$). In cross C 1 progeny had a higher MGR than the highest parent (t-test; $t_{10} = 6.00$, $p < 0.05$). It was not possible to detect segregating classes among the progeny.

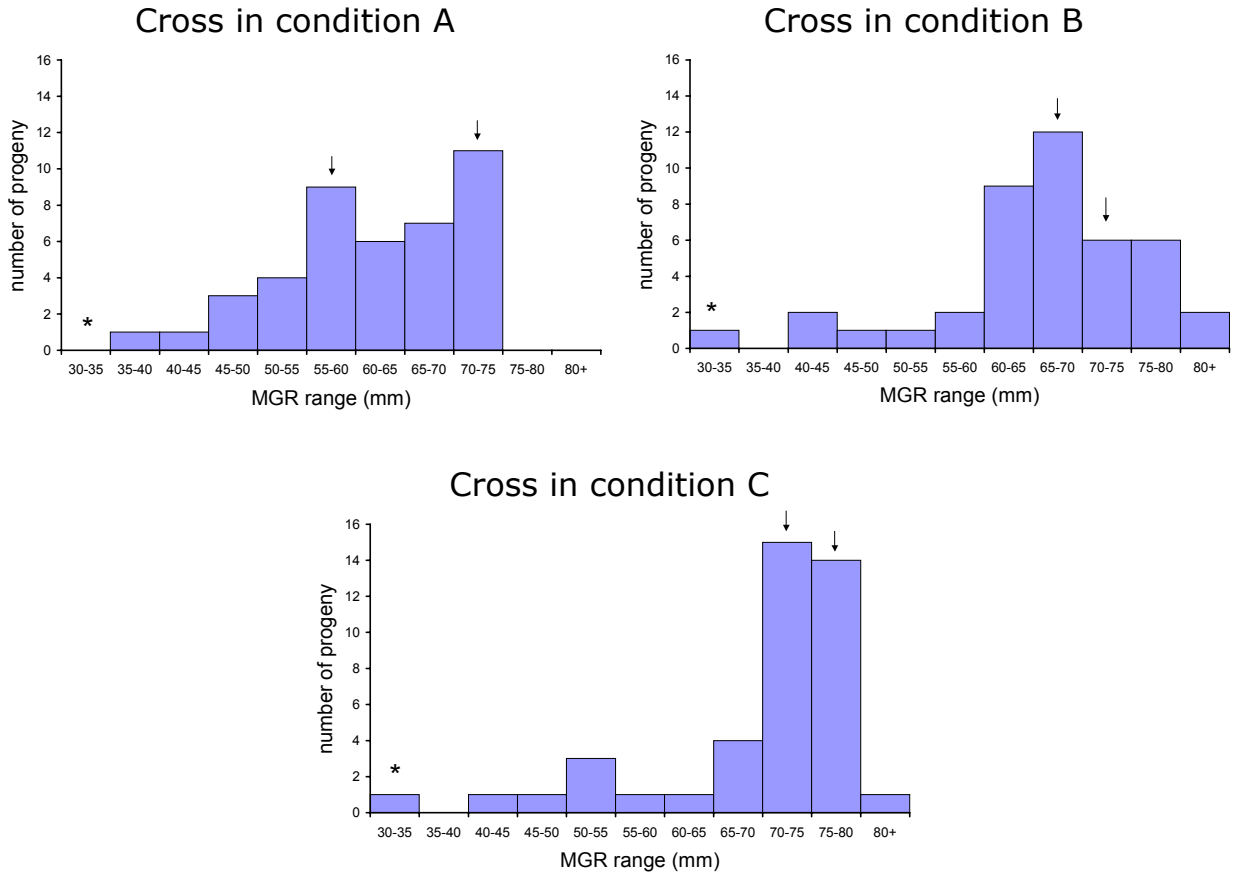

Figure 8. Of conditions A, B and C used in the evolution experiment (see Table 2), two evolved strains were crossed and the MGR of 40 progeny was measured together with the parents of the cross. Graphs show MGR ranges with the number of progeny. Arrows indicate the MGR class of the parents, * indicates the MGR class of the non-evolved resistant (WG561 and WG615).

Discussion

Experiments were performed to study the development of fludioxonil resistance and subsequent compensatory evolution in *Aspergillus nidulans*. Firstly, the prediction that resistance will carry a cost in the original fungicide-free environment was borne out. All resistant mutants showed a cost of resistance; the average mycelial growth rate (MGR) - which was used as fitness measure in our experimental procedure - of 30 resistant mutants was reduced by 56 %

compared to the sensitive ancestor when growing on fungicide free medium. A cost of resistance under drug-free conditions is commonly observed (Maisnier-Patin and Andersson 2004). One resistant mutant (WG561) was selected. This strain and WG615, which is isogenic for the resistance mutation and has equal MGR, were used to found the evolution experiment (Table 2).

The evolution experiment tested the prediction that a resistant strain will improve its fitness when cultured under conditions where resistance is not functional but causes negative pleiotropic effects on fitness. In all conditions tested, the MGR measured on medium without fungicide increases over the 3000 cell cycles. Variation in evolved MGR and the different trajectories found for different strains show the presence of multiple adaptive peaks (see Figure 4 and Figure 5). In all conditions, some of the evolved strains reached a higher fitness than the original or evolved sensitive ancestor (WG562), indicating that there are higher peaks in the fitness landscape than the one occupied by this ancestor, which did not evolve to such a higher peak in the course of our experiment (condition W), probably not being able to pass through an adaptive valley. Out of the 45 evolving fungicide resistant strains, 7 surpassed the original sensitive ancestor (WG562); the highest MGR was 1.24 (fitness relative to WG562; SEM = 0.005).

We studied the process of compensatory adaptation under three different experimental conditions: one constant condition (condition A, never fungicide) and two varying conditions (condition B with diminishing fungicide and condition C with alternating absence and presence of fungicide). In conditions B and C the evolving strains tended to show a more rapid adaptation and a higher final fitness than the evolving strains in condition A. A possible explanation in terms of adaptive fitness topography is that in our experiment the continuing change of environment has prevented strains from getting “stalled” on a relatively low fitness peak. In condition B, fitness tended to improve only when

the fungicide concentration approached zero. Possibly, compensatory mutations were only seen as escaping sectors (and thus selected) when the cost of resistance had become really high. In condition C the alternating presence of the fungicide appears not to have hampered compensatory adaptation in the fungicide-free environment. Interestingly, the strains evolving into generalists did as well as the specialists (Elena and Sanjuan 2003). This is perhaps suggestive of compensatory mutations that were neutral (or at least not clearly deleterious) under fungicide conditions.

To study epistatic interactions, the trajectory of several evolved strains was analyzed in more detail to investigate the process of compensatory evolution in detail. We used two methods to estimate the number of steps in fitness compensation. First, we measured MGR more precisely and looked for discrete and abrupt changes (Figures 5 and 6). Next, we made a sexual analysis by crossing an evolved strain with the non-evolved resistant (see Table 4).

In evolved strain WG619 a sexual cross showed the presence of one compensating locus. Recombinants, with the compensatory allele in the sensitive background showed that the compensatory allele has a general fitness enhancing effect, although this effect is greatest in the resistant background. We crossed evolved strain WG621 with the resistant ancestor WG561 and found two segregating compensatory mutations (see Figure 7). Here, we could study the interaction between compensatory mutations in the resistant background. Within the progeny there was a recombinant class having only the second compensatory mutation without the first. The fitness of this recombinant was higher than all other classes from this cross. Apparently, the second compensatory mutation has a greater effect on fitness, but in this particular case some of its beneficial effect is removed by the first compensatory mutation. Our data show that in all strains studied in detail the first step in fitness improvement was larger than subsequent steps. This is compatible with the idea that mutations

with large benefits are more likely in individuals that are distant from fitness optima and less likely in those close to optima (Burch and Chao 1999; Fisher 1930).

In two of the three crosses between evolved strains (see Figure 8) we found recombinant progeny with a higher fitness than the respective parental classes. Apparently, in the combinations studied, some of the independently selected compensatory mutations display additive effects on fitness. However, most of the progeny has a lower MGR than the parents, probably caused by a breaking-up of favorable combinations of compensatory mutations.

Based on our experiments, the prediction can be made that the use fludioxonil in agriculture can lead to resistant strains that by subsequent accumulation of compensatory mutations can achieve an equal or even higher mycelial growth rate than the original fungicide sensitive types. Our findings under the different environmental conditions suggest that prolonged presence of fungicide followed by gradual degradation of the fludioxonil (condition B) or re-application of fludioxonil in a cycle (condition C), do not retard the emergence of resistant types with a high fitness.

Mycelial growth rate (MGR) is an easily to assess and highly reproducible fitness measure for filamentous fungi (Pringle and Taylor 2002). Other possible fitness measures would be total (a)sexual spore production or the total production of biomass from a fixed quantity of carbon source. These fitness measures are more difficult to assess and give a larger variation between replicate measurements than the MGR. In another study it was found that MGR and these other two fitness measurements are highly correlated when studying fitness in *Aspergillus niger*, a related filamentous fungus (De Visser et al. 1997). By always including the same reference strain, MGRs of different experiments can be compared (Bruggeman et al. 2003). Under more natural conditions, MGR

clearly will not be the only trait under selection and is likely to be an inadequate measure of lifetime reproductive success. However, we stress that in our experiment we *choose* to define adaptive success exclusively in terms of MGR and that therefore MGR represented total fitness in our experiment.

In evolving asexual populations in liquid media and without recombination, clonal interference (competitive elimination of mildly beneficial mutations by a superior beneficial mutation) is an important factor that affects the rate of adaptation (Gerrish and Lenski 1998). In an expanding fungal colony, clonal interference may play a role in several ways. In contrast to global resource competition, which prevails in liquid cultures, physical interference and local competition are likely in a growing fungus. Beneficial mutations can become fixed in a sector, which is unlikely to affect the rest of the colony as a whole. Another beneficial mutation could start independently of the first mutation somewhere else in the mycelium. Our selection method implies maximal clonal interference; we select only one fit sector eliminating other potential sectors.

In addition to addressing questions regarding the development of resistance and the subsequent compensatory evolution, we have shown that the filamentous fungus *Aspergillus nidulans* is a useful organism for the experimental study of evolutionary processes. The spatially structured growth results in isogenic sectors with a beneficial mutation. Sexual crosses are a valuable tool for genetic analysis of evolved strains. Future work with this fungal experimental system will involve comparisons between evolution in haploids and diploids, and between sexual and asexual reproduction, for which this system had promising possibilities.

Acknowledgements

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3 Parasex enhances adaptation to novel environments

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Many fungi have a 'parasexual cycle' in which haploid nuclei may fuse to form relatively stable diploid nuclei. Diploid nuclei can spontaneously produce haploid recombinants by repeated loss of chromosomes, and/or diploid recombinants by mitotic crossing-over. The parasexual cycle is widely used for genetical analysis, both in fungi and in mammalian and human cell cultures (Käfer 1958; Pontecorvo and Käfer 1958; Roper 1966; Ruddle and Creagan 1975; Swart and Debets 2004). However, since many fungal species have a fully functional regular sexual cycle as well, its evolutionary function remains enigmatic (Caten 1981; Elliott 1994; Roper 1966). Here we show that the parasexual cycle can enhance adaptation to novel environments. In an experimental study of adaptation over 3000 cell cycles with isogenic haploid and diploid strains of the filamentous fungus *Aspergillus nidulans,* we demonstrate that evolved populations that started as diploid but reverted to haploidy have a higher rate of adaptation than populations that began and remained haploid or diploid. We propose that the parasexual cycle enables fungi to exploit an extended vegetative diploid phase to accumulate recessive mutations and subsequent haploidization to generate high-fitness recombinants.

Among the eukaryotes, many species exist as diploids and many others as haploids. The question whether there are any particular evolutionary advantages to being diploid or haploid has generated many studies, both theoretical (Crow and Kimura 1965; Jenkins and Kirkpatrick 1995; Orr and Otto 1994; Perrot et al. 1991) and experimental (Adams and Hansche 1974; Mable and Otto 1998; Paquin and Adams 1983; Zeyl et al. 2003). In the theoretical model studies, essential parameters are the level of dominance of a mutation and the mutation supply rate. The phenotypic effect of recessive mutations will be (partially) masked in diploids. Hence, diploidy will in the short term be favored in case of deleterious mutations but disfavored in case of adaptive recessive mutations. Haploids may evolve faster due to immediate expression of adaptive mutations, at least in large asexual populations (Zeyl et al. 2003). However, diploid nuclei contain twice the amount of DNA of haploid nuclei, suggesting a higher mutation supply rate in diploids, which can be an advantage when the supply of mutations is limiting the rate of adaptation.

We hypothesize that in fungi the parasexual cycle, which occurs naturally in fungi but also in distantly related oomycetes (Pipe et al. 2000), plays a role in adaptation by exploiting specific advantages of both ploidy levels. In particular, it may derive its evolutionary significance from the combination of an extended vegetative diploid phase and subsequent mitotic segregation and recombination. Recessive mutations may first accumulate in vegetative diploid nuclei, from which recombinants may segregate by parasexual processes. If several recessive mutations interact to produce particularly well-adapted genotypes, this process is likely to be more efficient than the regular sexual cycle in which the diploid phase is of very short duration and also more efficient than vegetative reproduction which lacks recombination.

Aspergillus nidulans is a well-studied model organism having an asexual, sexual and parasexual reproductive cycle and is well suited for evolutionary

experiments (Clutterbuck 1974; Pontecorvo et al. 1953; Swart and Debets 2004). When growing on a surface, *A. nidulans* forms a spatially structured mycelium consisting of a dense network of filaments (hyphae). A mutation increasing mycelial growth rate is easily detected as a sector in the colony with a higher growth rate (Figure 1).

Figure 1. An *Aspergillus nidulans* colony growing on a surface for six days after inoculation in the center. The mycelium consists of hyphae, which are divided into compartments by septa, each of which contains three to four nuclei (Gull 1978). A typical mycelium of several days old contains 10^8 nuclei. After the event of mutation in one nucleus, an isogenic mutant sector in the expanding patch of mycelium can arise (Chapter 2). In case of a beneficial mutation, this sector will have a higher growth rate than the surrounding mycelium and can easily be detected. The time interval between two successive nuclear divisions is around 90 minutes when growing at 37 ^{o}C (Chapter 2).

Diploid mycelium has the same architecture as haploid mycelium, but contains half the number of nuclei (Clutterbuck 1974; Fiddy and Trinci 1976; Gull 1978; Roper 1966). Thus the amount of DNA in a diploid colony is about the same as in a haploid colony of the same size. The parasexual cycle involves the following events. Haploid nuclei can fuse to form diploids that can propagate

vegetatively and revert to haploidy by repeated non-disjunction of whole chromosomes (Pontecorvo and Käfer 1958). Mitotic recombination can occur during the diploid phase at a rate 500 times lower than meiotic recombination (Pontecorvo 1958). In nature most isolates of *A. nidulans* are haploid, with 0.01 - 0.1 % diploids (Caten 1981; Pontecorvo 1958; Upshall 1981). In a growing mycelium haploid nuclei fuse to form vegetative diploids with a probability of 10^{-6} and diploid nuclei undergo spontaneous haploidization with a probability of 10^{-2} per mitosis (Clutterbuck 1974; Käfer 1961; Pontecorvo 1958).

In our study, 15 parallel isogenic haploid and 20 diploid strains evolved for over 3000 cell cycles. The experimental setup was identical to Chapter 2; see Methods. Fitness is defined as the *mycelial growth rate* (MGR) of a colony on the surface of solid medium (Pringle and Taylor 2002); propagation in the evolution experiment was achieved by inoculating a small part of the growing front of a colony with the highest MGR six days after incubation onto a fresh plate. The total experiment comprised of 25 transfers. The strains used carry a resistance mutation to the fungicide fludioxonil, and bear a cost of resistance in the form of a 50% reduction in fitness in the absence of fungicide (Chapter 2); we studied adaptive recovery during growth on fungicide-free medium.

Figure 2 shows the mean fitness trajectory of all haploid and all diploid strains. The rate of adaptation is given by the mean slope of fitness trajectories of replicate strains. Of the 20 initially diploid strains, four reverted to haploidy in the course of the experiment. Only the haploid segregants showed a mean rate of adaptation significantly higher than zero (t-test, $t_3 = 16.7$, $p < 0.001$) and had a higher rate of adaptation than those that retained haploid or diploid (t-tests; $p < 0.0001$). Fitness improvement was caused by second site mutations rather than reversion to fungicide sensitivity, since all evolved strains retained their resistance. Spontaneous haploidization of the diploid populations resulted in an instantaneous and dramatic increase in fitness. This occurred in the different

strains at different points in time during the experiment. Therefore, their mean fitness trajectory shows a gradual increase (Figure 2).

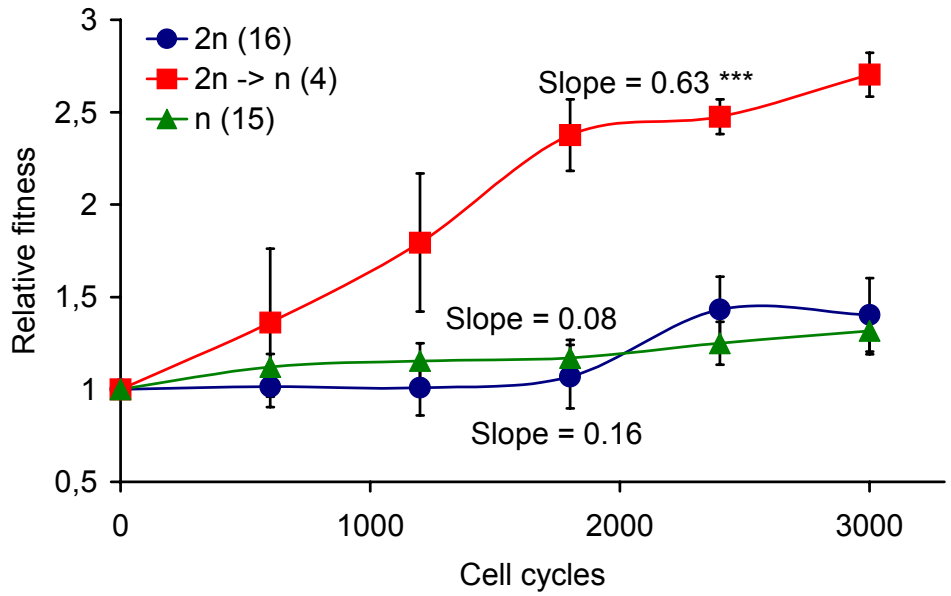

Figure 2. Fitness trajectories of haploid and diploid population. The speed of adaptation is given by the slope of the fitness trajectory. Of the 20 replicate populations beginning as a diploid, four reverted to haploidy in the course of the experiment; their mean trajectory is shown separately. One diploid population became homozygous for an adaptive mutation. Error bars represent the standard error of the mean (SEM).

We further analyzed the adaptive mutations present in the four haploid strains that had a diploid history. Firstly, diploids heterozygous for the adaptive mutation were constructed (Swart and Debets 2004). These diploids expressed the non-adapted phenotype showing that the adaptive mutations in the different populations were recessive.

Secondly, from the –80 ^{o}C stock where at several time intervals samples of all evolving populations had been stored for later analysis, the four diploid strains were retrieved 200 cell cycles prior to their spontaneous haploidization. Haploidization was induced (Käfer 1958) to see if recessive adaptive mutations

had already accumulated in the diploid nuclei. For one diploid population, we could identify a haploid segregant with the same MGR as the successful haploid revertant that arose spontaneously (analyzing the five most fit progeny out of 40 that were initially isolated). From the other three diploid populations the MGR of the segregants varied in between the level of the diploid ancestor and the spontaneous haploid segregant, see Figure 3. We conclude that also in those three diploids recessive beneficial mutations had already accumulated, but that either a particularly adaptive mutation had not yet arisen or that the particularly successful recombinants were not produced during the forced haploidization.

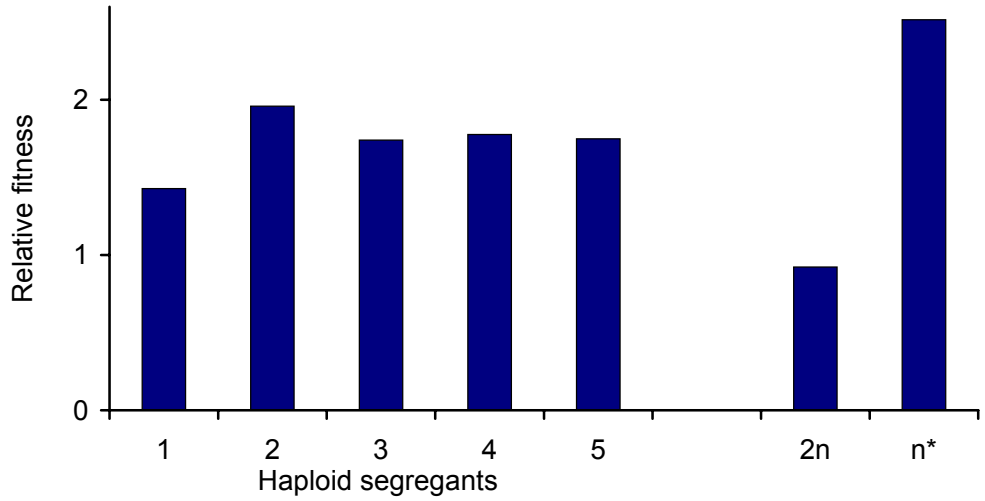

Figure 3. Relative fitness (MGR) of five forced haploid segregants, their ancestor (2n) and the spontaneously segregated haploid (n*). The diploid population was retrieved 200 cell cycles prior to spontaneous haploidization and forced to haploidize. From 40 segregants, five were selected on the basis of apparent vigorous growth. Their fitness (MGR) relative to the strain that founded the evolution experiment was measured. The forced segregants did not have the same MGR (ANOVA, $F = 31.6$, $df = 4$, $p < 0.001$).

Thirdly, the number of adaptive mutations present in the haploid revertants of evolving diploid populations was assessed by crossing them with the ancestor (Pontecorvo et al. 1953) as in Chapter 2. The MGR on fungicide free medium of 40 progeny was used to identify phenotypic classes. In one cross, two distinct

classes in the progeny could be detected, indicating the presence of just one major adaptive mutation. In the other three crosses, multiple classes were observed together with intermediate phenotypes, suggestive of the presence of multiple adaptive mutations and epistatic interactions.

Among the 16 diploid lines that remained diploid during the experiment, we found one line in which the MGR had increased by a factor 2.5, about the same level as the haploid recombinants. After induced haploidization, 20 haploid segregants were analyzed, all of which showed the same large MGR as their diploid progenitor. Furthermore, we performed a dominance test by combining one of these haploid segregants and a non-evolved fungicide resistant haploid strain (WG631). The resulting diploid did not show the elevated MGR phenotype on fungicide-free medium. From these observations we conclude that the single diploid line that showed a large increase in MGR during the experiment had become homozygous by mitotic recombination for a part of the genome that carried a recessive adaptive mutation with large effect, or – less likely - perhaps several linked recessive mutations producing the MGR increase. Our results support the view that adaptation may often involve non-additive interactions between genes (Wright 1982). If adaptation in our experiment had involved additive effects of several mutations, the lines that started as haploids should have shown the fastest response, since they allow immediate expression of recessive adaptive mutations. This is in contrast to the diploid lines in which these recessive mutations accumulate but are only expressed after parasexual segregation or recombination.

In conclusion, our experiments suggest an important role for parasexual processes (spontaneous haploidization by repeated non-disjunction and mitotic recombination) during the adaptation to novel environments. Soon after its discovery by Pontecorvo (Pontecorvo et al. 1953) the usefulness of the parasexual cycle for genetic analysis was quickly recognized, and initially its

role in natural conditions was seen as an alternative for sexual recombination. It must be stressed that the typical starting point of the parasexual cycle was considered to be the heterokaryotic state of a fungus. Clearly, when two different nuclear genotypes occur in a common cytoplasm, recombination by a parasexual process can generate substantial genetic variation. However, the enthusiasm for an important evolutionary role of the parasexual cycle has been replaced by skepticism (Caten 1981; Elliott 1994) once it was recognized that heterokaryons are rare in nature, due to the widespread occurrence of somatic incompatibility in fungi preventing anastomosis of different mycelia. We believe that our results justify a rehabilitation of the view that the parasexual cycle has an important evolutionary role in fungi, because also in homokaryons sufficient genetic variation appears to be generated by mutation to make genetic recombination effective. The important difference with the sexual cycle is the presence of an extended vegetative diploid phase allowing the accumulation of adaptive but recessive mutations in diploid nuclei.

Methods

Strains. The *Aspergillus nidulans* strains used in this study were derived from the original Glasgow strains (Clutterbuck 1974), see Chapter 2. From lab strain WG562 (*lys*B5) a spontaneous mutant resistant to the fungicide fludioxonil (Novartis; 0.2 ppm) was isolated (WG561; lysB5; III, *fld*A1: resistance to fludioxonil). There are fitness costs associated to the resistance (Chapter 2). Neutral genetical markers were introduced into the resistant mutant WG561 to construct WG615 (*w*A3; *fld*A1; *pyro*A4); WG561 and WG615 were used to construct the fungicide resistant diploid strain WG561//615. WG631 (*y*A2; *pro*A2; *fld*A1) was used in a cross to assess the number of adaptive mutations in haploidized originally diploid strains.

Media and culturing. Strains were cultured on solid Minimal Medium (MM), see Chapter 2, supplemented with lysine (2.0 mmol/l), pyrodoxin (0.1 mg/l) and proline (2.0 mmol/l) where needed. Strains were always incubated at 37 ^{o}C. Whether the evolved populations retained their resistance to fludioxonil was assayed by comparing the MGR of evolved and non-evolved populations on MM with fludioxonil (Novartis; 0.2 ppm, Chapter 2). Haploidization of diploids was induced (Käfer 1958), using 1.7 ppm of benomyl in Complete Medium (Chapter 2). Using the same technique, diploids (before and after adaptation) were distinguished form haploids and the moment at which spontaneous haploidization had occurred was assessed. Crosses were performed as described by Pontecorvo et al. (1953).

Fitness assays. We defined fitness as the *mycelial growth rate* (MGR) of fungal mycelium (Chapter 2; Pringle and Taylor 2002). After six days of growth, we determined the MGR by averaging the colony diameters as measured in two randomly chosen perpendicular directions; these measurements were done using three independent assays. The MGR was made relative to the non-evolved haploid (WG561 and WG651) or diploid ancestor (WG561//651) that founded the evolution experiment.

Due to physiological differences between haploids and diploids, the fitness in terms of MGR of isogenic haploid and diploid counterparts is not identical. We scaled the rates of adaptation during the evolution experiment by expressing the MGR relative to the initial value of the ancestral strain. However, if we take the initial differences in MGR into account (by multiplying the slope with initial MGR), the difference found between the four haploidized strains and the all time haploid and diploid strains remains highly significant (two-sided t-test; $p < 0.0001$).

Evolution experiment. For this experiment, eight strains were founded from WG561, seven strains from WG651 and 20 strains from WG561//615. All strains used carry a resistance to the fungicide fludioxonil resulting in a lowered fitness than wild type lab strains when growing on medium without fungicide due to the costs associated with the resistance (Bjorkman et al. 2000; Schrag and Perrot 1996). Adaptive recovery on solid medium without fungicide is measured by monitoring the *mycelial growth rate* (MGR) on the surface of a Petri dish (Chapter 2). For the experiment, the part of the growing front with the highest MGR was identified after six days of incubation. A sample of mycelium taken from the growing front containing between 10,000 and 40,000 nuclei was transferred to fresh medium. The total experiment comprised 25 transfers. The transferred mycelium is considered genetically homogeneous, so the effective population and bottleneck sizes are small. Every transfer, samples of all populations were stored in a non-evolving state (at –80 ^{o}C) and the MGR of all strains was measured. The MGR was made relative to the ancestor of the population; the rate of adaptation was calculated as the slope of the fitness trajectory (Zeyl et al. 2003). Every five transfers the ploidy for all diploid derived strains was assessed using benomyl (Käfer 1958; Swart and Debets 2004).

Acknowledgements.

S.E.S, A.J.M.D. and R.F.H. designed the experiments, which were conducted by S.E.S. and M.S.; S.E.S, A.J.M.D. and R.F.H. co-wrote the paper. The Netherlands Organization for Scientific Research (NWO-ALW) has funded this project.

4 Artificial and natural selection in compensatory evolution with experimental fungicide resistant strains of *Aspergillus nidulans*

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Abstract

In an experimental study of adaptation we have investigated the relative effectiveness of artificial selection versus natural selection on the rate of compensatory evolution in a fungicide resistant strain of the filamentous fungus *Aspergillus nidulans*. Using mycelial growth rate as a fitness measure, artificial selection involved the weekly transfer of the fastest growing sector onto a fresh plate. Transferring random samples of all the spores produced by the mycelium approximated natural selection. Fungicide resistant and fungicide sensitive haploid and diploid strains were used in an evolution experiment over 10 weekly transfers, which is equivalent to 1200 cell cycles. Two different environmental conditions were applied: a constant fungicide-free environment and a weekly alternation between presence and absence of fungicide. Results show that for all strains and conditions used, the transfer of a random sample of all spores leads to more rapid adaptation than the transfer of the visually 'fittest' sector. The rates of compensatory evolution in the constant and the alternating environment did not differ. Moreover, haploid strains tend to have a higher rate of adaptation than isogenic diploid strains.

Introduction

Antibiotic resistance easily develops in microorganisms by a single mutation. Subsequent compensatory evolution to ameliorate resistance associated fitness costs has been studied experimentally, initially using mainly bacterial systems (Andersson and Levin 1999; Lenski 1997). The process of fitness compensation has been shown to involve second site mutations rather than reversion to antibiotic sensitivity, resulting in resistant strains not only growing well in the presence of the antibiotic but also in its absence (Bouma and Lenski 1988; Moore et al. 2000b; Reynolds 2000; Schrag and Perrot 1996).

We recently presented a study of fungicide resistance development and subsequent compensatory evolution in the filamentous fungus *Aspergillus nidulans* (Chapter 2). In that study, one mutant resistant to the fungicide fludioxonil with associated fitness costs was selected for an experimental study of compensatory evolution over 3000 cell cycles where 40 parallel strains of this mutant evolved under three different environmental conditions. In that study we defined fitness as the *mycelial growth rate* (MGR). An adaptive mutation occurring in a nucleus in growing mycelium can be recognized as a sector in the mycelium with an enhanced growth rate (Figure 1). Strains were cultured by weekly transferring a fraction of the mycelium most distant to the point of inoculation onto fresh medium. We hypothesized that this artificial selection procedure would maximize the chance of an adaptive mutation to be selected. Our results showed that in all environmental conditions used the mutant strain was able to restore fitness. Genetic analyses of crosses between different evolved strains and between evolved and ancestral strains showed that a limited number of one, two or three major loci were involved in fitness compensation and revealed interaction between compensatory mutations.

Figure 1. An *Aspergillus nidulans* colony (WG561) after four days of growth showing a sector with enhanced growth rate. The formation of a colony starts after germination of an asexually or sexually derived spore or from mycelium containing at least one nucleus. A dense network of hyphae develops by expansion of mitotically active apical cells that contain up to 40 nuclei (Kaminskyj and Hamer 1998). Twenty-four hours after the formation of a hypha, the asexual cycle has completed with the formation of spores on conidiophores (Figure 2; Adams et al. 1998; Timberlake 1990). The sexual cycle commences around three days after termination of the asexual cycle. The production of sexual spores results in fruiting bodies (cleistothethia), each containing up to 100,000 spores (Adams et al. 1998; Casselton and Zolan 2002).

In the present study we used fungicide resistant *A. nidulans* strains to investigate the relative effectiveness of two modes of selection on the rate of compensatory evolution. Artificial selection by transplanting the 'fittest' sector of a colony to fresh medium at certain time intervals was compared with natural selection, which was approximated by transfer of random samples of 0.1 % of all the spores of the fungal mycelium. *A. nidulans* mycelium produces numerous asexual and sexual spores (up to 10^9 on the surface of a colony the size of a Petri Dish). Under natural conditions their dispersal and subsequent outgrowth is likely to be of much greater importance than mycelial extension, since most

mycelial colonies tend to be short-lived due to rapid exhaustion of the substrate. However, we hypothesize that this random selection procedure decreases the chance of an adaptive mutation to be directly selected in an evolution experiment, hence that the direct selection of adaptive mutations by selection of the 'fittest' sector, as practiced in our previous study (Chapter 2) overestimates the rate of compensatory evolution in nature.

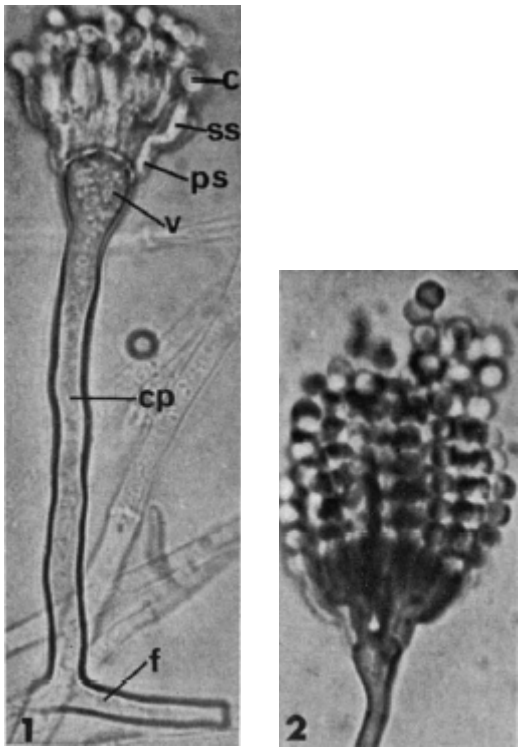

Figure 2. Condiophore carrying asexual spores. Sixteen hours after formation of mycelium, subapical cells in hyphea differentiate into foot cells which give rise to a stalk with a vesicle and serigmata that carry chains of asexual spores. The left figure gives an overview of the total apparatus; c: conidium; ss, secondary serigmata; ps, primary serigmata; v, vesicle, cp, conidiophore stalk; f, mycelial foot cell. The right figure shows a fully-grown conidiophore with up to 10,000 asexual spores. These pictures were taken from Clutterbuck (1969).

The genetics of *A. nidulans* are well studied (Adams et al. 1998; Clutterbuck 1974; Pontecorvo et al. 1953; Swart and Debets 2004; Timberlake 1990) and this fungus is a suitable model system to experimentally address evolutionary questions (Chapter 2). It combines several of the characteristics of unicellular microorganisms used in experimental evolution (Elena and Lenski 2003) with additional advantages of multicellularity in a somatic structure of growing mycelium containing nuclei in spatially defined positions. The *mycelial growth*

rate (MGR) is an easily to assess and highly reproducible fitness measure (Bruggeman et al. 2003; Pringle and Taylor 2002); Chapter 2. The number of cell cycles (successive nuclear mitotic divisions) in the growing front of the mycelium is up to 112 per week (Bergen and Morris 1983; Kaminskyj and Hamer 1998) and the parasexual and sexual cycle is of use for genetic analysis (Clutterbuck 1974; Käfer 1958). Samples taken at different time points can be stored in a non-evolving state at –80ºC and later used for comparative analysis. Vegetatively growing isogenic haploid and diploid strains can be used (Käfer 1958; Pontecorvo et al. 1953), see also Chapter 3.

We have studied the effect of artificial and natural selection in an evolution experiment over 10 weekly transfers (equivalent to 1200 cell cycles for the growing front of the colony) in different situations. Fungicide resistant and fungicide sensitive haploid and diploid strains were used growing in the absence of fungicide. Because of their cost of resistance, resistant strains are more distant from a fitness optimum than the fungicide sensitive strains. Therefore, mutations with large effect are more likely to be adaptive in resistant than sensitive strains that are on or around a fitness optimum (Fisher 1930). For the resistant strains, we expect the transfer of the mycelial sector directly pointing to an adaptive mutation to lead to a faster rate of compensatory evolution than the transfer of a random spore sample. For the sensitive strains, where adaptive mutations will have much smaller effects and therefore are likely to escape visual detection, we expect the transfer of random spore samples to be more successful.

We used isogenic haploid and diploid strains to assess the influence of ploidy on the rate of adaptation in the two modes of selection. A priori we do not know what outcome to expect comparing evolving haploid and diploid strains. There are physiological differences between haploid and diploid strains. Diploid strains only produce around 25% of the number of spores haploids produce per unit surface area of mycelium (see Methods), however, diploid hyphae expand

around 5% more rapidly than haploid hyphae (Schoustra and Slakhorst, unpublished observation). In diploid strains, the phenotypic effect of recessive adaptive mutations can be masked, however, successful combinations of adaptive mutations could be formed after haploidization due to chromosome loss by repeated non-disjunction or mitotic recombination of a diploid (Pontecorvo and Käfer 1958).

Compensatory evolution in fungicide resistant haploid and diploid strains was compared in two different environments: a constant fungicide-free environment and the alternating absence and presence of fungicide. In the alternating environment we expect the random spore sample propagation to lead to a higher rate of adaptation than the mycelial sector propagation, since the conditions after a transfer are different from the conditions where selection took place.

Methods

Strains. *Aspergillus nidulans* strains used were derived from the original Glasgow strains (Clutterbuck 1974). Haploid and diploid strains both sensitive and resistant to the fungicide fludioxonil (4-(2,3-difluoro-1,3-bezodioxol-4-yl)pyrrol-3-carbonitril) were used. WG562 (*lys*B5) and WG562//145 (*lys*B5 // *w*A3, *pyro*A4) were sensitive. WG561 (*fld*A1, *lys*B5) and WG561//615 (*fld*A1, *lys*B5 // *w*A3, *fld*A1, *pyro*A4) were resistant to the fungicide fludioxonil and had associated fitness costs.

Media and culturing. Strains were cultured in Petri dishes with solid Minimal Medium (MM) supplemented with lysine to a final concentration of 2.0 mmol/l or Complete Medium (CM). See Clutterbuck (1974) and Chapter 2 for full details. Spore suspensions were made in saline (distilled water with NaCl 0.8 g/l) supplemented with Tween80 (0.01%) by washing off all spores from the

surface of all mycelium on a plate with 5 ml saline-tween. Agar plates were inoculated in the center either with an inoculation needle or with 5 µl of spore suspension. Plates were always incubated at 37 ^{o}C. Two different growth conditions were used: fludioxonil-free MM and MM with weekly alternating absence or presence of fludioxonil (Novartis; 0.2 ppm). For the latter condition, only the resistant haploid and diploid strains were used. Ploidy levels of all initially diploid strains were assessed after the last transfer by growing them on CM with benomyl 1.7 ppm (Käfer 1958). Maintenance of resistance was assessed by growing all initially resistant strains on MM with fludioxonil (0.2 ppm).

Replication and propagation. Per strain and condition, twelve replicates of the same strain were used. After incubation of six days, all plates were placed at 4 ^{o}C for 24 hours after which strains were transferred to fresh medium using two ways of transfer. The first way was by transfer of a part of mycelium from the growing front most distant to the point of inoculation; in this way 10,000 to 40,000 nuclei are transferred. The second way was by washing off all spores from the surface of the colony with 5.0 ml of saline-tween of which 5 µl was used to inoculate fresh medium, giving a fixed bottleneck size of 0.1 %. Of the in total twelve replicate populations per condition, six were transferred with mycelium of the growing front of the fittest sector of the colony and six with a 0.1 % sample of all spores. After six days of growth, a fungicide sensitive haploid colony (WG562) produces on average around $1.6 * 10^9$ spores (n = 12; standard error of the mean = $2,4 * 10^8$); a diploid colony (WG562//145) $5.2 * 10^8$ spores (n = 12; SEM = $3.9 * 10^7$). The evolution experiment consisted of 10 weekly transfers, which amounts to approximately 1200 cell cycles for the growing front of the colonies.

Fitness assays. We defined fitness as the *mycelial growth rate* or MGR (Pringle and Taylor 2002); Chapter 2. We measured the MGR for all strains at every

transfer by averaging the colony diameter as measured in two randomly chosen perpendicular directions. The MGR of the fungicide sensitive strain WG562 (weekly taken from the –80 ^{o}C stock, grown in six fold as a non-evolving control) was used as a reference to calculate the relative fitness of the other strains. The slope of the fitness trajectory over the 10 transfers of a strain, computed from a linear regression analysis, defines its rate of adaptation in this evolution experiment.

Results

In an evolution experiment, artificial and natural selection have been compared using fungicide sensitive and resistant haploid and diploid strains in two different environmental conditions. Figure 3 shows the mean trajectories of the relative fitness of the replicate strains grown under the same conditions and with the same mode of transfer. The slope of the fitness trajectories of evolving populations in time represents the rate of adaptation. The mean slopes of all six replicates per treatment and ploidy are in Table 1.

Propagation by random spore samples resulted in more rapid adaptation than propagation by artificially selecting the 'fittest' sector. This was significant and independent of whether strains were fungicide sensitive or resistant, haploid or diploid, or whether environments were stable or changing (ANOVA, $F = 20.1$, $df = 1$, $p < 0.001$).

Overall, there was a significant increase in relative fitness (MGR) after 10 transfers (the mean slope of fitness all fitness trajectories is higher than zero, $\chi^2_1 = 20.5$, $p < 0.001$). However, this was not the case for all individual groups of replicate strains that were propagated with sectors, see Table 1. The fungicide resistant strains had a higher rate of adaptation than the fungicide sensitive strains (t-test, $t_{46} = 4.94$, $p < 0.001$).

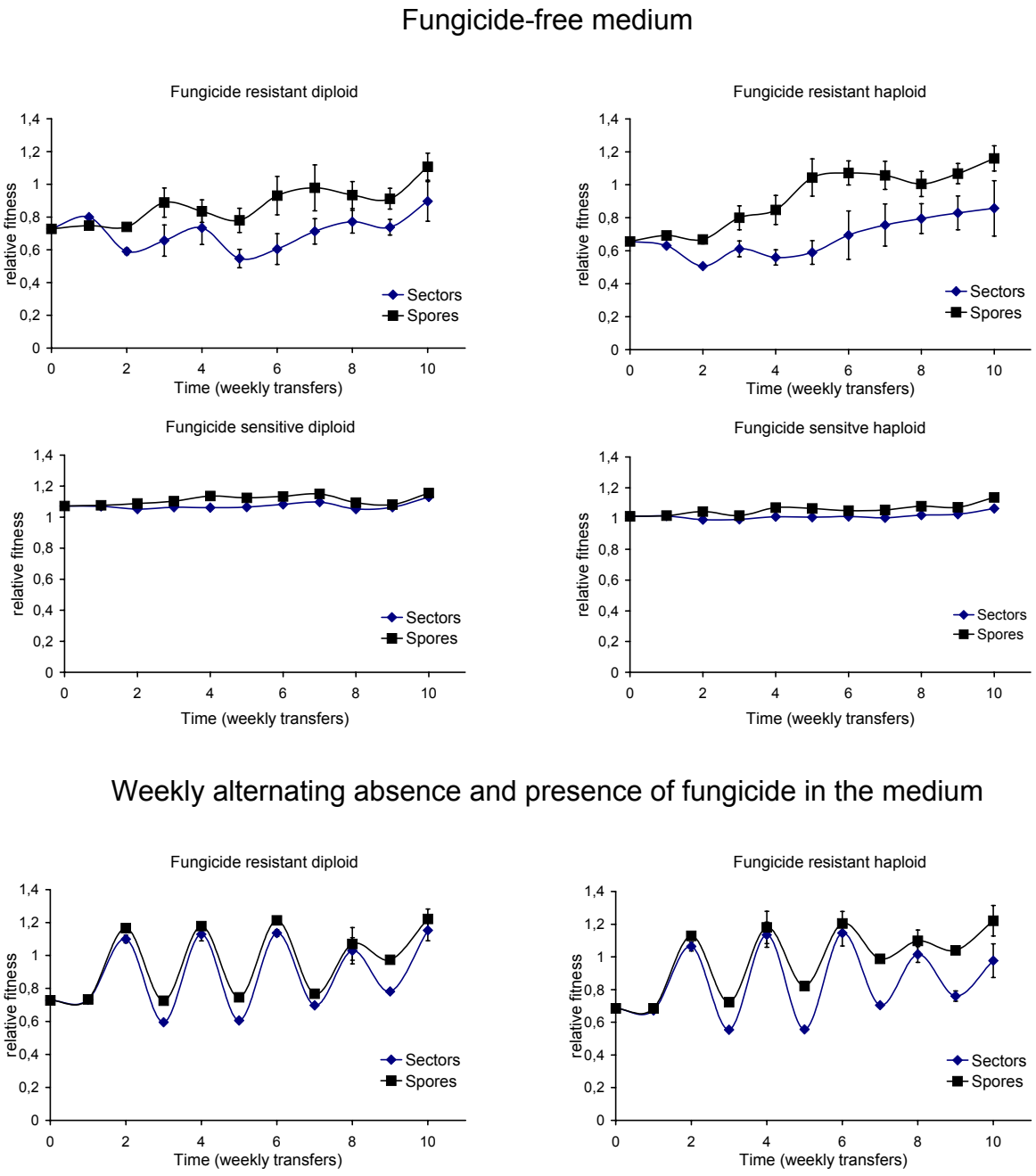

Figure 3. Fitness trajectories for the evolution experiment over 10 transfers (equivalent to 1200 cell cycles). Two environmental conditions were used as indicated by the heading of a group of graphs. The titles of individual graphs show the strains used. Twelve replicates of each strain were used in every condition, six of which were propagated by transferring the 'fittest' sector and six by transferring a random sample of all spores (0.1 %). Weekly the MGR of all strains was measured and made relative to a fungicide sensitive reference strain (WG562) growing parallel in the absence of fungicide. Each line in each graph represents the mean of six replicate independently evolving strains that had the same treatment. Error bars indicate the 0.95% confidence interval for the mean. In several cases, the error bars are smaller than the symbols.

Table 1. Rates of adaptation given by the mean slope (* 1000) of fitness trajectories of all replicate fungicide resistant or sensitive strains per environmental condition and mode of transfer. The value for the 0,95 % Confidence Interval (CI) of the mean is indicated. There were twelve replicate populations per growth medium per strain, six of which were transferred using the fittest sector and six using a random sample of all spores. Alternating growth medium is medium with alternating absence and presence of fungicide at each transfer. An asterisk * indicates that the Confidence Interval does not comprise zero ($p < 0.05$), showing that the mean rate of adaptation was greater than zero.

Strain	Environment	Transfer by 'fittest' sector	*0,95 % CI*	Transfer by random spore sample	*0,95 % CI*
Resistant diploid	Fungicide free	10,1	*13,8*	30,9	*11,8 **
Sensitive diploid	Fungicide free	3,2	*0,6 **	5,0	*1,6 **
Resistant haploid	Fungicide free	28,0	*24,4 **	52,4	*14,6 **
Sensitive haploid	Fungicide free	3,0	*1,2 **	9,0	*2,2 **
Resistant diploid	Alternating	3,6	*12,0*	21,4	*12,7 **
Resistant haploid	Alternating	8,2	*11,2*	43,4	*14,0 **
Average over all strains and media		9,4	*14,2*	27,0	*17,2 **

Comparing haploid and diploid strains over all treatments and conditions showed that haploids had a higher rate of adaptation than diploids (ANOVA, $F = 9.48$, $df = 1$, $p = 0.004$). However, comparison of haploid and diploid strains that had the same mode of transfer and growth conditions only shows statistically significant differences when strains are transferred with random spores samples, haploids having the highest rate of adaptation (t-tests, $df = 10$, $p < 0.05$). After the tenth transfer, we determined the ploidy of all originally diploid strains. Out of the 36 initially diploid strains one strain that had been propagated by transfer of sectors and one that had been propagated by transfer of spores had become haploid. The two haploidized diploid strains had a higher rate of adaptation than the mean of all diploid strains, however, this difference was non-significant (t-test, $t_{34} = 1.34$, $p = 0.19$).

The difference observed in the rate of adaptation of resistant strains evolving in fungicide-free and alternating environments is non-significant (t-test, $t_{34} = 1.67$, $p = 0.10$). For each strain only the first five transfers to medium without fungicide were taken into account for this comparison. After 10 transfers, all initially resistant strains had retained their resistance to fludioxonil, indicating that compensation for fitness costs was by second-site mutations.

Discussion

In an evolution experiment over 10 weekly transfers, we have investigated the relative effectiveness of artificial selection versus natural selection on the rate of adaptation in *Aspergillus nidulans* strains. Artificial selection involved the weekly transfer of the fastest growing sector onto a fresh plate (Figure 1), while transferring random samples of all the spores produced by the mycelium approximated natural selection. Fungicide sensitive and resistant haploid and diploid strains were allowed to evolve in two different environments: a fungicide-free environment and an environment with a weekly alternation between presence and absence of fungicide.

The results of the evolution experiments show that in all conditions and with all strains used the transfer of random spore samples resulted in the most rapid adaptation. Since a mycelial sector with enhanced growth rate directly points to a beneficial mutation of fairly large effect, (Figure 1), we hypothesized that for evolution starting under clearly sub-optimal conditions (resistant strains in a fungicide-free environment) artificial selection would result in a higher rate of adaptation than natural selection. However, our results clearly contradict this hypothesis.

A possible explanation for the more rapid adaptation when transferring random samples of all spores lies in the number of mitoses needed to produce a spore from a nucleus in the mycelium. After their development, some mycelial subapical cells differentiate into foot cells (Figure 2), from where a condiophore with chains of asexual spores are formed. Up to 100 mitoses take place between the formation of mycelium and the formation of the asexual spores (Clutterbuck 1969; Pontecorvo et al. 1953). Clearly, these additional nuclear divisions during spore formation allow more genetic variation to be generated by mutation than in mycelial hyphae only. Apparently, the generated random variation includes many adaptive mutations that can be efficiently selected during spore germination. Moreover, the fittest sector approach only samples nuclei that manage to form a sector, i.e. are located in the growing front of the mycelium at the moment they receive a favourable mutation. The more central mycelium of the colony is not involved in the expansion of the colony, but serves mainly metabolic functions. Advantageous mutations occurring in nuclei in the central part of the colony will not end up in a recognisable fast growing sector. However, they do have a chance to get incorporated in spores, as spore formation occurs on the whole surface of the colony, both in the central part and in the expanding growth front. It is therefore quite possible that in the random spore sample transfer procedure the net is cast much wider for catching a favourable mutation than in the fittest sector approach.

In two previous studies of compensatory evolution in *A. nidulans* strains (Chapters 2 and 3), the adaptive mutations found were recessive, in line with the general observation (e.g. (Mukai 1964) that most mutations are (partly) recessive. Mycelial cells consist of more than one haploid or diploid nucleus. The chances of establishing an isogenic sector in mycelium consisting of multi-nucleate cells will be lower for a recessive mutation then for a dominant mutation. Therefore, maintenance of genetic variation in single-nucleate spores could have a large impact on the rate of adaptation.

Since relative fitness is calculated as the MGR of a strain relative to the MGR of the fungicide sensitive haploid strain (WG562) used as a control, several strains do not have a relative fitness of 1 at the start of the evolution experiment. When isogenic, diploids have a higher MGR than haploids as thus a different relative MGR at the start of the evolution experiment. For resistant strains, the mycelial growth rate (MGR) on medium with fungicide is higher than the MGR of fungicide sensitive reference strain growing in the absence of fungicide. This makes their relative fitness greater than 1 (c.f. Figure 3).

On average not only resistant strains but also sensitive strains gained in fitness demonstrating that the sensitive strain was not optimally adapted to the experimental conditions we used. However, the resistant strains had a higher rate of adaptation than the sensitive strains, which can be explained by their lower initial fitness due to the cost of the resistance they carried. Because the resistant strains started at a lower relative fitness a larger fraction of all mutations is potentially adaptive than for fungicide sensitive strains that are closer to a fitness optimum (Fisher 1930; Moore et al. 2000b).

The comparison of rates of adaptation for haploids and diploids shows that haploid strains on average tend to have a higher rate of adaptation. This is in accordance with a similar study with haploid and diploid *Saccharomyces cerevisiae* strains (Zeyl et al. 2003) and the study described in Chapter 3. Out of the total of 36 diploid strains, there were two diploid strains that reverted to haploidy and had a higher rate of adaptation than the all time haploids and diploids, although these differences were non-significant. However, it may point to a potential evolutionary advantage for a vegetative diploid phase in otherwise haploid filamentous fungi. In nature, most isolates of *A. nidulans* are haploid, with 0.01 - 0.1 % diploids (Caten 1981; Pontecorvo 1958; Upshall 1981).

For the resistant strains, adaptation takes place at an equal rate in a stable fungicide-free environment and an environment with alternating absence and presence of fungicide. Apparently, mutations that are adaptive in the environment without fungicide do not have (severe) negative side effects in the presence of fungicide. Whereas mutations causing fungicide resistance have large trade offs in the absence of fungicide, its compensatory mutations do not, showing that the topography of the adaptive landscapes in these two cases is different (Elena and Sanjuan 2003; Wright 1982). There are practical implications of this finding for agriculture where fungicides are widely applied to control plant pathogens. Fludioxonil is widely used in agriculture to control emerging fungal infections on grape vines in particular (Cabras et al. 1997; Verdisson et al. 2001). Our results suggest that discontinuous and irregular use of fungicide does not reduce the risk of spread of resistance.

In summary, in experiments of adaptive evolution with *A. nidulans* strains ameliorating costs of fungicide resistance, artificial selection by selecting the fastest growing part of the mycelium does not overestimate the rate of adaptation in more natural circumstances where total spore production of the fungal colony is taken into account. On the contrary, selecting only on the basis of mycelial expansion may lead to an underestimation of the rate of adaptation.

Acknowledgements

We wish to thank Marc Maas and Klaas Swart for help, suggestions and comments. The Netherlands Organization for Scientific Research (NWO-ALW) funded this project.

5 *Velvet–* strain of *Aspergillus nidulans* exhibits non-circadian light inducible rhythm

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Abstract

The velvet allele (*ve*A1) is known to prevent the formation of banding patterns in spore formation in *Aspergillus nidulans*. In this study, the observed light inducible rhythm of a velvet- *A. nidulans* mutant strain in the formation of conidiospores and ascospores is further characterized. The rhythm appeared to be light entrainable, however needs to be induced by a cycle of light and darkness and therefore is non-circadian.

Introduction

We present the results of a (physiological) characterization of the rhythm in the formation of conidiospores and cleistothethia exhibited by a velvet- (*ve*A1) *Aspergillus nidulans* strain (Figure 1). Velvet- strains are known not to exhibit any light inducible rhythm in the formation of conidiospores and ascospores (Käfer 1977; Timberlake 1990). The mutant was found when studying the development of resistance to the fungicide fludioxonil and subsequent compensatory evolution in *A. nidulans* (Chapter 2). The resistance gene (*fld*A1) was assigned to chromosome III by parasexual analysis.

We investigated whether the rhythm found is a circadian rhythm. A circadian clock keeps organisms in synchrony with the 24-hour day-night cycle on earth and is wide spread. A rhythm is circadian if it has the following three characteristics (Johnson et al. 2003; Loros and Dunlap 2001): (1) It should persist in the absence of "zeitgeber"; these are temporal signals such a light-dark or temperature oscillations. A rhythm under constant conditions (usually constant darkness and temperature) is called free running and the period is referred to as free running period (FRP) and should be around 24 hours. The rhythm should also be independent of nutritional conditions. (2) It can be entrained, or reset, by external signals. (3) The free running period is independent of temperature (in a temperature range relevant for the organism).

In fungi, circadian rhythms have been demonstrated in *Neurospora crassa*; in this organism the phenotypic manifestation as well as the genetic background has been extensively studied (Loros and Dunlap 2001). The band (*bd*) mutation enhances the visualization of the rhythm that can be observed in asexual spore production. The band mutation renders the cells less sensitive to high CO_2 levels in race tubes and Petri dishes; high CO_2 levels inhibit the formation of conidia (Bell-Pedersen 2000; Sargent et al. 1966).

The presence of a circadian clock was demonstrated in *Aspergillus flavus* and *A. nidulans* (Greene et al. 2003). In *A. flavus*, an endogenous circadian rhythm in sclerotium production was observed. However, in *A. nidulans* the authors say to lack an easily observable rhythm in phenotype, they only observed a rhythm in expression of genes homologous to circadian rhythm regulation genes in *A. flavus* and *N. crassa*.

Figure 1. Velvet- (*ve*A1) *Aspergillus nidulans* strain demonstrating a rhythm in the formation of conidiospores and ascospores. There are clear sections of the colony were asexual sporulation predominates (green and fawn conidia). The parts with other color contain the sexual fruiting bodies (cleistothethia with red ascospores). The formation of conidiospores starts at "dawn".

Materials and Methods

Strains. The *Aspergillus nidulans* strains were derived from the Glasgow collection (Clutterbuck 1974), see Table 1 for a list of all strains. For the experiments, we used a mutant strain exhibiting a light inducible rhythm (WG622) and a control strain not exhibiting the rhythm (WG562). This mutant strain was derived from a fludioxonil resistant mutant (WG651), which underwent compensatory evolution for 3000 generations ameliorating costs

associated to fungicide resistance (Chapter 2). The uncompensated resistant mutant (WG561) exhibits the rhythm, however the compensated mutant does so in a more pronounced way, see figure 1.

Table 1. Strains used in this study and their genotypes.

Strain	Description	Genotype
WG652	Fungicide resistant strain	V: *lys*B5
WG651	Mutant strain resistant to fludioxonil (exhibits rhythm)	III: *fld*A1; V: *lys*B5
WG622	Mutant strain resistant to fludioxonil with compensation of fitness costs (exhibits rhythm)	III: *fld*A1; V: *lys*B5; ?: *flc*8
WG615	Fungicide resistant strain, used to construct diploids	I: *w*A3; III: *fld*A1; IV: *pyro*A4
WG044	Velvet+ strain, used to make a strain with the "banding-allele" in a *Ve*+ background.	VIII: *ve*A^{+}; *orn*B7; *fw*A1

*fld*A1: resistance to the fungicide fludioxonil, enhances the circadian rhythm.
*lys*B5: lysine deficiency.
flc8: compensatory mutation to eliminate fitness costs associated to fludioxonil resistance; this mutation is not needed to visualize the rhythm but does make it more clear.

Growth media and conditions. Minimal medium (MM) was used (Clutterbuck 1974), supplemented with lysine (2 mmol/l). To study the free-running period of the mutant race, tubes and Petri dishes (15 cm diameter) were used in constant darkness at different temperatures: 27, 30, 33, 35, 37, 39, 40.5 ^{o}C. After 10 days of incubation, we calculated the FRP by counting the number of bands present. To study entrainability an adjustable light-dark cycle was applied. The sugar concentration (100%, 30% and 10% of 15 g/l) was varied to assess its influence on the banding pattern.

Dominance. To study the dominance/recessiveness of the ability to exhibit the rhythm, diploids homozygous and heterozygous for the "banding allele" were constructed using WG615 (Swart and Debets 2004). Dominance was assessed

comparing the phenotype of the different homo- and heterozygous diploids with respective haploid strains.

Results

Free running period and entrainability. Growing in total darkness, the mutant strain (WG622) does not exhibit a rhythm in the production of condio- and ascospores. For it to do so, it needs a cycle of light and darkness. The amount of light needed is remarkably low; the very faint light passing trough a closed door of an incubator is sufficient. The rhythm is well entrainable (Johnson et al. 2003). Incubation with Light/Dark cycles of 10 hours light and 10 hours dark (10L/10D), 12L/12D and 18L/18D resulted in clear rings of ascospores and conidiospores; exactly one ring each per L/D cycle.

Different temperatures and sugar concentrations. When entrained, the strain exhibits the rhythm al all temperatures tested (27 ^{o}C, 30 ^{o}C, 33 ^{o}C, 37 ^{o}C, 39 ^{o}C and 40.5 ^{o}C) and at all sugar concentrations (CM with 100%, 30% and 10% of the normal sugar concentration).

Dominance/recessiveness. We constructed diploids heterozygous for the resistance mutation (*fld*A1), these heterozygous diploids do not exhibit a clear rhythm. Homozygous diploids do show the rhythm in a very clear way. The mutation enhancing the banding pattern is (partially) recessive.

***Velvet*+ background.** By parasexual recombination using strain WG044 (Swart and Debets 2004), we placed the "banding-allele" *fld*A1 in a velvet + background. In this background, the strain with the banding allele exhibits the rhythm.

Discussion

The results show that our *Aspergillus nidulans* mutant shows certain characteristics of a circadian rhythm. Although very faint light is sufficient to enhance the appearance of a clear banding pattern, the rhythm needs to be entrained, making the rhythm non-circadian. The banding pattern is clearly visible in a velvet- as well as in a velvet+ background, in contrast to the general observation that *ve*A1- strains do not exhibit any light inducible rhythm (Timberlake 1990). Greene *et al.* (2003) have shown that a number of genes homologous to genes associated with circadian rhythms in other organisms show an autonomous cycle in expression. They however could not find a phenotypical demonstration of cycled expression of genes. We did find a clear banding pattern in the growing colony, however it needs some form of entrainment.

Very clearly, the fungus produces the asexual conidiospores during "daytime" and switches to the production of sexual spores during the night. During day the chances of dispersal are highest due to more air movement, this could explain this switch between sexual and asexual sporulation.

The mutation conferring resistance to fludioxonil (*fld*A1) associated with light inducible rhythm may be of value to further investigate the circadian rhythm in *Aspergillus nidulans,* like the *band* mutation that lowers CO_2 sensitivity has been to the analysis of the circadian clock in *Neurospora crassa* (Bell-Pedersen 2000). The exact mechanism of the fungicide fludioxonil and its resistance is not known, but nothing suggests its association with CO_2 sensitivity (Hilber et al. 1995).

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6 The population dynamics and ecology of temperate phage: a killing and recruiting allelopath

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Abstract

We present a model and experiments on the population dynamics of lysogeny. The results of competition experiments with sensitive and lysogenic bacteria show that lysogens can successfully prevent invasion of sensitive bacteria and are able to invade in and recruit from a population of sensitive bacteria. This is consistent with the model we developed. The ecological role of lysogeny is discussed as similar to the ecological role of bacteriocins: temperate phage can act as allelopathic agents.

Introduction

Bacteria are not nice, not even to each other. They produce a variety of toxic agents that kill other closely related species of bacteria. The most famous of these are antibiotics, but there are bacteriocins and other antibacterial toxins as well. In many cases, the production of toxic agents is an altruistic character. The cells producing the agent are immune to the action of the toxic agent but are

killed by lysis when releasing the toxic agent in the environment. Only a limited fraction of the population will lyse and kill closely related competitors, liberating resources for the remaining isogenic relatives (Riley and Gordon 1999; Riley and Wertz 2002). Under what conditions will bacteria producing these allelopathic agents become established and be maintained in populations of susceptible bacteria (Wiener 1996)? Theory and experiments suggest that unless they have an intrinsic advantage, rare bacteria producing these allelopathic agents can only increase in frequency in populations of sensitive bacteria in physically structured habitats, such as surfaces and semi-solids. In liquid, allelopaths are able to invade and eliminate the sensitive population only when they start at a high enough frequency (Chao and Levin 1981; Czaran et al. 2002; Durrett and Levin 1997; Gordon and Riley 1999; Iwasa et al. 1998; Kerr et al. 2002).

Temperate phage can act as an allelopathic agent. A phage (from φαγειν, which is Greek for eating) is a virus that infects bacteria. When a temperate phage attacks a susceptible bacterium, there are two possible scenarios: it can go into a lytic or into a temperate phase (Campbell 1961; Stewart and Levin 1984). In the lytic phase, the phage is virulent and takes over the machinery of the bacterial cell. It then makes the bacterium produce many, up to several hundreds, new phage particles that are released in the environment by lysis and thus leads to the death of the bacterial cell. In the temperate phase, the phage DNA is inserted in the bacterial genome or can exist as a plasmid to form a semi stable association. The prophage in a lysogenized bacterium will replicate with the replicating bacterial cell and will be vertically transmitted. The lysogenic bacterium is immune to phage infection, however can be induced to go into the lytic phase, depending on the environmental conditions (Stewart and Levin 1984).

A temperate phage can act as an allelopath in the following way. In a competing population of lysogens and sensitive bacteria, a small fraction of the lysogens

could induce a lytic phase, release phage particles, and kill and recruit bacteria from the sensitive population giving an advantage to the remaining non-lysed lysogenic cells.

We have developed a model for the population dynamics of bacteria and temperate phage. By using relevant estimates of biological parameters, the dynamics of competing populations of sensitive bacteria, lysogens and free phage can be predicted. We designed experiments with the bacterium *Salmonella* Typhimurium (strain LT2) and its temperate phage *P22*. This is a well-studied bacterial phage system; in the last decade it has been primarily used for cloning and transformation purposes (Eriksson and Millar 2003; Grana et al. 1988; Levine et al. 1970; Schmieger 1971). We were able to compete populations of sensitive and lysogenic bacteria and free phage in both a well-mixed habitat in liquid culture and in a structured environment. The short-term as well as the long-term dynamics were studied by transferring stationary phase cultures to fresh medium with different initial densities of the subpopulations of lysogenic and sensitive bacteria.

We (1) demonstrate theoretically and experimentally that in both environments lysogens invade a population of sensitive bacteria, even when they are rare. They also prevent invasion when they are abundant. Both can occur even when they have a selective disadvantage relative to their sensitive competitors. (2) We consider and speculate with the aid of models on the conditions responsible for the evolution of lysogeny: *why be temperate*?

The model

The model used (see Figure 1) is based on the temperate phage model in mass action by Stewart and Levin (1984), which in turn was an extension of the

model by Levin, Lenski and Chao (Levin et al. 1977). We consider three populations of cells, each having their own initial density: (1) free temperate phage (T), (2) sensitive bacteria (S), and (3) lysogenic bacteria (L). Sensitive cells can be attacked by free temperate phage, which adsorb at a constant rate δ. After adsorption, the temperate phage can go into a lysogenic cycle with a constant probability λ, or go into the lytic cycle with probability (1-λ). For simplicity, we did not include a latent period. Lytic infections result in the production of new phage particles and eventually lysis of the bacterial cell; β is the number of new phage particles released and is referred to as the burst size. Lysogens are semi-stable; however, a lytic cycle can be induced with rate ξ. We assume there is no spontaneous loss of the phage from lysogens and that the burst size (β) is constant. The bacteria grow at a rate of ψ_S and ψ_L; the growth rate is dependent on the concentration of a limiting resource (R) and on the resource concentration where the growth rate is half its maximum value (*k*). The limiting resource is taken up by the bacterial cell and converted with efficiency *e*.

This leads to the following equations with respect to time (*t*):

$$dL/dt = [\psi_L * R/(k+R)] * L + \lambda * \delta * S - \xi * L \quad (1)$$

$$dS/dt = [\psi_S * R/(k+R)] * S - \lambda * \delta * S \quad (2)$$

$$dT/dt = (1-\lambda) * \delta * T * S * \beta + \xi * L * \beta - \delta * T * S \quad (3)$$

$$dR/dt = R_{at\ start} - e * [\psi_L * R/(k+R)] * L - e * [\psi_S * R/(k+R)] * S \quad (4)$$

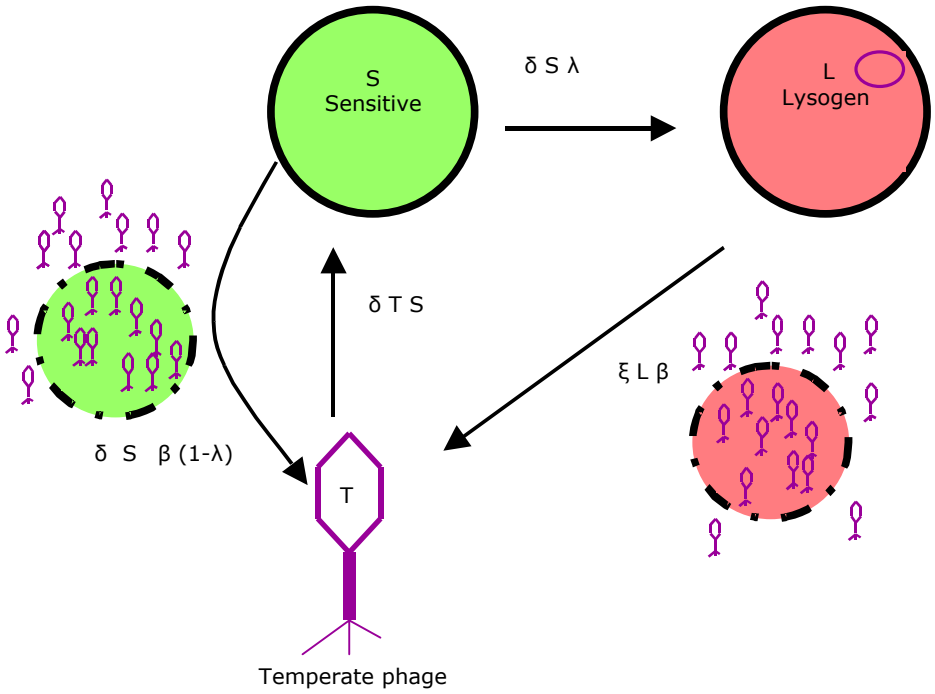

Figure 1. Temperate phage model, adapted from Stewart and Levin (1984), showing the interaction of free temperate phage and lysogenic and sensitive bacteria. See text for details and Table 2 for an explanation on the symbols and parameter values.

Materials and methods

Strains. We used two different strains of *Salmonella* Typhimurium LT2, each in a lysogenic and a non-lysogenic state, see Table 1. With selective media, we are able to detect the respective strains, even when they are rare. Additionally, TA plates reveal whether a strain is *ara*- or *ara*+ by colony color. We used temperate phage P22. By growing both strains in the presence of excess phage P22, we obtained lysogens of the two strains.

Media and culturing. For competition experiments in liquid and solid medium, M9 was used with glucose (M9 consist of Na_2HPO_4 6.0 g/l; KH_2PO_4 3.0 g/l; NaCl 0.5 g/l; NH_4Cl 1.0 g/l; 1M $MgSO_4$ 0.5 ml/l; 0.1 M CaCl 0.5 ml/l; 0.2%

thiamine 2.5 ml/l and glucose 500 μg/ml) We used liquid M9 to determine the relevant growth parameters. When estimating the number of the different bacteria in the cultures we used non-selective and selective media: LB (Difco); LB with nalidixic acid (0.1 %), Tetrazolium Agar (consisting of tryptone/peptone 10.0 g/l; yeast extract (1.0 g/l); sodium chloride 5.0 g/l; agar 16.0 g/l; arabinose 10.0 g/l and 5% triphenyltetrazolium chloride solution 1.0 ml/l), Minimal Arabinose (consisting of dibasic potassium phospate 7.0 g/l; monobasic potassium phosphate 2.0 g/l; ammonium sulfate 1.0 g/l; sodium citrate 0.5 g/l; agar 16.0 g/l; arabinose 4.0 g/l; thiamine 0.002 % and magnesium sulfate 1 %) and phage plates (consisting of tryptone 10.0 g/l; yeast extract 1.0 g/l; sodium chloride 8.0 g/l; agar 10.0 g/l; glucose 1.0 g/l and $CaCl_2$ 1M 2.0 ml/l.

To estimate the number of phage in a culture we used a lawn of sensitive bacteria, grown in liquid LB and plated on solid LB. In some assays, the phage culture was filtered (Ø 0.2 μm) prior to plating to eliminate lysogenic bacteria that could also form a plaque on a sensitive lawn.

Table 1. Strains used.

Code	Genotype			Growth rate (/hour)
A	ara-	nal R	non lysogen	0.79
B	ara-	nal R	lysogen	0.78
C	ara+	nal S	non lysogen	0.83
D	ara+	nal S	lysogen	0.85

ara: Arabinose; nal: Nalidixic acid; S: sensitive; R: resistant

Estimation of relevant growth parameters. *The growth rate* of the four strains was measured in an automated machine constantly measuring the optical density (OD) of the growing bacterial culture. The machine holds a micro titer plate with 96 wells. We grew up our cultures overnight in 10 ml of M9 starting from a

single colony. Then we diluted this overnight culture 1:100 in M9 and put 200 µl into a well. The growth rates of the strains used are in Table 1.

Burst size and latent period were determined in a one step growth experiment. 0.5 ml of an overnight culture of sensitive cells was transferred to 10 ml of M9. Phage suspension was added when the bacterial culture was at 1/3 of its maximum OD ($1 * 10^6$ phage/ml after adding to the culture). Samples of appropriate dilutions were taken of the culture every 5 minutes and plated to estimate the number of phage by plating on a sensitive lawn of bacteria. Prior plating cultures were filtered to eliminate lysogenic bacteria. From a sudden increase in phage numbers, the latent period and the burst size (β) were deducted.

Adsorption rate of the phage *P22* to *Salmonella typhimurium* LT2 was estimated by first transferring 0.5 ml transferring an overnight culture of sensitive bacteria to 10 ml of M9. The bacterial density is estimated when reaching 1/3 of the maximal density, then the bacteria are killed by placing the culture at 65 ^{O}C for 15 minutes and phage were added ($1 * 10^7$/ml). Samples of appropriate dilutions were taken every 2 minutes and plated for phage on a lawn of sensitive bacteria. The adsorption rate (δ) can be estimated from the following equation:

$$dP/dt = - \delta P N \qquad (5)$$

where P is the density of free phage and N the density of bacterial cells. Alternatively, δ can be calculated by dividing the slope of a plot of the natural logarithm of the phage density against time by –N.

Lysogeny rate and induction rate were taken from literature.

Competition experiments. In an effort to ascertain the conditions under which lysogens can invade a population of sensitive non-lysogens, the inverse and the longer-term outcome of the interactions, we performed competition experiment with lysogens and non-lysogens initiated at different initial relative frequencies. To control for background effects, we performed these experiments with reciprocal strains, so strain A versus D and strain B versus C, see Table 1. We performed these pair wise competition experiments starting with different ratios $1 * 10^2$, $1 * 10^4$ and $1 * 10^7$ in both liquid culture; 100 µl of the mixture in 10 ml M9 in 50 ml flasks and on surface cultures using the method presented by (Simonsen 1990).

After 24 hours, 0.1 ml of the liquid cultures were diluted and plated to estimate the densities of bacteria and free phage and 100 µl of these cultures were transferred 10 ml of fresh medium for a total of three transfers. The M9 agar with surface cultures was removed from the microscope slides, suspended in 10 ml saline and vigorously vortexed for a minimum of 30 seconds. The densities of bacteria and phage in these suspensions were estimated by diluting and plating and the culture maintained by spreading 100 µl on a fresh slide. The density of free-phage was estimated by passing the appropriated dilutions through 0.45 micro filters, mixing 100 µl with 100 µl of a lawn of sensitive bacteria in 2 ml of top agar which was poured onto LB plates. To distinguish lysogens from non-lysogens, using a template with stainless steel pins we replica plated the sampled colonies onto LB agar with and without a top agar lawn of sensitive bacteria. The lysogens in the master plate form colonies with a halo, non-lysogens form a halo-less colony on the lawn and free phage form colony-free halos.

Results

Parameter estimates. Estimations were made for the relevant parameters of the model. Some of them were determined experimentally (see Materials and Methods), while others were taken from literature, see Table 2.

Table 2. Overview of the parameters used in the model studies and experiments with their values. Some parameters were estimated experimentally (see Materials and Methods); others were taken from literature.

Parameter	Description	Value	Source
δ	Adsorption rate	$4.8 * 10^{-10}$ phage/bacterium	This study
λ	Lysogeny rate	$1 * 10^{-3}$ lysogens/adsorption	(Stewart and Levin 1984)
β	Burst size	12 phage/burst	This study
ξ	Induction rate	$1 * 10^{-3}$ inductions/lysogen	(Stewart and Levin 1984)
ψ_S	Growth rate sensitives	0.84 per hour	This study
ψ_L	Growth rate lysogens	0.79 per hour	This study
k	Carrying capacity	0.25	(Stewart and Levin 1984)
e	Conversion efficiency	$5 * 10^{-7}$	(Stewart and Levin 1984)
lp	Latent period (not in model)	30 to 40 minutes	This study

Model simulations. The model was programmed in the program BERKELEY MADONNA ™ (www.berkeleymadonna.com). With the model, we examined three outcomes of a competition between lysogenic and sensitive bacteria: one with populations initially at equal high numbers, one where lysogens are initially rare and one where the sensitives are initially rare. When programming, we assumed a well-mixed liquid culture with serial passage of 1% of the culture after 24 hours. The simulation results are in Figure 2.

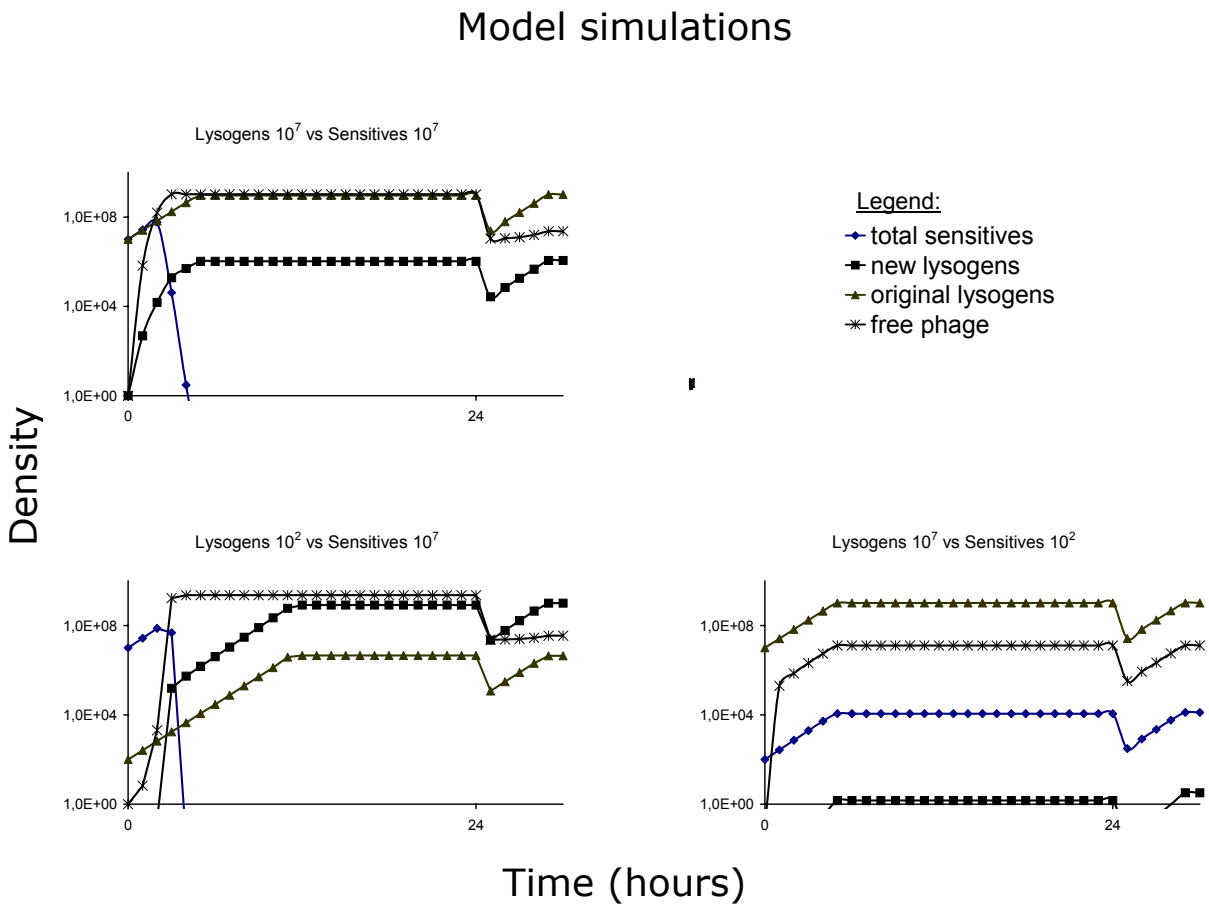

Figure 2. Model simulations of a competition between lysogenic and sensitive bacteria. The initial densities of the two competing populations are given above the graph. The ratios of the different populations did not change after a transfer; in the graph only the first 30 hours (including one transfer after 24 hours of 1% of the culture to fresh medium) are plotted.

The model simulations show that when the two populations start at equal high density, the sensitive population declines rapidly, partly due to conversion to lysogens. Even when the lysogenic population initially is rare, the sensitive population declines rapidly in favor of the growing lysogenic population included newly recruited lysogens from the sensitive population. Even in a simulation with an initial ratio of 1 lysogen to 1 * 10^7 sensitives, all sensitive

bacteria are eliminated within 6 hours (data not shown). For the temperate phage there are many sensitive cells to replicate on, so here the free temperate phage reach much higher densities than in the case where sensitives are rare. When lysogenic bacteria are initially present at a high density and the sensitive at low, the situation remains rather stable. The sensitive bacteria have a growth rate advantage to the lysogens but are not able to invade the lysogenic population. There are not enough sensitive cells to generate a high number of free temperate phage. If the growth rate advantage of the sensitive population is set to zero, the sensitive population is eliminated. In all cases, the dynamics during the first 5 hours are critical; after these 5 hours the densities remain stable, even after a serial transfer to fresh medium.

Experimental results. We experimentally studied the population dynamics of lysogenic and sensitive bacteria by growing populations of temperate phage and sensitive and lysogenic bacteria in competition, starting at several different ratios. We used the bacterium *Salmonella typhymurium* LT2 with its temperate phage *P22.* The lysogens and non-lysogens had different genetic markers. The reciprocal competition experiments (so strain A versus D and strain B versus C, see Table 1) had identical results, showing that the differences in growth rates found had no effect. The cultures were transferred to fresh medium (1%) every 24 hours and maintained for 72 hours. Different initial frequencies of lysogenic and sensitive populations were used; both were initially abundant and initially rare. In the liquid cultures samples were taken every 30 minutes, for surface slide cultures samples were taken every 24 hours during transfer.

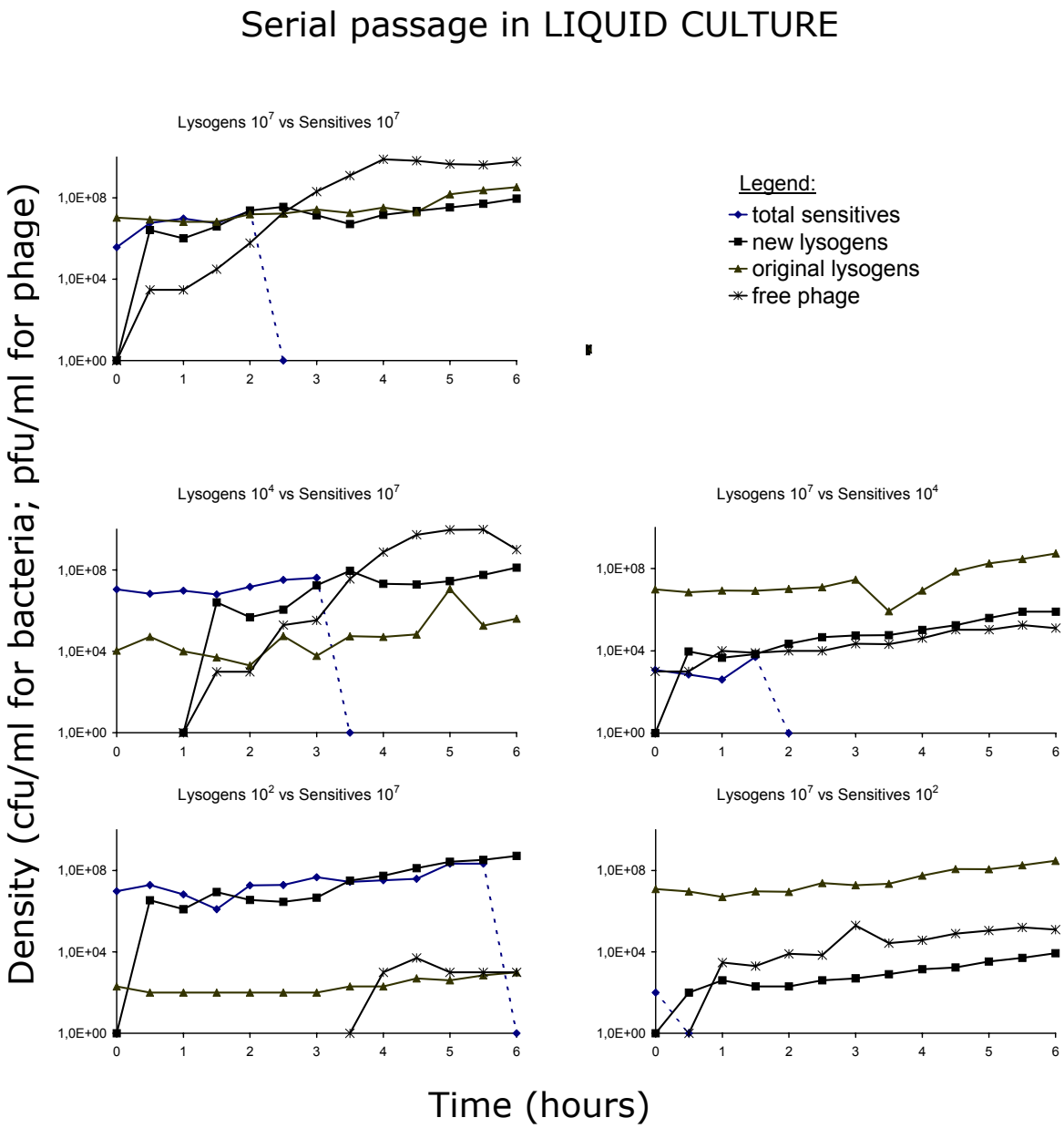

Figure 3. Results of the first 6 hours of a serial passage competition experiment between a lysogenic and a sensitive strain in liquid culture. After these 6 hours, the ratios of the populations remained stable during the remaining 66 hours of the experiment. The initial ratios of the lysogenic and sensitive population are given above the graph. Every 30 minutes samples were taken and plated for density measurements. A dotted line is shown when a population density was lower than the detection limit.

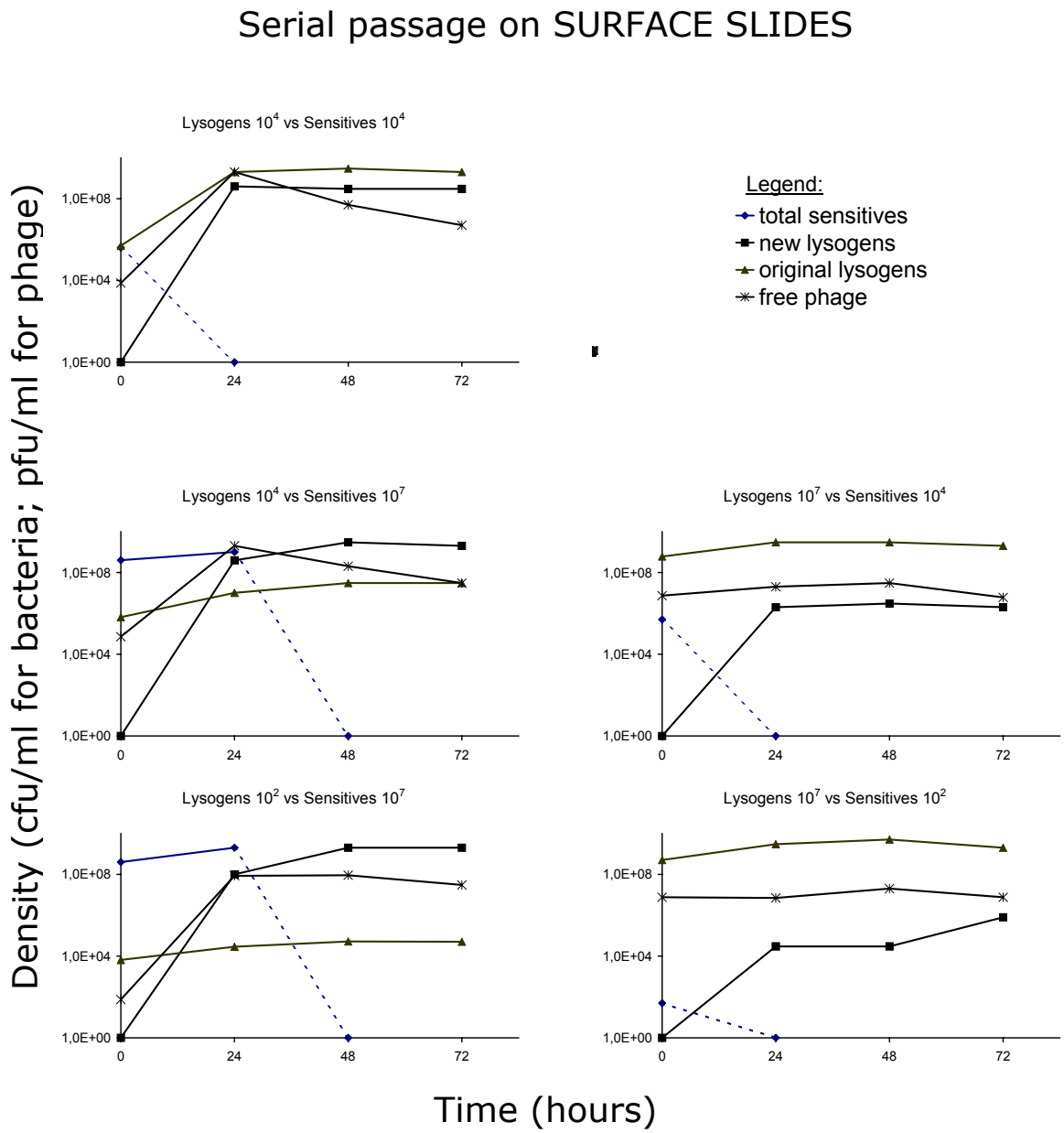

Figure 4. Results of a competition experiment between a lysogenic and a sensitive strain in a structured habitat on surface slides. The initial ratios of the lysogenic and sensitive population are given above the graph. During transfer of the cultures every 24 hours, samples were taken and plated for density measurements. A dotted line is shown when a population density was lower than the detection limit.

Liquid culture. First, we started an experiment in liquid culture over 72 hours, transferring every 24 hours. We soon discovered that, similar to the model, most if not all dynamics take place in the first 6 hours of the competition; the densities found at 24, 48 and 72 hours were identical to the densities found after 6 hours (data not shown). Figure 3 shows the dynamics of the first 6 hours when a sample was taken every 30 minutes. By (replica) plating, the number of sensitives, original lysogens, newly recruited lysogens and free phage were determined (see Materials and Methods). The detection of newly recruited lysogens is not 100% accurate in terms of raw numbers, however the trends as shown are. The best would be to have a phage with a detectable marker on it; this temperate phage is not available but would be worth constructing.

Results show replacement of all sensitives by (new) lysogens at all initial ratios tested. Recruitment of new lysogens from the sensitive population by the original lysogens occurs within 30 minutes and is the major cause of the decline of the sensitive population; however the original lysogens increase somewhat in frequency as well (see Figure 3). The density of free phage is dependent on the density of lysogens combined with the density of sensitives. A high density of lysogens means a high density of free phage by induction; a high density of sensitives means many cells to "feed" on.

Surface slides. The results of the competitions on surface slides are in Figure 4 and show the same dynamics as in liquid culture. In all competitions, the sensitive population declines in favor of the lysogenic population consisting of the original lysogens and the newly recruited lysogens. When the sensitive population starts at high density ($1 * 10^7$), it persists for one transfer.

Discussion

In this study, we explore the properties of temperate phage as allelopaths. A fraction of the lysogenic bacterial population can sacrifice itself by inducing a lytic cycle, liberating new phage particles that can in turn attack an invading or residential sensitive population. Lysogenic bacteria are immune to attacks by the phage they harbor. We developed a model and carried out competition experiments with lysogenic and sensitive bacteria and (free) temperate phage.

For the competition experiments, we used two environments: a well-mixed liquid culture and a structured medium on surface slides (Simonsen 1990) and different initial ratios of the respective populations. The competition had the same final result in both environments: the sensitive population was eliminated at any initial frequency tested. In the liquid cultures, the dynamics took place during the first 6 hours of the experiment after which the ratios remained stable even after transfer to fresh medium. In the structured habitat it took up to 48 hours for the ratios to stabilize.

The model we developed for well-mixed liquid culture (see Figure 1) is supported by the experimental liquid culture results. The sensitive population is eliminated by the lysogens in all cases studied, even when the lysogenic population has an intrinsic growth disadvantage and initially has a low density compared to the sensitive population. When the initial frequency of lysogens is low and that of sensitive bacteria high, the sensitive population is replaced within 6 hours by newly formed lysogens via recruitment. During this period, the density of free phage remained low. In the reciprocal experiment, the invading population of sensitive non-lysogens were either killed or recruited into the lysogen population within two hours.

The densities of free phage, as predicted by the model compared to the experimental results, are in qualitative agreement, however the model simulations predict too many free phage. The reasons for this remain to be explored. A possible reason is a not sufficiently accurate estimate for key parameters such as adsorption rate of free phage to sensitive cells, lysogeny rate, and induction rate. In the model, we assumed these parameters are constant; however, this may not be a valid assumption.

Similar studies have been carried out on the allopathic properties of bacteriocins, in particular colicins (Chao and Levin 1981; Durrett and Levin 1997; Fuhrman and Schwalbach 2003; Gordon and Riley 1999; Iwasa et al. 1998; Levin 1988; Nakamaru and Iwasa 2000; Wiener 1996). The results found are similar to the present study: the colicin-producing population can prevent invasion of sensitives. However, colicin-producing cells are able to invade a sensitive population only if their density is above a critical level, especially when growing in well-mixed liquid culture (Frank 1994). The critical frequency at which the colicinogenic population should be present to be able to invade a sensitive population is around $2 * 10^{-2}$ (Chao and Levin 1981). This was not observed in the present study with temperate phage; we show experimentally and in simulations that a lysogenic population can invade starting at low initial densities (frequency of $1 * 10^{-5}$). Our results are in qualitative agreement with Brown et al. (Brown et al. 2004).

Why be temperate? This study clearly shows that temperate phage can act as allelopathic agents, like bacteriocins and other toxins secreted by bacteria to target closely related species. How could the mechanism of temperate phage have evolved and why is it maintained? One hypothesis, set from the perspective of the bacterium and supported by the results of the present study, is that lysogeny evolved in response to selection for allelopathy, as an adaptation for bacteria to invade established communities of sensitive bacteria and to prevent

invasion of their habitat by sensitive bacteria (Stewart and Levin 1984). However, virulent phage, which can arise by mutation from temperate, can replicate on lysogens and sensitive bacteria. Does this mean that they will eliminate the lysogens and thus all temperate phage (Campbell 1961)? To address this question Stewart and Levin (1984) developed a model of the dynamics of phage and bacteria in continuous (chemostat) culture. Analysis of this model suggests that temperate phage could coexist or replace lytic phage if, (a) they are more fit (have higher adsorption rates δ and/or burst sizes β than lytic) or (b) they carry genes that augment the fitness of their lysogenic hosts.

Being temperate can be beneficial to the phage as a way to survive without harming the host when conditions are less favorable such as low densities of host bacteria to the phage (Abedon et al. 2001; Campbell 1961; Edlin et al. 1975). A lytic phage can invade a population of sensitive bacteria only when the density of the lytic population is high enough for the rate of phage replication to overcome the rate of phage loss. The loss of phage could be due to dilution of the medium and can be a problem specifically in environments with low host density. Lysogens, however, if they are adapted to a habitat, could invade and become established starting at any initial density. If the density of the sensitive bacterial population is too low for temperate as well as lytic phage, lysogens would not become established. However, with the appropriate oscillation in density, temperate phage could replace the virulent even when they are at a selective disadvantage (Stewart and Levin 1984).

For both hypotheses, lysogeny will evolve and be maintained if the temperate phage carry genes that augment the fitness of their host bacteria. Temperate phage have a role in bacterial recombination (Campbell 1961). There are phage-encoded characters, such as drug resistance and toxin production, which may be selected for under some conditions. The prophage may also enhance fitness in ways that are not known (Dykhuizen et al. 1978; Edlin et al. 1975).

Acknowledgements

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7 Summary and Discussion

This thesis aimed to experimentally unravel the initial phases of the evolution of fungicide resistance and compensatory changes that alleviate fitness costs of resistance mutations in the filamentous fungus *Aspergillus nidulans*. Fungicide resistance development not only forms a useful model for the study of adaptation, but also is of great practical importance (**Chapter 1)**.

In this thesis, the filamentous fungus *Aspergillus nidulans* was used as model organism. **Chapter 1** describes how its well-developed genetics can be exploited in laboratory studies (Clutterbuck 1974; Pontecorvo et al. 1953). The sexual and parasexual cycles are of use for genetic analysis; crosses can reveal the number of loci involved in a certain phenotypic trait and epistatic reactions. Parasexual analysis is a tool to assign loci to linkage groups (Clutterbuck 1974; Käfer 1958; Pontecorvo et al. 1953; Swart and Debets 2004).

In **Chapter 2**, the characteristics of *A. nidulans* as model organism in experimental studies of evolutionary processes are described. It has many of the experimental advantages of prokaryotic microorganisms (Elena and Lenski 2003; Lenski and Travisano 1994): a short generation time, easy culturing in the laboratory in large numbers and easy storage at –80 ^{o}C so that ancestral and descendant strains or populations can be compared simultaneously. The use of a filamentous fungus has additional advantages, such as multicellularity, a spatially structured mycelium and vegetative growth as a haploid and as a diploid. Key feature has proven to be the multi-cellular mycelium containing nuclei in spatially defined positions. The *mycelial growth rate* (MGR) is an easily to assess and reproducible fitness measure, the number of cell cycles for a nucleus in the growing front of the mycelium is up to 112 per week (Bergen and

Morris 1983; Kaminskyj and Hamer 1998). This thesis represents the first use of *A. nidulans* in experimental evolutionary studies and has shown that it is an organism well suited for that end.

Aspergillus nidulans strains were confronted with the fungicide fludioxonil (4-(2,3-difluoro-1,3-bezodioxol-4-yl)pyrrol-3-carbonitril). This fungicide is widely used in agriculture to control emerging fungal infections on grape vines in particular (Cabras et al. 1997; Verdisson et al. 2001) and is described in **Chapter 1**.

Chapter 2 shows that resistance to fludioxonil bears a fitness cost for *A. nidulans* strains when growing in the absence of the fungicide. On average for the 30 mutants analyzed, these costs were around 50 % compared with fungicide sensitive strains in terms of MGR; higher than in other known studies with bacteria where fitness costs are usually in the range of 5 to 20% (Cohan et al. 1994; Levin et al. 2000; Reynolds 2000; Schrag and Perrot 1996).

In several 'natural' environments such as hospitals and farms, the use of antibiotics has resulted in a persistent resistance in the organism targeted by the antibiotic, see **Chapter 1**. Since resistance comes with a cost in the absence of the antibiotic, fitness restoration by compensatory adaptation is essential for the resistance to persist in a natural population (Andersson and Levin 1999). **Chapter 1** describes several studies from literature with bacterial systems showing compensation occurs mainly by second site mutations (Maisnier-Patin and Andersson 2004; Reynolds 2000; Schrag et al. 1997).

Chapter 2 describes the study of compensatory evolution using a fludioxonil resistant mutant strain of *A. nidulans* that had resistance associated fitness costs. In an evolution experiment lasting for 27 weeks (which is equivalent to about 3000 cell cycles) 40 parallel strains evolved under three different environmental

conditions: (1) a constant fungicide-free environment; (2) initially high concentrations of fungicide in the environment that gradually decreased and reached zero; and (3) alternating total absence and presence of fungicide in the environment. Every week, part of the fungal colony most distant to the point of inoculation was selected and transferred to fresh medium, see front cover.

Results show that in all conditions used the mutant strain was able to restore fitness without losing its resistance to the fungicide. In 7 (out of the 40) cases, the evolved strain reached a higher fitness than the original sensitive ancestor. Fitness compensation occurred in one, two or three discrete steps. Genetic analysis of crosses between different evolved strains and between evolved and ancestral strains revealed interaction between compensatory mutations and provided information on the number of loci involved in fitness compensation. The results show that even in the conditions (2) and (3) where fungicide was present in the medium during stages of the evolution experiment, resistant strains were well able to restore the fitness costs that are only evident in the absence of fungicide. Their final fitness and rate of adaptation tended to be even higher these of strains that evolved under constant fungicide-free environments.

Chapter 4 describes an experimental study on the relative effectiveness of artificial selection versus natural selection on the rate of compensatory evolution in fungicide resistant strains, investigating whether artificial selection overestimated the rate of adaptation in more natural conditions. Using mycelial growth rate as a fitness measure like in the study described in Chapter 2, artificial selection involved the weekly transfer of the fastest growing sector of the mycelium onto a fresh plate. Natural selection was approximated by transferring random samples of all the spores produced by the mycelium. Fungicide resistant and fungicide sensitive haploid and diploid strains were used in an evolution experiment over 10 weekly transfers, which is equivalent to 1200 cell cycles. Here, two different environmental conditions were applied: (1)

a constant fungicide-free environment and (2) a weekly alternation between total absence and presence of fungicide in the environment.

Results show that for all strains and conditions used, the transfer of a random sample of all spores leads to more rapid adaptation than the transfer of the visually 'fittest' sector. This implies that artificial selection as applied in the study described in Chapter 2 does not overestimate the rate of adaptation in more natural conditions. The rates of compensatory evolution in the constant and the alternating environment did not differ, supporting the results described in Chapter 2. Moreover, haploid strains tend to have a higher rate of adaptation than isogenic diploid strains.

Chapter 3 reports a study where fungicide resistant haploid and diploid strains of *A. nidulans* were used to study the effect of ploidy on the rate of compensatory evolution. Our results implicated an important role for parasexual recombination. Many fungi have a 'parasexual cycle' in which haploid nuclei may fuse to form relatively stable diploid nuclei. Diploid nuclei can spontaneously produce haploid recombinants by repeated loss of chromosomes, and/or diploid recombinants by mitotic crossing-over. The parasexual cycle is widely used for genetical analysis not only in fungi but also in mammalian and human cell cultures (Käfer 1958; Roper 1966; Ruddle and Creagan 1975). However, since many fungal species have a fully functional regular sexual cycle as well, its evolutionary function remains enigmatic (Caten 1981; Elliott 1994; Roper 1966).

Results show that the parasexual cycle can enhance adaptation to novel environments. In an experimental study of adaptation over 3000 cell cycles with isogenic haploid and diploid strains of *A. nidulans,* we demonstrated that evolved populations that started as diploid but reverted to haploidy have a higher rate of adaptation than populations that began and remained haploid or diploid.

We propose that the parasexual cycle enables fungi to exploit an extended vegetative diploid phase to accumulate recessive mutations. Subsequent haploidization of these diploids can generate high-fitness haploid recombinants that phenotypically express the recessive adaptive mutations. The results of Chapter 3 are also relevant for the general question whether there are any particular evolutionary advantages to being diploid or haploid; this problem has generated many studies, both theoretical and experimental (Crow and Kimura 1965; Mable and Otto 2001; Orr and Otto 1994; Paquin and Adams 1983; Zeyl et al. 2003).

Chapter 5 describes a characterization of light inducible rhythms in *Aspergillus nidulans,* which was observed phenotypically in several populations obtained from the study described in Chapter 2. The velvet- allele (*ve*A1) is known to prevent the formation of banding patterns in the formation of conidiospores and ascospores (Timberlake 1990). In this study, a velvet- strain of *A. nidulans* that exhibits such a rhythm is further characterized. The rhythm in spore production is light entrainable, however needs to be induced by a cycle of light and darkness. The results of this study may be of benefit for future research on circadian and other rhythms that occur in biological systems (Greene et al. 2003).

Chapter 6 describes the possible allelopathic properties of temperate phage and is a project that was carried out in collaboration with professor Bruce Levin in Atlanta (USA). Phage (bacterial viruses) can either be virulent or temperate; in the latter case the phage incorporates itself into the bacterial genome forming a semi-stable association. A prophage as it is called then ensures resistance to the bacterial cell for infection of virulent phage; the bacterium is now lysogenic. However, the prophage can become virulent after some external or internal trigger and form many new phage particles that are released after lysis of the bacterial cell. These newly formed phage particles can attack sensitive bacteria.

Lysogeny can be regarded as an altruistic character; a fraction of a lysogenic population can lyse to release phage particles than can eliminate competing phage sensitive populations and free resources for the remaining lysogenic population.

Chapter 6 presents a model and experiments on the population dynamics of lysogeny. The results of competition experiments with sensitive and lysogenic bacteria (*Salmonella* thyphymurium LT2) and phage (P22) show that lysogens can successfully prevent invasion of sensitive bacteria and are able to invade and recruit from a population of sensitive bacteria. This is consistent with the model we developed. The ecological role of lysogeny is discussed as similar to the ecological role of bacteriocins: temperate phage can act as allelopathic agents.

Practical implications for fungicide resistance management

The results described in this thesis have implications for agricultural practice where many fungicides are used to control plant pathogens and where policies and strategies are being developed to manage resistance development (IFIC 2004). This thesis presents *in vitro* studies with the fungicide fludioxonil and model fungus *Aspergillus nidulans*, showing that fungicide resistance can emerge easily and at high frequency. The newly resistant mutants bear fitness costs in the absence of fungicide, i.e. in the absence of fungicide they have a lower growth rate than sensitive strains. In conditions where fungicide is not always present, like in fields where fungicides are applied at certain time intervals or in neighboring fields, compensation of fitness cost is needed for the resistant mutant to persist. Without compensation of fitness costs resistant strains would be eliminated by sensitive strains due to selection. Once resistant strains manage to compensate fitness costs associated with resistance, resistance may become persistent.

The results described in this thesis show that this compensation of resistance associated fitness costs is very likely to occur. Experimental studies of adaptation demonstrated that the fitness costs could effectively be compensated for by a limited number of second site adaptive mutations. In several evolved resistant strains, just one compensatory mutation was sufficient for total fitness compensation. Fitness compensation was shown to occur in several different environments: in the total absence of fungicide as well as in its alternating or decreasing presence. This has implications for future policies; applying fungicide in cycles or in different concentrations may not retard the process of fitness compensation.

For some strains a limited time (a limited number of cell cycles in the evolution experiment) was needed for total compensation, showing a sufficient supply of mutations with compensatory effect. This is also illustrated by the fact that compensation is not caused by the same mutation in the different compensated strains that were analyzed. When numbers of fungi are high as could be the case in a 'natural' situation on a field with an affected crop, the chances become even higher that in one of the newly resistant strains a compensatory mutation will arise and that resistance will persist.

This process of compensation was studied using artificial selection of adaptive mutations (Chapter 2), however the study described in Chapter 4 has shown that more natural selection acting on random samples of all spores produced by the fungal mycelium leads to even more rapid compensation of fitness cost. Dispersal of spores is an important mechanism for fungi to spread and could play a major role in fitness compensation in 'natural' environments.

In conclusion, this thesis has shown that attempts to manage the spread of resistance by retarding compensation of fitness costs associated to resistance are likely to fail. Therefore, a more effective way of fungicide resistance

management should focus on prevention of occurrence of resistance mutations. This occurrence of resistance mutations will in part depend on the specific fungicide applied and could be limited by reduced use. Alternative ways of pest control could be promoted, such as diversification of crops and field use and cultivation of seeds and crops less susceptible to fungal infections; this could help to reduce the size of fungal populations to which a fungicide may need to be directed. Organizations like the Fungicide Resistance Action Committee (see www.frac.info) recommend the use of mixtures of multiple fungicides in order to reduce the frequency at which resistance develops (Brendt and Hollomon 1998; Christ and May 2003). The effects of this strategy could be evaluated using a similar approach as the studies described in this thesis.

Future studies

Chapter 2 described the study of fungicide resistance development and subsequent compensatory evolution. By parasexual analysis we showed resistance to be associated with a single gene located on chromosome III. Crosses have revealed epistatic interactions among different compensatory mutations. The next step would be an attempt to find and characterize the genes associated with resistance and compensation. This could reveal what biochemical pathways are affected by fludioxonil resistance and its compensatory mutations. Up to now, the exact mechanism that makes fludioxonil a fungicide is not fully known.

Chapter 4 has shown that the highest rate of adaptation in an evolution experiment can be achieved using random samples of all spores produced by a mycelium for propagation. The experiment described in Chapter 4 consisted of 10 transfers and was a comparison of artificial selection using the fittest sector (see front cover) and natural selection with random spore samples. It would be

interesting to study the rate of adaptation in a continuation of that experiment. A possible outcome is that the situation after 10 transfers is final and no further adaptation occurs, which indicates all evolving strains had reached a fitness optimum (see Figure 2 in Chapter 1). Another possibility is that strains of different treatments (propagation with sectors or spores) still have a different potential for further adaptation and that the differences in rates of adaptation found after 10 transfers are not final. The strains may follow a different adaptive trajectory depending on the mode of transfer (c.f. Burch and Chao 1999 who studied the effect of bottleneck size on adaptive trajectories). An adaptive mutation with relatively large effect may (or may not) stall a strain on an adaptive peak that is relatively high but not the highest. Adaptation in smaller steps as could be the case when strains are propagated using sectors rather than spores may eventually direct to a higher fitness peak, assuming the topography of the adaptive landscape is identical for all strains and does not depend on the mode of transfer (Wright 1982). This is an interesting question regarding the fundamentals of the dynamics of adaptation.

Other future research could focus on the role of sexual recombination during adaptive evolution. In the experiments presented in Chapters 2, 3 and 4, asexual offspring was selected for propagation. However, *A. nidulans* has a fully functional sexual cycle, and allows the study of the effects of sex on compensatory evolution. By recombination, combinations of adaptive mutations may be formed and/or broken down (Crow and Kimura 1965; Fisher 1930; Muller 1932); the formation of favorable combinations of adaptive mutations could be one of the reasons for the persistence of sexual rather than asexual reproduction in many biological systems. Whether these favorable combinations are formed in adapting populations and their effects on the rate of adaptation can be assessed in an experiment with *A. nidulans.* This will provide experimental evidence for fundamental questions regarding the evolution of sex.

Chapter 6 has described competition experiments for the study of allelopathic properties of temperate phage that are in a resting state in lysogenic bacteria. In these experiments sensitive and lysogenic bacteria were competed and it was shown that new lysogens were recruited from the sensitive population. Constructing a temperate phage with a detectable marker would be essential for further studies.

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Het verminderen van fitnesskosten verbonden aan antibioticumresistentie

De laatste tientallen jaren hebben vele micro-organismen (bacteriën en schimmels) een resistentie ontwikkeld tegen antibiotica. Dit heeft tot gevolg dat antibiotica (een gedeelte van) hun genezende werking tegen microbiële ziekteverwekkers hebben verloren. Micro-organismen kunnen door één enkele mutatie in het DNA resistentie ontwikkelen tegen een antibioticum. In veel gevallen zijn er fitnesskosten verbonden aan resistentie: in de afwezigheid van antibioticum heeft een resistente stam een verminderde groei in vergelijking met niet-resistente stammen. Resistente stammen kunnen deze kosten echter compenseren met aanvullende mutaties in het DNA die de nadelige effecten van resistentie opheffen zonder de resistentie aan te tasten. Deze mutaties noemt men compenserende mutaties en het proces noemt men compenserende evolutie. Na compenserende evolutie is de resistentie blijvend geworden omdat resistente stammen zich kunnen handhaven naast sensitieve, ook zonder antibioticum in de omgeving. Dit vermindert de effectiviteit van antibiotica.

Dit proefschrift beschrijft onderzoek naar de ontwikkeling van antibioticumresistentie en daaropvolgende compenserende evolutie. Met behulp van de modelschimmel *Aspergillus nidulans* en het fungicide (antibioticum tegen schimmels) fludioxonil is dit proces experimenteel onderzocht onder verschillende omstandigheden. De resultaten laten zien dat resistentie zich gemakkelijk ontwikkelt en dat binnen korte tijd en onder alle onderzochte omstandigheden compenserende mutaties optreden. De belangrijkste conclusie is dan ook dat aangezien het proces van compenserende evolutie zo gemakkelijk optreedt na het gebruik van antibiotica, een blijvende resistentie moeilijk te voorkomen is.

Inleiding

Met de ontdekking van penicilline in 1928 door Alexander Flemming begon het tijdperk van de antibiotica. In de jaren '40 van de vorige eeuw werden antibiotica voor het eerst toegepast bij medische behandelingen. Sindsdien is gebleken dat antibiotica erg effectief zijn bij het bestrijden van microbiële infecties, niet alleen bij mensen maar ook bij landbouwgewassen. Dit heeft geleid tot een grootschalig gebruik.

De laatste (tientallen) jaren is duidelijk geworden dat het grootschalig gebruik van antibiotica resistenties met een blijvend karakter tot gevolg heeft. Een humane ziekteverwekker als *Staphylococcus aureus* is bijvoorbeeld ongevoelig geworden voor vele antibiotica en daarmee tegenwoordig lastig te behandelen (Kacica en McDonald, 2004).

In de landbouw worden de meeste microbiële infecties op gewassen veroorzaakt door schimmels. In de jaren '50 zijn de eerste fungiciden (antibiotica tegen schimmels) ontwikkeld. In eerste instantie werd vooral gebruik gemaakt van fungiciden met een breed werkingsspectrum zoals zwavel- en koper-verbindingen, resistentie kwam toen nog weinig voor. Vanaf de jaren '60 zijn er fungiciden ontwikkeld met een complexere chemische structuur die een specifieke werking hebben tegen één bepaalde schimmelsoort (phenylamiden, aminopyrimidinen; Russell 1999). In de eerste jaren na hun eerste toepassing zorgden de nieuwe fungiciden voor een grote vooruitgang in gewasbescherming. In de jaren '70 begonnen echter de eerste resistentie schimmels de kop op te steken. In de jaren '90 had 50% van de *Phytophthora infestans* stammen (veroorzakers van de aardappelziekte) een fungiciden-resistentie, met als gevolg dat schimmelinfecties lastiger te bestrijden werden. Hoewel *Phytophthora infestans* geen schimmel is maar een oömyceet, is wel eenzelfde problematiek van toepassing.

In Nederland worden fungiciden met name gebruikt bij de teelt van snijbloemen en aardappelen. De overheid werkt samen met organisaties als de Land- en Tuinbouw Organisatie (LTO) aan de beheersing van fungicidenresistentie met het beleid om het gebruik van fungiciden te verminderen. In 1990 is hiertoe het Meerjarenplan Gewasbescherming opgesteld met als doel een reductie in fungicidengebruik van 35%. De evaluatie van dit plan in 2001 liet zien dat het gebruik van fungiciden in Nederland in de periode 1990-2000 niet was gedaald, maar vrijwel gelijk was gebleven op 4.000.000 kilogram actieve stof per jaar (Ekkes en anderen 2001).

Dit proefschrift beschrijft experimenteel onderzoek naar de ontwikkeling van resistentie en de daarop volgende compenserende evolutie die fitnesskosten verbonden aan resistentie vermindert. Hierbij is gebruik gemaakt van de modelschimmel *Aspergillus nidulans* en het fungicide fludioxonil.

De gebruikte schimmel is een modelschimmel omdat de genetische architectuur geheel bekend is en er daardoor vele technieken beschikbaar zijn om vragen over genetische veranderingen te beantwoorden. Fludioxonil wordt onder andere gebruikt om schimmelinfecties op wijnranken te bestrijden.

Resistentieontwikkeling als evolutionair proces

Schimmels en andere micro-organismen zoals bacteriën kunnen met één enkele mutatie in het DNA (verandering van het erfelijk materiaal) een resistentie ontwikkelen en zich zo aanpassen aan een milieu met antibiotica. Mutaties in het DNA komen in alle organismen voor met een zeer lage frequentie van ongeveer één per miljoen celdelingen. Als een organisme al optimaal is aangepast aan zijn omgeving, hebben mutaties meestal een nadelig effect op de uitgroei en overleving van het individu dat de mutatie oploopt.

Suboptimale groeiomstandigheden zoals de aanwezigheid van een antibioticum, vergroten de kans dat een mutatie een gunstig effect heeft. Het feit dat micro-organismen vaak in populaties van grote aantallen voorkomen, vergroot de kans op het optreden van een dergelijke mutatie in tenminste één individu van die populatie. Dit resistente individu zal sneller groeien en meer nakomelingen produceren dan niet-resistente individuen en zo de overhand kunnen krijgen.

In de meeste, zo niet alle gevallen heeft een resistentie ook bijeffecten. Uit verschillende onderzoeken is gebleken dat een (micro-)organisme zich met een enkele mutatie niet aan alle omstandigheden tegelijk kan aanpassen. De aanpassing die groei mogelijk maakt in een milieu met antibiotica verlaagt tegelijkertijd diezelfde groei in een milieu zonder antibiotica. Men spreekt in dit verband van fitnesskosten verbonden aan antibioticumresistentie, waarbij fitness de groei en overleving in brede zin aanduidt. Vanwege deze fitnesskosten zou het goed mogelijk zijn dat resistentie vanzelf weer verdwijnt als het antibioticum niet meer wordt gebruikt; resistente stammen verliezen de concurrentie met niet-resistente stammen en worden op die manier geëlimineerd.

Micro-organismen blijken echter in staat te zijn de fitnesskosten verbonden aan antibioticumresistentie te compenseren door de ontwikkeling van aanvullende mutaties in het DNA, compenserende mutaties genaamd. Dit proces heet compenserende evolutie en kan tot gevolg hebben dat een resistente stam goed groeit (een hoge fitness heeft) in zowel de aan- als afwezigheid van het antibioticum in het milieu. Als dit eenmaal het geval is, is de kans groot dat een resistentie blijvend is geworden. Resistente stammen zullen niet worden weggeconcurreerd door niet-resistente stammen, ook niet tijdens de afwezigheid van het antibioticum in het milieu.

De ontwikkeling van resistentie met de daaropvolgende ontwikkeling van compenserende mutaties is een evolutionair proces aangezien de meest

succesvolle individuen binnen een populatie in frequentie toenemen door selectie.

Evolutie bestuderen in een experiment

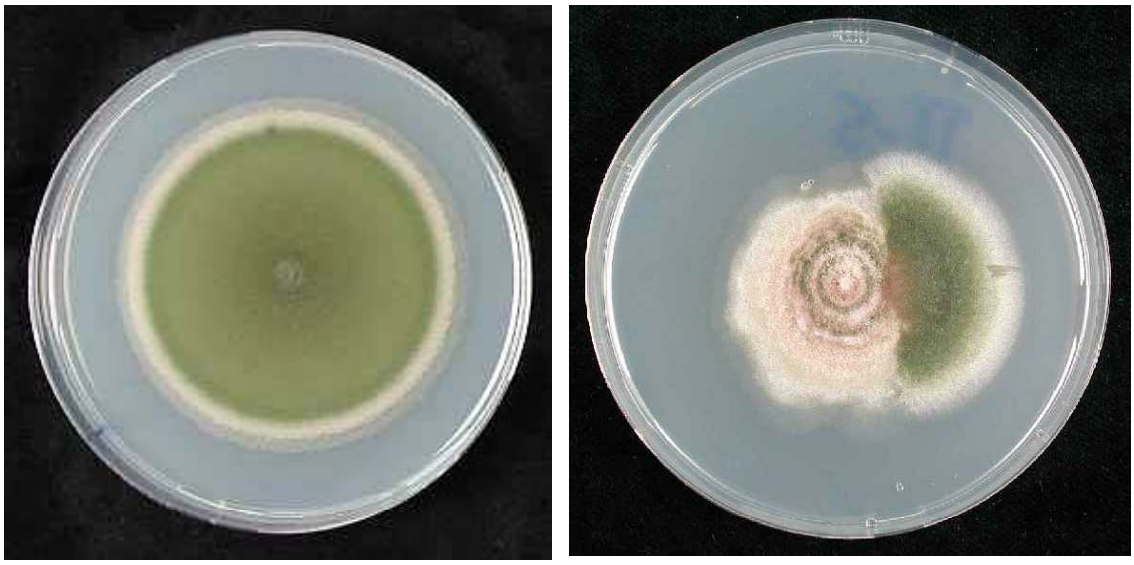
Met behulp van micro-organismen kunnen evolutionaire processen experimenteel worden bestudeerd. Omdat micro-organismen een zeer korte generatietijd hebben (meestal maar een paar uur of minder), kan na het optreden van een mutatie het effect op de volgende generatie snel worden vastgesteld. Ter vergelijking: een generatie bij mensen duurt in Nederland tegenwoordig ongeveer 30 jaar. Van micro-organismen kunnen gemakkelijk grote populaties van miljoenen individuen worden gekweekt. Ondanks de lage mutatiefrequentie is er dan toch een reële kans dat er inderdaad een mutatie optreedt in één of meer individuen gedurende enkele weken.

Experimentele uitwerking en resultaten

De schimmel *Aspergillus nidulans* groeit aan het oppervlak van een groeimedium en vormt een netwerk van schimmeldraden (mycelium). Onder normale omstandigheden groeit de schimmel netjes radiaal uit vanuit het punt van aanenten, zie Figuur 1. Onder suboptimale omstandigheden (zoals groeimedium met fungicide erin) is er een reële kans dat er tijdens het uitgroeien een mutatie optreedt met een groeiverhogend effect. Vanaf het punt waar de mutatie optreedt zal het mycelium sneller gaan groeien en een sector met verhoogde groei vormen, zie Figuur 1 en de omslag van dit proefschrift.

We hebben door de schimmel te laten groeien op een medium met fungicide verschillende resistente mutanten kunnen oppikken die goed groeien op het

medium met fungicide. Genetische analyse heeft aangetoond dat er één enkele mutatie aan deze resistentie ten grondslag ligt. De resistente stammen hebben fitnesskosten op medium zonder fungicide, hier groeien ze gemiddeld half zo snel uit als niet-resistente stammen. Dit betekent dat voor resistente stammen medium *zonder* fungicide erin een suboptimale groeiomstandigheid is geworden.

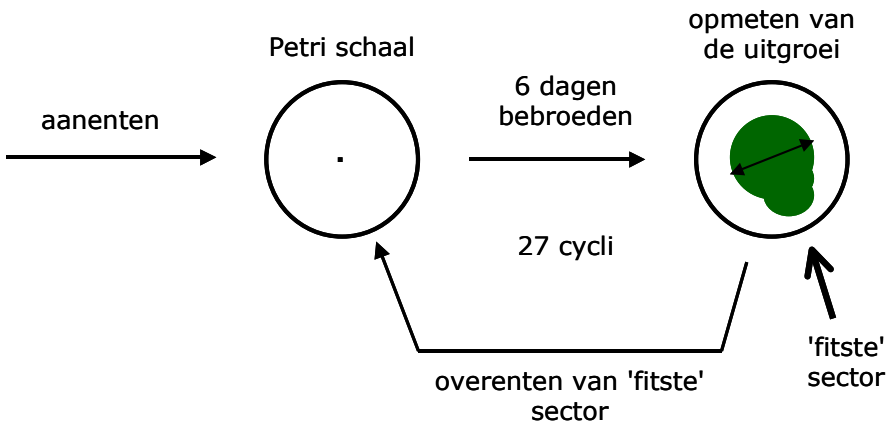


Normale groei Suboptimale groei

Figuur 1. Optimale (links) en suboptimale groei (rechts) na zes dagen van de schimmel *Aspergillus nidulans* op een oppervlak. Onder suboptimale omstandigheden kan er een mutatie optreden met een gunstig effect op de uitgroei. Na een dergelijke mutatie wordt een sector gevormd die sneller uitgroeit dan de rest van het mycelium.

We hebben één resistente stam geselecteerd voor een experiment om compenserende evolutie te bestuderen. Tijdens dit experiment werd deze stam geënt op vast medium. Na uitgroei van zes dagen werd het verst uitgegroeide mycelium geselecteerd. Hiervan werd een klein gedeelte overgebracht naar vers medium, zie Figuur 2. Deze cyclus werd meerdere malen herhaald. Door bij elke keer overenten de uitgroei op te meten, kon het proces van evolutie worden gevolgd in de tijd.

Van de resistente stam werden meerdere replica's gebruikt (in totaal 40) die onafhankelijk van elkaar dezelfde behandeling van overenten kregen. De groeiomstandigheden waren niet voor alle replica's gelijk; de replica's zijn verdeeld over drie verschillende groeiomstandigheden om de gevolgen van verschillende manieren van fungicidengebruik te onderzoeken. De verschillende omstandigheden waren: (1) medium zonder fungicide, (2) medium met eerst veel fungicide dat bij elke keer overzetten iets afnam om na 12 keer overzetten op nul te komen en (3) medium waar elke eerste drie keer geen fungicide in zat maar elke vierde keer wel.



Figuur 2. Opzet van het experiment van compenserende evolutie. De resistente stam werd aangeënt in het midden van een Petri schaal. Na zes dagen bebroeden (zie ook Figuur 1) werd het verst uitgegroeide stukje van het mycelium geselecteerd en overgebracht naar vers medium. Dit experiment werd uitgevoerd met 40 replica's, verdeeld over drie verschillende groeicondities.

De resultaten laten zien dat onder alle gekozen groeiomstandigheden de replica's gemiddeld genomen na 27 keer overenten sneller groeien dan aan het begin van het experiment. De snellere groei is veroorzaakt door compenserende mutaties die bij de verschillende replica's op verschillende momenten in de loop van de 27 cycli zijn ontstaan. Er zijn verschillen in groeisnelheid ontstaan tussen de replica's onderling tijdens de 27 keer overenten. Niet alle replica's hebben hun

uitgroei kunnen verbeteren, er zijn echter ook zeven replica's die hun groei zo veel verbeterd hebben dat ze nu op medium *zonder* fungicide zelfs beter groeien dan de niet-resistente stammen.

De resultaten laten zien dat er geen invloed is van de verschillende omstandigheden die gekozen waren. De wisselende aan- en afwezigheid van fungicide blijkt geen vertragend of versnellend effect te hebben op het proces van compenserende evolutie.

Genetische analyse heeft laten zien dat 1, 2 of 3 mutaties met duidelijk waarneembaar effect verantwoordelijk zijn voor het proces van compenserende evolutie.

Gevolgen voor de praktijk van fungicidengebruik en resistentiebeheersing

Dit onderzoek heeft laten zien dat er twee stappen zijn in het ontstaan van een blijvende fungicidenresistentie. De eerste stap is het optreden van de resistentie-mutatie. De tweede stap is het proces van compenserende evolutie dat ervoor zorgt dat ook in afwezigheid van fungicide, resistente stammen zich kunnen handhaven naast niet-resistente. Dit vermindert de effectiviteit van fungiciden als middel om schimmelinfecties te bestrijden.

Beheersing van fungicidenresistentie en de bijbehorende problematiek richt zich in de praktijk onder andere op het voorkomen of vertragen van tenminste één van beide stappen. Dit onderzoek laat echter zien dat deze aanpak weinig kansrijk zal zijn. Voor resistentie is slechts één enkele mutatie nodig. Aangezien schimmels vaak in grote aantallen voorkomen, is het waarschijnlijk dat ten minste in één individu een resistentiemutatie zal optreden. Voor een blijvende resistentie is compenserende evolutie nodig. Dit onderzoek heeft laten zien dat

voor dit proces slechts een beperkt aantal mutaties nodig is en dat er geen vertragend effect is van verschillende groeiomstandigheden die de toepassing van verschillende spuitregimes hebben nagebootst. Ook het ontstaan van deze mutaties is moeilijk te verhinderen, ook met de toepassing van verschillende spuitregimes.

Dit leidt ertoe dat het essentieel is om methoden van gewasbescherming te bevorderen die niet of minder gebruik maken van fungiciden. Gedacht kan worden aan het gebruik van gewassen die minder gevoelig zijn voor infecties door schimmels en aan diverse vormen van gewasdiversificatie zodat een schimmelinfectie minder snel om zich heen zal grijpen.

Ander onderzoek in het kader van dit project

Naast het onderzoek naar resistentieontwikkeling en compenserende evolutie zijn er gedurende dit onderzoeksproject ook andere aspecten van evolutie onderzocht. Hoofdstuk 3 beschrijft onderzoek naar de evolutionaire functie van de parasexuele cyclus, een bijzondere manier van voortplanten die alleen bij schimmels voorkomt. Hoofdstuk 4 beschrijft onderzoek met schimmels naar de invloed van kunstmatige en natuurlijke selectie op hun snelheid van adaptatie aan nieuwe omstandigheden. Hoofdstuk 5 beschrijft de observatie van een ritme in het produceren van twee soorten vruchtlichamen in *Aspergillus nidulans*. Hoofdstuk 6 beschrijft het project dat is uitgevoerd in het laboratorium van professor Bruce Levin in de Verenigde Staten met als thema de interactie tussen bacteriën en hun virussen.

Terugblik

Gaandeweg mijn studie levensmiddelentechnologie werd mij duidelijk dat mijn hart ligt bij het doen van toegepast biologisch onderzoek waar een fundamentele vraag aan ten grondslag ligt. De problematiek van antibioticum-resistentie is een duidelijk voorbeeld van een dergelijk onderzoek en sprak me daarnaast aan vanwege de duidelijke koppeling met een maatschappelijk probleem.

Inmiddels voel ik me naast levensmiddelentechnoloog volwaardig geneticus. Ik kijk met veel plezier en voldoening terug op mijn promotieonderzoek in de voor mij altijd prettige en inspirerende omgeving van het laboratorium voor Erfelijkheidsleer. Mijn dagelijkse begeleiders Rolf Hoekstra en Fons Debets waren altijd zeer geïnteresseerd en betrokken. Op vrijwel elk moment waren zij aanspreekbaar voor overleg, ook kwamen zij vaak in het lab kijken naar alle Petri schalen met schimmels. In de laatste fase waren zij erg behulpzaam bij het schrijfwerk. In 2003 verhuisde ik naar Zambia waar Saskia een baan had gevonden. Hoewel ik vanaf toen alleen af en toe in Wageningen was en verder vanuit Zambia emailde of belde, nam hun interesse en betrokkenheid niet af.

Marijke Slakhorst heeft een essentiële en waardevolle bijdrage geleverd aan het project door het accuraat en enthousiast uitvoeren van vele experimenten en bepalingen, zeker tijdens de Zambia periode. Nadat Arjan de Visser bij onze leerstoel kwam werken heb ik ook met hem veel over het onderzoek gepraat en nagedacht. De discussies met Judith Bruggeman over de nukken van onze schimmel heb ik erg gewaardeerd. Anita Mulder verwerkte mijn grote hoeveelheden gebruikt glaswerk in de keuken zonder ooit te morren. Saskia, Hanneke en Eddy Ivens en Emma Kazeze hielpen in de slotfase met het bijschaven van verschillende teksten. Het niet te onderschatten administratieve

gedeelte van alles rond het project werd altijd zeer adequaat afgehandeld door Aafke van der Kooi samen met Corrie Eekelder.

Gedurende een groot deel van de tijd van mijn project had ik ook een deeltijdaanstelling van 20% als docent. Het geven van onderwijs heeft mijn promotieonderzoek duidelijk een extra dimensie gegeven. De combinatie van onderzoek doen en lesgeven vond ik erg leuk en inspirerend, mede dankzij de samenwerking met Klaas Swart, Hans de Jong, Edu Holub, Rolf en Fons en daarnaast zeker ook dankzij het contact met alle studenten.

De goede en constructieve sfeer binnen de groep en de vele sociale activiteiten zorgden er ook voor dat het zeer prettig werken was. De mensen die hieraan bijdroegen waren naast de al genoemde collega's mijn kamergenoten Marc Maas, Ronny Schoonenberg, Bertha Koopmanschap en Merijn Salverda en verder Pieter Wijngaarden, Henk Dalstra, Peter van Baarlen, Elaine de Souza, Karoly Pal, Michelle Habets, Danny Rozen en Stefan Bosmans. Een ander sociaal hoogtepunt was de activiteitencommissie waar ik samen met Aafke en Corrie Hanhart een jaar in zat en met wie ik nu nog steeds geregeld uit eten ga.

In 2002 werkte in gedurende een half jaar in Atlanta (Verenigde Staten) in het laboratorium van Bruce Levin. Het onderzoek daar deed ik samen met Andrea McCollum en Kim Garner, verder waren er Renata Zappala en collega en fijne huisgenoot Camilla Wiuff. Ik heb een geweldige en zeer waardevolle tijd gehad in Atlanta, zowel door het verloop van het onderzoek als door de warme ontvangst die ik heb gehad.

Deze terugblik laat duidelijk zien dat er vele mensen hebben bijgedragen aan het prettige en succesvolle verloop van mijn promotieonderzoek. Ik wil iedereen dan ook zeer bedanken **!!!**

Levensloop

Ik ben geboren op 17 juni 1974 in Den Haag. De lagere schooltijd bezocht ik eerst in Singapore en later in Oman (Midden Oosten). Daarna zat ik op het Sorghvliet Gymnasium in Den Haag waar ik in 1992 mijn eindexamen haalde. Aansluitend volgde ik de studie levensmiddelentechnologie aan de Landbouw-universiteit Wageningen. Mijn afstudeerprojecten deed ik bij de leerstoel Levensmiddelenmicrobiologie waar ik darmbacteriën onderzocht, bij de wijnfaculteit van de Universiteit van Bordeaux waar ik één van de fermentatiestappen in de wijnbereiding onderzocht en bij de leerstoel Biochemie in Wageningen waar ik de driedimensionale structuur van een enzymcomplex uit de glycolyse nader in kaart bracht. Ook liep ik stage bij de Heinz ketchup fabriek. Na mijn afstuderen in 1997 werkte ik anderhalf jaar als toegevoegd docent bij de leerstoel Levensmiddelenmicrobiologie, eerst aan een Franstalig algemeen collegedictaat over levensmiddelentechnologie voor de Universiteit van Burkina Faso, later ook aan nieuwe collegedictaten en ander lesmateriaal voor Wageningse studenten.

In 1999 begon in aan mijn promotieonderzoek bij de leerstoel Erfelijkheidsleer. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. In de eerste fase van mijn onderzoek deed ik een aantal doctoraalvakken over algemene en schimmelgenetica. Hiernaast volgde ik tijdens mijn gehele promotieonderzoek verschillende aio-curssusen om mijn kennis op specifieke gebieden van de (evolutie) biologie en bepaalde vaardigheden te vergroten. Ook haalde ik een onderwijsbevoegdheid. In 2002 deed ik gedurende een half jaar onderzoek in het laboratorium van en in samenwerking met Bruce Levin bij de universiteit Emory in Atlanta (Verenigde Staten). Hier werkte ik niet aan schimmels en resistentie maar aan bacteriën en bacteriofagen.

Tijdens mijn promotieonderzoek werd ik verschillende keren uitgenodigd om mijn werk mondeling te presenteren bij congressen. Ik gaf onder andere een presentatie bij de European Society for Evolutionary Biology (ESEB) in 2001 in Denemarken en in 2003 in Engeland; in 2002 sprak ik bij de Nederlandse Mycologische Vereniging. Daarnaast gaf ik op uitnodiging lezingen aan uiteenlopend publiek waarin ik het principe van genetische manipulatie en modificatie uitlegde.

Gedurende een groot deel van mijn onderzoek had ik een deeltijdaanstelling van 20% als docent bij Erfelijkheidsleer en gaf ik onder andere de vakken basiscursus genetica, microbiële genetica, moleculaire en evolutionaire ecologie en cursussen aan internationale studenten bij het Internationaal Agrarisch Centrum (IAC) in Wageningen. Ook gaf ik les in Atlanta.

Curriculum Vitae

Name	**Schoustra**, Sijmen Ecco
Birth details	Born on 17 June 1974 in The Hague, the Netherlands
Nationality	Dutch

Education

1999 - 2003	**Courses** in the field of evolutionary biology, genetics and in research skills at several universities.
1998 - 2002	Courses resulting in the Wageningen University **Certificate for Lecturing.**
1992 - 1997	**Food Science,** Wageningen University. Main subjects: microbiology and (bio)chemistry. Master of Science (MSc/ir) degree in September 1997.
1979 - 1992	Primary and secondary school.

Professional experience

1999 - 2004	**PhD project,** Laboratory of Genetics, Wageningen University (the Netherlands) with Professor dr. Rolf Hoekstra and Associate Professor dr. Fons Debets. Resistance development and compensatory evolution. Dissertation and four oral presentations at international conferences.
2000 - 2003	**Lecturer,** Laboratory of Genetics, Wageningen University. Different courses in Genetics to Dutch and international Bachelor and Master students and students of the International Agricultural Centre (IAC).
2002	Research project, *Ec*LF Laboratory, **Emory University** (Atlanta, USA; 5 months) with Professor dr. Bruce R. Levin. Allelopathic properties of temperate bacteriophage.
1998 - 1999	**Lecturer,** Laboratory of Food Microbiology, Wageningen University. Restructuring of Bachelor and Master courses of the laboratory. Editor and author of new course books.
1997	Master's thesis, Laboratory of **Biochemistry,** Wageningen University (5 months). The three dimensional structure of the pyruvate dehydrogenase complex.

1997	Practical period, **Heinz ketchup factory** (Elst, the Netherlands; 3 months). Research on yeast and bacteria in the microbial laboratory.
1996	Master's thesis, Faculté d'OEnologie, **Université de Bordeaux** (France; 5 months). Role of bacteriophages in the malolactic fermentation in wine making.
1996	Master's thesis, Laboratory of **Food Microbiology**, Wageningen University (5 months). Effect of pre- and probiotics on intestinal flora.

Publications

2004	**Schoustra, S.E.** Reducing fitness costs associated with antibiotic resistance – Experimental evolution in the filamentous fungus *Aspergillus nidulans.* Thesis Wageningen University, ISBN 90-8504-106-6.
2004	Chapters 2, 3, 4, 5 and 6 of the thesis have been or will be submitted for publication in Scientific Journals.
1999	Hengeveld A.F., **S.E. Schoustra**, A.H. Westphal and A. de Kok. Pyruvate dehydrogenase from *Azotobacter vinelandii,* properties of the N-terminally truncated enzyme. European Journal of Biochemistry 256:1-11.
1999	Hartemink R., **S.E. Schoustra** and F.M. Rombouts. Degradation of guar gum by intestinal bacteria. Bioscience and Microflora 18:17-25.
1999	**Schoustra, S.E.** De schimmel als fabriek. Voedingsmiddelen-technologie 32:16-18.
1998	**Schoustra, S.E.** (Editor), Food Fermentation, pp. 186, Department of Food Technology, Wageningen University.
1998	**Schoustra, S.E**. (Editor), Inleiding in de Levensmiddelen-microbiologie en –hygiëne, pp. 208, Department of Food Technology, Wageningen University.

Language

Dutch (mother tongue), English (excellent), French (good), Frisian (good), German (good).

Hobby's

Playing the violin, singing classical music, swimming, playing bridge.

PE&RC PhD Educational Statement Form

With the educational activities listed below, the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology (PE&RC), which comprises of a minimum total of 32 ECTS (= 22 weeks of activities).

Review of literature

- Haploids and Diploids in Evolution (2003)

Post-Graduate Courses

- Population Genetics (Dalfsen, 2000)
- Life History Theory (Schiermonnikoog, 2001)

Deficiency, Refresh, Brush-up and General courses

- Basic Genetics (Wageningen, 2000)
- Microbial Genetics (Wageningen, 2000)
- Scientific Writing (Amsterdam, 2001)
- Writing a Grant Proposal in Evolutionary Biology (Switzerland, 2002)
- Basic Didactics (Wageningen, 2002)

PhD discussion groups

- Genetic Resources and Diversity (2000 - 2003)
- Plant-Insect Interaction (1999 - 2001)
- Journal Club of the Program in Population Biology, Ecology and Evolution (2002)

PE&RC annual meetings, seminars and Introduction days

- Annual meeting Research School of Functional Ecology (Texel, 2002)
- Annual meetings Research Schools PE&RC and Experimental Plant Sciences (Wageningen, 1999, 2001)

International symposia, workshops and conferences

- Biannual meeting of the European Society of Evolutionary Biology (Denmark, 2001)
- European Meeting of PhD Students in Evolutionary Biology (Germany, 2001)
- Annual meeting of the Netherlands Mycological Society (Utrecht, 2002)
- Gordon Conference on Microbial population genetics (USA, 2003)
- Biannual meeting of the European Society of Evolutionary Biology (UK, 2003)

Laboratory training and working visits

- Laboratory of Professor B. R. Levin (Emory University, USA, 2002 - 2003)
  Subject: the ecology of bacteria and phage

The work presented in this thesis was carried out at the Laboratory of Genetics, Wageningen University, the Netherlands and partly at the *Ec*LF Laboratory, Emory University, USA with financial support of the Netherlands Organization for Scientific Research (NWO-ALW), grant number 809.64.021.

Lusaka, Zambia, September 2004.