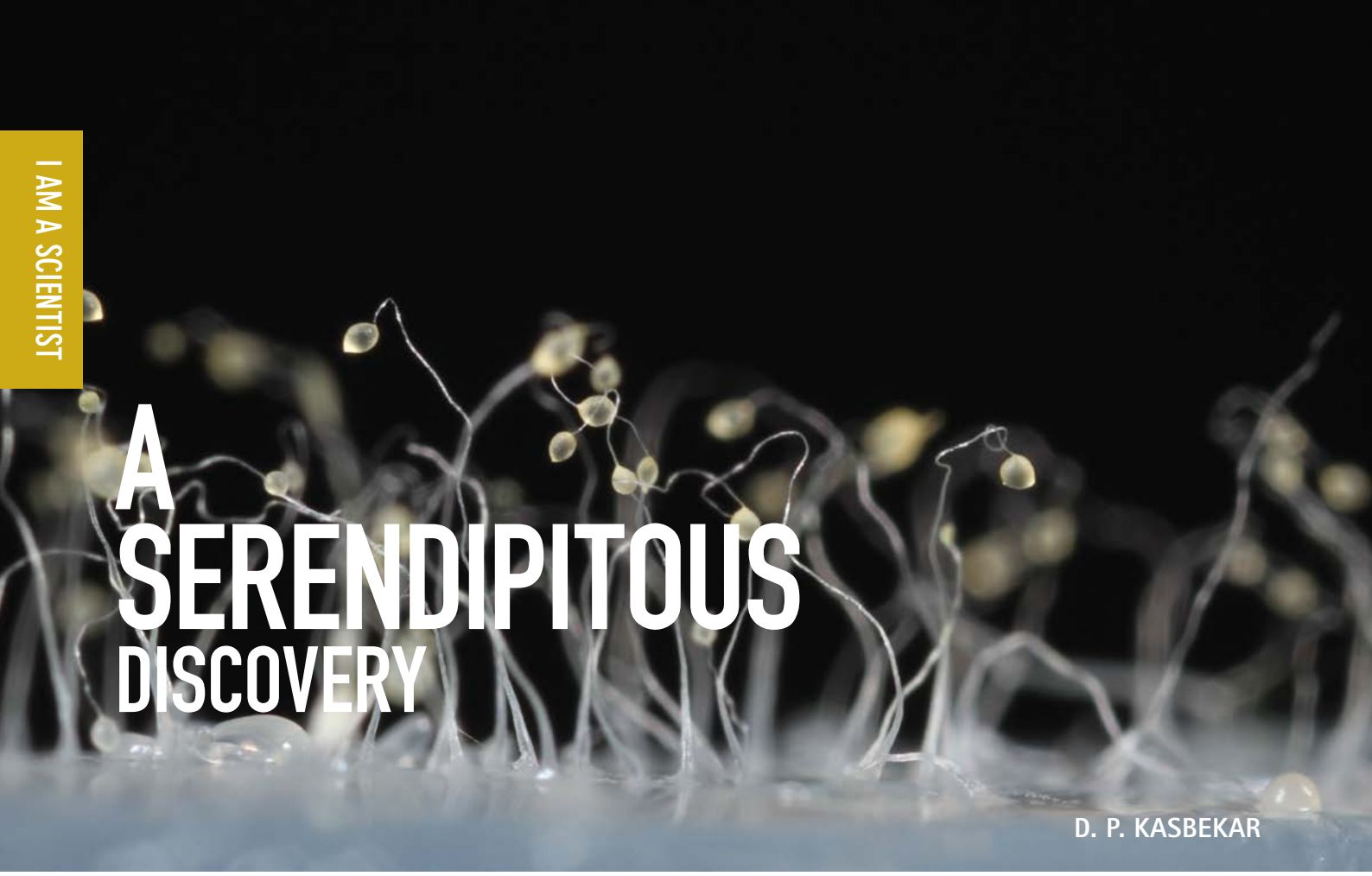

A SERENDIPITOUS DISCOVERY

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How often do we associate luck or chance with scientific discoveries? A scientist shares how his reluctance to throw some extra cell culture plates into the trash helped him see a failed experiment in a new light, and led to an interesting discovery.

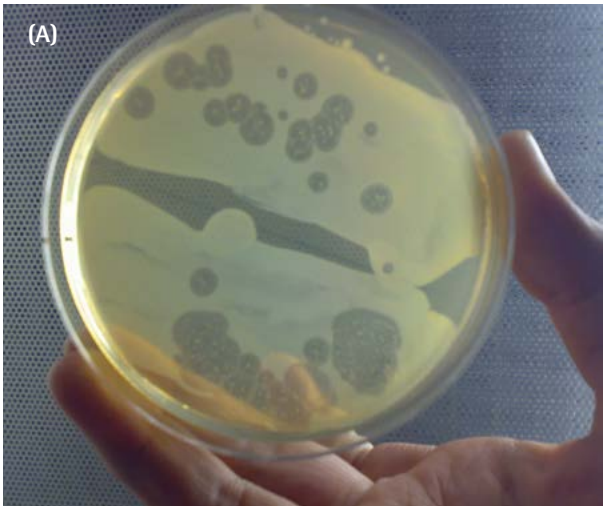
Sterols are ringed lipids that play an important role in maintaining the integrity of cell membranes in eukaryotes like us. For example, studies show that errors in the biosynthesis of cholesterol, one of the most widely-known examples of sterols, cause serious defects in human embryonic development that almost invariably result in foetal death. This raises the question—do sterols play a role in cellular differentiation and morphogenesis in eukaryotes?

One way to answer this question is by studying the effects of mutations that cause errors in the sterol biosynthesis pathway of a fast-growing, easy to maintain, eukaryotic multicellular (model) organism. This was the aim of my doctoral research (1978-1984). I sought to obtain mutants of the free-living soil amoeba *Dictyostelium discoideum*, in which normal membrane sterol was replaced by a precursor sterol (see **Box 1**). These mutants would allow us to evaluate the importance of normal membrane sterols in the development of the *Dictyostelium* fruiting body (see **Fig. 1**).

How do we obtain such mutants? At the time, the easiest way to do this was to select for amoebae resistant to the antifungal nystatin. Nystatin is related to the antifungal amphotericin B, which is used to treat the COVID-19-associated mucormycosis (black fungus) infection. Both antifungals bind to the normal membrane sterols, forming sterol-antifungal complexes. These complexes puncture holes in the cell membrane, destroying its integrity and causing cell death. A mutation that replaces normal membrane sterol with a precursor sterol alters this outcome. Since nystatin does not bind with the precursor sterol very well, these mutants are more nystatin-resistant.

Nystatin resistance can be the result of recessive or dominant mutations. While members in the lab I worked in had already isolated amoeba with some recessive mutations, my aim was to obtain ones with dominant mutations. Why? We knew, from previous work, that recessive mutations often completely inactivate genes, and the complete inactivation of some sterol biosynthetic

Fig. 1. Growing amoeba in the laboratory.



(A) What are lawns and plaques? Much of the agar medium surface of the Petri dish is covered by a turbid bacterial lawn. The circular clear zones in the lawn are plaques formed by *Dictyostelium* amoeba feeding on the bacteria. The plaques enlarge and merge with each other. Within a plaque, starvation induces the amoebae to aggregate into multicellular clumps, which then transform into fruiting bodies. The aggregates and fruiting bodies are just about visible to the naked eye.

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(B) Visible towards the left of this award-winning image is the edge of an amoebal plaque on a bacterial lawn, as seen at 100X resolution. The bacteria (visible as a concave blurry layer on the far left of the image) have been cleared by aggregating amoebae. Further right, these aggregates can be seen transforming into fruiting bodies, each with a slender stalk that is a few millimetres tall and holds aloft a ball of vegetative spores.

Credits: Dr. Dirk Dormann, MRC LMS, Imperial College, London and Nikon Small World. URL: https://www.nikonsmallworld.com/galleries/2009-photomicrography-competition/life-cycle-of-the-social-amoebae-dictyostelium-discoideum. License: Protected by Copyright. Used with permission of the rights owners.

genes might produce only inviable amoebae. In contrast, dominant mutations may either retain partial gene activity or induce novel patterns of gene activity. Thus, for some sterol biosynthetic genes, dominant mutations might be the only way to obtain viable amoebae. There was also a chance that such mutants (with dominant mutations) would help identify new genes affecting sterol biosynthesis.

Since both dominant and recessive mutations affect the same pathway, how would one differentiate between the two? This difference becomes evident in diploid amoeba (see **Box 2**). If a nystatin-resistant (mutant) haploid cell and a nystatin-sensitive haploid cell fuse, and the resulting diploid cell is nystatin sensitive (in genetics-speak, it shows the 'nystatin-sensitive phenotype'), then we can infer that the mutation is recessive. In contrast, if the diploid cell shows nystatin resistance, then we can conclude that the mutation is dominant.

Box 1. ***Dictyostelium discoideum:***

In the wild, *Dictyostelium* amoebae feed and multiply on bacteria that grow on decaying vegetative matter. When the amoebae run out of bacteria to feed on, they gather in hundreds of thousands to form visible multicellular aggregates. The aggregates transform into fruiting bodies, each a few millimetres tall. Each fruiting body is composed of a slender stalk bearing a droplet of spores. Small fauna, such as ants and earthworms, disperse the spores to new food sources where they germinate to release amoebae. These newly released amoebae go on to repeat the growth-division-dispersal cycle.

That these fast-growing, haploid, eukaryotic, unicellular amoebae can be induced to form multicellular aggregates makes them very useful as model organisms to study cell-cell interactions in development. Also, they are easy to grow in the laboratory—they can be plated on a bacterial lawn (which, in turn, is grown on an agar-based medium in a Petri dish). Therefore, *Dictyostelium discoideum* is used by several developmental biologists to study cell differentiation and morphogenesis during fruiting body development.

Box 2. Diploidy in ***Dictyostelium:***

Cells with two sets of the genome are called diploid, and those with only one set are called haploid. For example, most human cells are diploid, but our sperm and eggs are haploid.

While *Dictyostelium* amoebae are commonly haploid, they can occasionally fuse to form diploid amoeba. Fusion occurs among starving amoebae and it might reflect a form of proto-cannibalism (cannibalism was observed in the related species *Dictyostelium caveatum*). Once formed, these diploid amoebae are stable through several cell divisions, but, eventually, lose the extra set of chromosomes and revert to haploidy.

Earlier scientists had obtained their mutants by exposing haploid amoebae to nystatin, and isolating those that showed resistance to it. I set out to obtain mine from diploid amoebae. Why? For a diploid cell to show nystatin resistance due to a recessive mutation, two independent mutations would have to occur—each inactivating one copy of the same gene. In contrast, only a single dominant mutation would be sufficient to confer nystatin resistance. This meant that the probability of getting resistance in diploid cells due to a single dominant mutation was much higher than that of getting resistance due to two recessive mutations (see **Fig. 2**).

I started my experiment by preparing Petri dishes with agar medium to which nystatin was added. Since *Dictyostelium* feeds on bacteria, I transferred cells of the bacterium *Klebsiella aerogenes* to these plates and allowed them to grow into a lawn that covered the surface of the agar. Most bacteria do not contain sterols, and hence they are unaffected by the nystatin. I also transferred about 100,000-500,000 amoebae to these plates. The vast majority of these amoebae were killed by the antifungal. A very small number of amoebae (typically, 1 in 100,000) survived the antifungal, fed on the bacterial lawn, and formed a plaque or colony (this is a clear circular zone from which all the bacteria are cleared). For these to survive, they would have to carry a mutation that prevented the biosynthesis of the normal membrane sterol. In this way, I was able to readily obtain several nystatin-resistant diploid amoebae.

In the next stage of my experiment, I induced each of these colonies to revert to haploidy by growing them on normal plates without nystatin (see **Box 2**). Next, I plated the haploid derivatives on a bacterial lawn—those that retained their nystatin resistance in the haploid state would be likely to carry a dominant mutation; while those that showed nystatin sensitivity were likely to carry the unmutated gene.

Unexpectedly, none of the haploid derivatives survived on the nystatin plates. To check for errors, I repeated these experiments several times. Each time, the nystatin-resistant diploids produced only nystatin-sensitive haploid derivatives. I began to worry that my colleagues might think I was an incompetent researcher who was squandering lab resources.

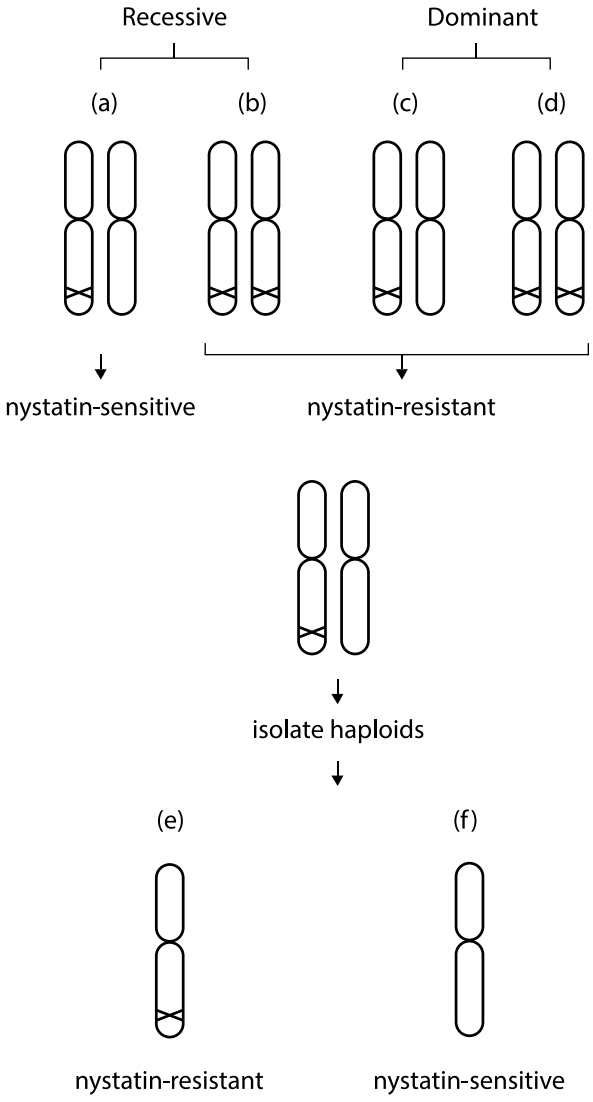

Fig. 2. The difference between dominant and recessive mutations. The elongated figures of "8" represent chromosomes. Each chromosome schematically represents one set of the genome. "X" marks a mutation. "a", "b", "c", and "d" are diploid cells (i.e., they contain two sets of the genome), whereas "e" and "f" are haploid cells (i.e., they contain one set of the genome). **Upper panel:** The recessive mutation is present in only one genome set in "a", making "a" nystatin-sensitive. It is present in both sets in "b", making "b" nystatin-resistant. In contrast, a dominant mutation confers nystatin resistance regardless of whether it is in one set as in "c", or in both as in "d". Selection for nystatin resistance in a diploid is more likely to select "c" type cells with a single dominant mutation, rather than "b" or "d", in which two independent mutations inactivate both gene copies. **Middle and lower panels:** When a "c" type nystatin-resistant diploid reverts to haploidy, haploid cells with the mutation ("e") are nystatin-resistant and those without the mutation ("f") are nystatin-sensitive. That we found no "e" type haploids following haploidization on nystatin-free medium cast doubt on whether the nystatin-resistant diploids were in fact type "c". Later, I showed that the nystatin-resistant diploids were not "c" type cells produced by mutation, but instead were induced by a novel nystatin-dependent nystatin-resistance (NDNR).

The answer to these puzzling findings came much later when I was setting up a different experiment. The aim of this experiment was to isolate nystatin-resistant mutants with recessive mutations, and then screen the mutants for their relative sensitivity towards azasterol, another antimicrobial. I had previously found that one type of nystatin-resistant mutant was sensitive to azasterol; another, like the wild type, was not. I used a preliminary control experiment to verify that the wild-type haploid amoebae could grow on agar plates supplemented with azasterol. In the second step, I transferred these amoebae onto nystatin-agar plates to isolate nystatin-resistant mutants with recessive mutations. As it turned out, I happened to run out of wild-type amoebae. This meant that I had a few extra nystatin-agar plates in hand. Meanwhile, the wild-type amoebae had grown well on the azasterol supplemented plates I had used in the control experiment. So, instead of tossing out the extra nystatin plates, I transferred some of these control amoebae onto them, and labeled the plates accordingly.

I expected the wild type amoebae grown on azasterol-supplemented plates to respond to nystatin in the same way as the ones grown on plates without azasterol. Why? I knew that azasterol blocked a step in the biosynthesis of sterols. Thus, much like the nystatin-resistant mutants, amoebae grown in its presence accumulate a precursor sterol in place of the normal sterol. This precursor sterol would make these amoebae resistant to nystatin if it were added to the same plates. But when these amoebae were transferred to nystatin plates without azasterol, they would regain the ability to synthesize normal membrane sterols, which would make them nystatin sensitive.

To my surprise, in a couple of days, the wild-type amoebae transferred from azasterol-supplemented plates showed exuberant growth on the nystatin plates. In contrast to the sensitivity of the wild-type amoebae transferred from normal plates, these amoebae had grown as well as the recessive nystatin mutants. That the azasterol-derived amoebae had remained nystatin-resistant seemed to imply that the nystatin in their growth medium was capable of inducing resistance to itself.

This nystatin-dependent-nystatin-resistance (NDNR) could also explain the results of my experiment to identify nystatin-resistant amoebae with dominant mutations. When the wild-type diploid amoebae were plated directly on nystatin supplemented media, most were rapidly killed by the antifungal. Only some amoebae survived and developed nystatin resistance. Since this happened at frequencies low enough to be comparable to the mutation frequency, I had assumed that this resistance was due to mutations in their sterol biosynthesis genes. However, it now seemed possible that these surviving amoebae had acquired nystatin resistance (in the absence of a mutation), which remained only as long as they were maintained on the nystatin supplemented media. This would explain why this resistance was lost when these amoebae were taken off nystatin in the course of obtaining haploid derivatives. It would also explain the exuberant growth of the amoebae transferred from the azasterol-supplemented plates to the extra nystatin-supplemented ones (the plates that I did not toss out). Since azasterol induced the replacement of the wild-type sterol by the precursor sterol, a much higher percentage of these amoebae would survive the initial nystatin killing long enough to acquire NDNR.

This was an entirely new observation—one where nystatin (and not a mutation) seemed to induce amoebae to become resistant to its killing effect. Many questions still remain unanswered. What is the molecular basis of NDNR? Can NDNR occur in fungi or human cells? Does this phenomenon extend to amphotericin B? The one that baffled my supervisor was—what inspired me to transfer the amoebae grown on the azasterol-supplemented plates to the nystatin-supplemented ones? It seemed such a crazy thing to do! Both my 'failure' to obtain dominant mutants and the fact that I did not toss out the extra plates were integral to this serendipitous discovery of NDNR. I believe it was Samuel Goldwyn who said *"... the harder I work the luckier I get".*

Notes:

1. The research described here was published in Antimicrob. Agents Chemother. 27: 974-976, 1985. URL: https://journals.asm.org/doi/10.1128/AAC.27.6.974.
2. To read more about cannibalism in slime moulds, refer: Waddell D. R. 1982. A predatory slime mould. Nature 298, 464-466.
3. Source of the image used in the background of the article title: Slime Mould. Credits: Usman Bashir, Wikimedia Commons. URL: https://commons.wikimedia.org/wiki/File:Dictyostelium_discoideum_43.jpg. License: CC-BY-SA.

D. P. Kasbekar is a retired scientist.