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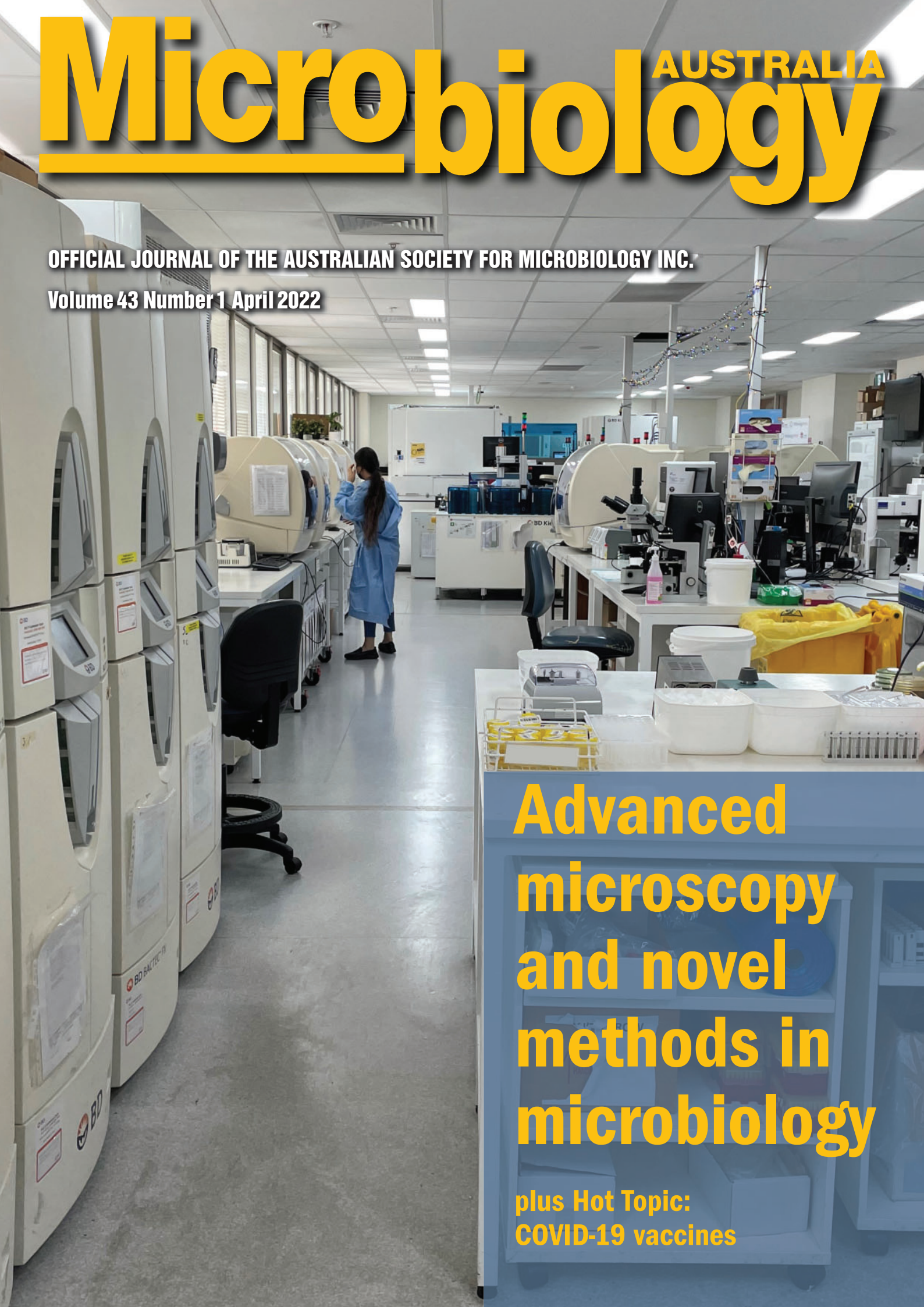

Advanced microscopy and novel methods in microbiology

plus Hot Topic:
COVID-19 vaccines

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Contents

Cover image: Courtesy of Tom Olma (Westmead Hospital).

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Vertical Transmission

Dena Lyras
President of ASM

I wish you a belated but warm welcome to this new year, 2022. For many of us, this year is already looking very different to the past 2 years. However, the dominance of microbes and microbiology in the media, and in demanding attention from the public, continues. As well as the continuing attention to COVID-19 (aren't we microbiologists all just dreading the appearance of the next variant?), we are now seeing the emergence of Japanese encephalitis virus (JEV) in multiple locations in Australia. As the COVID-19 pandemic has shown, our relative isolation is not a safeguard from the risks of global health crises. Sadly, history has taught us that war and a rise in infectious diseases go hand-in-hand because of the dismantling of existing health systems and control programmes, such as diagnostics and vaccination, the displacement of populations, overcrowding, and lack of clean food and water. The war in Ukraine is terrifying for many reasons, including the human suffering and death that is happening by the hour, and the implications for continued disruption of global relations, but the addition of escalating infectious diseases adds a layer of complexity to an already fraught situation. As microbiologists, we appreciate that human behaviours, good and bad, can have major consequences on the activities of our microbial partners.

We are pleased to be bringing you this issue, focussed on microbiological diagnostics, and we have many exciting topics to cover this year. Please get in touch if you have ideas for articles to contribute, or special issues to put together, or any hot topics you would like to see covered. This is your journal and you can play a role in shaping the content of upcoming issues.

Don't forget to go to our ASM Community portal, which allows members to connect with one another, to join special interest groups, have discussions with members who have similar interests, and to keep up to date on all ASM matters. To join the ASM community, go to https://community.theasm.org.au/ and click on the icon at the top RHS of the screen to set up your profile. Select 'Communities' from the banner menu to join a Special Interest Group community. Our other platforms, including our website www.theasm.org.au, ASM on Twitter, @AUSSOCMIC, or on Facebook, are also very active. We encourage and welcome your engagement using any option that suits you.

Microbiology Australia updates

Microbiology Australia (MA) continues to gain greater recognition globally, now being listed by the *Directory of Open Access Journals* (DOAJ), an online directory that indexes and provides access to high-quality, open access, peer-reviewed journals. Inclusion in DOAJ has the benefits of raising visibility of MA content and, importantly, demonstrates best practice editorial and publishing standards (which also helps distinguish MA from the plethora of predatory open access journals out there). More information on the benefits of indexing in DOAJ can be found at: https://doaj.org/apply/why-index/#why-your-journal-should-be-indexed-in-doaj

Other databases listing MA include Scopus and Clarivate's Emerging Sources Citation Index (ESCI). MA is a member of COPE (Committee on Publication Ethics) and requires authors to list conflicts of interest and sources of funding.

Most importantly, MA aims to meets the needs of members of *The Australian Society for Microbiology*. Melinda and Tess (pictured here) were proud to receive the print copy of the most recent issue.

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EDITORIAL
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Reflections from Melbourne, the world's most locked-down city, through the COVID-19 pandemic and beyond

Ian Macreadie

Melbourne, Victoria's capital city, has had more lockdowns (Table 1) due to the COVID-19 pandemic than any other city in the world. With its population of 5.5 million people Melbourne has a well developed economy based on high technology services, education, sports, arts, events and tourism. Most of these activities were all deeply affected by 262 days of lockdowns with Melburnians issued stay-at-home orders except for permitted activities.

Lockdown measures varied somewhat on each occasion. Some are listed in Table 2.

The adverse effect of lockdowns on morale and mental health was experienced in Melbourne and other places where there were lockdowns, however, the sheer number of lockdowns, their duration, and the uncertainties due to different rules in different jurisdictions made the lockdown experience in Melbourne even tougher. The Victorian government provided financial support for most workers who had lost income from their regular work due to the restrictions. However, the criteria for assistance was not met by many who worked in casual positions. These people included the many foreign students who normally supported themselves through casual employment. It also included many senior students who practiced their skills as laboratory demonstrators.

By late 2021, Melbourne moved out of lockdowns and into a highly enviable position, becoming one of the most vaccinated places in the world (90% vaccinated as of late November 2021). Now Melbourne provides an example of what can be achieved with a high vaccination rate.

What led to the high vaccination rate?

The major reasons for Melbourne's high vaccination rate are likely to be the following.

(1) Lockdowns: The lockdowns (Table 1) were a major reason for high vaccination rates. Prior to vaccines, elimination or suppression of transmission were the only options for people to avoid COVID-19. Australia is an island continent that was relatively easy to isolate from the rest of the world for almost 2 years by international border controls. Australians were ordered 'Do Not Travel' and 'essential travellers' arriving were made to undergo hotel quarantine for 2 weeks. In addition, to restrict internal movements, the six states and two territories frequently closed their borders during 2020 and 2021 to limit SARS-CoV-2 spread. In some instances, just one infected person being identified in the community led to lockdowns. As a result several states experienced very few cases of community-acquired COVID-19.

Lockdowns often had very harsh restrictions (Table 2) and were unsustainable in the long term: health authorities realised a high vaccination rate was the only possible option and the government promised a roadmap out of lockdowns. The roadmap was tied directly to vaccination rates so people in locked-down Australian states (NSW and Victoria) where new delta infections numbered 1000–2000 cases per day, quickly sought vaccines in the third quarter of 2021.

(2) Mandatory vaccinations: The Victorian Government mandated that many work groups had to be vaccinated. This included health-care workers, educators, child carers, police, construction workers, hospitality workers and many others.

(3) Vaccine availability: Australia initially faced delays in obtaining vaccines, leading many people to see the protective benefits in other countries and anxiously await their availability in Australia. People keenly waited for their risk group or age group to become eligible to receive the vaccine. By the latter part of 2021 COVID-19 vaccines (Astra Zeneca, Pfizer BioNTech and Moderna) were widely and freely available from vaccination hubs, general practitioners and many pharmacies.

(4) Endorsements of vaccines: There was a good advertising program with endorsements of vaccines by many high-profile public figures and correct information provided to the public. [On the downside there was widespread misinformation available that led to vaccine hesitancy. Much of the misinformation on social media was eventually censored and much of the misinformation was disproven by the fact that vaccinated people are obviously healthy and leading normal lives.]

Coming out of lockdowns with high vaccination rates

With 80% of the population over 12 years of age being fully vaccinated, the international 'Do Not Travel' restriction was finally removed in November 2021. This roadmap restriction

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Table 1. Melbourne's 262 days of lockdowns.

Lockdown	Start dates (duration)
1	31 March 2020 (43 days)
2	9 July 2020 (111 days)
3	12 February 2021 (5 days)
4	28 May 2021 (12 days). Arrival of delta variant
5	16 July 2021 (12 days)
6	5 August 2021 (79 days). Schools and playgrounds closed, night curfew. Earlier lockdowns ended when infections were controlled. This lockdown ended when Victoria's eligible population was 70% vaccinated.

Table 2. Some of the lockdown rules.

- Four (or five) reasons to leave home: getting food, exercising for up to 2 h, care or care giving, for authorised work or education (to get vaccinated).
- Work from home where possible.
- One person per household per day allowed to shop.
- Curfew from 9 pm to 5 am.
- Mask to be worn outside the home.
- No travel beyond 5 km from one's home.
- No visitors to homes.
- No visitors to old-aged care homes.
- No one to leave Greater Melbourne. There was a period when this was enforced by police roadblocks supported by the Australian Defence Force, a so called 'ring of steel' around Melbourne.

had been in place since March 2020, barring all international travel except for the few who could obtain exemptions. Being fully vaccinated I made use of the newfound privileges in November, taking the opportunity to visit relatives in Germany, where there was no need to have a negative PCR result prior to travel or quarantine on arrival. However, in December, when I returned to Australia and with the announcement of the omicron variant, new regulations were suddenly introduced. I needed to have a negative PCR result prior to travel, quarantine for 72 h on arrival and to have negative PCR tests after arrival and on days 5–7. On day 6 I was sent a text message indicating the person sitting next to me on the plane had tested positive, so I had to quarantine until I received a negative PCR result. Fortunately, my test results were all negative.

In contrast, Germany had good COVID-19 controls with lockdowns and excellent vaccine availability in the early stages of the COVID-19 pandemic but it restored freedoms with a meagre 60% vaccination rate. Movement was allowed subject to 3G entry conditions (**G**eimpft (vaccinated), **G**enesen (recovered), **G**etestet (tested)), i.e. entry required proof of vaccination, recovery or a negative antigen (or PCR) test. However, the fully vaccinated population was below 70% and this resulted in record numbers of COVID-19 cases in December: >100 000 per day (https://www.worldometers.info/coronavirus/country/germany/). Tragically, hospitals were filled to capacity, and turned some people away. In 2020 Germany was accepting COVID-19 patients from other countries but in the December 2021 wave some German patients were being sent elsewhere, including Italy. Many places in Germany moved to 2G restrictions, deleting the third option that contributed, in part, to lower vaccination rates. The dire state in Germany prompted the German Health Minister, Jens Spahn, to dramatically state that in a few months 'Germans will be either vaccinated, cured or dead.'

Coming out of lockdowns

In December 2021 vaccination rates in Victoria surpassed 90% and more restrictions were eased for vaccinated people. However, the new freedoms and the arrival of the omicron variant saw case numbers rise to astounding levels. Following this were increased hospitalisations and deaths, although many fewer than there would have been without the vaccines. While COVID-19 seemed a mainly overseas disease to many Australians in 2021, most Australians now know people who have had COVID-19. With the substantial protection afforded by vaccines, Victoria and NSW committed to staying open despite the omicron surge and its pressures on supply chains and health-care systems.

For the unvaccinated the Victorian government maintained restrictions, forcing many out of their employment and barring them from non-essential services. It is questionable whether this is now warranted or is it a punitive measure of the government? A rate of 90% vaccination tremendously reduces community risk so that it becomes reasonable to offer more liberties for the unvaccinated. Vaccination is a personal choice and provides a personal benefit for the vaccinee and for the community. While some were concerned about the rushed development of novel COVID-19 vaccines, it is now clear that the current vaccines are safe: over 7 billion vaccine doses have been administered (https://covid19.who.int/). In 2022, a greater range of vaccines are available, some more conventional, and this may lead to less vaccine hesitancy.

High vaccination rates need to be a priority for all countries. We do not just live in one region of the world. We are affected by what goes on in other countries in relation to cultural exchange, trade and tourism, and others need to regain freedoms as well. Wherever there are low vaccination rates, there will be continued major disruption to lives, overwhelmed healthcare systems, loss of life and the effects of long COVID, which can cause significant morbidity for young people who may have initially just had mild COVID-19 disease.

In an ideal world it is desirable to let people make their own choice about being vaccinated, but these should be educated choices. However, when a highly communicable disease like COVID-19 threatens a population, everyone should do their best to contribute towards its control. When 10–20% or more choose not to be vaccinated there are major repercussions for society.

It is important that we learn from the this pandemic and maintain our vigilance of other emerging microbes and of their development of antibiotic resistance. Microbiologists have been sounding warnings for years so COVID-19 comes as no surprise.

GUEST EDITORIAL
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Advanced microscopy and novel methods in microbiology

D. İpek Kurtböke, Wieland Meyer and Linda L. Blackall

Microbiology, like all the sciences, is founded upon the twin pillars of craft techniques and philosophical speculation. Without the empirical observations of the first, the subject would be but a meaningless verbiage, and without the organizing hypotheses of the second, would be but a collection of descriptions and receipts.

Patrick Collard, 1976

The beginning of modern microbiology starts from the late 1800s building on the advances made in chemistry, physics, and evolutionary cell biology and development of experimental and microscopic methods in the preceding centuries. Antoine van Leeuwenhoek did the pioneering work in microscopy in the 1670s and contributed toward establishment of microbiology as a scientific discipline. Microbiology has been through many different eras starting from the 'speculation' like the 'spontaneous generation' to current genome level understanding. In the first golden age of microbiology (1857–1914) causative agents of diseases (e.g. the Germ Theory of Disease), the role of immunity in preventing and curing disease (e.g. smallpox) and the chemical activities of microorganisms (e.g. fermentation) were identified. Key techniques like culturing microorganisms and subsequent microscopy as well as development of vaccines (e.g. rabies) and aseptic surgical techniques (e.g. the use of phenol) were introduced. In the second golden age of microbiology (1910 onwards) discovery of antibiotics and 'chemotherapy' eventuated with the key contributions from Alexander Fleming and Howard Florey who were able to produce the first antibiotic for clinical use. In the third golden age of microbiology molecular tools have significantly advanced our understanding of microorganisms at the genome level. Molecular diagnostic tools rapidly revolutionising the medical field and the treatments for the better: examples include shotgun metagenomic sequencing pathogens, microsatellite instability analysis systems, nanotrap virus capture kits as well as increasing affordability of PCR-based assays.

In this special issue of *Microbiology Australia*, we thus highlight advances in microscopy and novel diagnostic and microbiological methods. These technologies provide advances in understanding microorganisms, including their interactions among each other as well as with higher organisms. Their further exploitations provide valuable products for the needs of society.

In this special issue of *Microbiology Australia*, we thus highlight advances in microscopy and novel diagnostic methods. Tom Olma highlights the automation in diagnostics, Sarah Kidd and Gerhard Weldhagen communicate the diagnosis of dermatophytes. The article on the role of long read sequencing in fungal identification and diagnosis of mycoses reflects on the superior features of the methods by Laszlo Irinyi, Minh Thuy Vi Hoang and Wieland Meyer. Microbiology has provided the knowledge for industrial advancements: an example is shared by Ian Macreadie and Sudip Dhakal in an article titled 'The awesome power of yeast' as a cell factory for valuable proteins, including vaccines and new therapeutics. An example of microscopic techniques is covered by Pranali Deore, Iromi Wanigasuriya, Sarah Jane Tsang Min, Douglas R. Brumley, Madeleine van Oppen, Linda L. Blackall and Elizabeth Hinde with an article on the non-traditional approach to study microbial ecophysiology using fluorescence lifetime imaging microscopy (FLIM). İpek Kurtböke shares her experiences with actinomycete imagery dating back to 1980s. Douglas R. Brumley reveals the improvements in understanding processes in microbial ecology using mathematical modelling and quantitative video microscopy. A laboratory report is communicated by Laura G. Dionysius, Peter R. Brooks and İpek Kurtböke on the use of streptophages to control odorous streptomycetes on nuts. The issue concludes with the hot topic article on COVID-19 vaccines by Paul Selleck and Ian Macreadie covering a summary of technologies developed to produce 140 COVID-19 vaccines, which are recognised by the WHO as being in various developmental phases.

COVID-19 once again highlighted the importance of rapid diagnosis, vaccine and antimicrobial drug development. Super-fast vaccine delivery was possible due to the collaboration between scientists, manufacturers and distributors as well as ongoing background research before the virus has appeared. SARS (2002) and MERS (2012) investigations gave vaccine designers a head start. Ehrlich's vision 'the goal is to find chemical substances that have special affinities for pathogenic organisms and that, like magic bullets, go straight to their targets' only became a reality after 40 years of research. Accordingly, investment into research and development is a must for the generation of sound knowledge platforms that will provide the base for future discoveries.

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Microbiology Australia

Automation in diagnostic microbiology

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ABSTRACT

Automation in diagnostic microbiology has provided accessibility to technology overcoming the delays inherent in culture-based technology and providing options to centralisation via use of digital imaging to interpret and report culture plates which enables cultures to be shared over the internet opening the virtual diagnostic microbiology laboratory. Automation is a continuous process which precipitates faster results and invites the diagnostic laboratory into a continuous processing, 24/7 world optimising what it has to offer for improved turnaround time (TAT) and improved workload management. While molecular technology was able to decrease TAT of results it was initially specialised and not freely available to the diagnostic microbiology laboratory becoming more a centralised subspeciality. The automation of molecular methods released the technology to be able to be decentralised and provide greater local access and community benefits from the technology and providing equity of community access to the technology. Thanks to automation, diagnostic microbiology is at the forefront of opening the availability of advances in testing to the broader community via decentralising technology and sharing technology in the virtual diagnostic microbiology laboratory.

Keywords: automation, continuous, culture automation, diagnostic, on-demand, microbiology, remote plate reading, shared technology, virtual.

My thanks to the guest editors for the invitation to share my journey and insights regarding automation in diagnostic microbiology (ADM). ADM is not that novel. It is more 'old' news rather than breaking news. It is not my aim to compare and contrast. What I would like to do is to focus more on how automation in the diagnostic microbiology laboratory (DML) can be used, its impact on service delivery and subsequently on the profession. When you stand at the door of a DML (Fig. 1), what do you see? The past or the future? Do you see a lot of instruments or hardly any? Is the space recognisable as a DML or does it look more like a biochemistry laboratory? Just as importantly – what can't you see? A virtual DML evolving out of the ADM.

Certainly, instrumentation is a feature of the modern DML, typically performing multiple microbiology diagnostics in the same space. Instrumentation has brought it together by automating testing, whether urine microscopy, blood cultures, identification and susceptibility (ID&S), molecular testing and now even culture-based technologies. These have become more common place tools for the ADM laboratory managed through integration with the Laboratory Information Management System (LIMS). The impacts are staff savings, faster standardised results, interfacing LIMS, automatic reporting and possibly interpretation. Integration of automation (e.g. blood culture with ID&S, molecular, and MALDI-TOF) opens further opportunities for the provision of more rapid, meaningful clinical information. For most ADMs, this has been an evolutionary process driven more by a desire for efficiency and to proactively manage increasing workloads and restrictive budgets. The efficiency gains however precipitate consolidation. The same driver creating local health districts with governance over several hospitals and organisations to achieve consolidation efficiencies.

The options available for consolidating testing to take advantage of automation efficiencies are limited – either centralise the test and it is not performed on site or provide the technology on site. Molecular methods were seen as needing specialised skills and culture technology was hamstrung by growth requirements making referral a viable option. The cost of the technology was a further limiting factor given the smaller volumes on-site and availability of suitable logistics.

A centralised approach to consolidating DMLs had occurred within our region around 2008 and a number of regional and metropolitan DMLs closed. Regional units performed very limited tests on site and the rest were centralised, leveraged by suitable logistics, the

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Fig. 1. Panorama view of a diagnostic microbiology laboratory.

24/7 operations in our laboratory and the high level of automation in place which combined to minimise the clinical impact of transferring microbiology specimens off site. There was a distinct feeling of loss for these sites that extended to an actual loss of expertise and professional development of staff as well as to the clinicians and community. The appeal to work in regional sites diminished further adding to the difficulty to attract staff.

With the development of culture based automation (CBA) using digital imaging in the DML we were presented with a realistic alternative to centralisation that was previously unavailable – remote access. In October 2015 we went live with the BD Kiestra™ Total Laboratory Automation (BDKTLA) and journeyed into a different world of microbiology. The principles are the same, but they are achieved through automation of processes and digitisation of images using monitors connected to the system. A more inclusive approach is to share the technology off site. Ironically, forwarding specimens for processing on CBA has the same impact for all sites including the host. Everyone forwards their specimens to the CBA for processing. Unlike centralised models, the referring laboratory can read plates digitally as if they had the automation physically on site. It also has access to the other host technologies – instrument-based susceptibilities, MALDI-TOF and molecular methods. The plate reading and interpretation is decentralised, and they become inclusive with enhanced professional development. The technology can also separate the work into folders that are site specific. There is minimal difference to having the technology on site. Participating sites have internet access to the technology and associated efficiency benefits. The virtual laboratory. They are advantaged by freeing up staff to expand on-site service and utilise quiet periods to support the remote reading. Staff are rostered over the 24/7 period to read and report plates digitally rather than in hand. Instead of the laboratory being just 30 sq m it becomes 6000 sq km and is at the forefront of ADM and the evolving virtual DML. It is a work in progress, not without continuing challenges needing significant recalibration of mindsets, change management, workflow review, communication and adjustments. It does not really stop.

Today we have evolved into a continuous processing, on demand DML operating 24/7 – testing, plate reading, ID&S and reporting. Everything on our scope is available over the internet and remotely. With two participating regional sites and a team of staff to work from home providing 24/7 support remotely – virtually. They are mutually complementary with flexible working arrangements making it easier to accommodate changing circumstances and commitments. This was especially helpful during the COVID-19 pandemic.

Due to the historically specialised nature of molecular processing and equipment use, molecular biology was developing into a separate subdiscipline, centralising molecular testing. However, automation of molecular methods into self-contained instruments integrating extraction, amplification and detection without introducing contamination has simplified the technology. It became accessible and affordable for the DML. This provided the opportunity to place the technology at referring sites to not just improve turnaround times (TATs) but to better manage workload to the referral site. It challenges the traditional centralised mindset of work efficiency to a decentralised model with improvements in TAT and equity of service access for remote communities. The opportunity for mini hubs over shorter distances has emerged providing local referral of specimens. This was certainly evident during the COVID-19 pandemic with coronavirus testing performed at regional sites on automated molecular instrumentation providing rapid results for regional public health units and enabling workload to be better shared than gridlocked into a central distant location.

Consolidating our platforms to one simplified automated molecular platform also allowed molecular methods to be integrated into our routine 24/7 service workflow to provide these assays on demand, without batching and easily used by out of hours DML staff. The samples were prepared ready to load when the platform was ready for the next run. Molecular testing evolved into a continuous process with no restrictions on menu availability with tests available on demand 24/7 in stark contrast to batch driven centralised models.

The 24/7 laboratory moved us beyond just providing rapid urgent results on request. It synchronised the DML to the continuous nature of automated testing, expanding our function over three shifts and the internet. This has avoided double/triple handling of specimens that occurs with batching. The added benefit was faster more relevant results that initially exceeded clinical expectations but quickly became the new norm feeding customer addiction to the improvements.

The shift by NSW Health to decentralising medical services and establishing Rural Referral Hospitals impacted not

just workload but expectations of on-site services for regional and rural laboratories. The ADM opened applications to assist with workload and improvements in TAT via molecular automation and access to culture automation. This in turn provided offsets to expand local services by shifting the specimen processing off site but still retaining the reading and reporting – with the added advantage of access to the latest technologies such as MALDI-TOF. The rural community was receiving equity of service comparable to metropolitan and city communities.

The benefits were more obvious during the COVID-19 pandemic with the addition of coronavirus testing to Regional and Rural NSW. Timely rapid COVID-19 results were achieved enabling rapid tracking to protect the community the equal of any large teaching hospital and better than most. Regional testing also took a lot of workload pressure off referral laboratories. The same assays were used. The community felt significant support and not disadvantaged by delays in referring specimens to central testing laboratories. In turn additional support was provided by the referral laboratory via its automation of culture processing using the BDKTLA. This freed staff to focus on COVID testing while still maintaining current microbiology services.

Consideration of how ADM is implemented needs to be applied to the future. How it is implemented and how the profession of diagnostic microbiology will evolve and develop? Traditional centralisation could potentially decimate the profession limiting positions and training. It is the easiest option but not necessarily the best option. It is exclusive and no doubt appeals to some. It does not mean that the technology and what it offers should be ignored. So much would be lost. It is a case of how it is managed and the alternative approaches. As a discipline there is a broader consideration, the freeing up of sites, broadening service and buy into the technology sharing through the Virtual DML. An inclusive approach. Like all things there is a balance.

Automation in diagnostic pathology has typically been defined by the laboratory physical dimensions and location. Laboratories refer specimens to the central laboratory, which processes and sends out results. No further input by the referring laboratory. However, ADM has changed this and offers real alternatives.

The virtual laboratory is an inclusive laboratory sharing the technology amongst those that have access. Enabling enhanced professional development and improvement in services to the community. This can be fine-tuned, enhancing access to technology that was otherwise unaffordable and unavailable. There are undeniable challenges in sharing technology but these are surmountable.

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Biography

Tom Olma is NSWHP, Pathology West and Regional and Rural NSW Microbiology Discipline Network Supervising Scientist, in-charge of Core Microbiology, CIDMLS, ICPMR, Westmead, NSW. He is a BSc graduate from UNSW and started his career at Blacktown Hospital, NSW, as a hospital scientist to become a Senior Hospital Scientist in charge of Microbiology before consolidation with ICPMR Westmead where he has been in charge of what today is known as Core Microbiology for the past 26 years. His career is closely aligned to automation and rapid methods in diagnostic microbiology. Tom has a self-professed passion for proactive diagnostic microbiology in patient management. He is a past Committee member, Secretary, Chairperson of ASM NSW branch and a previous chairperson of hosting the ASM National Conference in Sydney.

Future issues of *Microbiology Australia*

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Diagnosis of dermatophytes: from microscopy to direct PCR

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ABSTRACT

Dermatophyte fungi are a common cause of skin, nail and hair infections globally, ranging from mild to cosmetically disfiguring, or even invasive infections in rare cases. Specimens requiring fungal microscopy and culture for suspected dermatophyte infection make up a significant portion of the workload in diagnostic microbiology laboratories. Whilst still considered the gold standard, a dermatophyte culture-based method is labour intensive, has poor sensitivity, slow result turnaround time and requires significant expertise for identification of the fungi. Molecular diagnostics, especially real-time PCR, have the potential to improve diagnostic sensitivity, reduce labour requirements and decrease result turnaround times. Despite these advantages, a PCR-based approach may present some difficulties and disadvantages, most notably its diagnostic range and incompatibility with oral therapy prescribing requirements under the Pharmaceutical Benefits Scheme. Here we review current best practices and future prospects for laboratory diagnosis of dermatophyte infections, including the role of microscopy, culture and direct PCR.

Keywords: dermatophyte, *Epidermophyton*, fungal culture, medical mycology, microscopy, *Microsporum*, onychomycosis, real-time PCR, tinea pedis, *Trichophyton*.

Introduction

Dermatophyte fungi are a common cause of skin, nail and hair infections globally. The most common agents are *Trichophyton rubrum* and *T. interdigitale*,[1–3] but other dermatophytes belonging to the genera *Arthroderma*, *Trichophyton*, *Epidermophyton*, *Microsporum*, *Nannizzia*, *Paraphyton*, and *Lophophyton* (the latter three genera formerly classified within *Microsporum*) also cause infection. Infections commonly present as tinea pedis and onychomycosis, but can affect any keratinised area of the body, having low clinical acuity. However, some infections may be debilitating or invasive in immunocompromised or elderly patients.[4,5] The prevalence of onychomycosis is approximately 10% in the general population, but may increase to 50% in those aged >70 years.[6] Infections are usually transmitted by direct or indirect human contact, but may also be acquired from animal sources or soil, depending on the species. The prevalence of these infections and the species that cause them appears to vary significantly by geographic region, and is well reviewed by Nowicka and Nawrot.[3]

Occasionally, yeasts such as *Candida* spp. and non-dermatophyte moulds such as *Scopulariopsis* spp., *Aspergillus* spp., *Fusarium* spp., and *Acremonium* spp. may cause onychomycosis, but the diagnosis is complicated by these fungi also being common environmental contaminants.[7,8] Diagnostic guidelines require at least two subsequent isolations of these fungi in the absence of a dermatophyte and in the setting of direct microscopy exhibiting fungal hyphae not resembling dermatophytes.[9]

Effective antifungal agents are available for treatment of dermatophyte infections in topical or oral formulations, with the latter being necessary to treat refractory nail infections. However, for oral formulations to be prescribed through the Pharmaceutical Benefits Scheme (PBS), patients need to fulfil clinical requirements and laboratory diagnostic criteria including microscopy and dermatophyte culture.[10] Therefore, these infections form a significant laboratory workload and reliable, sensitive, and specific diagnostic methods are needed for effective treatment.

While much of mycology continues to utilise conventional methods such as microscopy and culture, the diagnosis of dermatophyte infections is increasingly becoming modernised through the use of real-time PCR assays. Here we review current best practices and future prospects for laboratory diagnosis of dermatophyte infections, including the role of

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Table 1. Summary of available diagnostic methods for dermatophyte infections.

Method	Direct microscopy[3,11–14]	Culture[3,14,15]	Culture identification by MALDI-ToF[16–18]	Culture identification by ITS sequencing[19]	Direct detection by RT-PCR[20–26]
Depth of identification	• Differentiates dermatophytes from other moulds and yeast	• Dependent on staff expertise and available culture media	• 37 species level identifications • 4 genus level identifications (Bruker filamentous fungi database v. 4.0)	• Both genus and species level identification of dermatophytes represented in validated/curated genetic data repositories	• Most commonly to genus level • Species and species complex level identification dependent on assay design
Advantages	• Fast result turnaround time • Good sensitivity when an optical brightener is used	• Grow the aetiological agent for identification and subsequent studies • Identify non-dermatophyte causes of onychomycosis • Data may be used for epidemiological purposes	• Direct identification from semisolid media possible • Cost effective • Reduced need for morphological identification • Species library regularly updated • Fungal taxonomy regularly updated • Creation of in-house species library possible	• Gold standard – results are unequivocal • Sequence data can be shared and compared between laboratories • Online databases are publicly available	• Reduced sample preparation • Increased sensitivity • Reduced turnaround time • Reduced requirement for morphological expertise • TGA approved commercial kits available • In-house assay detection chemistry can be suited to a specific demographic • Use some existing laboratory equipment
Disadvantages	• Low specificity • Differentiating dermatophyte hyphae/elements from those of other fungi requires significant expertise	• Low sensitivity • Takes 2–4 weeks to grow, and additional time for identification • Morphological identification requires significant expertise	• May need subculture to improve spectral analysis – increases workload and turnaround time • Species library is restricted to research use only (RUO) for many laboratories	• Requirement for additional equipment and dedicated laboratory space • Needs highly trained and skilled staff • Databases may not be adequately curated (e.g. GenBank) • Costs frequently exceed Medical Benefit Scheme remuneration	• Detection chemistry may not be compatible with high throughput laboratories • Requirement for additional equipment and dedicated laboratory space • May detect DNA from non-viable dermatophytes post-treatment • May not detect species causing non-dermatophyte onychomycosis • Methods not included in current PBS prescribing criteria

microscopy, culture and direct PCR, and Table 1 summarises the advantages and disadvantages of each.

Microscopy

Direct microscopy of specimens is an essential component of the diagnostic pathway for cutaneous fungal infections. It provides a relatively fast result demonstrating the presence or absence of fungal elements indicative of infection. More importantly, a skilled microscopist can differentiate dermatophyte hyphae from that of non-dermatophyte moulds and yeast pseudohyphae, and the budding yeast cells of *Candida* spp. from those of *Malassezia* spp. This alone may be sufficient for a diagnosis of dermatophyte and dermatophyte-like infections. However, observation of fungal elements does not necessarily indicate causation of infection or demonstrate viability of the fungal elements and it is not possible to determine the genus or species. Therefore, microscopy is a low-specificity diagnostic technique.

Traditionally, skin and nail specimens are digested in 10–20% potassium hydroxide (KOH) as a clearing agent for 3–16 h prior to microscopy, having a false-negative rate of 5–15%.[11] Staining with chlorazol black E can provide additional contrast for visualising the fungal elements (Fig. 1*a*). However, optical brightener stains such as Calcofluor White or Blankophor, which bind to cellulose and chitin in the fungal cell wall and fluoresce under UV light (Fig. 1*b*), significantly improve the sensitivity of microscopy (82–91% vs 74–85% KOH preparation alone)[12,13] and allows more rapid scanning of slides. Optical brightener stains are recommended in laboratory guidelines for dermatophyte studies.[14]

Culture

Culture remains the gold standard for diagnosis of dermatophyte and other fungal infections, yielding a specific aetiological agent that can be identified to species level. However, culture has low sensitivity,[15] requires 2–4 weeks

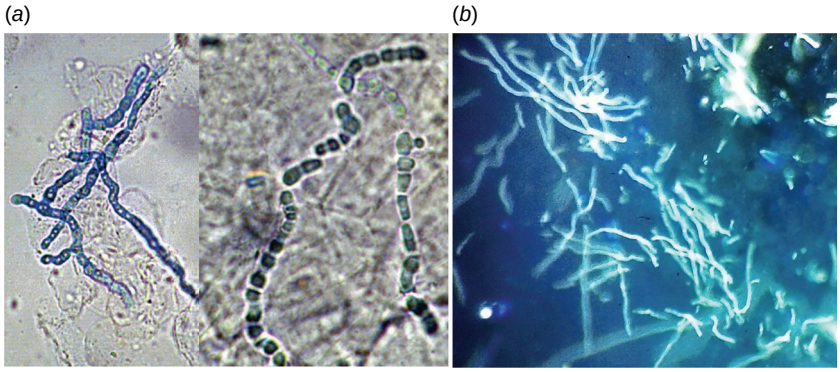

Fig. 1. KOH wet mounts of nail tissue containing dermatophyte hyphae with arthroconidia, stained with (*a*) chlorazol black, viewed under a light microscope, and (*b*) calcofluor white, viewed under a UV microscope. Image credit: (*a*) courtesy of David Ellis, Mycology Online (www.mycology.adelaide.edu.au).

for growth, and potentially a further 2 weeks for identification. Species identification needs detailed examination of colony and microscopic morphology, requiring specialised media and significant expertise.

Culture media for dermatophyte studies should include a specialised isolation medium such as Lactritmel or Dermatophyte Test Medium, containing antibiotics and cycloheximide; this minimises growth of fast growing bacterial and fungal contaminants, allowing the slower growing dermatophyte to grow. Sabouraud's dextrose agar containing antibiotics but not cycloheximide is also recommended for nail specimens to allow growth of potential causes of non-dermatophyte onychomycosis.

The identification of dermatophytes and other fungi requires specialised training and expertise, something of a dying art in the era of laboratory automation. Commercial matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-ToF) databases have the potential to identify common dermatophyte species; however, many species, for example, *Trichophyton benhamiae*, *T. equinum*, *T. erinacei*, *T. eriotrephon*, *T. interdigitale*, *T. mentagrophytes* and *T. tonsurans*, cannot be reliably differentiated.[16] Optimised protein extraction methodologies combined with supplemented databases may be far more accurate,[17,18] although significant initial setup is required. DNA sequencing for species identification using the Internal Transcribed Spacer (ITS) rDNA region may be useful in some cases, but sequence databases contain numerous entries for dermatophytes with incorrect identifications that complicates making a reliable identification, and particularly between *T. interdigitale* and *T. mentagrophytes*.[19]

Real-time PCR assays

Given the slow and labour-intensive nature of conventional methods, many laboratories are looking towards real-time PCR for detection of dermatophytes in clinical samples.[20–26] The primary advantage of this approach is the decreased turnaround time over culture, with results typically available within 1–3 days of receipt in the laboratory. Additionally, the sensitivity of PCR is around 20–30% higher than culture, in part because dermatophyte DNA can be detected even in the presence of fast-growing fungal contaminants that might overgrow the slower-growing dermatophytes in cultures.[15,20,21,25] PCR-based identification reduces the need for morphologists, who could be utilised more effectively diagnosing fungi from life-threatening infections.

PCR assays may be commercial or in-house designed. In-house assays have the benefit of being customisable to existing laboratory platforms, as well as to include species that best represent the local epidemiology,[23] and fungi involved in non-dermatophyte infections of the skin and nails. Commercial assays typically have the benefit of regulatory approval, lessening the validation requirements on the laboratory. The sensitivity of dermatophyte PCR assays has invariably been shown to exceed that of culture.[20–26]

Some commercial assays include pan-dermatophyte detection and/or specific primers for detection of a limited number of species. For example, the Dermatophyte PCR Test (SSI Diagnostica, Denmark), utilises pan-dermatophyte primers targeting the CHS1 gene as well as *T. rubrum*-specific ITS2 primers, the DermaGenius Nail multiplex assay (PathoNostics, The Netherlands) contains species-specific primers for *T. rubrum*, *T. interdigitale* and *C. albicans* only, and the Dermatophytes Real-Time PCR (EurobioPlex, France) is designed to detect six dermatophyte species. The 'Dermatophytes and other Fungi' multiplex tandem PCR (MT-PCR) (AusDiagnostics, Sydney) detects at least 14 dermatophyte species, as well as four *Candida* species and two non-dermatophyte moulds, with species identification made possible by specific primers and melt curve analysis (Fig. 2). This assay does not currently differentiate all of the species that it detects,[21,22] but differences in melt curve may be suggestive of certain species, requiring further validation. Given the limitations of species differentiation with PCR assays, it may only be possible to report the species complex, genus, or simply 'dermatophyte detected'. This loss of species resolution limits insight into the potential origins of infection (i.e. anthropophilic vs zoophilic), but for most clinicians the faster turnaround time coupled with increased sensitivity, appears to be an acceptable trade-off.

PCR assays may not detect non-dermatophyte fungi that cause onychomycosis, which have an incidence of around 2–20%.[7,8,27,28] The AusDiagnostics panel includes targets for *Aspergillus* spp., *Scopulariopsis* spp., and four *Candida* species but not *Fusarium* spp., *Acremonium* spp., and *Neoscytalidium* spp. Since these may be environmental contaminants or opportunistic skin flora, they may be detected in the absence of infection and should be reported with caution, potentially utilising reflex cultures where non-dermatophyte onychomycosis is suspected.

No.	Sa	Gene	Call	Corrected melt	Take-off	Calculated ct	Concentration
N1	2...	*Trichophyton* spp.	Present	84.71	12.52	20.33	39 758
N2	2...	*T. rubrum* complex	Present	84.77	12.65	20.46	36 546
N3	2...	*Mentagrophytes*					
N4	2...	*Microsporum* spp.					
N5	2...	*Microsporum canis*					
N6	2...	*E. floccosum*					
N7	2...	*Nannizzia gypsea*					
N8	2...	*Scopulariopsis* spp.					
N9	2...	*Aspergillus* spp.					
N10	2...	*Candida* \ Meyeroz...					
N11	2...	Candida2	Present (C. parapsil...	79.72	24.55	32.36	18
N12	2...	SPIKE	Present	81.8	14.67	22.48	10 000

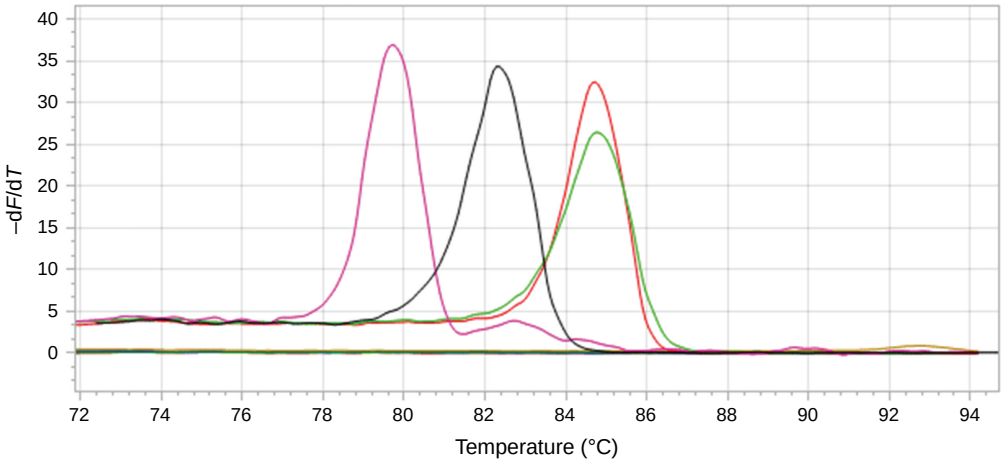

Fig. 2. Screenshot of Dermatophytes and other Fungi MT-PCR result display (AusDiagnostics Multiplex-Tandem PCR Results Software ver. 1.7.14) from a nail scraping, demonstrating melt curve detection of *Trichophyton* species (red), further identified as *T. rubrum* complex (green), trace quantities of a *Candida* species (pink) likely representing skin flora, and an internal PCR control (black).

Real-time PCR detection of squalene epoxidase mutations conferring terbinafine resistance has recently been described.[29] However, the significance and clinical utility of such assays are currently limited, as susceptibility breakpoints for dermatophytes remain tentative or unavailable.[30]

Despite the superior sensitivity of PCR compared to culture, dermatophyte detection in nail specimens requires confirmation by either microscopy or culture in order to satisfy Australian PBS requirements for prescription of oral terbinafine. This represents additional work, and does not always provide the necessary confirmation. Some of these cases may be attributed to the detection of DNA from non-viable dermatophytes (i.e. already inactivated by antifungal treatment).

Conclusions

PCR detection of dermatophyte infection brings many benefits to laboratories that are increasingly stretched for resources. However, currently, such advances come at the cost of accurate speciation, and the associated ecological and epidemiological information that accompanies it. Current PBS requirements and the increasingly apparent role of non-dermatophyte fungi in cutaneous infections means that there remains a role for microscopy and culture in routine diagnostic laboratories.

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Data availability. Data sharing is not applicable as no new data were generated or analysed during this study.

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Gerhard Weldhagen is the supervising pathologist for the National Mycology Reference Centre at SA Pathology. He completed his undergraduate studies in 1993, receiving a Bachelor's degree in Medicine and Surgery (MBChB) from the University of Pretoria, South Africa. This was followed by a Master's degree in Clinical Microbiology (MMed (Path) cum laude) and subsequently a PhD in Microbiology, conferred by the University of Pretoria in 2002 and 2005 respectively. After settling in Australia during 2009, a Fellowship of the Royal College of Pathologists of Australasia (FRCPA) was attained during the same year. Current interests include the role of molecular assays in diagnostic microbiology, including mycology.

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Long-read sequencing in fungal identification

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ABSTRACT

Long-read sequencing is currently supported by sequencing platforms from Pacific Biosciences and Oxford Nanopore Technologies, both of which generate ultra-long reads. Metabarcoding and metagenomics are the two approaches used when implementing sequencing. Metabarcoding involves the amplification and sequencing of selected nucleic acid regions, while in a metagenomic approach extracted nucleic acids are sequenced directly without prior amplification. Both approaches have associated advantages and disadvantages, which, in combination with long-read sequencing, provide a promising new approach for fungal identification and diagnosis of mycoses, on which we will reflect in this short review.

Keywords: diagnostics, DNA barcoding, fungal identification, long-read sequencing, metabarcoding, metagenomics, mycoses, next generation sequencing.

Long-read sequencing

Long-read sequencing technologies are characterised by the potential to generate ultra-long reads over 10 kb in one run.[1] Pacific Biosciences (PacBio) first released their long-read sequencing instrument in 2011 and most recently released the Sequel IIe (Fig. 1).[2] PacBio sequencing is based on the conversion of fluorescent signals produced when nucleotides are bound to a template strand via a polymerase. The template double-stranded DNA has two hairpin adaptors bound to each end and so sequencing continues around the template for the duration of the polymerase lifetime. PacBio sequencing has achieved high accuracy, which is reported to be 99.8%, and produces a high throughput with parallel sequencing of millions of template strands.[3] However, PacBio sequencing is a trade-off between read length and read quality: as longer template strands are sequenced, there is often a less accurate consensus sequence. Additionally, library preparation is estimated to be 1 day, which may not be suitable for time sensitive applications. Oxford Nanopore Technologies (ONT) began commercial release in 2015 with their MinION sequencer and has since released instruments and flow cells for a variety of throughput requirements (Fig. 2).[4] The basis of ONT sequencers are biological nanopores fixed in membranes, of which nucleotide strands travel through to cause a current change, which is then translated into a sequence. ONT sequencing read length is only limited by sample DNA length, quantity, and purity and as they provide a wide array of library preparation kits and throughput options it can be scaled to any potential use intended. Additionally, the low initial investment and small portable size of some ONT sequencers allow sequencing to be performed in locations outside the laboratory.[2] The major drawback of ONT is the lower read accuracy reported, although computation intensive bioinformatic tools are available to increase accuracy to 99%.[5] Samples that result in low DNA quantity, quality, and short read lengths do not take full advantage of ONT sequencing and impede sequencing by blocking and inactivating pores. These sequencing technologies are further discussed in other studies.[2]

Studies using PacBio and ONT long-read sequencers to sequence and identify fungal species using metabarcoding and metagenomic approaches are described below to demonstrate the potential use for long-read sequencers in fungal identification.

Metabarcoding

Metabarcoding involves the sequencing of targeted nucleic acid regions within environmental and clinical samples. This approach combines DNA barcoding with high throughput sequencing of specific taxonomic regions (barcodes). These barcodes, which have

Fig. 1. Pacific Biosciences Long Read Sequencing Platforms. The first commercial long read sequencer Sequel was released in 2015, supporting the SMRT Cell 1 M. The newer instruments Sequel II and Sequel IIe support the SMRT Cell 8 M, capable of simultaneous sequencing of 8 million template DNA strands. The Sequel IIe improves upon the previous iterations through software upgrades resulting in a reduction in analysis, file transfer and data storage requirements. Instrument pictures from the manufacturer's webpage (https://www.pacb.com/).

Fig. 2. Oxford Nanopore Long Read Sequencing Platforms. The flagship product, the MinION, is a palm-sized device supporting MinION flow cells containing 4 × 125 nanopore channels. GridION and PromethION are the higher throughput instruments, supporting 5 and 48 flow cells respectively. The Flongle flow cell contains 126 nanopore channels and is compatible with the MinION and GridION. A flow cell for the PromethION containing 2675 nanopore channels is also commercially available. Instrument pictures from the manufacturer's webpage (https://nanoporetech.com/).

high taxonomic coverage and high resolution, are amplified and sequenced (Fig. 3).[6] The generated barcode sequences are clustered into operational taxonomic units (OTUs) based on sequence similarity and compared against databases containing reference barcode sequences to provide an accurate representation of the microbial population. The advantages of this approach are the low level of genomic material required and faster and less complex computational analysis. However, as only specific regions are sequenced, information is limited to identification, although this may not be an issue for all studies. Prior amplification of a sample also potentially introduces bias, which may result in the inaccurate representation of the microbial community.[6] Previous microbial metabarcoding studies with short-read sequencing technologies used micro-barcodes that spanned less than 600 bp, which were often shorter than the full-length barcoding regions.[7] Long-read sequencers can span beyond the full-length barcode regions and resolve longer structural variations that are challenging for short-read sequencers, leading to higher discriminatory power.

Long-read fungal metabarcoding studies primarily use the full-length internal transcribed spacer (ITS) region of the rRNA gene cluster to identify fungal species, although shorter barcode regions are also used. To validate the ability of long-read sequencers to identify fungal species, ONT and PacBio have been used to identify members of mock fungal communities. The full-length ITS and the ITS1 regions were shown to accurately identify 16 and 26 fungal species within mock communities respectively using PacBio sequencing.[8,9] The full-length ITS region in conjunction with ONT sequencing has also successfully identified fungal species in mock communities.[10,11] The ITS region was found to be the superior locus for fungal identification in nanopore sequencing although, in a mock community with varying abundance of 16 fungal species, species level identification was only achieved for 1/3 of fungal species.[7] Metabarcoding of clinical samples with nanopore sequencing of the ITS region, has been explored extensively. Pathogens were identified from nine positive blood culture bottles which were then verified by routine diagnosis, one of which was a *Candida albicans* infection.[5] Type strains of five *Candida* species were also identified to sufficient (100–200×) coverage and nanopore sequencing errors did not affect correct species identification.[5] Full-length ITS nanopore sequencing has also identified potential pathogens in patient samples previously negative by traditional diagnostic methods.[11,12] PacBio has also been used in the clinical space to characterise the gut mycobiome of 14 healthy individuals with the ITS1 locus.[9] PacBio sequencing has primarily been applied to metabarcoding of environmental samples and has been demonstrated to outperform nanopore sequencing in such applications due to nanopore sequencing errors.[13] Metabarcoding of samples, such as tree roots,[14] soil,[13] mangrove sediments,[15] and lake water,[7] have revealed the potential of PacBio targeting the full-length ITS region to be used in broad ecological studies requiring accurate characterisation of the mycobiome of complex environmental samples. Metabarcoding of fungi using long-read sequencing has been established to be a promising avenue for the identification and characterisation of fungal species in clinical and environmental samples.

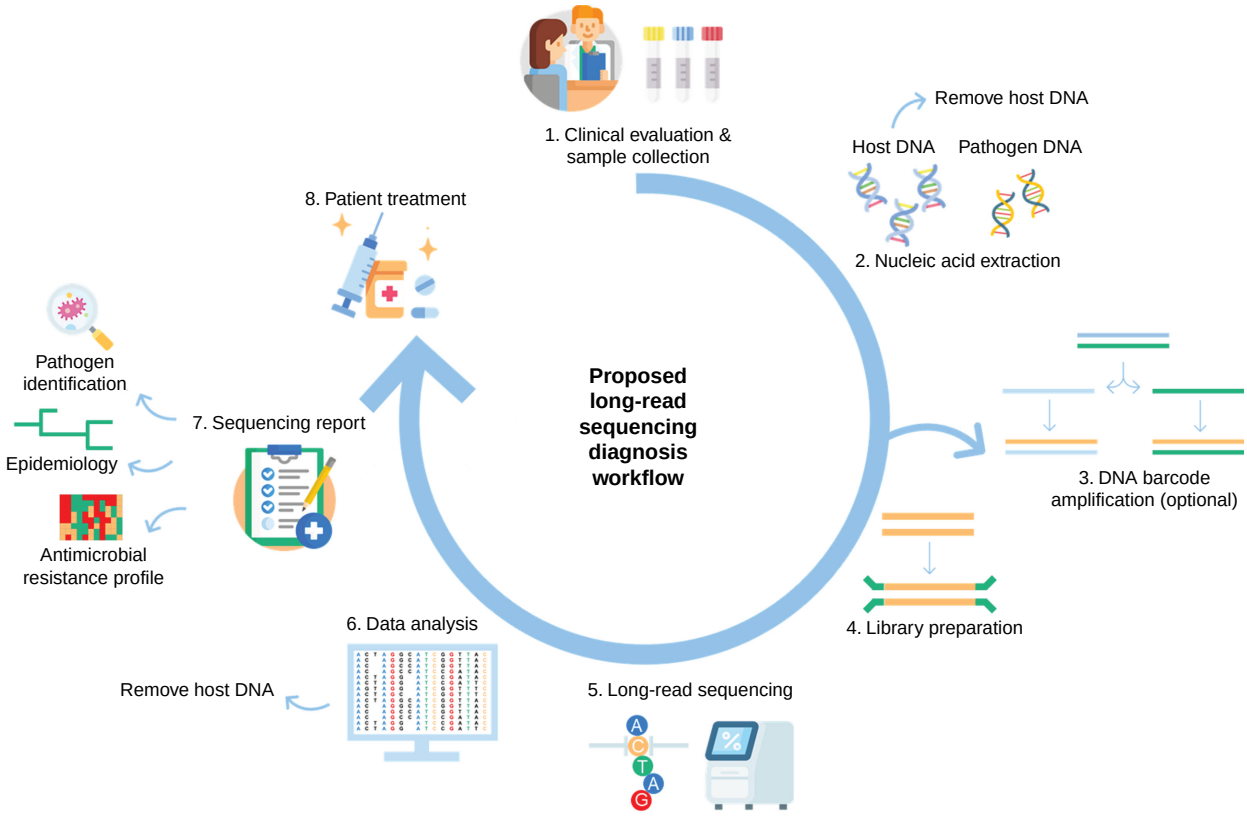

Fig. 3. Proposed Long-read Sequencing Diagnosis Workflow. Diagnosis of fungal infections would begin with clinical evaluation and patient sample collection. Nucleic acids would then be extracted from patient samples containing both host and pathogen DNA. Additional removal of host DNA may occur at this stage, and/or in metabarcoding studies. This is followed by DNA barcode amplification. In both metagenomic and metabarcoding studies, library preparation takes place before sequencing with long-read sequencers. Analysis of sequencing data would then be performed to generate a sequencing report which may include pathogen identification, epidemiological information, or an antimicrobial resistance marker (specific known mutations that confer resistance) maybe identified during metagenomic sequencing. This information in conjunction with the clinical evaluation then provides a diagnosis from which a treatment plan for the patient can be developed. Icons made by https://www.flaticon.com/authors/freepik.

Metagenomics

Metagenomic sequencing, also known as shotgun sequencing, aims to sequence all genetic material within a sample. In this approach, all genetic material (DNA or RNA) is extracted from the primary samples, which are then fragmented and undergo library preparation to suit the sequencing technology. The sequencing library then undergoes in-depth sequencing and data analysis (Fig. 3).[16] An advantage of metagenomics over metabarcoding is higher resolution, as more parts of the genomes of every organism in the sample are sequenced, potentially generating information beyond identification, such as antimicrobial resistance and virulence. The direct sequencing of genetic material eliminates the need for prior culturing and amplification, reducing the time from sample collection to identification, eliminating any bias that may occur due to these additional steps, resulting in a more accurate representation of the community composition.[17] In metagenomic sequencing studies, the overwhelming amount of background DNA compared to microbial DNA remains a challenge and methods to enrich microbial DNA have been developed.[18] Additionally, the cost and bioinformatic requirements of metagenomic sequencing is generally higher than those of metabarcoding studies. For full use of metagenomic sequencing reads, robust reference whole genome sequences are required. Long-reads generated in metagenomic studies are more correctly mapped to reference genomes and give high discriminatory power for accurate identification. However, the main current hurdles for long-read metagenomics based fungal identification are the fact that current genome databases lack adequate coverage for all fungal species.[19]

Metagenomic studies with long-read sequencers to identify fungal species are currently limited. However, preliminary studies have demonstrated their promising potential. PacBio sequencing of skin samples using a metagenomic approach identified a similar microbial community to short-read sequencing.[20] Additionally, metagenomic PacBio sequencing has been used in conjunction with short-read sequencing for genome assembly of fungal species in complex lichen samples.[21,22] Metagenomic nanopore sequencing identified pathogens from 87 patient samples in a single hospital study from a range of infections, including fungal infections, achieving sensitivity and specificity of 90.9 and 100% respectively, outperforming short-read sequencing.[23] The same approach was applied to three patient samples positive for *Pneumocystis jirovecii* and three negative respiratory samples.[19] All positive samples returned reads identified as

P. jirovecii. However, *P. jirovecii* was also detected in negative samples. Furthermore, fungal species were identified that are geographically restricted to areas that did not align with the patient's travel histories. These likely misidentifications were attributed to issues applying the bioinformatics tools to fungal identification. Additionally, all samples reported 77–95% *Homo sapiens* reads aside from one outlier (10%) demonstrating the high abundance of human background DNA.[19] Studies involving methods to overcome the limitations of metagenomic sequencing have emerged. The combination of metagenomic sequencing and whole genome amplification has been utilised to increase DNA quantity whilst maintaining the community composition to characterise the microbiome on the surface of oil paintings.[24] A method to deplete human DNA in clinical samples has been applied to respiratory samples and confirmed an *Aspergillus* infection previously diagnosed by culturing.[25] Additionally, this method also identified fungemia caused by *Candida glabrata* within 24 h whilst an extended culturing time (48–72 h to actionable results) was required for traditional identification.[25] Although there are currently drawbacks, with methods to overcome its limitations, long-read metagenomics is a favourable prospect for fungal identification.

Conclusion

Sequencing of genetic material has taken a significant step forward with the release of PacBio and ONT long-read sequencers. Their initial applications to fungal identification have indicated the promising potential for routine use in clinical diagnostics, microbiome characterisation, whole genome assembly and more, if the limiting factors of DNA extraction, low fungal-human sample DNA range, lack of reference sequences (DNA barcodes and whole genomes), and lack of bioinformatic tools, can be overcome. Implementation of long-read sequencing to diagnosis of fungal infections additionally requires a standardised workflow (Fig. 3). If successfully introduced into routine diagnosis of fungal infections it would drastically reduce turnaround time, from currently several days/weeks to less than 24 h (Fig. 3), enabling a timely and accurate induction of antifungal treatment, reducing mortalities, treatment and hospitalisation costs.

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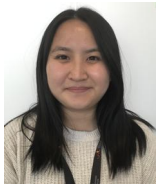

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'The awesome power of yeast'

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ABSTRACT

Yeast is one of the most useful microorganisms in society. Aside from the well known traditional uses in beer, wine and bread making, yeast is currently providing new opportunities for our society. This article examines some of those new opportunities which include using yeast as a model organism, yeast as a cell factory for valuable proteins, including vaccines and new therapeutics, and yeast as a very convenient tool for teaching.

Keywords: ageing research, cancer research, drug discovery, gene engineering, mitochondrial function, Nobel prizes, teaching, yeast-derived vaccines.

'The awesome power of yeast genetics' was a term generally believed to have been first used by Ira Herskowitz (1946–2003).[1] With thousands who have experienced the power of yeast genetics the phrase thrived and continues in new contexts today, not just yeast genetics. To date five Nobel prizes have been awarded to yeast researchers for their discoveries of important cellular mechanisms (Table 1). In this article we describe that awesome power that is available today (Fig. 1).

Yeast as a model organism

Yeasts are the best studied eukaryotes. Our advances in genetics, molecular and cell biology have been greatly aided by yeast, in particular *Saccharomyces cerevisiae*, whose genome was the first eukaryotic genome to be sequenced. Much of the information on the contributions of yeast to biology is readily available on the *Saccharomyces* Genome Database.[2] This information has aided the characterisation of many genes and proteins in humans. Functional complementation of yeast gene deletants with human genes is often observed in yeast, further allowing insights into human proteins in health and disease. To a lesser extent, several *Candida* species have been similarly exploited and the information compiled on the Candida Genome Database.[3]

Due to the conservation of important fundamental processes of eukaryotes from yeasts to humans, yeast have played crucial roles in the study of several human diseases.[4] The similarities such as age-associated loss of proteostasis makes them valuable models for diseases involving age-related proteinopathies. Through advanced synthetic biology approaches it is also possible to reconstruct yeast for the study of diseases like Alzheimer's disease, Parkinson's disease, Huntington's disease, prion disease, and cancers.[5–7]

Yeast also offers unique opportunities to study diseases involving mitochondrial defects since it can grow with defective mitochondria.[8] In addition, the conservation of cellular signalling and pathways from yeast to higher eukaryotes make it more highly useful in studying diseases involving a multitude of processes. For example, these attributes have been used in recent studies that have been outstanding in identifying compounds that can modify mitochondrial health.[9,10]

Yeast as a host for vaccines

The first viral subunit (for HBV and HPV) vaccines were made in yeast and they have been used safely for more than three decades. Since then, several important therapeutic proteins including vaccines have already been synthesised using yeast as a cell factory (Table 2).[11] In the 1980s, Macreadie and colleagues at CSIRO were involved in the development of the 2nd viral subunit vaccine for a poultry virus, infectious bursal disease virus.[12]

Can yeast be used to produce a COVID-19 vaccine? So far progress to the development of a yeast-derived COVID-19 vaccine has been slow compared to the many other COVID-19 vaccines. However, the efficacy of a yeast-derived receptor binding domain (RBD)-based COVID-19 vaccine looks to be promising.[13] A challenge with producing human proteins in yeast is the type of carbohydrate added to proteins (glycosylation).[11] Human glycosylation is complex, while yeast adds high mannose. This challenge has now been met, by synthetic biology approaches. *Pichia pastoris* was re-engineered with genes involved in human glycosylation to

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Table 1. Nobel prizes awarded to yeast researchers.

Year	Authors	Subject category	Discovery	Yeast species
2001	Leland H. Hartwell, R. Timothy (Tim) Hunt and Paul M. Nurse	Physiology and medicine	Key regulators of the cell cycle	*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*
2006	Roger D. Kornberg	Chemistry	The molecular basis of eukaryotic transcription	*Saccharomyces cerevisiae*
2009	Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostak	Physiology and medicine	Discovery of how chromosomes are protected by telomeres and the enzyme telomerase	*Saccharomyces cerevisiae*
2013	James E. Rothman, Randy W. Schekman and Thomas C. Südhof	Physiology and medicine	Machinery regulating vesicle traffic, a major transport system in our cells	*Saccharomyces cerevisiae*
2016	Yoshinori Ohsumi	Physiology and medicine	The discovery and elucidation of mechanisms underlying autophagy	*Saccharomyces cerevisiae*

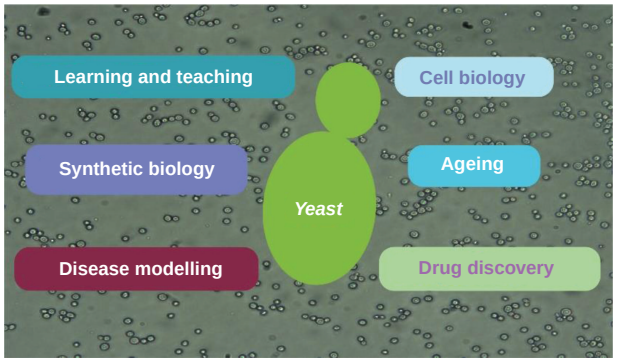

Fig. 1. Some applications of the awesome power of yeast. The background image shows yeast viewed by light microscopy.

produce up to 16 different types of mammalian glycosylation patterns. This has revolutionised the pharmaceutical industry and the use of *P. pastoris* for vaccine production.[14] Corbevax is an example of a *P. pastoris* expressed subunit vaccine, for COVID-19. It is patent free and is regarded as an effective, low cost vaccine candidate.[15]

Two hybrid technology and drug screening

Protein interactions are immensely important for assembly of protein complexes and for cell signalling. The yeast two hybrid system, pioneered by Fields and Song (1989) has provided technologies to discover human protein–protein interactions, including their precise molecular interaction interfaces.[16] Further, it is possible to use yeast displaying human protein interactions to find small molecules that disrupt these interactions.[17] This is a powerful approach for drug screening since molecules found in such an approach must already have a degree of bioavailability.[18]

Ageing

Yeast is also a useful model to study aging. Throughout their life most yeast species produce progeny by budding. With each bud that is released a bud scar is left at the surface and these scars can readily be stained with calcofluor white.[8] In every generation, 50% of the population are newborn yeast, while the remainder have one or more bud scars. Flow cytometry is a very convenient means to be analyse or isolate yeast populations, which provides a convenient means of observing the ageing of yeast and can facilitate study of the expression of ageing-related genes.[19]

Table 2. Some therapeutic proteins expressed in yeast for commercial purposes.[11]

Host yeast species	Therapeutic proteins	Target disease
Saccharomyces cerevisiae	Vaccine antigens	Hepatitis A and B
		Diphtheria
		Tetanus
		Pertussis
		Polio
		Haemophilus influenza type B infection
		Human papilloma virus (HPV) infection
	Lepirudin	Heparin-induced thrombocytopenia type II
	Insulin	Type I diabetes
	Glucagon	Hypoglycemia
	Human growth hormone	Dwarfism, pituitary turner syndrome
Pichia pastoris	Insulin	Type I diabetes
	Human serum albumin	Blood volume expansion
	Ecallantide	Hereditary angioedema
	Interferon-α 2b	Hepatitis C and cancer
	Anti-IL6R antibody	Rheumatoid arthritis
	Anti-RSV antibody	Respiratory syncytial virus infection
Yarrowia lipolytica	Pancrelipase	Exocrine pancreatic insufficiency
Hansenula polymorpha	Hepatitis vaccine	Hepatitis B

As noted by Dhakal (2021) proteostasis declines with aging and can readily be observed in yeast having the Alzheimer's disease protein, amyloid beta.[8] Anti-ageing drugs can readily be tested in such yeast models. For example, a study to screen several bioactive compounds found two excellent candidates (baicalein and *trans*-chalcone) that have potential to treat and prevent AD. Additionally, the synergistic activity of these two compounds in improving ageing health in the yeast that expressed amyloid beta was identified using such yeast models.[20]

Yeast as a tool in teaching

Yeast is GRAS (generally regarded as safe), fast growing and can be used as a convenient organism for teaching aseptic technique, metabolism, synthetic biology and cell biology.[11] Yeast are usually grown in rich media and the total absence of antibiotics, even for selection of transformants. Therefore, proper technique is required.

Yeast are eukaryotes and have the organelles of eukaryotes. There is beauty in the sight of mitochondria in live yeast, being able to look at them as structures resembling roots in a plant pot, rather the textbook image: an oval shape showing their cross section. Furthermore, the effect of mitochondria on growth can be readily demonstrated on media with a non-fermentable carbon source, and yeast lacking the ability to grow on such media can be observed. This lack of growth can happen when a mitochondrial inhibitor is added to the media (e.g. antimycin, oligomycin, erythromycin, chloramphenicol) or when the yeast contains a defect in its mitochondrial genome. This defect can include a point mutation or a deletion of a portion or all of the mitochondrial genome. Such mutants are known as mit$^-$ or petite mutants.

Conclusion

So far, yeasts have proved to be an awesome research tool for studying human diseases, a very convenient biological factory for production of several important therapeutic proteins and a platform for screening drugs against human diseases. Important discoveries made in yeast have always provided solutions to big problems in simpler ways.

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Data availability. Data sharing is not applicable as no new data were generated or analysed during this study.

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Biographies

Ian Macreadie has a 45-year history of yeast genetics. He was a research scientist at CSIRO for 24 years and then taught at RMIT University until 2020. He is currently an Honorary Professor of RMIT University. He is Editor-in-Chief of *Microbiology Australia*.

Sudip Dhakal is a PhD researcher and a tutor/instructor at the School of Science, RMIT University, whose research focuses on investigating therapeutic strategies against Alzheimer's disease using yeast models.

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Fluorescence lifetime imaging microscopy (FLIM): a non-traditional approach to study host-microbial symbioses

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ABSTRACT

Corals and their photosynthetic endosymbiotic algae (Symbiodiniaceae) produce a strong autofluorescent signal that spans the visible to near-infrared (NIR) spectrum. However, this broad-spectrum emission hinders the use of fluorescence *in situ* hybridisation (FISH) for the study of bacterial heterogeneity within the different niches of corals and Symbiodiniaceae, because FISH fluorophores also fluoresce within the visible to NIR spectrum. A solution to this impediment is to use fluorescence lifetime imaging microscopy (FLIM). The 'lifetime' property of fluorophores is a feature that enables sample (e.g. coral/Symbiodiniaceae) autofluorescence to be distinguished from FISH-labelled bacteria. In this manner, the location of bacteria around and within Symbiodiniaceae can be quantified along with their identity and spatial distribution. Furthermore, the 'lifetime' of the host and associated microbe cellular autofluorescence can be analysed in terms of endogenous fluorophore composition (e.g. metabolic co-factors, aromatic amino acids) and serves as information for symbiotic versus parasitic host-microbe association.

Keywords: autofluorescence, confocal microscopy, fluorescence lifetime imaging microscopy, label-free detection, microbial ecology, microbial symbiosis, phasor analysis, visualisation.

Introduction

Our understanding of microbes is largely based on classical phenotypic assays, advanced multi-omics, and optical microscopy methods. The discovery of genetically encoded fluorescent proteins (e.g. green fluorescent proteins), alongside advances in chemical fluorophore synthesis, has enabled exogenous incorporation of fluorescent molecules into a range of microbes, and development of fluorescence microscopy methods to visualise particular species within a complex biological system.[1] However, fluorescence labelling and visualisation of multiple microbial species within a mixed community remains a challenge, because of difficulties in establishing pure cultures[2] that are required for probe optimisation, and the presence of broad spectrum autofluorescence from different chemicals present in microbial consortia. In this review we explore how fluorescence *in situ* hybridisation (FISH) coupled with fluorescence lifetime imaging microscopy (FLIM) presents a unique opportunity to circumvent the latter technical hurdle in uncovering the *in situ* location and identity of bacteria in Symbiodiniaceae.

Fluorescence microscopy in microbial studies

FISH is a method that was first used to visualise bacteria in 1989[3] and it involves the hybridisation of fluorescently labelled oligonucleotides (~20 nucleotides) to the *16S* rRNA or *23S* rRNA in ribosomes, so that specific bacteria can be detected and their spatial location determined.[1,4] The fluorophores commonly available for FISH exhibit excitation–emission properties that span the visible to near-infrared (NIR) spectrum. However, since this spectral range overlaps with the broad spectrum autofluorescence of samples such as cyanobacteria or algae,[5] microbial biofilms,[6] and coral or Symbiodiniaceae cells,[7] selection of suitable FISH fluorophores to localise bacteria within these environments via conventional epifluorescence or confocal laser scanning microscopy (CLSM) has proven difficult. Nonetheless, there is a strong interest in the use of fluorescence microscopy methods that can distinguish FISH fluorophores from sample

autofluorescence. In addition to FISH-based visualisation of microbes, fluorescent dyes have been used to directly count microbes in environmental samples,[8] study microbial respiration activity using redox dyes,[9] determine proteins involved in peptidoglycan synthesis using synthetic fluorescently labelled D-amino acids,[10] and evaluate oxidative stress using enzymatically cleavable fluorophores.[11]

Fluorescence lifetime imaging microscopy

A fluorescence microscopy method that offers the opportunity to distinguish FISH fluorophore emission (and fluorescent dyes more broadly) from sample autofluorescence is fluorescence lifetime imaging microscopy (FLIM). FLIM is a method that measures the fluorescence *lifetime* of a fluorophore's emission rather than its fluorescence *intensity*. The fluorescence lifetime of a fluorophore is defined as the time spent in the excited state upon absorption of a photon, before emitting the photon and returning to the ground state (Fig. 1*a*). FISH fluorophores typically exhibit a fluorescence lifetime on the order of nanoseconds, depending on their individual chemical structure and molecular environment,[12] and in most cases this time is distinct from the fluorescence lifetimes of autofluorescent chemicals in a host organism, for example, Symbiodiniaceae (Fig. 2*a*, *c*). Thus, FLIM offers the capacity to readily detect FISH-labelled microbes within Symbiodiniaceae autofluorescence (Fig. 2*b*), despite their significant spectral overlap. Additionally, the fluorescence lifetime of holobiont (the collection of a host and its associated microbiome) autofluorescent endogenous chemicals (e.g. metabolic co-enzymes, structural proteins, vitamins, pigments and amino acids) can be determined and analysed to inform on the different molecular environments that host FISH-labelled bacteria. In particular, moieties of chlorophyll *a*, phycobiliproteins, nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), flavins, aromatic amino acids, porphyrins[13] all contribute to the fluorescence lifetime recorded throughout a holobiont, and these signals can be used as a label-free record of host and/or symbiont physiology.

FLIM imaging of FISH-labelled bacteria can be achieved using fluorescence (typically CLSM) microscopes and the time- or the frequency-domains (Fig. 1*b*, *c*). In the time-domain, the detection of fluorescence is coupled to the excitation source that is pulsed (one or two-photon) and a detection unit that can measure the arrival time of the emitted photons (e.g. via use of time-correlated single photon counting (TCSPC)). The successive photon arrival time within each FLIM image pixel is then represented in the form of a histogram and fitted to an exponential (or multi-exponential) that reports the characteristic fluorescence lifetime.[12] In the frequency-domain, the excitation source is sinusoidally modulated and the detection unit measures the phase delay and change in amplitude that the fluorescent emission undergoes with respect to the excitation source (demodulation). Both time- and frequency-domain data can be transformed into a phasor representation (Fig. 1*d*) which is a fit free approach for lifetime analysis that facilitates multiple-component analysis within each pixel of a FLIM image.[11] A representative phasor plot associated with Symbiodiniaceae and FISH labelled bacteria is shown in Fig. 2*d*.

Use of FLIM in microbial studies

So, what does this mean for microbiologists? Although FISH can enable taxonomic affiliation, quantification and localisation of probed microbial communities, distinguishing FISH probe fluorescence from host (e.g. Symbiodiniaceae) autofluorescence can be provided by FLIM (Fig. 2). The *in situ* determination of physiological and chemical processes

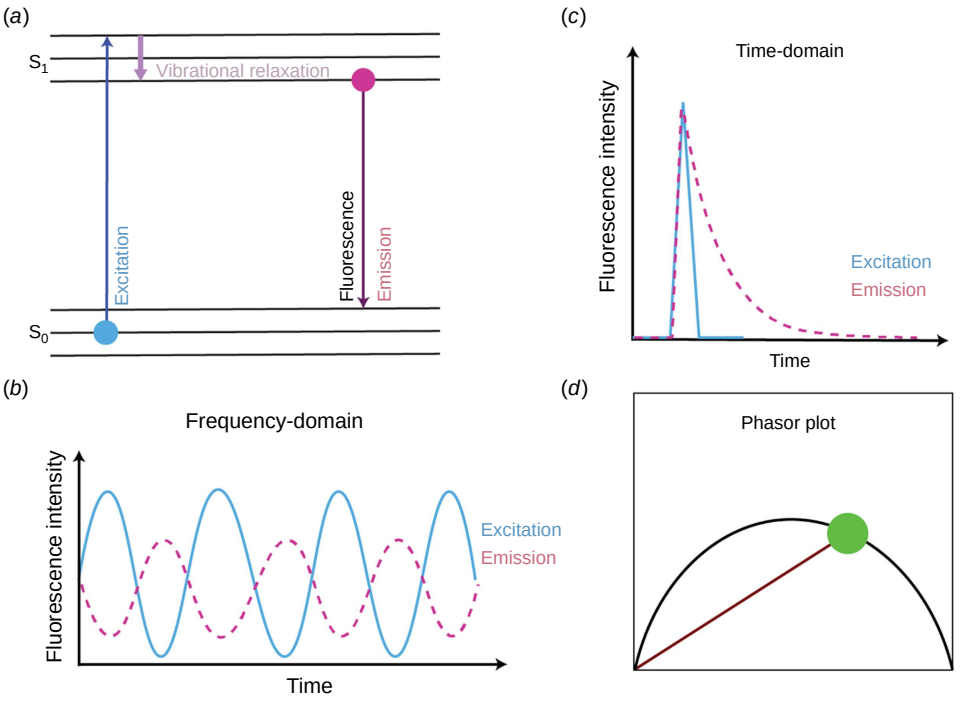

Fig. 1. (*a*) Schematic of molecular fluorescence (Jablonski diagram). An electron in the ground state (blue circle) upon absorption of a photon is excited to higher electronic state, where upon vibration relaxation, it emits a fluorescent photon (purple circle) and returns to the ground state. The fluorescence lifetime is the time spent in the excited state. (*b*) and (*c*) principle behind measurement of a fluorescent molecule's lifetime in the time- (*b*) versus frequency- (*c*) domain. (*d*) Graphical representation of the fluorescence lifetime recorded in the time or frequency domain transformed into a phasor within a phasor plot.

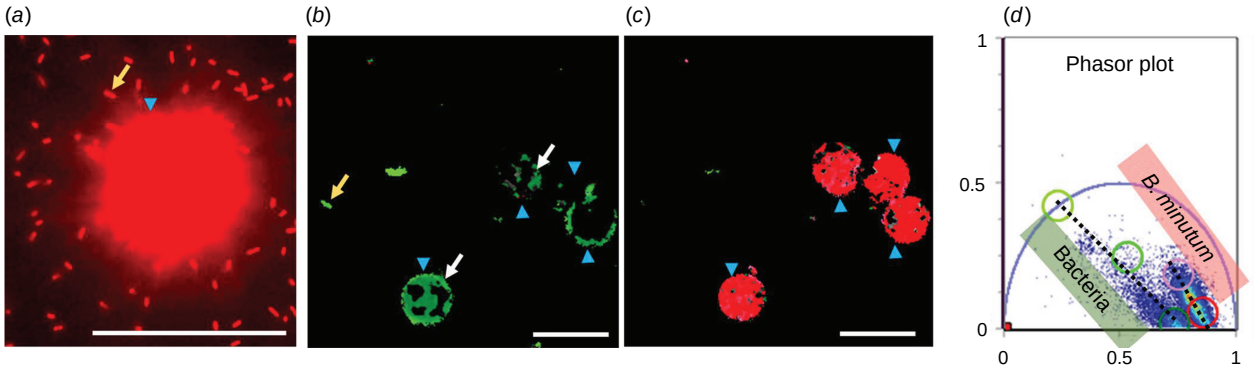

Fig. 2. Symbiodiniaceae (*Breviolum minutum*) and associated bacteria. (*a*) Epifluorescence microscopy image of bacteria (yellow arrow) associated with *B. minutum* (blue arrowhead). Bacteria were labelled with the EUBmix probe suite targeting the *16S* rRNA using FISH and labelled with ATTO 647N. The image was taken at excitation 640 nm and emission 656–806 nm. Any bacteria intracellular in *B. minutum* are masked by *B. minutum* autofluorescence (blue arrowhead). (*b*) Fluorescence lifetime imaging of intra- (white arrows) and extra-cellular (yellow arrow) bacteria (green colour) after FISH with EUBMix probe labelled with DY490 (laser excitation 488 nm, emission 500–550 nm). Four *B. minutum* cells (no visible autofluorescence) containing intracellular bacteria (green colour) are marked by blue arrowheads. (*c*) Fluorescence lifetime imaging of *B. minutum* autofluorescence (same four cells as in (*b*), blue arrow heads) (laser excitation 488 nm, emission 580–640 nm). Note that DY490 emission maximum is at 551 nm, so bacteria labelled with EUBMix-DY490 would not be visible at these conditions. (*b*) and (*c*) are fluorescence lifetime images pseudo-coloured according to the palette defined in the phasor plot (*d*). In the phasor plot the linear combination of fluorescence lifetimes that underpin DY-490 labelled bacteria green versus *B. minutum* autofluorescence are defined by a green and pink-red palette (respectively). Scale bar = 10 µm.

requires metabolic probing of cells,[4] which can be done by FLIM of endogenous metabolites. The lifetime property of fluorophores is influenced by the fluid viscosity, pH, temperature and ion concentration in the fluorophore vicinity, which provides information about fluorophore behaviour and function.[12] This is vital for monitoring cellular heterogeneity and changes in the local microenvironment of microbes. For example, NAD(P)H is an important co-factor in numerous biosynthetic pathways and has a characteristic lifetime signature at 340 nm excitation, 470 nm emission. The autofluorescence lifetime of free (0.4 ns) and enzyme bound NAD(P) (1–5 ns) can be monitored using two-photon excitation in a FLIM setup providing information on the physiological variation among microbial cells.[14]

Monitoring the metabolic activity of NAD(P)H is a particularly promising method to study host invasion[15] by beneficial (symbionts) and detrimental microbes (e.g. pathogens). For example, the acquisition of Symbiodiniaceae by coral larvae or early recruits is an important stage in the establishment of symbiosis. The variation in fluorescence lifetimes of endogenous compounds, including NAD(P)H and chlorophyll *a*, could help to determine physiological fitness of Symbiodiniaceae during its colonisation phase. In corals, climate change and other environmental disturbances are hypothesised to cause oxidative stress due to excessive build-up of reactive oxygen species (ROS), which leads to the separation of Symbiodiniaceae from the host,[16] known as coral bleaching. The autofluorescence of endogenous NAD(P)H has been used for co-localisation and quantification of oxidative stress using two-photon-FLIM.[17,18] Monitoring of enzyme-bound and free NAD(P)H dynamics is particularly important given the hypothesised role of NAD(P)H oxidase in ROS production in corals and Symbiodiniaceae.[19] These approaches could be implemented for visualisation and tracking of spatiotemporal dynamics of ROS to better understand their role in coral bleaching. FLIM could also be used to link bacterial associations (intra-versus extracellular endosymbionts) with Symbiodiniaceae by measuring the *in situ* heterogeneity during varying physicochemical conditions such as of temperature, pH,[12] and oxygen gradients[20] at the single-cell level.[21]

Another procedure that can measure elemental distribution in a sample is nanoscale secondary ion mass spectrometry (NanoSIMS),[22] which involves use of stable isotopes and FISH to visualise the incorporation of labelled substrates into single microbes in complex microbial communities. However, FLIM is a non-invasive procedure in comparison to the destructive technique of NanoSIMS.

The study of microbes in a spatially constrained environment using a fabricated microfluidic platform[23,24] combined with FLIM can aid in understanding biogeochemical processes at the microscale level, even in mixed microbial scenarios.

Several labelled and label-free FLIM detection approaches using specific (e.g. FISH) or non-specific fluorophores (e.g. Syto 13, DAPI, Hoechst 33342) and autofluorescent moieties (e.g. NAD(P)H and chlorophyll *a*) are outlined in Table 1.

Final remarks

Real time FLIM of labelled (via FISH) and label-free (via endogenous autofluoresence) microbes will provide a deeper understanding of the succession of endosymbionts within a host. The success of this prolonged live cell FLIM experiment will depend on the photostability of fluorescent probes and the stability of viable microbial cells. In recent years, advancement has been made in the use of photostable quantum dots (QDs), which are nanoparticles with long fluorescence lifetimes. QDs can be conjugated to FISH probes adding stability and flexibility to conventional FISH and FLIM.

Table 1. Examples of FLIM approaches to study physiological features of prokaryotes.

Application	Feature	FLIM technique	Reference
Monitoring of active biofilms	• Lifetime of porphyrin molecules to detect presence of biofilm forming *Helicobacter pylori* in the intracellular and extracellular matrix	• TCSPC using porphyrin autofluorescence	25
	• Enhanced spectral distinction of diatom and bacteria in ~40 µm thick lotic biofilm	• TP-FLIM using Syto13 stain and chlorophyll *a* autofluorescence	6,26
	• Change in metabolism as determined by fluorophore lifetime in biofilms formed under reduced, normal and elevated nutrient levels and in biofilms comprising distinct chemoautotrophic and heterotrophic microbes		
Metabolic activity	• Discrimination of bacterial species (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Bacillus subtilis*) and their growth stages	• TP-FLIM and Phasor-FLIM using NAD(P)H autofluorescence	14,27
	• Mapping metabolism via free versus enzyme bound NAD(P)H indicating physiological state of *E. coli* under selective pressure of bacteriostatic and bactericidal agents		
	• Tracking metabolic turnover of NADH in *P. aeruginosa* cells transitioning from planktonic (low virulence) to host-surface attached (virulent to amoebae)	• TP-FLIM using NAD(P)H autofluorescence	28
	• Mapping central carbon metabolism (citrate, pyruvate, glycerol, citrate as substrate) in *P. aeruginosa*		
Growth kinetics	• Lifetime mapping as an indication of early exponential, mid-exponential and stationary phase of pure cultures of *E. coli*	• TP-FLIM using nucleic acid label, SYTO 13	26
Membrane viscosity	• Estimation of lipid bilayer viscosity in live cells, sphaeroplast and liposomes containing lipid extracts of *E. coli*	• TCSPC using membrane specific dye, BODIPY C10 dye	29
Viability phenotyping	• Classification of live and dead (heat killed and chemically fixed) cells of *Streptococcus mutans*	• TP-FLIM using membrane voltage sensitive dye and lifetime and phase variable combined classifiers	30
Photosynthesis	• Temporal and cellular heterogeneity in the photosynthetic ability of the cyanobacterium *Acaryochloris marina*	• TSCSPC using autofluorescence of photosynthetic pigment, chlorophyll *d*	31
	• Distinct lifetimes of 'open' and 'closed' reaction centres, and estimation of rate kinetics of electron transfer		

TCSPC, time-correlated single photon counting; TP-FLIM, two-photon fluorescence-lifetime imaging microscopy; TSCSPC, time- and space-correlated single photon counting.

Additionally, the linear association between the QD's fluorescence lifetime and pH could be exploited to monitor intracellular pH in response to cellular disturbances. The study of endogenous metabolic co-enzymes, structural proteins, vitamins, pigments, and amino acids by their autofluorescence via FLIM has been a major experimental approach in the eukaryotic biomedical field but has not yet been widely employed in prokaryotic fields. Endogenous autofluorescent chemicals permit label-free detection of microbial associations, including studying actively dividing cells, in environmental scenarios. We envisage that the quantitative information obtained by phasor-based FLIM analysis of these probes, combined with multivariate tools can help discriminate between symbiont enriched and/or depleted zones in natural ecosystems.

The additional modalities of FLIM such as Förster resonance energy transfer (FRET) that measures differences in the lifetime of a fluorophore arising from either donor and acceptor molecules, will pave the way to understanding physical interactions between symbiont and host cell or between two different microbial cells in consortia. In FRET, the fluorescence emission shift occurs in instances where donors transfer energy (due to physical proximity and interaction) to acceptor fluorophores. The FLIM-FRET technology is a promising means of studying environmental microbial associations (e.g. protein or metabolite translocation between symbiont and host or between two different microbes) and is yet to be explored.

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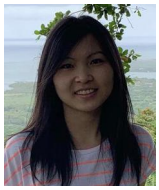
Dr Pranali Deore is an environmental microbiologist, and her current research focuses on developing advanced bio-optic tools for visualisation of microalgae-bacteria symbiosis. Her expertise is in microalgal photo-physiology and use of Fourier transform spectroscopy to study microbial interactions. She is currently a post-doctoral research fellow at the University of Melbourne.

Dr Iromi Wanigasuriya is a molecular biologist and a research fellow at The University of Melbourne. She is currently using advanced microscopy techniques to understand dynamic epigenetic mechanisms.



Sarah Jane Tsang Min Ching has a Master of Science and is a research associate at The University of Melbourne. She uses advanced molecular biology and microbiology techniques for isolation and identification of microbial symbionts of corals. Her past research involves understanding metabolomic changes in sea anemones (laboratory model of corals) transplanted with heat evolved Symbiodiniaceae strains.

Dr Douglas Brumley BSc (Hons), PhD (Cantab) is a Senior Lecturer at The University of Melbourne. He leads an interdisciplinary research group which utilises mathematics, microfluidics and microscopy to study a range of dynamic processes in biology including bacterial motility, symbioses, nutrient cycling and flows around coral reefs.

Professor Madeleine J. H. van Oppen is an ecological geneticist with an interest in microbial symbioses and climate change adaptation of reef corals. Her early career focused on evolutionary and population genetics of algae and fish, and subsequently corals. Currently, her team is using bioengineering approaches aimed at increasing coral climate resilience and the likelihood that coral reefs will survive this century. These interventions include coral host hybridisation and conditioning, directed evolution of microalgal symbionts and bacterial probiotics.

Professor Linda L. Blackall is an environmental microbial ecologist, who has studied many different complex microbial communities ranging from host associated through to free living in numerous environments. One of her research fields is the microbiota of corals and sponges. The numerous methods she develops and employs in her research allow elucidation of microbial complexity and function in these diverse biomes.

A/Professor Elizabeth Hinde is a cellular biophysicist with an expertise in fluorescence lifetime imaging microscopy and fluorescence fluctuation spectroscopy. She develops methods to spatially map live cell nuclear architecture and is using this technology to uncover the impact this dynamic structural framework has on DNA target search.

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Correct interpretation of actinomycete imagery using scanning electron microscopy

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ABSTRACT

Antibiotic discovery was one of the most significant advances in therapeutic medicine following the advances in fermentation technology owing to Howard Florey and his associates. The 'Golden era' of antibiotics following the first discoveries in the laboratory of Waksman and his colleagues from a group of microorganisms known as actinomycetes lasted for 34 years (1940–1974). These fascinating microorganisms especially the members of the genus *Streptomyces* gave us the majority of the known antibiotics we use today, like streptomycin, kanamycin, neomycin, gentamicin, vancomycin and many more. To be able to produce these antibiotics in large-scale, the producer actinomycetes had to be selectively isolated. This resulted in a collaboration of over 40 laboratories from 18 different countries called 'The International *Streptomyces* Project (ISP)'. The isolates generated in this project were studied in-depth including their morphologies together with their bioactivity. One of the components of these investigation was the correct interpretation of actinomycete morphology including the use of scanning electron microscopy. At the end of the first European Actinomycete Conference in Bradford University in England (1984), I had the opportunity to be trained by late Professor Cross on actinomycete growth morphologies. Thirty-eight years later when I witness the frequent difficulties students encounter in the interpretation of the actinomycete SEM images, I decided to write this paper and pass the skills given to me by late Professor Cross to the younger generation.

Keywords: *Actinomycetales*, Actinomycetes, *Actinomycetia*, *Actinomycetota*, growth morphology, SEM imagery, *Streptomyces*, taxonomy.

In Memoriam, Professor Tom Cross, University of Bradford, UK.

Current classification of actinomycetes

First discovery of a species within this group of bacteria dates to Harz in 1877[1] by the description of *Actinomyces bovis*. Later Buchanan named the order *Actinomycetales*[2] and in the subsequent year named the family Actinomycetecaea.[3] Stackebrandt *et al.*[4] proposed a new Class called Actinobacteria under the Domain Bacteria, followed by the creation of the phylum with the same name.[5,6] In this restructure order, *Actinomycetales* was confined to the original Hartz cluster that is comprised of non-mycelial taxa (e.g. *Actinomyces*).

The use of the same name both for the phylum and the class was, however, not preferable, and recently a new class named Actinomycetia was proposed by Salam *et al.*[7] covering all members of the former order *Actinomycetales*.[8] Here caution must be exercised as currently the order *Actinomycetales* covers the family Actinomyceteceae (Buchanan original description).[9] So, families like Streptomyceteceae that formerly belonged to this order[10] cannot be located under this order any longer. In addition, in 2021 the name of the phylum Actinobacteria revised again into Actinomycetota.[9] In summary, the phylum Actinomycetota is the former phylum Actinobacteria. The proposed Class Actinomycetia is the former class Actinobacteria. Class Actinomycetia also covers the members of the former order *Actinomycetales*, which is different to the current order *Actinomycetales*. Finally, to conclude, every member under the class Actinomycetia is an actinomycete (actinomycetes in plural) in a general term that has been in use over 70 years.[10]

Another important point is that RNA oligonucleotide studies showed that the possession of branched hyphae (e.g. *Thermoactinomyces*) should not automatically place a bacterium within the class Actinomycetia, nor should the inability of an organism to form branching

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Table 1. Distinctive growth morphologies of actinomycetes.[12,13]

Growth morphology group	Substrate mycelium	Aerial mycelium	Colony texture on agar medium	Example genera
Group 1 (see Fig. 1*a*)	Soon fragmenting into various-sized rod-coccoid elements	None	Soft, bacterial-like	*Agromyces*, *Oerskovia*, *Rhodococcus*
Group 2 (see Fig. 1*b*)	Substrate mycelium is not fragmenting	None	Tough	*Micromonospora*, *Actinoplanes*, *Dactylosporangium*
Group 3 (see Fig. 1*c*)	Substrate mycelium is fragmenting into various sized rod-coccoid elements	Dry, powdery-cottony aerial mycelium is formed but breaks down sooner	Moderately soft	*Nocardia*, *Nocardioides*
Group 4 (see Fig. 1*d*)	Substrate mycelium is not fragmenting, stable	Dry, powdery-cottony aerial mycelium is formed	Tough	*Streptomyces*, *Actinomadura*, *Thermomonospora*, *Microbispora*, *Microtetraspora*, *Planomonospora*

filaments (e.g. *Arthrobacter*, *Cellulomonas* and *Rothia*) necessarily exclude it from this taxon. Accordingly, genus *Thermoactinomyces* with its low guanine and cytosine content and endospore forming ability was removed from the actinomycete cluster and placed into the family *Bacillaceae*.[11]

Distinctive growth morphologies of actinomycetes

Whichever family they belong to, actinomycetes only display four different types of growth morphologies (Table 1). Full understanding of these structures is imperative during interpretation of the SEM images.

It is also important that intact material is not damaged during processing for the SEM such as when gradual dehydration in alcohol is done prior to critical point drying. Any damage during processing can result in wrong interpretation of the image e.g. spore surface morphology, especially for the structures 'rugose' and 'warty'.[8,14]

Another important aspect is the timing of the examination for growth morphologies. Different microscope slide preparations should be prepared and examined in consecutive days. Young colonies will exhibit different morphologies as full maturation is not complete as well as too old colonies will result in collapse of spore chains. Spore surfaces also should be examined at right times (this differs for all above listed four different growth morphologies) to be able to identify spore surface structure correctly. As an example, a smooth looking spore surface might change into a 'hairy' or 'spiny' structure[8] later in the growth cycle (Fig. 2).

As noted by the late Professor Cross (1989),[15] choice of media is important as most sporoactinomycetes would require special media to allow differentiation and development of characteristic spores and pigments. His examples include, the transformation of pale, shiny, hard colonies of a *Streptomyces* species on nutrient agar into bright yellow colonies with a powdery white aerial mycelium and spirals of arthrospores when the organism is subcultured onto a more suitable growth medium such as oatmeal or inorganic salt starch agars.[16] More recent examples of such differences in growth can be seen in recent publications of English *et al.*[17] and Kurtböke.[18]

Again, the late Professor Cross (1989)[15] highlighted that the actinomycete outgrowths starts from fragments of mycelium and develop into hyphae that penetrate the agar forming the substrate mycelium and hyphae that branch repeatedly and become cemented together on the surface of the agar to form a tough, leathery colony. He also added that actinomycete growth can be slow, a branching mycelium growing at the surface of transparent agar can be seen with the aid of a microscope after 24 h, and visible colonies may appear in 3–4 days, but mature aerial mycelium with spores may take 7–14-days to develop, and some very slow growing strains may require up to a month of incubation. Lengthy incubation times can result in evaporation of the medium, so thick agar plates are required. Thermophilic species incubated at high temperatures require a humid incubator.

A useful diagram of the developmental life cycle of a *Streptomyces* species is provided by Barka *et al.*[19] illustrating growth from sporulation to development of substrate (vegetative) and then aerial mycelium leading to septation and formation of spores.

Sample preparation for SEM

Streaking actinomycete spores/hyphal fragments on agar medium

At all times a 'rough, stiff loop is essential for abrading the colony and collecting sufficient mycelial fragments for an efficient transfer'. Spore suspensions can also be prepared first and used for streaking.[15] They can be prepared 'by detaching the spores from aerial hyphae with a loop or scraper and placing them in a suspending medium containing a wetting agent'. The arthrospores of streptomycetes are hydrophobic because of the enveloping sheath, and the wetting agent aids their even suspension in the diluent. Free spores may also be removed from lawns of aerial mycelium by rolling glass beads or agar cylinders over the surface.[15]

Inclined cover slip technique

Best results can be achieved using inclined glass coverslip technique[15] and Oatmeal agar supplemented with yeast extract.[12] Once the actinomycete is streaked plated onto this medium, round cover slips can be embedded onto the

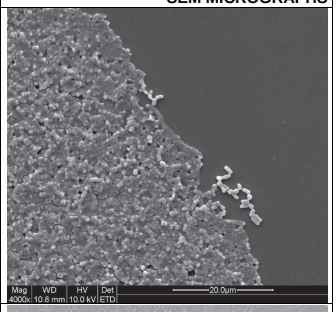
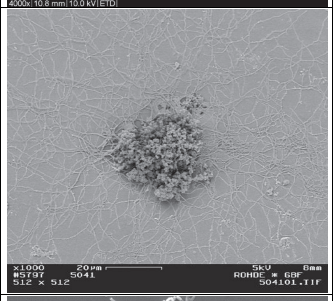
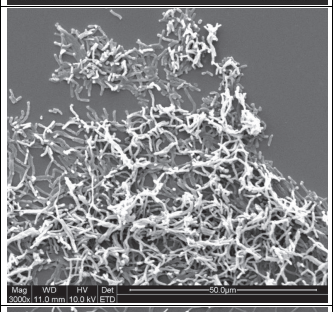
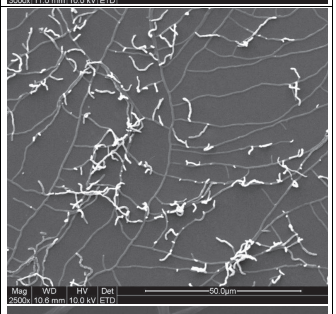
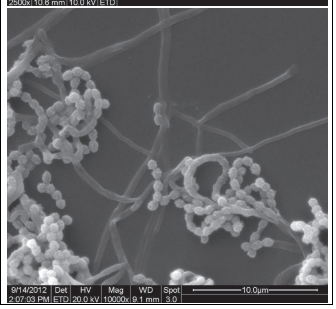
Growth Morphology Group	SEM MICROGRAPHS
Group 1 *Rhodococcus* species (USC-10041)(*a*) Substrate mycelium soon fragmenting into various-sized rod-coccoid elements. No aerial mycelium	
Group 2 *Micromonospora* sp. (USC-5041)(*b*) Substrate mycelium is not fragmenting, stable, no aerial mycelium, spore clusters are on the substrate mycelium	
Group 3 *Nocardia* sp. (USC-10003)(*c*) Substrate mycelium fragmenting into various sized rod-coccoid elements, aerial mycelium is formed, however, breaking down quicker than the category 4	
Group 4 1] *Streptomyces* sp. (USC-50006)(*d*-1) Substrate mycelium stable, not fragmenting, aerial mycelium is formed 2] *Streptomyces* sp. (USC-633)(*d*-2) Substrate mycelium stable, not fragmenting, aerial mycelium is formed and matured into spore chains followed by spore formation	 

Fig. 1. Growth morphologies of actinomycete groups captured using SEM.

streaked lines with a 45° angle. Growth starts and developing mycelia simultaneously moves onto the coverslip. Multiple cover slips would allow replication and observations to be conducted at different growth times.

Once the growth is sufficient the cover slip can be removed, place onto SEM stubs, fixed with osmium tetroxide, gradually dehydrated in alcohol before being subjected to critical point drying and subsequently coating with gold.[20,21] In this final stage, it is imperative that experienced technical

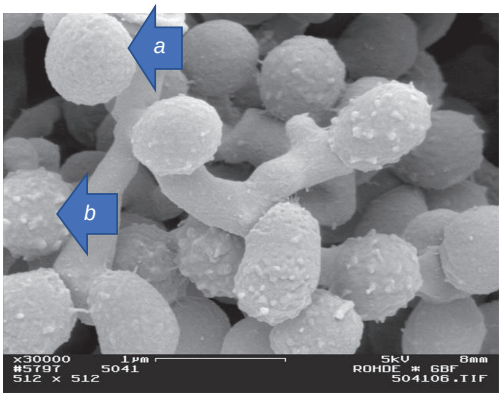


Fig. 2. Spore maturation of a *Micromonospora* species (USC-5041): (*a*) early stage, (*b*) mature stage where spines are formed.

officers who have acquired knowledge on the use of SEM assist the researcher who is engaged in image interpretation.

Modified techniques were also developed, such as the one by Prakash and Nawani[22] in which lyophilisation is used rather than chemical fixatives and dehydrating agents.

Conclusions

Again, as stated by the late Professor Cross,[15] 'one requires patience when working with actinomycetes, and the ability to plan and run several experiments concurrently to avoid wasting time'. I would like to add the importance of students understanding the long and arduous route from being a novice to becoming an expert. Hard work and perseverance is important to patiently build their knowledge and gain laboratory skills.[23] What is presented here is the outcome of 40 years of continuous learning and skill building in the field of actinomycetology.[24] Understanding the value of team work as well as appreciating the roles of other disciplines and experts such as technical staff members in EM units, without whom the quality micrographs cannot be produced, is also imperative for novices.

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Data availability. Data is embedded in the text as SEM micrographs.

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Biography

Dr Kurtböke is currently a senior lecturer at the University of the Sunshine Coast (USC) in Australia and one of the members of the Genecology Research Centre of the USC, conducting research in applied, industrial and environmental microbiology. She is an internationally reputed actinomycetologist and she has been in the field of biodiscovery since 1982 conducting research into discovery of novel and potent therapeutic compounds produced by actinomycetes in Turkey, Italy, the UK, and Australia with leading pharmaceutical companies. She has been an Executive Board member of the World Federation of Culture Collections (WFCC) since 2000, currently serving her second term as the President of the Federation. She is also one of the members of the International Committee on Taxonomy of Viruses (ICTV)'s, Bacterial Viruses Subcommittee. She has editorial duties in different journals including *Marine Drugs, Diversity and Frontiers Marine Science/Marine Biotechnology*.

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Feedback loops between mathematics and microbiology

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ABSTRACT

The combination of mathematical modelling and quantitative video-microscopy provides exciting opportunities for elucidating the mechanisms behind key processes in microbial ecology, ranging from cell navigation and nutrient cycling to biofilm establishment and symbioses. Central to this approach is the iterative process, whereby experiments and modelling inform one another in a virtuous cycle: vast quantities of experimental data help to test and refine mathematical models, the predictions from which feed back to the experimental design itself. This paper discusses recent technologies, emerging applications, and examples where calibrated mathematical models enable calculation of quantities that are otherwise extremely difficult to measure.

Keywords: applied mathematics, chemotaxis, fluid dynamics, microbial ecology, microfluidics, motility, navigation, video-microscopy.

Microorganism behaviour and interactions unfold at the microscale and are inherently dynamic. Ecological processes depend on spatial structure of the environment, as well as the arrangement and behaviour of microbes. For example, the capacity for microbes to actively navigate heterogeneous environments using chemotaxis can dramatically influence their nutrient acquisition and establishment of symbioses;[1] ubiquitous fluid flows in the environment (e.g. ocean, groundwater, gut, mucus) influence the motility of microbes and reshape the environment;[2] and Brownian motion can affect encounter rates between microorganisms.[3] While functional complexity of microbes is well documented, it can be very difficult to examine spatiotemporal processes using traditional tools in microbiology. The combination of visualisation in controlled environments and mathematical modelling is uniquely positioned to resolve dynamic features of microbial lifestyles, and the ways in which these scale up to ecosystem-level processes.

The growing use of microfluidics and video-microscopy has facilitated the direct imaging of microbial behaviour in a range of realistic controlled microenvironments. Typically fabricated from flexible, optically transparent elastomers such as polydimethylsiloxane (PDMS), microfluidic devices can be used to create precisely controlled physicochemical conditions.[4] Steady gradients or transient pulses of oxygen, amino acids, and sugars can be used to interrogate the chemotactic ability of microorganisms.[5,6] Microscale fluid flows can be generated with exquisite precision using ultraslow syringe pumps, with flow fields exactly solvable using known dimensions. Various other additional stimuli – including light illumination patterns, electric and magnetic fields[7] – can readily be overlayed. Taken together, this enables the user to accurately recreate physical and chemical features of realistic microenvironments – for example from the ocean or soil – in a device small enough to fit on a microscope slide (Fig. 1).

Mathematical modelling has been applied extensively over many decades to model phenomena in microbial ecology. These approaches can involve directly simulating the motion of individual cells (agent-based models); abstracting organisms or chemicals as continuous fields which vary in space and time (continuum models); modelling sensory pathways or physical processes using reduced-order dynamical systems; and using statistical models to process and interpret sequencing data. For example, understanding of pattern formation in growing domains,[8] limits to chemoreception,[9] and motility patterns of swimming microorganisms[10] stemmed from mathematical models. More broadly across microbiology, mathematical modelling has been used to investigate metabolism in microbial communities and the transmission of infection in disease epidemiology. The success of mathematical models hinges on accurately identifying the essential features of the biological system that shape the ecological processes, and detailed parameterisation of the model.

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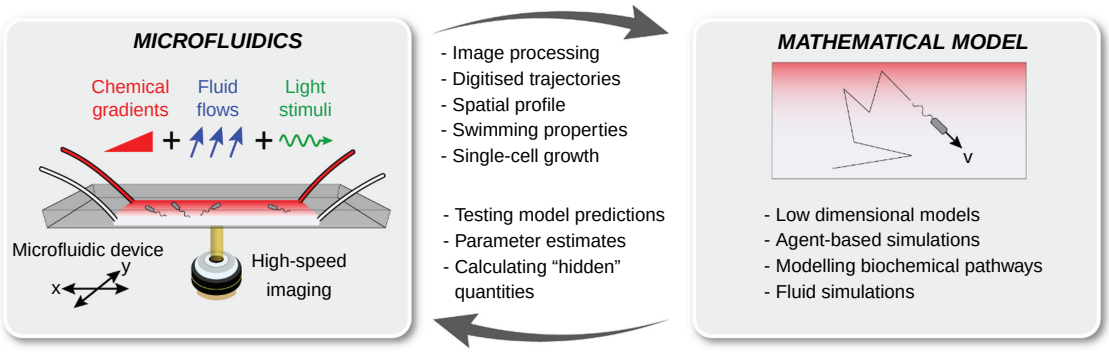

Fig. 1. Feedback loop between microfluidic experiments and mathematical models. High-speed imaging of microbes in precisely controlled microenvironments provides data that can be analysed using image processing algorithms. The digitised trajectories provide vast quantities of single-cell data, for example, spatial distribution of cells, individual swimming properties over time and single-cell growth rates across the population. These data inform mathematical models, enabling robust model testing, parameter estimates, and the calculation of quantities that are extremely difficult to measure experimentally. These model outputs facilitate refinement of experimental design.

The feedback loop between experiments and mathematical modelling is essential for understanding various processes in microbial ecology. Previous works have highlighted the utility of microfluidic devices for visualising dynamic processes.[4] However, these technologies realise their full potential when combined with mathematical modelling, particularly through iteration between experiments and theory. Because experimental environments (e.g. chemical gradients, flow, light, fields) can be independently and continuously varied, the predictions of mathematical models can be thoroughly scrutinised. Where differences occur, models can be updated, and further experiments conducted (Fig. 1).

What are the key elements that facilitate this iterative loop? Video-microscopy of microbes in carefully controlled arenas enables collection of vast quantities of data, for example in the form of high-speed movies, time-lapse photographs, or fluorescence intensity. Image processing algorithms – often conducted in MATLAB, Python, ImageJ – result in digitised trajectories, for example, the position of all motile bacteria at all time-steps[11] or the growth dynamics and lineages of cells throughout a biofilm.[12] From these data, cell concentration profiles, swimming speeds, turning angles, and attachment events[13] can be quantified with great precision. Although visualisation is a key step, this methodology is not merely 'observational'. The extracted information can be either used to parameterise mathematical models or directly compare with the predictions of simulations or modelling efforts.

Many aspects of microorganisms' environment and lifestyle can be modelled explicitly using known governing equations and physical principles. The Navier–Stokes equations can be used to precisely calculate how fluid flows and local shear transport and rotate microbes;[14] the transport and spread of dissolved organic matter can be solved using the advection-diffusion equation; and buoyancy forces, gravitational torques and magnetic fields can be readily included.[15] Explicit calculation of the hydrodynamic flow fields around swimming microorganisms[16] allows one to determine how organisms physically interact with one another[17] as well as in dense suspensions.[18] Mathematical modelling can also shed light on complex biochemical pathways and whole cell dynamics. These range from low-dimensional models, where a full sensory pathway can be abstracted as a dynamical system with few parameters,[19] through to systems biology approaches that involve high-dimensionality modelling of many processes in a cell.[20] Despite their apparent simplicity, minimal models have elucidated how different bacteria navigate chemical profiles using for example logarithmic sensing[21] or fold change detection,[22] and how the discrete molecular nature of chemical attractants places limits on gradient detection.[23] These models, which enable prediction of microbial dynamics in arbitrary settings with great accuracy (~1% fitting error)[24] despite few parameters, were developed and validated with the large quantities of data obtained through microfluidics and microbial tracking.

To close the feedback loop, model predictions must be able to inform refinements of experimental design. In the simplest case, this can be using mathematical models to identify the key regions of parameter space to be studied experimentally, for example, determining the domain size, imaging timescales or nutrient concentrations at which specific phenomena are likely to occur. Pioneering work of Berg and Purcell,[9] later extended by others, calculated the strength of a chemical gradient necessary to elicit a chemotactic response. Experiments can also be designed to test the robustness of the model – for example, would a model for chemotaxis still work if the gradients were increased by a factor of 10 or 100? Challenging models through a suite of different experiments removes the likelihood of coincidental agreement between experiments and modelling.

Mathematical models can reveal specific phenomena that might not otherwise be seen in experimental systems. Knowledge of how fluid gradients reorient cells suggested that swimming microbes exhibit different behaviours as fluid shear is varied; advection–diffusion modelling suggests that external flow can dramatically influence quorum sensing of microbial communities;[25] and theoretical work on confined active suspensions[26] hinted that confining boundaries could stabilise bacterial suspensions into vortical flow patterns.

Mathematical modelling enables one to calculate important parameters and quantities that cannot currently be measured directly. Marine nutrient cycling and

biogeochemistry is underpinned by the concerted action of microbial populations, and determining precisely how microbial behaviour (e.g. motility, navigation) influences nutrient uptake, represents a key challenge. Resource acquisition is readily measured using bulk techniques, for example by examining cell growth rates and dry mass measurements. At the single-cell level, Nanoscale secondary ion mass spectrometry (NanoSIMS) and other quantitative imaging tools[27] provide highly quantitative measurements for nutrient uptake, but can be prohibitively expensive and time-consuming. However, in microfluidic arenas where the nutrient landscape can be spatially controlled and the bacterial positions readily measured,[24] it is straightforward to calculate single-cell nutrient exposure across the entire bacterial population. Quantities such as the energetic cost of swimming, which can be extremely challenging to measure biochemically, can be readily calculated using fluid dynamical models which consider the shape, swimming speed, and hydrodynamic drag on a cell body.[28] This enables quantitative predictions about optimal strategies for microorganisms in various realistic environments. For example, the competition between non-motile and chemotactic bacteria is shown to depend sensitively on the local ocean productivity.[29]

Perhaps the most powerful aspect of mathematical modelling is the capacity to investigate the implications of varying key physical parameters and examining how sensitive results are to changes in microbial behaviour. For example, how does the nutrient uptake change as chemotactic sensitivity is modified, and is there an optimal value? How does IgA-mediated agglutination of pathogens vary as antibody stickiness and cell growth rates change? Mechanistic models can either examine key metrics for given parameters, or allow these parameters to vary over time, known as *in silico* evolutionary experiments.

Future directions. We have discussed the iteration between experiments and modelling, communicated through data and model outputs. The greatest advances thus far have been in relation to single microbial species or simple communities. Community analysis is typically performed at a very high level, e.g. through metagenomics, but is not well suited to exploring spatial and temporal effects, and physical processes involved in the microbial lifestyles. An important area for future exploration will be examining interaction between multiple species and assessing the role of spatial heterogeneity and resource diversity in natural communities.

Another clear future area of research is the application of machine learning (ML) and artificial intelligence (AI). In the shorter time, this is likely to assist with the extraction of data from experimental results (e.g. image segmentation, cell identification), which can be fed to mathematical models as outlined earlier. However, in the longer term, AI will likely assist in the modelling phase itself, proposing and testing reduced order models, or guiding human intuition.[30] Through exposure to large datasets, AI may be able to 'learn' how microorganisms behave, either as individuals (e.g. chemotaxis, motility patterns, growth rates), or communities (e.g. symbiosis partnerships, community dynamics). This offers the tantalising possibility of extrapolating behaviour from small numbers of different species – for example, chemotaxis towards specific compounds, symbiosis between pairs of organisms – to complex communities with multiple metabolic interactions. Despite these possibilities, ML and AI find it difficult to provide reasoning for their predictions. So, there will be a trade-off between accurate prediction of dynamics and revealing underlying mechanisms or physical principles (at least in the short term). Taken together, the combination of quantitative video-microscopy, mathematical modelling, and machine learning, is promising for providing quantitative understanding of how complex microbial communities behave.

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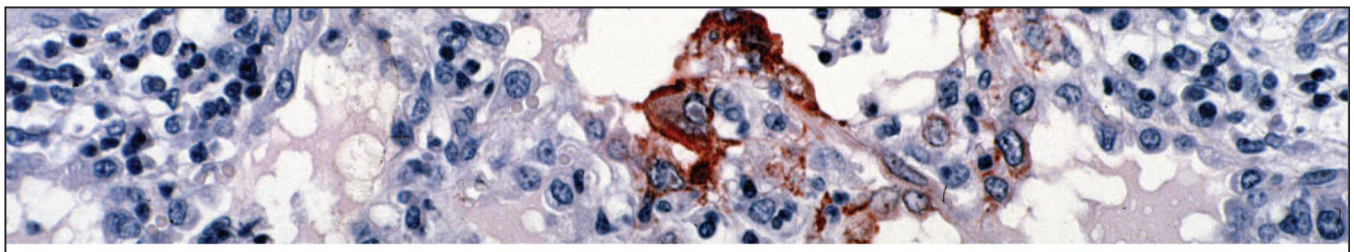

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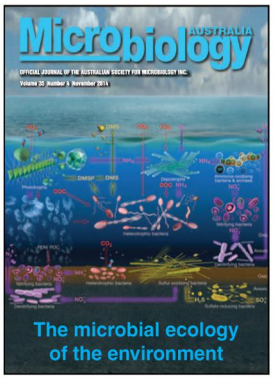

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Microbiology AUSTRALIA

Detection and control of off-flavour compound-producing streptomycetes on locally produced nuts using streptophages

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ABSTRACT

Members of the phylum Actinomycetota are the most prominent part of the soil microbiota, more specifically the species within the genus *Streptomyces* of this phylum. Key functions of *Streptomyces* species (or streptomycetes in general terms) include nutrient cycling and plant growth promotion and disease protection. However, these species can also produce volatile organic compounds, predominantly geosmin, which is responsible for musty and mildew scents that are unpleasant to humans and can negatively impact the nut crop industry as odorous nuts generally lose their market value. Bacterial viruses, called bacteriophages have been previously used successfully in agriculture and aquaculture to remove such odorous species and they may therefore be applied to the nut industry. To eliminate these compounds, the producer streptomycetes may be selectively removed from nut surfaces using streptophages. The removal of *Streptomyces* species from nut surfaces can then be expected to minimise geosmin production, therefore removing the unpleasant off-flavours and benefiting the nut industry.

Keywords: actinomycetes, bacteriophages, food taints, geosmin, nuts, Streptomyces, streptophage, volatile organic compounds.

Introduction

Streptomycetes as the producers of volatile organic compounds

Over a thousand microbial volatile organic compounds (VOCs)[1] have been identified from streptomycetes, many of which are acids, alcohols, aldehydes, alkenes, benzenoids, esters, ketones, pyrazines, and terpenes.[2] VOCs produced by *Streptomyces* species can benefit agriculture via the production of bioactive compounds, which can assist in bacterial and fungal growth inhibition, plant growth promotion or inhibition, and invoke resistance mechanisms.[2]

Geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol), 2-methylisoborneol (2-MIB; 1,2,7,7-tetramethyl-exo-bicyclo-heptan-2-ol), and dimethyl disulfide are three of the major VOCs produced by *Streptomyces* species.[3–5] Geosmin and 2-MIB are semi-volatile and terpenoid secondary metabolites.[6] Streptomycetes produce these compounds via the 1-deoxy-D-xylulose 5-phosphate/2-*C*-methyl-D-erythritol 4-phosphate (DOXP/MEP) and melavonic (MVA) pathways.[7] Likewise, dimethyl disulfide is a volatile sulfur compound produced via methionine degradation followed by methanethiol oxidation.[8,9]

Taste and odour compounds in environment

VOCs are also known as taste and odour compounds (T&Os) due to their detectability by humans. Humans can detect these T&Os-VOCs at concentrations of 4 ng/L, due to their olfactory sense.[4,10] Furthermore, geosmin synthase genes, which enables geosmin production, are broadly distributed within the members of the genus *Streptomyces*.[4,11] These compounds arise in food products, such as nuts and fish, via bioaccumulation from plant debris, soil, and water use.[6,12,13] Further accumulation of geosmin can occur in storage silos if left unmaintained due to continued growth of streptomycetes, not only resulting in strong odours but also giving rise to organic dust toxic syndrome.[14] Geosmin has an earthy flavour,[15,16] yet there has been no successful technique to remove these VOCs due to the ineffectiveness and high cost of current methods.[17] A recent study conducted at the University of the Sunshine Coast (USC) aimed to remove VOCs on locally-produced and openly sold nut samples using streptophages.

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Bacteriophage safety in agricultural products and humans

Bacteriophages have seen a rise in use as biocontrol agents in agriculture, bioprocessing, and healthcare.[18,19] Bacteriophages are regarded as safe for animals, humans, and plants, further promoting their use in the previously mentioned industries.[20–22] Chibani-Chennoufi *et al.*[23] reported that mice exposed to an oral four-phage cocktail did not experience a decline of their commensal *E. coli* biota. Bruttin and Brüssow[24] also reported that human volunteers orally exposed to phage T4 maintained their commensal *E. coli* population.

Findings of an example study from the Sunshine Coast region

Streptomycetes

Eight streptomycetes were isolated from seven different locally produced and openly sold nut samples using two different isolation methods (air compaction using an air sampler[25] and conventional serial dilution[26]) and incubation temperatures (28°C and 37°C) to maximise the chances of detection of these odorous species. Details of these isolates are given in Table 1.

Streptophages

Like bacteria, bacteriophages are present in agricultural environments where the host bacteria reside.[27] Usually bacteriophages are host specific, however, they also display polyvalency within the host's taxonomic rank.[22] The three major families of actinophages are Myoviridae, Siphoviridae, Podoviridae. Siphoviridae morphology is the most abundant one, particularly in soil among the actinophages.[28,29] This group of phages is mostly polyvalent within the Streptomycetaceae family to which the genus *Streptomyces* belongs. They are commonly known as streptophages[30] and morphologically consist of a long and flexible noncontractile tail. The siphoviridae heads contain portal protein at the vertices, which connects the head and tail segments, while the other vertices contain capsid proteins[31] and the tails usually are 100–400 nm in length depending on the species.[31]

Bacteriophages target host populations via phenotype modification, predation, and lysogeny.[27] Soil is the major reservoir for actinophages, and they most commonly target actinomycete genera *Streptomyces*, *Actinoplanes* and *Mycobacterium*.[27]

Application of streptophages onto nut samples and testing for the presence of the VOCs

Nine different polyvalent streptophages from USC's Microbial Library[32] were selected and used to create a composite phage suspension (Fig. 1) at a concentration of 10^8 pfu/mL. A composite streptomycete suspension was also created by mixing all eight streptomycete isolates at a concentration of 10^4 cfu/mL. This concentration was selected as it represents unacceptable contamination value determined by the NSW Food Authority in their guidelines[33]

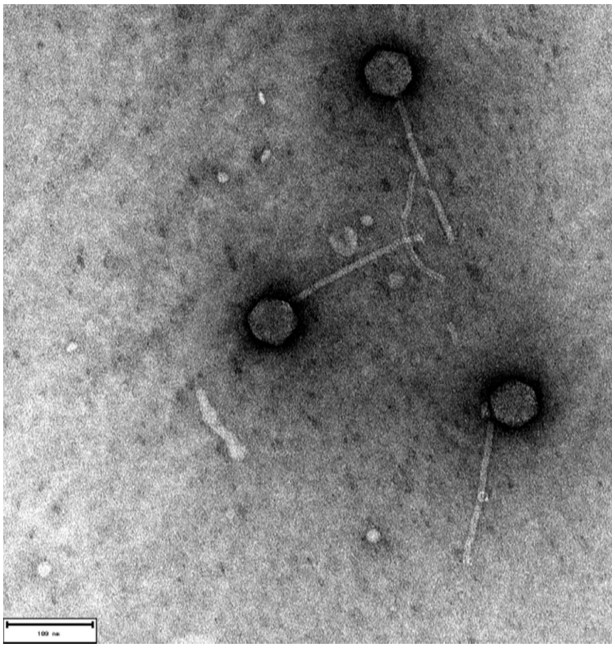

Fig. 1. TEM micrograph of the streptophages in the composite phage suspension displaying typical siphoviridae morphologies.

Table 1. Key characteristics of the Streptomyces isolates from nut samples.

Isolate code	Nut type and isolation method used	Closest relative identified using *16S* ribosomal RNA gene, partial sequence Blast analysis https://blast.ncbi.nlm.nih.gov/Blast.cgi
USC-7000	Corn kernels, air sampler,[A] 28°C	*Streptomyces* sp. strain 219202
USC-7001	Roasted peanuts, air sampler,[A] 28°C	*Streptomyces* sp. strain GS10
USC-7002	Raw peanuts, air sampler,[A] 28°C	*Streptomyces* sp. strain HBUM206355
USC-7003	Raw peanuts, air sampler,[A] 28°C	*Streptomyces* sp. strain HBUM206419
USC-7004	Raw almonds, air sampler,[A] 37°C	*Streptomyces werraensis* strain IIPR:KR05:01
USC-7005	Raw peanuts, air sampler,[A] 28°C	*Streptomyces werraensis* strain IIPR:KR05:01
USC-7006	Raw peanuts, serial dilution, 37°C	*Streptomyces werraensis* strain IIPR:KR05:01
USC-7007	Roasted peanuts, air sampler,[A] 37°C	*Streptomyces werraensis* strain IIPR:KR05:01

[A]Sampl'air Lite (https://www.biomerieux-usa.com/industry/samplair).

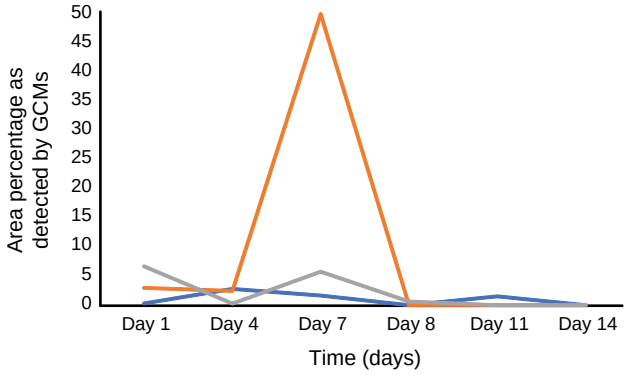

Fig. 2. Decreased values of geosmin after phage application. Key: blue – geosmin production (control); orange – geosmin production with streptomycetes application; grey – geosmin production with streptomycetes application then streptophage application after day 7.

for microbiological quality of ready to eat foods in Australia. Surface sterilised and UV irradiated nut samples using the methods by El-Tarabily[34] and Thomas and Puthur[35] were deliberately infected with this composite sample of streptomycetes. After 3 days of incubation streptophage composite sample was applied onto the streptomycete inoculated nuts with a host/phage ratio of 1:2. This ratio was selected due to past successful applications of phages onto hosts.[36] VOC production was examined using Headspace-Gas chromatography mass spectrometry (HS-GC/MS) throughout the 14 days of incubation in tightly capped bottles. A mixed standard of Geosmin and 2-MIB (Sigma-Aldrich) was used to detect Geosmin, which is known to be detected at 8.83 min.

Findings indicated a sharp decrease immediately in geosmin production after the composite phage suspension application onto streptomycete infected nut samples (Fig. 2). After day 8, the geosmin levels were near zero and streptomycete cfu/mL came down to the acceptable levels by the NSW Food Authority ($<10^2$).

2-MIB was not detected on the streptomycete or streptomycete plus phage treated nut samples at any stage of this study. Yanxia *et al.*[17] reported a strong correlation between geosmin and 2-MIB concentration, indicating that 2-MIB may be dependent on geosmin production so the sharp decrease in geosmin might be the reason of its absence.

Conclusions

The continual rise in demand of agricultural products has resulted in an increase in preferences in the use of environmentally and human health friendly methods replacing other synthetic agents. Therefore, bacteriophage treatments gained attention to minimise product losses and nutritional properties from disease causing bacteria. Like the previous successful treatments of potatoes[37–39] and strawberries,[40] the observed success of streptophage treatment on nuts in this study may indicate similar positive outcomes might be possible. Therefore, information generated via the studies like the one presented here can contribute toward development of effective phage biocontrol methods targeting different problems in agriculture. Such methods might subsequently reduce the economic losses of the growers due to unmarketable product including the ones possessing earthy-musty smells.

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Dr D. İpek Kurtböke is currently a senior lecturer at the University of the Sunshine Coast (USC) in Australia and one of the members of the Centre for Bioinnovation of the USC, conducting research in applied, industrial and environmental microbiology. She is an internationally reputed actinomycetologist and she has been in the field of biodiscovery since 1982 conducting research into discovery of novel and potent therapeutic compounds produced by actinomycetes in Turkey, Italy, the UK, and Australia with leading pharmaceutical companies. She has been an Executive Board member of the World Federation of Culture Collections (WFCC) since 2000, currently serving her second term as the President of the Federation. She is also one of the members of the International Committee on Taxonomy of Viruses (ICTV)'s Bacterial Viruses Subcommittee. She has editorial duties in different journals including *Marine Drugs*, *Diversity* and *Frontiers Marine Science/Marine Biotechnology*.

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Vaccine technologies used to develop COVID-19 vaccines

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ABSTRACT

In December 2019, cases of atypical pneumonia were diagnosed in hospital patients in Wuhan, Hubei province, China. The disease, now known as COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As case numbers increased and spread across the planet many companies scrambled to develop vaccines to control the infection and disease. Prior research on SARS-CoV-1, new vaccine technologies and unprecedented funding have allowed vaccines to be developed and approved in record time, without the usual pauses and bypassing any of the requirements of the vaccine approval process. This paper is a review of the current literature on some of the vaccines targeting SARS-CoV-2 and of the new technologies used to produce them.

Keywords: cell culture, COVID-19 vaccines, expression systems, recombinant virus vaccines, SARS-CoV-2, subunit vaccines, vaccines, vaccine technologies.

Introduction

As of 29 January 2022, the WHO reported 364 191 494 confirmed cases of COVID-19, including 5 631 457 deaths globally. As of 18 January 2022, a total of 9 571 502 663 vaccine doses have been administered worldwide.[1] A November 2021 study by the WHO Regional Office for Europe and European Centre for Disease Prevention and Control estimated that 470 000 lives have been saved in the 60 years and over age group by the COVID-19 vaccination campaign in 33 countries across the WHO European Region.[2] Over 800 000 Americans have died so far during the U.S. COVID-19 pandemic, with more than half those deaths occurring during 2021. Without vaccines there would have been approximately 1.1 million additional COVID-19 deaths and an additional 10.3 million additional COVID-19 hospitalisations in the U.S. by November 2021.[3] The WHO reports that 140 vaccines (Table 1) are at the clinical development stage and 194 vaccines at the pre-clinical development stage.[4] The Australian Department of Health has approved four vaccines for use in Australia, Vaxzevria (AstraZeneca), Comirnaty (Pfizer) and Spikevax (Moderna) and Nuvaxovid (Novavax).[5] The AstraZeneca vaccine is a non-replicating recombinant adenovirus, the Pfizer and Moderna vaccines are messenger RNA vaccines and the Novavax vaccine is a protein subunit vaccine.

Vaccine technologies

The aim of vaccination is to induce an immune response that neutralises the virus and prevents its replication. This is achieved by exposing the host to an appropriate viral protein. There are three general approaches to developing virus vaccines. Vaccines use either whole viruses, individual viral proteins or genetic material so that the vaccine directs production of the viral protein. The whole virus can be live, attenuated or killed, viral proteins can be full-length or truncated and the nucleic acid can be DNA or RNA. Protein-based vaccines can have adjuvants to enhance the host immune response and nucleic acid vaccines can have promotor sequences to enhance protein production. These also have the advantage of not requiring live virus for vaccine production or secure laboratory facilities appropriate to safely handle the agent.

Protein subunit

A protein subunit vaccine uses one or more individual viral proteins to produce an immune response against the virus. The target proteins usually contain the neutralising epitopes of the virus and are produced in either yeast, *E. coli* or insect cells. These vaccines contain a

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Table 1. COVID-19 – novel coronavirus vaccine candidates worldwide as of 21 January 2022. Modified from WHO.[4]

Platform	Number
Protein subunit	47
Viral Vector (non-replicating)	19
DNA	16
Inactivated virus	19
RNA	23
Viral vector (replicating)	4
Virus-like particle	6
Live attenuated virus	2
Viral Vector replicating + Antigen-presenting cell	2
Viral Vector non-replicating + Antigen-presenting cell	1
Bacterial antigen spore expression vector	1
Total	140

genetically engineered, non-infectious virus protein rather than the whole virus and have been around for many years. Many of the standard childhood vaccines are protein subunit vaccines so the technology has been thoroughly tested.[6] The active ingredient of Novavax is a spike protein, SARS-CoV-2 rS (NVX-CoV2373) produced by a genetically engineered baculovirus. The baculovirus is grown in SF9 moth cells where it expresses the SARS-CoV-2 spike protein produced in moth cells. The spike protein is collected and assembled into synthetic nanoparticles. The final product consists of the nanoparticles and saponin-based Matrix-M adjuvant.[7]

Viral vector (non-replicating)

Viral Vector non-replicating vaccines use a genetically modified virus that has been rendered unable to replicate, usually by knocking out one or more key genes, to deliver a viral protein into the body. The gene for the viral protein of interest is inserted into the genome of the non-replicating vector virus together with a promotor. Once injected, the body's cells transcribe and translate the viral gene into copies of the viral protein that produces an immune response against the virus.[6] The AstraZeneca vaccine is a recombinant, non-replicating chimpanzee adenovirus vector (ChAdOx1) with the full-length SARS-CoV-2 spike glycoprotein gene inserted into the adenovirus genome. The AstraZeneca vaccine is derived from material cultured in an immortalised cell line, derived approximately 50 years ago from human embryonic kidney cells (HEK293). The cells contain the adenovirus genes necessary for viral replication, and the vaccine is purified to remove cellular material.[8]

DNA

DNA vaccines consist of a genetically engineered plasmid containing a DNA sequence coding for a viral protein of interest and promotor sequences. The DNA is transfected into cells, where the viral gene is transcribed into RNA, which is then translated into the viral protein. The body then mounts an immune response to the foreign protein. DNA vaccines are a relatively new vaccine technology. Several veterinary vaccines have been developed but before COVID-19 none had been approved for human use. Because of the COVID-19 pandemic DNA vaccine technology has developed rapidly and at least one COVID-19 DNA vaccine has been approved for use in humans and many are at the clinical development stage.[6]

Inactivated virus

Inactivated virus vaccines are one of the oldest technologies for producing vaccines and have been proven to be effective over almost 100 years. The virus is grown in a culture system such as cell culture, embryonated eggs or in animals. It is then inactivated using chemicals, heat or radiation to prevent the agent causing disease. Formaldehyde and β-propiolactone are now the most common inactivating chemicals used. Many human vaccines, including influenza and polio are produced this way. In the case of the influenza vaccine the virus is grown in eggs, partially purified and then killed (split) with formaldehyde to inactivate the virus. Inactivated viral vaccines often have an adjuvant added to enhance the host immune response. Inactivated virus vaccines are relatively easy to produce, can be made in reasonably large quantities, but the time of production can be quite long and can only be done in laboratories with bio-secure facilities appropriate for the agent being handled.[6]

RNA

RNA vaccines are a new technology. The RNA in vaccines is messenger RNA, which can be directly translated to protein by the transfected cells. As with DNA vaccines the mRNA is embedded in lipid nanoparticles. Even though mRNA vaccines are a new technology several mRNA vaccines have been approved for use to control the spread of COVID-19.[6] The Pfizer mRNA vaccine contains 30 µg of BNT162b2 mRNA embedded in lipid nanoparticles encoding the SARS-CoV-2 spike protein.[9] A prime dose of the Moderna mRNA vaccine contains 100 µg of mRNA embedded in SM-102 lipid nanoparticles. The booster dose contains 50 µg of mRNA.[10] The RNA for both vaccines was produced using cell-free, *in vitro* transcription from the corresponding DNA template. The cells that take up the lipid nanoparticles are destroyed, along with the RNA.

Viral vector (replicating)

This type of vaccine is similar to viral vector (non-replicating) vaccines, however the vector virus replicates in the host. The vector virus contains one or more inserted pieces of nucleic acid coding for the SARS-CoV-2 proteins of interest that, during the replication of the vector virus, expresses SARS-CoV-2 proteins to which the host produces antibodies. While the host is protected from COVID-19, as only SARS-CoV-2 proteins are produced, rather than the whole virus, a replicating vector virus may pose a risk of infection. These vaccines

can be developed rapidly once the technology has been established.[6]

Virus-like particles

Virus-like particles (VLPs) are protein multimer structures spontaneously assembled from viral structural proteins and containing no genetic material. VLPs have been produced by many virus families and assemble into high protein density structures, often in the identical configuration to that in the native virus. They may also contain the viral proteins responsible for cell attachment and entry resulting in efficient cell entry. VLPs have become commonly used in vaccinology as they present both B-cell and T-cell epitopes and provide a delivery system that generates both antibodies and cell-mediated immunity. It is possible to insert foreign proteins into VLPs where they can combine good safety and strong immunogenicity.[6] The widely deployed hepatitis B vaccine is an example of a VLP vaccine where the 22 nm particles comprising HBV surface antigen are produced in recombinant yeast. The HPV vaccine is another example of a VLP vaccine: its development at The University of Queensland involved Ian Frazer and Jian Zhou.

Others

Vaccine delivery strategies are constantly being researched, developed and refined. Recent advances in molecular biology have made recombinant organisms, such as viruses, bacterial and other vectors, potential delivery systems for vaccines. *Bacillus subtilis* endospores expressing foreign antigens have been used for oral and intranasal immunisation and were shown to generate mucosal and systemic responses in a murine model.[11] Antigen presenting cells have been used in presenting SARS-CoV-2 spike protein via MHC Class 1 to $CD8^+$ cells and via MHC Class II to $CD4^+$ cells in recombinant viral vectors.[12]

Conclusion

A multitude of COVID-19 vaccines have been developed or are currently undergoing pre-clinical and clinical development. Many variants of the SARS-CoV-2 virus have evolved since the virus was first identified and the evidence, and nature of the virus, suggests that evolution of new antigenic and genetic variants will continue. Therefore, the most useful vaccination strategies will be those that are easily adapted to the new variants of concern (VoC), can be produced in large volumes, have high titres and low cost. Vaccines alone will not eliminate the virus, and may even enhance its evolution, so other measures to reduce transmission, including frequent hand washing, wearing a mask, physical distancing, good ventilation and avoiding crowded places or closed settings, will continue to work against new variants by reducing the amount of viral transmission and therefore also reduce opportunities for the virus to mutate.

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Data availability. Data sharing is not applicable as no new data were generated or analysed during this study.

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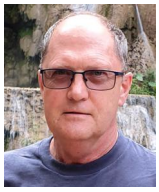

Paul Selleck has been at the Australian Animal Health Laboratory, now the Australian Centre for Disease Preparedness since, 1983. In this time he was head of the Avian Disease Diagnostic Laboratory, incorporating the National, OIE and FAO Reference Laboratory for Avian Influenza and Newcastle Disease and an OIE Reference Expert for Avian Influenza and Newcastle Disease. He was also involved in the Australian equine and swine influenza outbreaks in 2007 and 2009 respectively and has worked with Hendra, Nipah and SARS at physical containment level 4. Paul now works extensively in Asia, running training courses on biosafety and biosecurity and laboratory diagnosis. He also audits laboratories and runs training courses on quality systems and ISO laboratory accreditation.

Ian Macreadie is an Honorary Professor of RMIT University and is Editor-in-Chief of *Microbiology Australia*. In the 1980s with colleagues at CSIRO he developed a viral subunit vaccine for infectious bursal disease virus, using a novel yeast expression system. He maintains a keen interest in preventatives for SARS-CoV-2.

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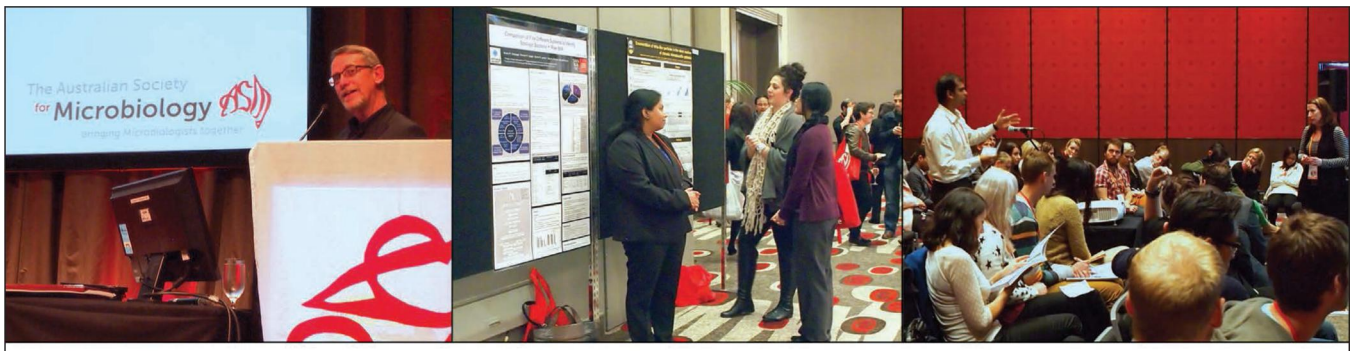

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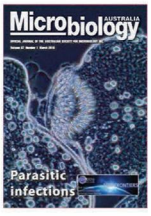

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Microbiology Australia

Jocelyn Robert Lane Forsyth: 03/09/1939–03/02/2022

Cheryl Power

Scholar, teacher, storyteller, conservationist and avid traveller

Dr Jocelyn Forsyth, known to many as Dr F or Joc, was a man of many facets. He was a larger-than-life character who grabbed everything he took on by the horns and changed it for the better. He commenced his 26 years of service in the Department of Microbiology and Immunology at the University of Melbourne in 1968 as Assistant Director of the Microbiological Diagnostic Unit, invariably referred to as the MDU. He subsequently became Director in 1977, a position previously held by the Head of Department. After his retirement in 1994 he remained a regular visitor while expanding on his book on the history of the MDU, which he initially co-authored with Alan Woodgyer in 1997.

Dr Forsyth was a man of great integrity and had an infectious enthusiasm for public health microbiology with a particular passion for parasites. He was a longstanding member of the ASM Parasitology and Tropical Medicine Special Interest Group and established the Parasitology program for the Royal College of Pathologists of Australia. He was also a founding member of what was originally known as the Venereology Society of Victoria, and is now known as the Sexual Health Society of Victoria. He served a term as the ASM Victorian branch chair from 1967 to 1968. He always encouraged his staff at MDU to be actively involved in an appropriate professional society, either as members of the ASM or AIFST.

During his time at MDU he oversaw a period of great change, both with multiple state government restructures and the type of work undertaken. The microbiological landscape dramatically changed with the ease of overseas travel and the influx of immigrants and Dr Forsyth used this as an opportunity to position the MDU as a highly respected microbiological reference centre. He set MDU up as the enteric reference laboratory for Victoria and much of Australia. He initiated a close working relationship between the Victorian Department of Health and the laboratory, a model that is unique to Victoria. Invitations to the MDU Christmas lunches were highly valued and served to forge the bond between the two organisations. He turned MDU into a national and international reference centre, supported by real time data, and played an integral role in establishing a number of surveillance schemes including NEPPSS for enteric pathogens and the Neisseria surveillance testing program. One of his most important legacies was his initiative to preserve the bacteria responsible for various outbreaks and for specific infections, so providing an invaluable resource for future researchers.

Dr Forsyth could have been seen by some as a hard task master. His work ethic involved long hours and he expected others to share his dedication to getting the job done and done well. He saw his work as a vocation rather than as paid employment and his employees as his extended family. On Saturday mornings he was invariably in his office or the lab and made tea for everyone as well as entertained them with fascinating stories. He enjoyed engaging with his staff and many of them benefited enormously from his teaching and mentoring, continuing on to life-long careers in Microbiology. This willingness to share knowledge and enthuse others extended to generations of medical students where his lectures to them were greatly enlivened by his antics, including mimicking patient's clinical signs and relating graphic personal experiences. He was particularly proud of the demanding exam questions that he set, which required students to work through problems not previously sighted. Three of the current jurisdictional Chief Health Officers were subjected to, and presumably survived, what was regarded as a formidable challenge.

Many fellow microbiologists and public health practitioners found Joc to be a mine of information, a source of knowledge and a gifted problem solver. He was always thinking about how to do things better and how to position MDU to best meet the public health needs of Victoria and Australia. Others found him a supportive and generous colleague. He had a great sense of humour and a booming laugh to match. He was an extraordinary man and will be greatly missed.

This tribute was compiled with help from many former friends, staff and colleagues, with major contributions from Professor Benjamin Howden, Professor Roy Robins Browne, Agnes Tan and Peter Traynor. Sadly there is not space to include the plethora of wonderful personal stories that were shared but they all emphasised that Joc was a real gentleman, and did, as directed by the founder of Scouting, Lord Robert Baden Powell, '…leave this world a better place than you found it so when your turn comes to die you can die happy in feeling that you have not wasted your time but have done your best'.

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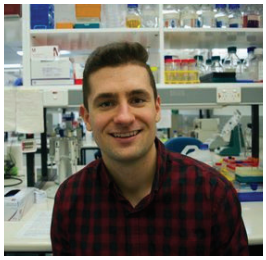

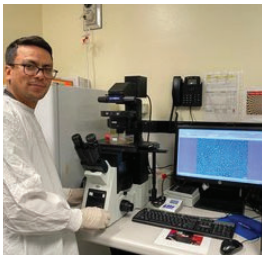

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