Hot off the Press

Microbiology Today Editor Meriel Jones takes a look at some papers in current issues of the Society's journals which highlight new and exciting developments in microbiological research.

What the eye can't see

For almost a century, microbiologists have relied on being able to grow bacteria from a habitat as one of their best strategies for identifying what is there. With the advent of molecular biology methods that rely on detecting minute traces of DNA, it has become apparent that this gives a very biased impression. For example, despite all the attention lavished on the inhabitants of the human gastrointestinal tract, most of the approximately one million million (10^{12}) cells in each gram of faeces represent bacteria that cannot yet be grown in the laboratory. Their interactions with each other, and their healthy host are equally poorly understood.

And although molecular methods provide a way to study these unculturable organisms, they raise new problems. There are many molecular methods and it is not obvious which is best for measuring the populations in a particular habitat. Researchers at Helsinki University in Finland have been working on this question by comparing three different methods to record the numbers of particular faecal bacteria. Their results show the value of the technique called real-time PCR. The polymerase chain reaction (PCR) is the basis of many molecular identification methods. It allows the presence of very small amounts of DNA with a specific sequence to be detected through adding reagents that make copies of the sequence, if it is present, until there is enough to be detected. These sequences can be unique to a species, or a genus. Real-time PCR records how the number of copies increases during the experiment, rather than simply checking at the end. Scientists think that this can be used to estimate of the number of bacteria that are present in the original sample, and the Finnish workers wanted to test whether this was correct for the faecal microflora.

They tested a series of reagents and experimental conditions for detecting *Bacteroides fragilis*, *Ruminococcus productus* and *Bifidobacterium longum*, representing groups of bacteria that are reported to be abundant in human faeces. As a contrast, they also tried to estimate numbers of *Escherichia coli* and *Lactobacillus acidophilus*, a minor part of the gut microflora, and of *Bifidobacterium lactis* which does not belong in the human gut but is increasingly used in dairy foods as a probiotic that may confer health benefits. The researchers could test how sensitively they could detect both purified DNA from these species, and DNA from these bacteria in authentic human faeces. Using real-time PCR the researchers could detect purified DNA from as few as 200–400 bacteria, or the presence of these species if they formed a mere 0·01 % of the bacterial population in a sample of faeces. When they measured their samples with an older, well-established method for studying faecal microbes, they could only detect bacteria that formed at least 3 % of the population.

Malinen, E., Kassinen, A., Rinttilä, T. & Palva, A. (2003). Comparison of real-time PCR with SYBR Green I or 5′-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology* **149**, 269–277.

Impact of meningococcal vaccine

Meningitis is a serious disease of children and young people, caused by the bacterium *Neisseria meningitidis*. Although prompt treatment with antibiotics is usually effective, the initial symptoms are not obvious, so medical attention may be delayed. Many people carry the bacterium within their nasal passages but remain healthy, and these carriers are essential to the spread of the disease. Several different varieties of bacterium circulate in this human reservoir, occasionally causing outbreaks of meningitis.

The Galicia region of north-west Spain has around half a million young people between the ages of 5 and 19 among its 2·7 million inhabitants. Meningitis is endemic, and by 1995/6 the incidence had risen to 11 cases per 100,000 in the population, caused by *N. meningitidis* serogroup C. After a review of all possible control measures, this situation was sufficiently serious for the Galician Regional Public Health Authorities to decide on a vaccination campaign, targeting everyone between 18 months and 19 years. Between December 1996 and January 1997 they vaccinated 472,465 children and young people using a vaccine that gave protection from serogroups A and C. By 1998, the incidence of meningococcal disease had declined to 4·3 cases per 100,000. The authorities decided to find out whether the number of healthy carriers had also decreased since these could be the source of any future disease.

The study examined children aged between 5 and 19 in two regions of Galicia that had different incidences of the disease before the vaccination campaign. The National Neisseria Reference Laboratory at Majadahonda, Madrid, tested about 14,500 nasal swabs, and worked out that the prevalence of carriers had decreased from 1·51 to 0·79 % after vaccination in the high-incidence area, and from 0·94 to 0·32 % in the low-incidence region. The tests allowed the researchers to check the serotypes of the bacteria, and they realized that only the decrease in serotype C carriers in the high-incidence region was significant. The number of carriers between 10 and 19 had decreased by over 70 %, but the number between 5 and 9, although small, had increased by 20 %. This fitted with earlier reports that the vaccine is less effective among the youngest children.

Interestingly, there was no increase in the carriage of serotype B one year after vaccination, which might indicate that this variety had failed to replace serotype C in the now-asymptomatic carriers. In addition, there had actually been a statistically significant rise in the carriage rates for serotypes other than B and C. Overall, the researchers are confident that despite these changes in the incidence of other serotypes, their data indicate the effectiveness of the 1996 vaccination campaign, especially since the incidence of disease caused by serotype C continued to decrease in 1997 and 1998.

Fernández, S., Arreaza, I., Santiago, I., Malvar, A., Berrón, S., Vazquez, J.A. & Hervada, X. (2003). Impact of meningococcal vaccination with combined serogroups A and C polysaccharide vaccine on carriage of *Neisseria meningitidis* C. *J Med Microbiol* **52**, 75–77.

OPPOSITE PAGE:
E. coli cells viewed under the microscope. The green cells are viable gfp-labelled cells, while the red cells are non-viable cells stained with propidium iodide.

COURTESY S. TOZE, CSIRO CENTRE FOR ENVIRONMENT AND LIFE SCIENCES, WEMBLEY, AUSTRALIA

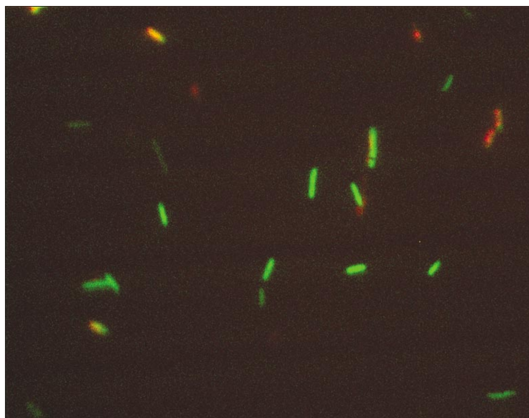
Persistent pathogens

Much of Australia has a low rainfall, and irrigation using recycled water is essential for agriculture. The water can be stored by injecting it into aquifers until it is needed. Bacteria in natural habitats often grow on surfaces in slimy layers called biofilms and during a pilot project in South Australia to store recycled sewage effluent, biofilms formed around the injection site. Researchers at CSIRO Land and Water and the University of Western Australia wondered whether this could lead to public health problems. The indigenous micro-organisms were unlikely to be a problem, but sewage water might contain pathogens. Tests for bacteria like *Escherichia coli*, a species found in faeces, are used as a way to check whether sewage has leaked into a water supply, but tests are sometimes positive without any obvious source of contamination. This has led researchers to think that pathogens can persist in the biofilms that sometimes coat the inside of water pipes. No-one knows whether the same happens in a natural environment like an aquifer.

Simon Toze and his colleagues decided to use a laboratory system designed to simulate porous rock to study the survival of both *E. coli* and *Pseudomonas aeruginosa*, a ubiquitous water-borne micro-organism and opportunistic pathogen. A very dilute nutrient solution flowed gently through a series of glass flasks containing microscope coverslips, and soon coated everything with patches of biofilm. Nitrogen gas was bubbled through the liquid to simulate the anaerobic conditions in an aquifer. To make the test bacteria easy to see, the researchers used strains that produced a green fluorescent protein. Then they simply had to retrieve a coverslip and check it for glowing green bacteria to see whether the test species had grown into the biofilm. If the bacteria did not join the biofilm, they should gradually be washed out of the flasks with the growth liquid.

The researchers managed to detect *E. coli* in the flasks long after it should have been washed away if it had not joined the biofilm, except when the liquid was recycled sewage effluent. Perhaps surprisingly, the *E. coli* cells died away more rapidly in this more nutritious medium. Earlier researchers have indicated that biofilms of indigenous river water bacteria can reduce the persistence of *E. coli*. The reason may be the presence of many more bacteria, all better adapted to this environment than *E. coli*. *P. aeruginosa* survived much better in the sewage effluent than the *E. coli* cells. These experiments indicate that although adding nutrient-rich effluent to an aquifer may stimulate the development of a biofilm, bacterial species will vary in their ability to survive within it. Pathogens like *E. coli* which are adapted to the human gut may not survive well, while others like *P. aeruginosa*, which are better adapted to a watery environment, may flourish.

Banning, N., Toze, S. & Mee, B.J. (2003). Persistence of biofilm-associated *Escherichia coli* and *Pseudomonas aeruginosa* in groundwater and treated effluent in a laboratory model system. *Microbiology* 149, 47–55.



Rabies alert in Australia

Until May 1996, everyone thought that Australia was free of rabies and rabies-like disease. Then, a virus with distinct similarity to the rabies virus was isolated from a juvenile black flying fox, and the situation became very murky. Since then this virus, named Australian bat lyssavirus (ABL), has been detected in all the common species of flying foxes in Australia, and also in the yellow-bellied sheath-tailed bat, which is insectivorous. Of additional concern is the fact that two people in Queensland have died from an illness with all the symptoms of rabies following contact with bats infected with ABL. Although vaccines against rabies are thought to provide protection against illness caused by ABL, more information was needed.

A collaboration between Australian scientists at the Queensland Department of Primary Industries, University of Queensland, and the Queensland Health Scientific Services and researchers at the University of Oxford in the UK, has investigated ABL to determine whether the virus is identical in all the infected bats, and to gauge the public health risk. ABL consists of a strand of nucleic acid encased in a protein shell surrounded by a lipid envelope. The lipid envelope is studded with glycoprotein spikes and the ABL researchers focused on the gene for this glycoprotein, as it is the first part of the virus to come into contact with a new host. Not only is it the major target for antibodies from the host's immune system, but it also has the function of allowing the virus to enter a host cell and so start an infection. Its structure is crucial in allowing ABL to infect a suitable host, such as a bat or person.

The researchers used samples of brain or salivary gland tissue collected from Queensland bats that had been caught after behaving abnormally – particularly if they had shown aggression towards humans or domestic animals. If the tissue tested positive for ABL with a fluorescent stain, the scientists attempted to extract the virus and analyse its genes. They analysed 22 isolates of ABL from both flying foxes and insectivorous bats, and compared them with other lyssaviruses from around the world, including rabies virus.

The ABL isolates divided into two groups, one from the fruit-eating flying foxes and the other from insectivorous bats. This is similar to the situation for bat lyssaviruses in other parts of the world, where bats with the same lifestyle harbour similar lyssavirus strains. In this study, ABL isolates collected from flying foxes caught 1,400 km apart were found to be virtually identical. Not only do flying foxes migrate over extensive distances, but thousands of them roost together during the day in trees. Under these circumstances, one strain of the virus therefore has a good opportunity to infect many bats. In contrast, the insectivorous bats travel much shorter distances, stay in groups of less than 30 and are unlikely to mix with flying foxes. The clear segregation between virus variants also suggests that ABL is rarely transmitted to other animals, but this is one aspect that the Australian scientists want to investigate further. While it has been suggested that the lack of variation between ABL isolates from the same bat species may be due to the virus becoming adapted to its most commonly encountered host, it has also been proposed that these viruses may have evolved to a point where they have become 'viral generalists' able to infect many different cell types such that they have a selective advantage when exposed to new host environments.

Guyatt, K.J., Twin, J., Davis, P., Holmes, E.C., Smith, G.A., Smith, I.L., Mackenzie, J.S. & Young, P.L. (2003). A molecular epidemiological study of Australian bat lyssavirus. *J Gen Virol* 84, 485–496.

Spore-forming Gram-negative bacterium

Four years ago Ryozo Iriye and Yukio Doi reported a rather strange bacterial species from activated sludge in a domestic wastewater treatment tank in Saku, Japan. The cells produced a red pigment, and contained structures called endospores. These are a characteristic of a few genera of bacteria that are within a larger grouping called the Gram-positive bacteria. When these species experience unfavourable conditions, such as a lack of nutrients, the interior of the cell transforms into a multi-layered structure around the bacterial DNA. This is the endospore, which can survive through adverse environments to grow again once conditions improve. The scientists, not unnaturally, assumed that they had found another species within this group, probably a member of the genus *Bacillus*, but were intrigued by some of its other features. They and their colleagues have now carried out a battery of tests on the organism and confirmed that it may be an example of something very interesting indeed.

Their tests show, without a shadow of a doubt, that the bacterium is a subspecies of *Serratia marcescens*. This is a member of the very large group of Gram-negative bacteria, none of which are known to produce endospores. However, their tests show equally clearly that pure cultures of the organism can produce structures very like the endospores of *Bacillus megaterium*, even down to the chemical dipicolinic acid that is only found in endospores. Cells containing these structures could survive a heat treatment of 62 °C for 15 minutes, while all the cells of an authentic strain of *S. marcescens* died at 60 °C. Authentic *Bacillus* endospores should have no difficulty in surviving these, and higher, temperatures. The researchers think that the most sensible explanation of their results is that they have isolated a strain of *S. marcescens* that has acquired the genes for endospore production from a *Bacillus* species within the environment of an activated sludge tank. If they are correct, an exciting example of natural gene transfer between two very distantly related organisms has been found.

Ajithkumar, B., Ajithkumar, V.P., Iriye, R., Doi, Y. & Sakai, T. (2003). Spore-forming *Serratia marcescens* subsp. *sakuensis* subsp. nov., isolated from a domestic wastewater treatment tank. *Int J Syst Evol Microbiol* **53**, 253–258.

Chimeras' raise their heads

One of the most effective molecular biological methods to discover what bacteria are present within an environment is to extract DNA from it and then use the polymerase chain reaction (PCR) to amplify one particular DNA sequence. This is the 16S rRNA gene sequence, which is an essential part of the cell's protein synthesis machinery. Decades of study of this molecule by hundreds of scientists have ensured that its characteristics are known in exquisite detail. Some parts of the 16S rRNA gene are invariant in all bacteria, while other regions are distinctive of particular groupings, or even individual species. By carefully choosing which sections to amplify, researchers can use PCR of the 16S rRNA gene to search for the bacteria of their choice, including new species.

However, Philip Hugenholtz and Thomas Huber from the Advanced Computational Modelling Centre at the University of Queensland have pointed out, in a recent issue of IJSEM, that this tool comes with a distinct drawback. It is possible to produce chimeric 16S rRNA gene sequences. These are artefacts of PCR that are caused by the amplification products of two separate 16S rRNA sequences becoming joined together. This results in a sequence with parts from two distinct organisms. If the researchers do not realize what has happened, their analysis will suggest the presence of a novel organism. However, this organism is actually non-existent, and the 16S rRNA gene sequence generated has the potential to confuse subsequent taxonomic studies. Scientists have been aware of this problem for over a decade, and attempt to both prevent chimeras forming and detect any that slip through. However, when Hugenholtz and Huber searched public databases of 16S rRNA gene sequences, they quickly found a number of chimeras. The authors suggest that these, and any more that come to light, should be removed from the databases, split into their component halves and then resubmitted. As an aid to identifying chimeras, they have created a program called BELLEROPHON (http://cassandra.visac.uq.edu.qu/perl/bellerophon.pl).

Hugenholtz, P. & Huber, T. (2003). Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *Int J Syst Evol Microbiol* **53**, 289–293.

TB from the 18th century

Tuberculosis is caused by the bacterium *Mycobacterium tuberculosis*. It may have been responsible for a quarter of all deaths in Europe between the sixteenth and eighteenth centuries, and then spread worldwide. Researchers in the UK and Netherlands, working with staff at the Hungarian Natural History Museum, have been finding out about this ancient disease. The opportunity came following the chance discovery of a sealed crypt in the Dominican church at Vác in Hungary during repairs in 1994. It turned out to be the last resting place of members of prominent local families and clerics who had died between 1731 and 1838. Many of the bodies had become naturally mummified, and from examining the remains, as well as local family records, it seemed possible that many of the people were suffering from tuberculosis at the time of their death.

The unusually well-preserved state of the bodies allowed computer tomography to be used to confirm the symptoms of tuberculosis in one of them. Because the body tissues were so well preserved, the researchers sought permission to take some samples to carry out a detailed study of the mycobacterial DNA. Although mycobacterial DNA has been detected in old human remains, it is usually so fragmented that researchers can do little more than be confident that it is present. The complete sequences of several modern strains of *M. tuberculosis* and its relatives are now known and researchers want to understand how the bacterium has evolved. As well as purely scientific interest, this might indicate why it is such an effective pathogen.

The researchers took tissue samples from a mother, who died in December 1793 at the age of 55 and her two daughters. The younger daughter, aged 14, died in March 1795 and the older on Christmas day 1797, aged 28. To be confident about their findings, the researchers took very careful precautions against contaminating their samples. In addition, they obtained poorer results when they tested for longer sequences of DNA, which was an additional indication that they were really dealing with fragmented pieces of 200-year-old DNA, rather than a contaminant of fresh DNA that would be substantially intact.

One question that the researchers wanted to answer was whether the tuberculosis was in fact caused by *M. tuberculosis* or by its close relative *M. bovis*. This species infects cattle, but can infect people, particularly through dairy products. In the Europe of the past, when people lived closer to their domestic animals, many more people may have been infected with it than today. However, the researchers' tests found no evidence of *M. bovis* in the three bodies. Instead, several tests showed that the women were infected with *M. tuberculosis* similar to two modern strains. These included characteristics that are found across the globe today, which is consistent with the idea that the modern tuberculosis epidemic began in Europe in the 1700s and then moved to the New World and Africa.

Fletcher, H.A., Donoghue, H.D., Taylor, G.M., van der Zanden, A.G.M. & Spigelman, M. (2003). Molecular analysis of *Mycobacterium tuberculosis* DNA from a family of 18th century Hungarians. *Microbiology* **149**, 143–151.