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REVIEW

The value of cultures to modern microbiology

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Abstract Since the late nineteenth century, pure cultures have been regarded as the cornerstone of bacteriology. However, not all bacteria will multiply sufficiently to produce visible colonies on solid media; some cells will produce micro-colonies that are invisible to the naked eye. Moreover, the proportion of culturable cells that produce visible growth will vary according to the species and the state of the cells—are they actively growing or comparatively inactive? The latter have a poorer rate of recovery in terms of cultivability. It is unclear whether or not an individual colony is always derived from a single cell; it is possible that organisms in close proximity to each other may multiply and come together to produce single colonies. Then, the resultant growth will most certainly be derived from more than one initial cell. Although it is generally assumed that streaking and re-streaking on fresh media will purify any culture, there is evidence for microbial consortia interacting to form what appear to be single pure cultures. As so-called pure cultures underpin traditional microbiology, it is relevant to understand that the culture does not necessarily contain clones of identical bacteria, but that there may be variation in the genetic potential of the component cells, i.e. the cells are not homogeneous. Certainly, many bacteria change rapidly upon culturing, with some becoming bigger and less active. It is difficult to be sure if these changes reflect a loss or change of DNA or whether standard culturing methods select faster growing cells that are effectively not representative of the environment from which they were derived. These concepts are reviewed with an emphasis on bacterial fish pathogens.

Keywords Cultivability · Culture-independent techniques · Culturing · Micro-colonies

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Introduction

As microbiology moves into the 21st century, there is an argument simmering about the continued need for pure bacterial cultures; specifically, the discussion is focusing on the use of culture-independent versus culture-dependent approaches in microbiology. Clearly, there has been a move towards culture-independent techniques, notably embracing modern developments in molecular biology, particularly sequencing of the 16S rRNA gene, which do not need intact, viable bacterial cells. The advantage to these culture-independent techniques is that the bacteria may be studied regardless of whether or not they may be grown in the laboratory. There is a high level of specificity (=accuracy), and this is especially important when diagnosing disease. Moreover, this is a distinct advantage for those bacteria which have not been cultured in vitro; topical examples include taxa

classified as '*Candidatus*' (Austin and Austin 2016). Thus, pathogens may be identified directly in the natural environment or in pathological material without the need for time-consuming and often unsuccessful culturing efforts. However in the case of the culture-independent approaches, it may be unclear whether a positive reaction indicates the presence of intact, viable (or non-viable) cells or whether the DNA has been released into the environment, such as by cell autolysis/fragmentation. Could positivity suggest the presence of active or non-active, dormant or senescent cells, resident, transients or contaminants? Moreover, there may be confusion from the molecular data about the precise location of the cells and their role in the environment. With the numerous publications appearing that describe the presence (e.g. bacterial diversity in a municipal dumpsite as determined by 16S rRNA sequencing; Mwaikono et al. 2016) and even naming of organisms (e.g. '*Candidatus* Syngnamydia Venezia' which causes epitheliocystis in broad nosed pipefish [*Syngnathus typhle*]; Fehr et al. 2013) as a result of culture-independent techniques, it is surely appropriate to question whether or not the bacterial culture is approaching a situation where it could be regarded as redundant/superfluous to microbiology. Yet, cultures still form an integral part of public and private culture collections (e.g. American Type Culture Collection), and are necessary for many biotechnological applications including their use in vaccines and in food, e.g. yoghurts. Nevertheless, culture-independent techniques do generally enable rapid and accurate disease diagnoses to occur. Therefore from the wide range of culture-independent approaches, including both serological (e.g. the enzyme linked immunosorbent assay) and molecular techniques that are currently available, the end user may have access to reliable information to allocate names to organisms, although the relevance of the information may be unclear.

Historical perspectives of culturing

Since the late nineteenth century, microbiology has relied on the acquisition of pure bacterial cultures, which have been central to allied studies, including taxonomy, ecology and pathology. The developments, which persist to the present day, and have been summarised in Brock (1961), include the development of the petri dish (by Julius Richard Petri, a German bacteriologist, who was an assistant of the German microbiologist Robert Koch; Petri 1887), agar as a gelling agar (attributed to Angelina Hesse, who was the wife of Walther Hesse—another assistant of Koch; she used agar in her jellies and puddings, and gave the idea to her husband during a picnic one summer when her desserts did not melt in the hot sun), and the pressure cooker = autoclave (invented in 1879 by the French microbiologist Charles Chamberland, who worked with Louis Pasteur). The autoclave enabled bacteriological media to be sterilised, and therefore freed from possible contamination (Madigan et al. 2012). Previously, Koch had grown bacterial cultures on potato slices, which had their own problems, including the observation that not all bacteria would grow on potato, and when they did grow there were major issues of purification with the bacteria growing and developing as an amorphous mass on the moist surface (Madigan et al. 2012). Agar, which is derived from seaweed, replaced the use of gelatin, which melted at 37 °C, i.e. the temperature of choice for the culture of bacteria from human and animal sources, and can be rapidly degraded by a wide range of gelatinase-producing micro-organisms (Koch 1882). The pre-eminence of agar as a gelling agent remains although some alternatives have been suggested, including guar gum (Jain et al. 2005) and GELRITE (Shungu et al. 1983; Lin and Casida 1984), which are particularly suited for use with thermophiles, and Pluronicpolyol F127, a copolymer of polypropyleneoxide and ethyleneoxide (Gardener and Jones 1984).

Culturing techniques

It is apparent that only a small proportion of the bacteria in any habitat will grow in the laboratory, with reasons reflecting the choice of medium, incubation temperature and duration, and atmosphere, i.e. aerobic, anaerobic or micro-aerophilic. If the conditions are inappropriate for the organism, then growth to develop visible colonies is highly unlikely to occur. In addition, there are many organisms which do not produce visible colonies on laboratory media. For example, incubation at mesophilic temperatures will exclude the growth of psychrophiles and thermophiles; a comparatively short incubation period of

24–48 h will preclude slower growing organisms from growing (e.g. Mallory et al. 1977). The presence of comparatively high levels of organic nutrients in media may inhibit the development of oligotrophs (Mallory et al. 1977). Media containing components from the natural habitat, such as river, estuarine or seawater for aquatic bacteria (e.g. Mallory et al. 1977; Cho and Giovannoni 2004; Sowmya and Sachindra 2016) or soil extracts for terrestrial bacteria (e.g. Nishioka et al. 2016) improve cultivability. The uncultured majority may inevitably exert important roles in ecology such as contributing to nutrient cycling, synthesising novel molecules that could be relevant to biotechnology, and influencing other components of the surrounding microflora (Stewart 2012). With the inability of many bacteria to grow on conventional laboratory media, other approaches have been evaluated including co-culture with other microorganisms, dilution-to-extinction culturing (e.g. Stingl et al. 2008; Sosa et al. 2015; Yang et al. 2016) and micro-cultivation, that have shown promise with the study of these uncultured organisms, such as involving the identification of critical growth factors (Stewart 2012). These techniques are the starting points to learn more about the ecology of the difficult-to-culture bacteria.

Most microbiology laboratories will have access to a range of agar plates and broth media, although these media may bare little relationship to the nutritional status in the environment from which the bacterial cells are obtained. As an over-simplification, the inoculum containing the microbes is either:

1. introduced onto/into the medium usually by means of an inoculating loop or by a cotton-tipped swab, and spread over the surface of the solid medium (=spread plates), or
2. a known volume is incorporated into molted cooled agar media, mixed thoroughly by gently rotating the petri dishes, and allowed to set (=dilution plates). Organisms that are susceptible to the temperatures of molten cooled agar will be unlikely to grow with this technique.

Incubation will then be at a specified temperature for one or more days (e.g. Madigan et al. 2012). Essentially, the procedure is not so dissimilar to that developed in Robert Koch's laboratory in the nineteenth century. By these methods with the use of environmental samples (including soil and water), the desired outcome for statistical reasons after incubation is the development of 30–300 colonies/plate (Association of Official Agricultural Chemists [AOAC] 1999). With <30 colonies/plate, there could be concerns about the impact of contamination; >300 colonies/plate lead to issues of overcrowding (AOAC 1999). With pathological material derived from diseased animals or plants, the outcome of culturing is often dense growth covering most of the isolation plates (Austin and Austin 2016). The question is which colony/area of growth is to be used to derive the pure culture? With spread or dilution plates containing 30–300 colonies/plate a random approach may be used to avoid bias. However, dense/confluent growth of the type obtained from diseased tissues may be more troublesome for the establishment of pure cultures of relevance to the disease situation. Whatever the choice, the pure culture will be derived by streaking and re-streaking the bacterial growth several times onto fresh media with the aim of achieving monocultures whereby all the colonies appear to be homogeneous (Fig. 1). A complication is with those organisms, e.g. *Aeromonas salmonicida* (Austin and Austin 2016) and *Corynebacterium diphtheriae* (Murphy 1966), that may develop any of multiple colony types on solid media.

Certainly, pre-incubation in broth may lead to better recovery than direct plating onto agar-containing media (Olson 1978). An explanation is that damaged/dormant cells are allowed to recover, and become active again during this pre-incubation phase. Thus, there is the inference that not all bacterial cells will readily grow after inoculation onto solid media possibly reflecting a need for the organisms to adjust to the conditions in the new environment including nutrient availability and surface characteristics (Rolfe et al. 2012).

One of the underlying aims of culturing on solid media is to ensure the presence of well-isolated bacterial cells that will be cloned to develop clearly visible colonies. The assumption is that each bacterial cell is cloned to produce a single colony, which contains a homogeneous/identical population of cells (Jeanson et al. 2015). However, this has been challenged insofar as it has been reported that cells may mutate, swap and/or share genes (e.g. Pennisi 2002). Another issue is the relationship of cultured cells to those in the environment from which isolation occurred. For example, it is not unusual for cells to be

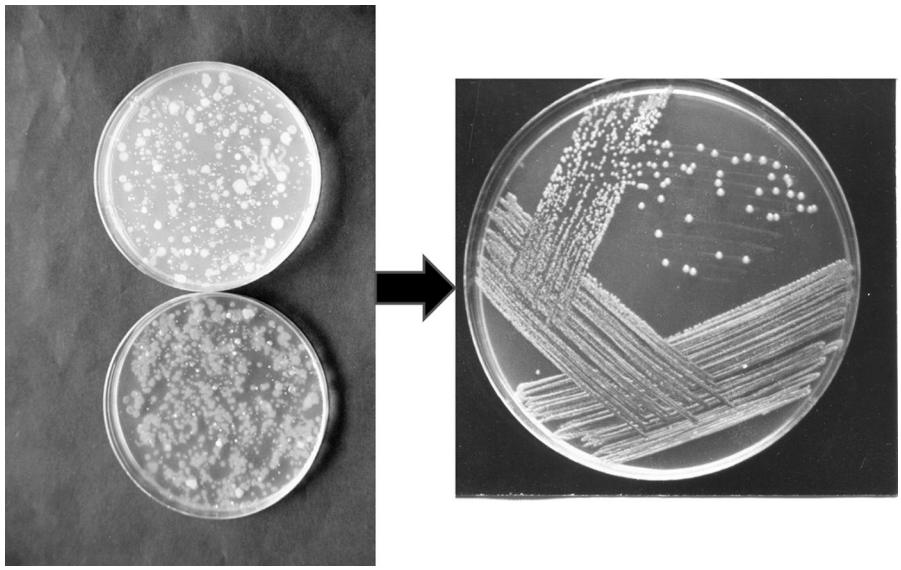

Fig. 1 Mixed culture growth from estuarine water on marine 2216E agar (*left*) with the resultant single pure culture (*right*). The reasons for the choice of colony/growth used to develop the pure culture are often unclear, and may reflect operator bias

bigger in laboratory media than their counterparts in the natural (especially marine) environment (Torrella and Morita 1981); does this reflect the better nutrient status of cultured cells? Then all too often traits are lost on subculture, e.g. the ability to ferment sugars, such as lactose (Zamenhof and Eichhorn 1967; Koskiniemi et al. 2012), although whether this reflects the loss of DNA by gene deletion or from plasmids and bacteriophages, or the switching off of specific genes is unclear. Another possibility is that cells that are better suited for growth on the laboratory medium outcompete those with the original demonstrable characteristics in the population therefore effectively diluting out the original cells, i.e. establishing what are effectively laboratory cultures that may have only limited resemblance to those of the original habitat.

What are the chances that bacterial colonies are really derived by multiplication of single cells? Considering the size of bacteria, i.e. 1–3 μm in length on an agar plate, in relation to the diameter of visible bacterial colonies [3–4 mm diameter is not uncommon] then there has to be a realistic possibility that more than one bacterium could be in the area occupied by the developing colony. This raises the question about whether or not the single colony could have the progeny from more than one cell, possibly as a result of co-aggregated cells being deposited at the same site on an agar plate. If the colony appears to be mixed, then the microbiologist would continue the purification stages of re-streaking onto fresh media. However if the contributing cells were closely related and appeared to be morphologically identical then the outcome could be a colony that appeared to be pure but was not. It is speculative how much effort the microbiologist may then take to continue the purification with a culture that appeared to be a single colony type, if at all. Another possibility is that some of the cells within the area occupied by the colony may be dormant or produce only limited growth, and therefore contribute little to the population in the developing colony, but nevertheless remain within the confines of the growing culture.

It is generally assumed that streaking and re-streaking a few times on fresh media will result in a single colony type that has the appearance of being pure. However, there is some evidence that some cultures considered to be pure actually contain the cells of a different taxon. In one example, eight seemingly pure cultures of purple-pigmented bacteria were isolated from sediment in a Scottish freshwater lake and stored on plates of tryptone soya agar (TSA) with subculturing at weekly intervals for 4 weeks before transfer to TSA slopes for storage at 4 °C. Four weeks later, three of the cultures were observed to have produced brown diffusible pigment in localised areas in the medium below the growth of the purple-

pigmented cultures. Colonies producing brown-diffusible pigment were subsequently purified, and equated with the smooth colony variant of the fish pathogen, *A. salmonicida* (Austin et al. 1998). In this case, the purple pigmented bacteria senesced during storage at 4 °C, leading to the recovery of *A. salmonicida* (Austin et al. 1998). It is speculative if this was a case of contamination or was it an example of a co-culture between the purple-pigmented bacteria and aeromonad.

Clearly not all the cells inoculated onto a medium will grow to produce visible colonies. Some cells could remain inactive but viable, whereas others may undergo limited growth, developing what are essentially micro-colonies that would not be readily seen with the naked eye, but which could be enumerated using special techniques, such as on-chip microscopy (Jung and Lee 2016). It is unclear if these cells are slower growing or whether they are incapable of producing larger colonies. Micro-colonies are less likely to be purified and studied further. However, it is argued that colonies on laboratory media are not natural, and do not represent the true nature of the bacteria in their normal habitats.

It is appropriate to question which macro-colonies become the focus of attention during the purification process to achieve the resultant pure culture. The microbiologist may take an inoculum from a group of identical-looking colonies or select one "representative" colony for purification? This raises the question about what constitutes a representative colony? A random approach would eliminate operator bias but depending on the amount of the growth on the agar plate this might be difficult to achieve meaningfully. Microbiologists may well select colonies, possibility unintentionally, according to a personal preference, including texture, shape and colour characteristics. This initial choice may have implications in subsequent studies.

The pure culture needs to be subjected to long-term storage as quickly as possible after initial isolation using a variety of techniques, including lyophilisation and cryopreservation at −70 or −80 °C in cryopreservant, namely 15–20% v/v glycerol (To and Etzel 1997). This is especially important if the culture has an interesting feature, such as antibiotic production. Maintenance on agar slopes at room temperature is not usually suitable to maintain the long-term viability of the culture in its original state; bioactivity may be quickly lost by phenotype instability (Berditsch et al. 2007) or effectively diluted out by cells better adapted to the medium/temperature.

Could lack of cultivability of some bacteria reflect lack of suitable osmotic support, i.e. could cells be more osmotically fragile than realised? There is some evidence that osmotically fragile cells (=sphaeroplasts; L forms) of some bacterial pathogens may exist in pathological material, and require special media, containing serum and sucrose, for growth. Thus, L-forms of *A. salmonicida* and *Yersinia ruckeri* (McIntosh and Austin 1990), *Lactococcus garvieae* (Schmidtke and Carson 1999) and possibly *Renibacterium salmoninarum* (Hirvelä-Koski et al. 2006) have been recognised. Conceivably osmotically fragile organisms would be missed by conventional culturing methods.

Culturing of pathogens

One of the goals of disease diagnostics and research is the acquisition of pure cultures of the pathogen. These may be used to determine pathogenicity in whole animals or cell cultures, or by detecting specific pathogenicity factors, such as haemolysins (Zhang et al. 2001). Pure cultures are invaluable for determining the relevance of pathogens in an epidemiological context. For example, multilocus sequence typing revealed a clonal lineage of *Aeromonas hydrophila*, which was associated with an epidemic of motile *Aeromonas* septicaemia in cyprinid fish in China (Zhang et al. 2014). Also, cultures are invaluable for determining antibiotic sensitivity patterns. A basic premise is that pathological material may be inoculated onto a solid or into a liquid medium with incubation at some specified temperature for a predetermined interval when individual cells of the pathogen will be cloned into dense culture growth, which will then be subjected to further study (Austin and Austin 2016). In the case of fish pathology, however, the range of media used is not extensive and lacks imagination in terms of the characteristics of the host. For aerobic, heterotrophic bacterial pathogens, the media are typically based on the presence of protein, carbohydrate and mineral salts, and include TSA or tryptone soya broth, brain heart infusion agar or broth, and marine equivalents containing elevated levels of sodium chloride or artificial sea salt mixtures

(Austin and Austin 2016). In addition, some specialised media have been designed for fastidious pathogens, such for *R. salmoninarum* and *Mycobacterium* spp., which are the causal agents of bacterial kidney disease and mycobacteriosis, respectively, with the former containing foetal calf serum and cysteine (Evelyn 1977), and the latter fresh egg, potato starch, glycerol and asparagine in the case of Löwenstein-Jensen medium (Austin and Austin 2016). Other media may be supplemented with blood, typically 5–7.5% v/v horse or sheep (Austin and Austin 2016). Also, there are dilute media in terms of ingredient concentration, e.g. cytophaga agar, which are suitable for flavobacteria or gliding bacteria (Anacker and Ordal 1959). Moreover, the incubation regimes that are often adopted may have little relevance to the growth conditions of the fish in aquaculture, with 37 °C used by many laboratories, regardless of the temperature at which the aquatic animal was reared (Austin and Austin 2016). The desired outcome is the development of dense virtually pure growth, which is taken as indicative of recovery of the pathogen (Austin and Austin 2016). The data do not usually enable the diagnostician to decide if the same organism started and continued the infection, or whether there might be a succession of micro-organisms involved with the disease cycle.

Is the culture representative of the actual pathogen or could it be a contaminant or a saprophyte growing on already damaged tissue? Moreover, it is unlikely that culturing on a single occasion would identify microbial population succession within a disease cycle. Also, conventional techniques are unlikely to recognise when two or more discrete organisms work synergistically or sequentially to produce a single pathology. A second organism could be a secondary coloniser, as was the case with *A. hydrophila* infecting fish suffering with columnaris, which is caused by *Flavobacterium columnare* (Scott and Bollinger 2014). In other cases, it was difficult to determine the relative importance of each organism. Examples include *Shewanella putrefaciens* and *Vibrio anguillarum* in loach (*Misgurnus anguillicaudatus*) (Qin et al. 2014), *Pseudomonas anguilliseptica* and *Delftia acidovorans* in European eels (Andree et al. 2013), *Edwardsiella ictaluri* and *F. columnare* in striped catfish (*Pangasianodon hypophthalmus* (Dong et al. 2015), and *Moritella viscosa* and *Aliivibrio wodanis* in winter ulcer disease (Hjerde et al. 2015).

Recovery of comparatively fast growing organisms may well mask fastidious (e.g. *Piscirickettsia* or *Chlamydia)*, slow growing (e.g. *Mycobacterium* or *R. salmoninarum)* or other organisms, e.g. '*Candidatus*' taxa that are incapable of growing on current laboratory media. Indeed, many '*Candidatus*' species have been described principally by sequencing of the partial 16S rRNA gene, which for several fish pathogens has pointed to a relatedness with the *Chlamydiaceae* (Austin and Austin 2016). There have been description of many species, including '*Candidatus* Arthromitus', '*Candidatus* Clavochlamydia Salmonicola', '*Candidatus* Syngnamydia Venezia', *Candidatus* Similichlamydia latridicola' and '*Candidatus* Piscichlamydia salmonis', which are associated with epitheliocystis/gastro-enteritis in salmonids in Europe and North America (e.g. Austin and Austin 2016; Stride et al. 2014). It is speculative how many more examples are awaiting recognition, and how many times have saprophytes or secondary invaders been blamed erroneously for pathological conditions that should have been attributed to more fastidious organisms.

Lack of cultivability

Some organisms have never been grown on laboratory media, with examples including *Mycobacterium leprae* (Pattyn 1973) and *'Candidatus* Syngnamydia Venezia*'* (Fehr et al. 2013; Austin and Austin 2016). Metagenomics may provide data on the organisms independent of the ability to culture them (Stewart 2012), such as the physiological properties of individual organisms. Based on genome-derived or physiological experiments with pure cultures, it is possible to make conclusions about bacteria in their natural environments. However, metagenomics or more appropriately metatranscriptomics may be more efficiently analysed if cultured representatives are present. Certainly, an attitude prevails that the culture may be representative of the organism in its natural environment, and should be stable during storage in the laboratory. The assumption that cultivability was akin to viability was challenged Xu et al. (by 1982), who observed intact, viable cells of the human pathogen *Vibrio cholerae* in the aquatic environment, in the absence of cultivability on media normally used for the isolation and maintenance of the pathogen. The concept of viable but non-culturable cells (VBNC)

was established (e.g. Wolf and Oliver 1992), and subsequently shown to occur with a wide range of Gram-negative and Gram-positive bacteria, including *A. salmonicida, Campylobacter jejuni*, *Cronobacter* spp., *Enterobacter* spp., *Enterococcus* sp., *Escherichia coli, Helicobacter pylori*, *Klebsiella* spp., *Listeria monocytogenes, Morganella* spp., *Mycobacterium tuberculosis*, *Providencia* spp., *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *V. vulnificus* and *Yersinia enterocolytica* (Oliver 2005, 2010; Nascutiu 2010; Li et al. 2014; Ramamurthy et al. 2014; Pinto et al. 2015). The development of the VBNC state may reflect a switch to the new conditions in a changing environment, and is of concern to the food and pharmaceutical industries whereby the presence of biocide leads to the development of the viable uncultured state rather than complete cell inactivation (Oliver 2005, 2010; Nascutiu 2010; Li et al. 2014; Ramamurthy et al. 2014; Pinto et al. 2015). The possibility of VBNC cells regaining pathogenicity in a susceptible host is a major concern, especially with pathogens such as *V. cholerae* (Colwell 1993).

The relevance of stock cultures

It is questionable whether stock cultures, i.e. those that have been maintained for a large number of years, provide much useful information about the biology of the bacteria, insofar as there may be a mismatch between the properties of the stored culture compared with fresh isolates. For example, with $>$ 100 cultures of *Vibrio harveyi*, which were initially regarded as pathogenic to fish or shellfish on primary isolation, only two isolates were markedly virulent after years in the laboratory (Zhang and Austin 2000). Here, virulence of the two isolates was associated with possession of double haemolysin genes; the other less pathogenic isolates possessed only single genes (Zhang et al. 2001). Conceivably, the non-pathogenic cultures lost haemolysin genes during storage. In addition, virulence has been linked to bacteriophage (Oakey and Owens 2000; Austin et al. 2003) and bacteriocin-like substances (BLIS; Prasad et al. 2005). *V. harveyi* myovirus like (VHML) bacteriophage enhanced virulence of the pathogen to Atlantic salmon, and enhanced haemolytic activity (Austin et al. 2003). Also, there was evidence that infection of *V. harveyi* with VHML led to a return to virulence of fish and invertebrates (Austin et al. 2003). Therefore, caution is needed before drawing any sweeping conclusions about the characteristic of stored cultures.

Minimum number of cultures for taxonomic studies

Scrutiny of the bacterial taxonomy literature since the start of the 21st century reveals that the overwhelming majority of new species are described after the examination of single cultures. The emphasis is often on the DNA rather than viable cultures. This is logical for the organisms that have not been cultured, but a polyphasic approach is appropriate for those organisms that may be cultured. Indeed, cultures are very important for good taxonomy with phenotypic descriptions of taxa relying on information gained from examinations of pure cultures, a representative of which will comprise the type strain of each [cultivable] species. A concern may be raised about whether it is appropriate to use single cultures for the description of new taxa (Christensen et al. 2001; Janda and Abbott 2002; Drancourt and Raoult 2005). Although a consensus has not been reached, it has been recommended that a minimum of five strains [that are pure and authentic] from a variety of locations and habitats should be used for the description of new species (Christensen et al. 2001; Janda and Abbott 2002). This approach reduces the risk that the single culture, and thus type strain of the new taxon, will be subsequently recognised to be an outlier rather than the centroid as more cultures are obtained and studied. Thus, by using multiple cultures, it is possible to gain reliable information about the overall characteristics of a taxon. Every effort should be made to obtain multiple cultures of taxa where only single cultures are available, although it is recognised that this process may take years to achieve.

Conclusions

Culturing still has a role in modern microbiology. However:

- Current culturing methods tend to select bacteria best suited for life in the laboratory. With storage,

the continued relevance of the culture to the cells in the habitat from which they were acquired is questionable.

- Environmentally relevant bacteria may be overlooked in a strictly cultivation-dependent approach. To avoid this situation, it should become the norm to combine cultivation with a cultivation-independent approach, mainly 16S rRNA gene amplicon sequencing and perhaps the direct fluorescence in situ hybridization (FISH) detection of microorganisms directly in the habitat (Moter and Gobel 2000; Bouvier and del Giorgio 2003; Rohde et al. 2015; Deshmukh et al. 2016).
- Efforts are needed to devise laboratory conditions appropriate for the cultivation of the more fastidious organisms that are difficult or impossible to grow in vitro.
- More needs to be done to preserve the original features of bacterial cells in laboratory cultures. This may involve maintaining the cells in a condition as close to the original habitat as possible.

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