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Walk on the wild side: The application of environmental isolates in microbiological testing

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Abstract

Environmental isolates are commonly used in the pharmaceutical sector to challenge microbial test methods and to release culture media. Does the use of these organisms add value? There are arguments in favour of this practice (broadening the test panel and with the assumption that some 'wildtype' characteristics are retained) and against (such as environmental isolates being difficult to standardise and the expectation that 'wildtype' characteristics are lost at some time point or they are not retained at all with the first subculture onto laboratory media). This paper considers these arguments.

Introduction

Across the past decade there has been a trend for environmental isolates to be used as part test regimes within pharmaceutical microbiology laboratories. Examples are with the qualification of microbiological methods (such as method suitability testing), for the quality control testing and release of culture media, and to evaluate disinfectant efficacy (1). The main driver towards the use of environmental isolates has come from regulators (there appears to be no pre-dating literature prompting the adoption of environmental isolates). According to Booth, this regulatory expectation for using environmental isolates to challenge culture media can be demonstrated in observations issued by the U.S. FDA as indicated in warning letters (2). The use of facility isolates is also connected to the realisation by many microbiologists that they need to broaden the test panel of organisms in order to improve the overall representativeness of the microbial challenge, as a means of increasing method robustness by demonstrating the recovery of a wider spectrum of organisms. However, considering that test panels can be broadened through use of type cultures from an approved culture collection there is more to be examined in the arguments calling for the use of facility isolated strains.

Environmental variations

That phenotypic variations occur is not disputed and this confers an evolutionary advantage, from adapting to new environments to persister subpopulations surviving exposure to concentrations of antimicrobials or disinfectants. The argument in favour of using pharmaceutical plant environmental isolates rests on the assumption that such facility isolates will exhibit different growth characteristics and will thereby be someway 'hardier' than laboratory strains derived from a culture collection. Here there is evidence that bacteria subjected to a changeable environment will show greater adaptation to a novel condition (3). In turn, this is based on the notion that such isolated organisms possess 'wildtype' characteristics which are retained for a duration of time or within a set number of passages. By 'wildtype' this infers that either selection has occurred (the survival of better adapted individuals) or that the isolated organisms exhibit different phenotypic characteristics, as observable traits (where variations have occurred between individual cells). These would be something observed by the microbiologist and not necessarily to alterations taking place within the microbial genome. Furthermore, no two species will adapt the same way under the same set of environmental stressors (there are variances with phenotypic plasticity) and within the same species, subpopulations will behave in different ways (4). There is no

consensus as to which phenotypic characteristics are of importance for demonstrating microbial recovery and these are likely to vary by species and different types of stressors to which the organism has been subjected. Examples include: Cell size (5); a requirement for different or modified levels of nutrients (diauxic shift) (6); and a diversification between motile and non-motile cells (7). Perhaps the greatest trait of interest is the growth rate, given the compendial focus on 'time to grow' as part of the acceptance criteria for microbiological methods.

Should such traits be presented, another area where there is a lack of consensus among microbiologists is whether such characteristics continue to be present once the organism has been subcultured onto nutrient rich laboratory media or whether the organism becomes phenotypically heterogeneous, displaying different traits according to different environments (8). Furthermore, should these traits continue, then for how long or through how many subculture steps do the wildtype characteristics remain significant influencers of growth rates.

On representation and culturability

The test panels of organisms recommended by the major pharmacopeia and within national and international standards (compendia) are in place to ensure reproducibility between laboratories when the same tests are conducted. In the case of most standards, these are designed to be multi-application and sometimes multi-industry. However, the range of isolates quoted may or may not be suitable for the application, and many quoted panels have not changed in recent decades. This is the product of evolving information about microbial risks, where pharmacopeia or a CEN or ISO standard may not have kept pace. One important role of the microbiologist is to review the panel and decide whether to supplement it with additional isolates. For example, there is a cogent argument for including *Micrococcus luteus* in the microbial test panel for evaluating media used for environmental monitoring, given the bacterium's common association with the microbiome of the outer layers of human skin (9) as well as the Micrococcaceae accounting for a high proportion of organisms recovered from cleanrooms (10). Ubiquity alone is not necessarily a criterion for inclusion. Those less set on widening test panels may counterpoint that a species of *Staphylococcus* is already recommended by the pharmacopeia and on this basis is there any merit in including a *Staphylococcus* isolated from a facility cleanroom? Perhaps if the recommendation was to replace the pharmacopeia strain of *Staphylococcus aureus* with *Staphylococcus epidermidis* given there are very few differences between microorganisms of the same genera when it comes to the determinants of growth upon culture media (as with different species of *Staphylococcus*, with few phenotypic differences supported by few genetic differences, as drawn from reviews into 16S rRNA base sequencing) (11). Yet outside of the species group, different families have different requirements and *Micrococcus* species have an ability to survive for prolonged periods in cleanrooms. As Baird-Parker observed back in 1963, the micrococci are saprophytes which are nutritionally less exacting and morphologically and biochemically more variable than the staphylococci (12).

While the reasons for expanding the test panel have merit on the basis of improved representativeness, should these additions be environmental isolates? An argument used by environmental isolate adherents relates to the expectation there will be different growth

requirements, growth rates, and cultural characteristics for organisms isolated from a defined niche compared with those long supported on laboratory culture media. To consider this issue, we need to consider culturability and environmental stressors.

The term 'viable but non-culturable' is well established (13, 14). That there are organisms within the environment that cannot be grown on culture media is known through the application of culture-independent molecular based methods. Such methods provide information about the genetic diversity of microorganisms (15) and suggest that more organisms globally are unculturable than are culturable (16, 17), a point that, as an aside, testifies as to why, within cleanrooms, maintaining a state of environmental control will always be more important than simply undertaking environmental monitoring. Situations where organisms maintain their viability but where they are unable to grow on laboratory media can be due to three reasons. First, it may be that no culture medium has yet been (or can be) developed to enable their culture (18). Secondly, the organisms may be culturable, but the culture medium used, or the incubation parameters selected are not conducive for growth (19). Thirdly, organisms may be culturable under one set of conditions, but they are not under a different set of conditions due to the activity of some type of 'stressor' that prevents or slows the growth necessary for cultivation at a given point in time. Stressors include variables of the environment, such as extreme temperatures, nutrient starvation, pH changes, osmotic stress, reactive oxygen species, exposure to preservatives, disinfectants or radiation, many of which will be active within the cleanroom environment. The response to stressors can trigger spore formation in some organisms (20) and a low-energy use or dormancy strategy in others, where cells remain metabolically active but are not dividing (21-23). In other cases, growth may occur, but a prolonged time is required due to phenotypic changes. This may include cells undergoing a form of change, such as cell dwarfing (24).

The presence of stressors is likely to be commonplace and many pharmaceutical environments, such as water systems and cleanrooms, will be challenging not only for microbial survival but also for microbial recovery and growth. Microorganisms confronted with suboptimal conditions will often adapt and re-allocate energy in order to maintain homeostasis when resources are scarce (25). Some taxa have the flexibility to generate ATP using different combinations of electron acceptors and electron donors, whereas other organisms are specialized in the assimilation of unique substrates or the conservation of energy by suspending their metabolic activities (26, 27). Other bacteria, such as those classed as saprophytic, can scavenge dead cells to meet their maintenance requirements and even to support limited cellular division (so termed 'necromass recycling') (28, 29). These alternations to cell functions will affect the recovered diversity of small populations of microorganisms (30). Such conditions can also lead to the acceleration of population mutations (31, 32), with the more prolonged starvation leading to the greatest number of genetic and phenotypic changes (33) as well as there being more strains harbouring mutations that increase or decrease persister (dormant cells) frequency (34).

Consideration of environmental isolates

That organisms will express phenotypic variations under different environmental conditions is established. Proponents for incorporating environmental organisms into test regimes maintain that specific stress responses and survival mechanisms shown by different

organisms leads to different growth patterns (35, 36). This effect could be to the extent that certain organisms appear fastidious to the level of 'no growth' being recorded from a test when in fact recovery may have been possible with longer incubation times, due to the reversal of non-culturability requiring extended resuscitation in order to overcome a given stress factor (37). This becomes an argument for including environmental isolates and, based on amassed data, possibly modifying the standard test protocol to account for a longer incubation time in order to produce sufficient biomass for the organism to become visible with the naked eye (38). Time does not only relate to the start of incubation but also to the point when transfer onto culture media occurs, since there will be a point after which recovery is no longer possible. With this, there will be variation between organisms since longevity and extinction are taxon specific (39).

Attempting to answer whether a longer incubation is required to recover environmental isolates is not straight forward. Within pharmaceuticals, there has been minimum published research examining the cultivation and growth of environmental isolates. Research, has nonetheless, taken place in other fields, examining survivor curves according to different ecological niches and laboratory recovery rates (40). For example, a study into the growth rates of *Pseudomonas aeruginosa* isolates from different ecological niches, including rivers and from homes, found high variability in growth rate among isolates on culture media (41). Hence, the time taken for incubation depends upon the species of organism and the physiological state of the organism. Such findings have not been noted by all researchers, however, with other studies showing a rapid adaptive response and a short generational time loss of any wildtype characteristics (42, 43). Different organisms will have awakening processes in response to encountering the nutrients and environmental conditions necessary for growth. These processes will be connected to different metabolic pathways. In addition, different triggers will be required to move an organism from a state of dormancy.

Research questions

This leads to the necessity of probing the research questions: 'how long should media be incubated for?' and 'do environmental isolates require a longer incubation time compared with compendial strains?' If it can be shown through study that the growth rates are similar, then the need to specifically include facility isolates is weakened, although the arguments for expanding microbial test panels remain. Whereas, if it can be shown through study that growth rates are longer, then the case to include facility isolates is strengthened. If this occurs, this is most probably due to an extended cell lag phase taking place before exponential growth begins.

It may also be, taking the point further, that the tests that are described in the compendia or according to local procedures may have incubation times that are too short and extended periods may be required to promote recovery. Alternatively, it may be necessary to introduce an enrichment step. At this level, the environmental isolate inclusion complicates things.

Whether this is or is not an issue should be the matter of study and given that there will be different organisms in different states and subject to different stressors, added to the fact that culture media formulation varies, then such studies would need to be conducted at the level of the individual plant.

Conclusion

This paper has been written as a stimulus piece. The aim was to weigh up the arguments for and against the use of environmental isolates within pharmaceutical facilities for both the release of culture media and for inclusion in media release systems. In balancing the arguments, relevant literature has been referred to. In coming down on one side, then to do so requires empirical evidence. Either way, the microbial test panel probably needs to be more representative to make laboratory methods more robust. It is just a question of whether this panel expansion is made up of culture collection strains or facility isolates, or whether it matters at all?

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