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Examination of the growth rates of environmental isolates compared with compendial strains

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Abstract

For some years there has been a regulatory drive for microbiology laboratories to use environmental isolates for media quality control and for the incorporation into method suitability studies. Where these organisms are included in testing regimes, do they take longer to grow? This paper assesses the growth rates of environmental isolates in comparison with compendial recommended cultures. The research presented here finds that environmental isolates do take slightly longer to grow compared with laboratory strains, yet this time difference was within the recommended incubation times of each test type. Therefore, microbiologists should generally expect slower growth but test methods may not need to be adapted to compensate.

Introduction

Within pharmaceutical microbiology the microbial panels quoted in the compendia offer the minimum acceptable challenge in terms of the range of species presented. There are many arguments for improving method robustness by expanding the panel [1]. A regulatory focus is with the use of environmental isolates for this purpose rather than a wider use of strains derived from a culture collection. This is based on the premise that the wildtype characteristics developed by the organism to its environment make the organism more fastidious and therefore a method that can recover environmental isolates is in some way more robust than a method that cannot and that the laboratory (and presumably the regulator) will gain greater confidence with the ability of the method to recover any microbial contamination present in a test sample.

There have been few published studies looking into incubation times and the origin of microorganisms and therefore attempting to answer whether a longer incubation is required to recover environmental isolates is not straight forward. Within pharmaceuticals, there has been no published research examining the cultivation and growth of environmental isolates. Work undertaken in other fields shows variation between different ecological niches and laboratory recovery rates [2] [3]. From this it can be inferred that incubation time is dependent upon the species of organism and the physiological state of the organism [4]. The physiological state will influence the triggers necessary to move out of a state of dormancy and the speed of the awakening processes once the organism encounters the nutrients and environmental conditions necessary for growth [5]. The growth rate itself will be the product of different metabolic pathways [6].

This discussion leads to the present inquiry: If ‘wildtype’ characteristics are retained, then do environmental isolates grow more slowly than laboratory cultures? Then, if they are shown to be more challenging to grow, is an extended incubation time required and different acceptance criteria required? And, if this is the case, does this create any ramifications for media quality control or method suitability testing in relation to maximum incubation times?

This paper studies the question of incubation time by examining growth rates of facility isolates against type cultures sourced from a recognised culture collection. For the evaluation

common types of culture media used within pharmaceutical microbiology were selected. The research questions set were: 'How long should media be incubated for?'; and 'Do environmental isolates require a longer incubation time compared with compendial strains?'

To answer these, the examination assessed pharmacopeia recommended cultures against facility isolates, noting growth times. As far as the author can ascertain, this is the first time such data has been presented in the form of a science paper.

Method

For the study into microbial growth rates, five culture media commonly used in pharmaceutical microbiology laboratories were selected. These media were:

- Tryptone soya agar (equivalent to soyabean casein digest medium, sometimes called tryptic soy agar, and abbreviated to TSA). This is a medium for the isolation and cultivation of non-fastidious and fastidious microorganisms.
- Tryptone soya broth (equivalent to soyabean casein digest medium, sometimes called tryptic soy broth, and abbreviated to TSB). This is a medium for the isolation and cultivation of non-fastidious and fastidious microorganisms. This medium is used for sterility testing and as a general growth broth.
- Reasoner's 2A agar (R2A). This is a low nutrient agar used for water testing and the cultivation of heterotrophic microorganisms
- Fluid thioglycollate medium. This medium is used for the growth of bacteria (aerobic and anaerobic). It is primarily used for sterility testing.

The TSA medium was presented with and without neutralisers (adding neutralisers to the formulation is designed to reduce any inhibitory effect from any disinfectant residuals). The medium was also presented as two different fills: 9cm plate and a domed plate with a 25cm^2 surface area for surface sampling (a contact plate). Examples of the use of each medium are presented below (in Table 1), together with the maximum test incubation times stated within the relevant test monograph, as taken from the European Pharmacopeia.

Table 1: Culture media evaluated

Medium	Example of use	European pharmacopeia recommended incubation time
9 cm TSA	Microbial Limits Test, Environmental monitoring	5 days for Microbial Limits Test
9 cm TSA (with neutraliser)	Environmental monitoring e.g. finger dabs	Not specified, but 3-7 days is typical.
TSA 25cm^2 contact plates	Surface sampling	Not specified, but 3-7 days is typical.
R2A agar	Water testing	5 days for Water for Injections testing
100mL TSB	Sterility Testing	14 days
100mL FTM	Sterility Testing	14 days

For the study, different lots of each medium were subjected to growth promotion testing (a required pre-release test). The growth promotion testing was conducted using compendial strains (as recommended in pharmacopeial monograph as per Table 2) and isolates from a manufacturing facility composed of cleanrooms, located in south-east England.

The standard compendial strains are:

Table 2: Recommended test organisms, as per the European Pharmacopeia

Microorganism	American Type Culture Collection (ATCC) Reference	Originally isolated from
Staphylococcus aureus subsp. *aureus*	ATCC 6538	Human lesion
Bacillus subtilis subsp. *spizizenii*	ATCC 6633	Earth
Pseudomonas aeruginosa	ATCC 9027	Outer ear infection
Clostridium sporogenes	ATCC 19404	Gas gangrene
Candida albicans	ATCC 10231	Man, with bronchomycosis
Aspergillus brasiliensis	ATCC 16404	Blueberry, North Carolina

The use of the above strains (Table 2) was against the test media as follows (Table 3):

Table 3: Test regime for study media against compendial strains

Media	*S. aureus*	*Bs. subtilis*	*Ps. aeruginosa*	*C. sporogenes*	*C. albicans*	*A. brasiliensis*
TSB	Ö	Ö	Ö	N/A	Ö	Ö
TSA	Ö	Ö	Ö	N/A	Ö	Ö
FTB	Ö	N/A	Ö	Ö	N/A	N/A
R2A agar	Ö	Ö	Ö	N/A	N/A	N/A

where the '√' denotes testing performed.

Each of the organisms used in the study were mesophiles ("middle loving"), that is organisms adapted to moderate temperatures, with optimal growth temperatures ranging from room temperature (about 20°C) to about 45°C. The test conditions used to incubate the inoculated media are shown in Table 4.

Table 4: Incubation temperatures for inoculated media

Medium	**Incubation temperature**
9 cm TSA (with neutraliser)	30-35°C
9 cm TSA (without neutraliser)	30-35°C
TSA contact plates	30-35°C
R2A agar	20-25°C and 30-35°C
TSB	20-25°C
FTB	30-35°C

The number of different lots of media tested and the types of environmental isolates used varied, as stated in the results section below. The variation arises because the study was conducted over a one-year period and different environmental isolates were selected, in some cases, for different media. The policy of the laboratory was that the selected wildtypes are used for approximately twelve months, after which a further set of isolates are used for the next twelve-month period. It is possible, based on facility microbiota reviews that the identified isolates will be the same species across successive years.

For both compendial and facility organisms, the challenge inoculum was targeted to fall between 10 and 100 CFU in a 0.1mL suspension. The application of the microbial challenge suspension to plate media was by spotting liquid inocula onto the surface of the plate and applying the culture across the plate using a sterile spreading device. The inoculation into broth media was direct, using a syringe and needle. All microorganisms were stored using the Microbank Storage System, where cells were placed onto beads and the beads placed into a cryopreservation fluid and held at -80°C. Cells were selected when they were most probably within the stationary phase. This was performed to reduce probability of mutations occurring and promote a longer survival rate. A further step to help to minimise change was to restrict the number of passages below 5. A single bead was used to prepare the challenge culture and the target inoculum was achieved by undertaking serial dilutions using peptone water. Post-incubation, for plate media, colonial counts were confirmed by counting colonies using a colony counting device equipped with a white light source lamp. For broth media, growth was

confirmed through visual inspection (an examination for signs of turbidity, sedimentation, or the presence of a pellicle). During the course of the study, all samples were examined every 12 hours.

Results

The test results for each medium are detailed in a series of tables and graphs.

Table 5: 9cm Tryptone Soya Agar (without neutraliser)

Number of batches tested	Environmental isolates used	Morphological type	Isolated from
19	*Micrococcus luteus*	Gram-positive coccus (GPC)	Human skin (via a finger dab)
	Pseudomonas fulva	Gram-negative rod (GNR)	Purified water system
	Bacillus cereus	Gram-positive sporing rod (GPSR)	EU GMP Grade C cleanroom surface
	Pseudomonas stutzeri	Gram-negative rod (GNR)	Purified water system
	Microbacterium maritypicum	Gram-positive rod (GPR)	EU GMP Grade C cleanroom surface
	Stenotrophomonas maltophilia	Gram-negative rod (GNR)	Mains water system
	Pseudomonas composti	Gram-negative rod (GNR)	Water for Injections sink outlet
	Bacillus subtilis	Gram-positive sporing rod (GPSR)	Transfer hatch
	Staphylococcus epidermidis	Gram-positive coccus (GPC)	Human skin (via a gown plate)

The test data from Table 5 has been represented graphically (Figure 1), for the mean compendial strain against the means of the tests for the different environmental isolates. For each graph, the mean time was graphed with outliers stated.

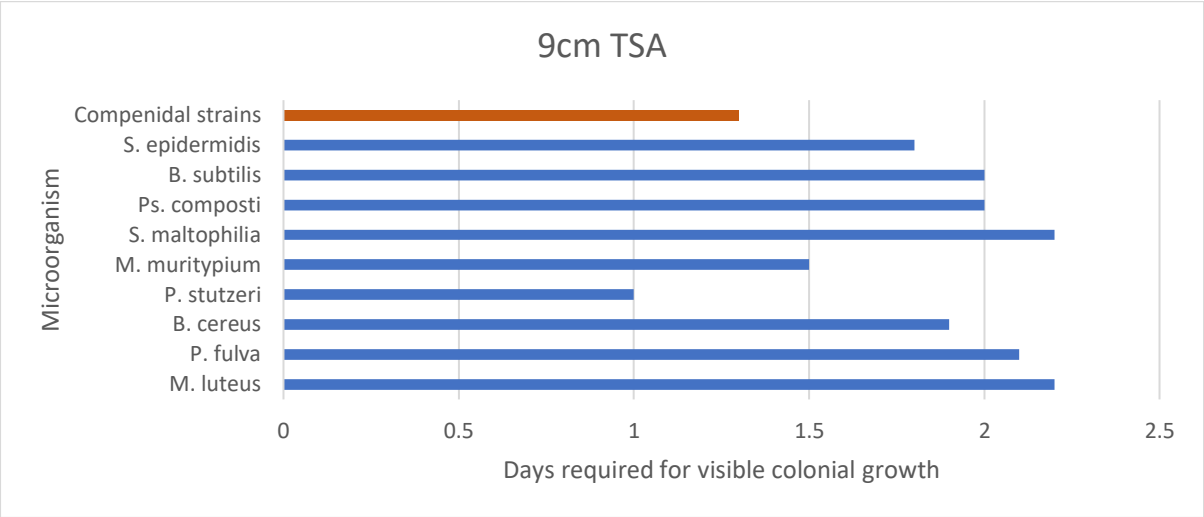

Figure 1: Growth promotion data for TSA plates (without neutraliser)

Table 6: 9cm Tryptone Soya Agar (with neutraliser)

Number of batches tested	Environmental isolates used	Morphological type	Isolated from
33	*Micrococcus luteus*	Gram-positive coccus (GPC)	Human skin (via a finger dab)
	Pseudomonas fulva	Gram-negative rod (GNR)	Purified water system
	Bacillus cereus	Gram-positive sporing rod (GPSR)	EU GMP Grade C cleanroom surface
	Microbacterium maritypicum	Gram-positive rod (GPR)	EU GMP Grade C cleanroom surface
	Stenotrophomonas maltophilia	Gram-negative rod (GNR)	Mains water system
	Pseudomonas composti	Gram-negative rod (GNR)	Water for Injections sink outlet
	Bacillus subtilis	Gram-positive sporing rod (GPSR)	Transfer hatch
	Staphylococcus epidermidis	Gram-positive coccus (GPC)	Human skin (via a gown plate)

The test data from Table 6 has been represented graphically (Figure 2), for the mean compendial strain against the means of the tests for the different environmental isolates.

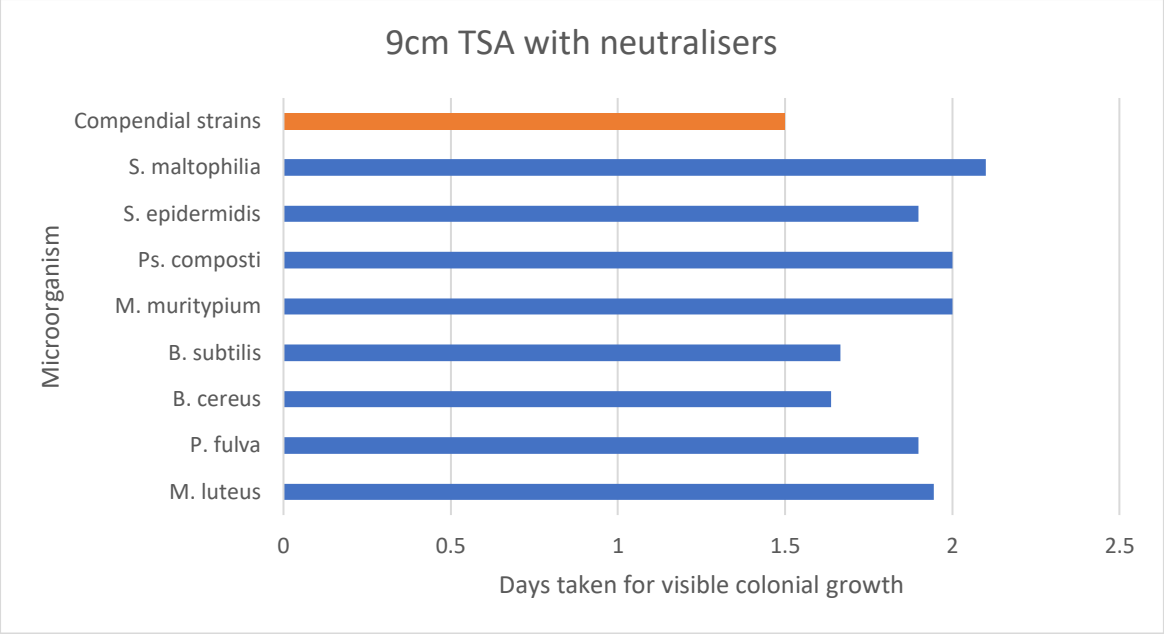

Figure 2: Growth promotion data for TSA plates (neutraliser)

Table 7: TSA contact plates

Number of batches tested	Environmental isolates used	Morphological type	Isolated from
16	*Micrococcus luteus*	Gram-positive coccus (GPC)	Human skin (via a finger dab)
	Pseudomonas fulva	Gram-negative rod (GNR)	Purified water system
	Bacillus cereus	Gram-positive sporing rod (GPSR)	EU GMP Grade C cleanroom surface
	Corynebacterium lipophiloflavum	Gram-positive rod (GPR)	EU GMP Grade D changing room
	Stenotrophomonas maltophilia	Gram-negative rod (GNR)	Mains water system
	Pseudomonas composti	Gram-negative rod (GNR)	Water for Injections sink outlet
	Bacillus subtilis	Gram-positive sporing rod (GPSR)	Transfer hatch
	Staphylococcus epidermidis	Gram-positive coccus (GPC)	Human skin (via a gown plate)

The test data (Table 7) has been represented graphically (Figure 3), for the mean compendial strain against the means of the tests for the different environmental isolates.

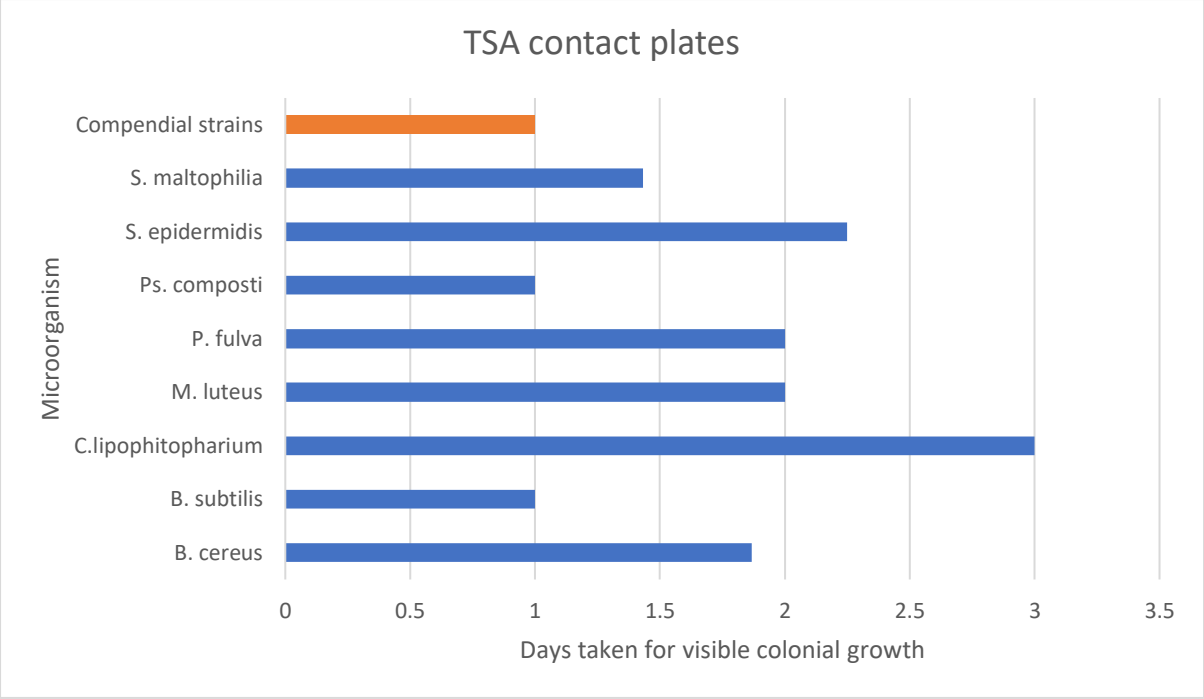

Figure 3: Growth promotion data for TSA contact plates

Table 8: R2A agar

Number of batches tested	Environmental isolates used	Morphological type	Isolated from
12	*Micrococcus luteus*	Gram-positive coccus (GPC)	Human skin (via a finger dab)
	Pseudomonas fulva	Gram-negative rod (GNR)	Purified water system
	Bacillus cereus	Gram-positive sporing rod (GPSR)	EU GMP Grade C cleanroom surface
	Stenotrophomonas maltophilia	Gram-negative rod (GNR)	Mains water system
	Pseudomonas composti	Gram-negative rod (GNR)	Water for Injections sink outlet
	Staphylococcus epidermidis	Gram-positive coccus (GPC)	Human skin (via a gown plate)

The test data (Table 8) has been represented graphically (Figure 4), for the mean compendial strain against the means of the tests for the different environmental isolates.

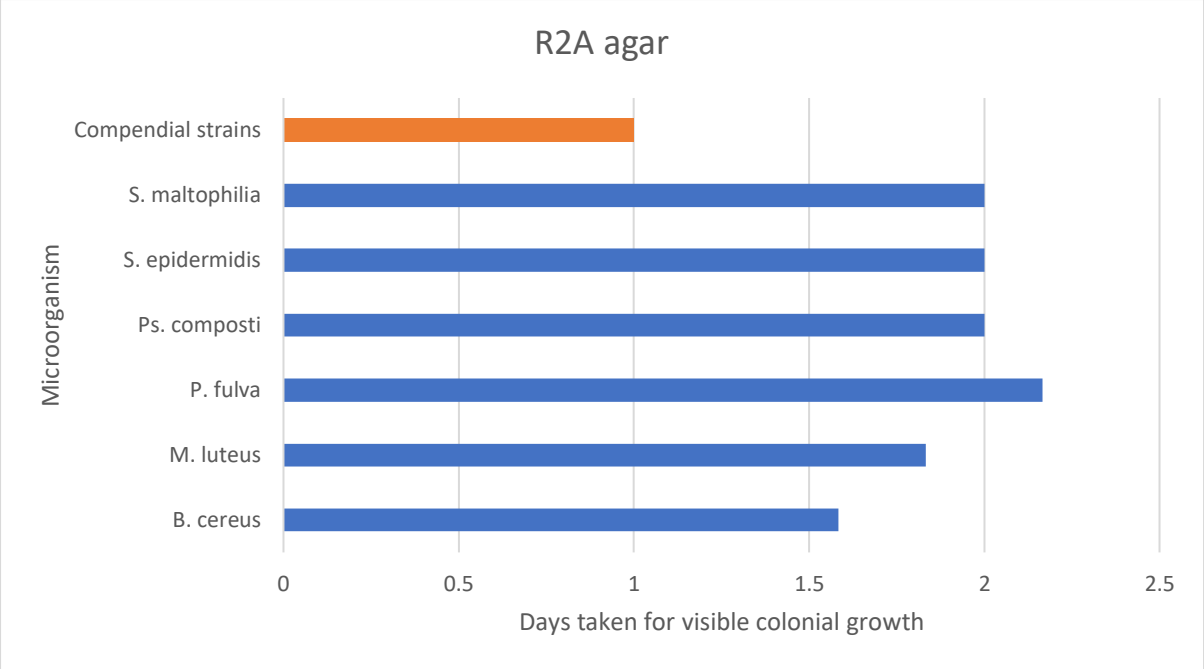

Figure 4: Growth promotion data for R2A agar plates

Table 9: Fluid thioglycolate medium

Number of batches tested	Environmental isolates used	Morphological type	Isolated from
10	*Pseudomonas fulva*	Gram-negative rod (GNR)	Purified water system
	Bacillus cereus	Gram-positive sporing rod (GPSR)	EU GMP Grade C cleanroom surface
	Corynebacterium lipophiloflavum	Gram-positive rod (GPR)	EU GMP Grade D changing room
	Stenotrophomonas maltophilia	Gram-negative rod (GNR)	Mains water system
	Staphylococcus epidermidis	Gram-positive coccus (GPC)	Human skin (via a gown plate)

The test data (Table 9) has been represented graphically (Figure 5), for the mean compendial strain against the means of the tests for the different environmental isolates.

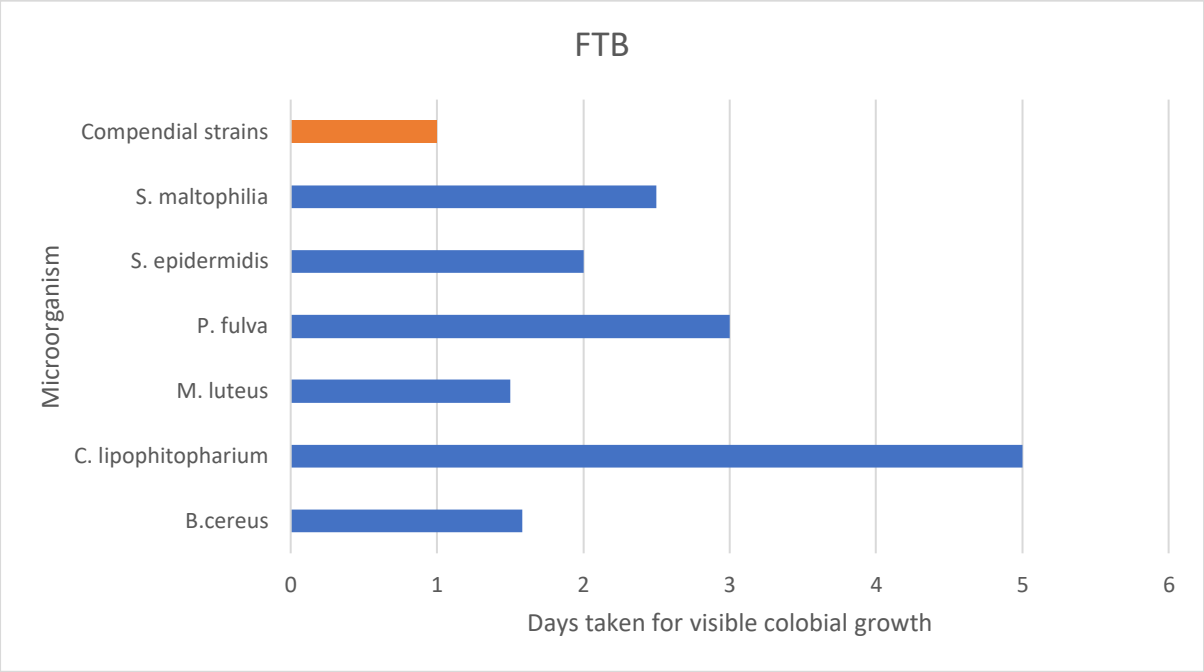

Figure 5: Growth promotion data for FTB

Table 10: Tryptone soya broth

Number of batches tested	Environmental isolates used	Morphological type	Isolated from
11	*Micrococcus luteus*	Gram-positive coccus (GPC)	Human skin (via a finger dab)
	Pseudomonas fulva	Gram-negative rod (GNR)	Purified water system
	Bacillus cereus	Gram-positive sporing rod (GPSR)	EU GMP Grade C cleanroom surface
	Corynebacterium lipophiloflavum	Gram-positive rod (GPR)	EU GMP Grade D changing room
	Stenotrophomonas maltophilia	Gram-negative rod (GNR)	Mains water system
	Staphylococcus epidermidis	Gram-positive coccus (GPC)	Human skin (via a gown plate)

The test data (Table 10) has been represented graphically (Figure 6), for the mean compendial strain against the means of the tests for the different environmental isolates.

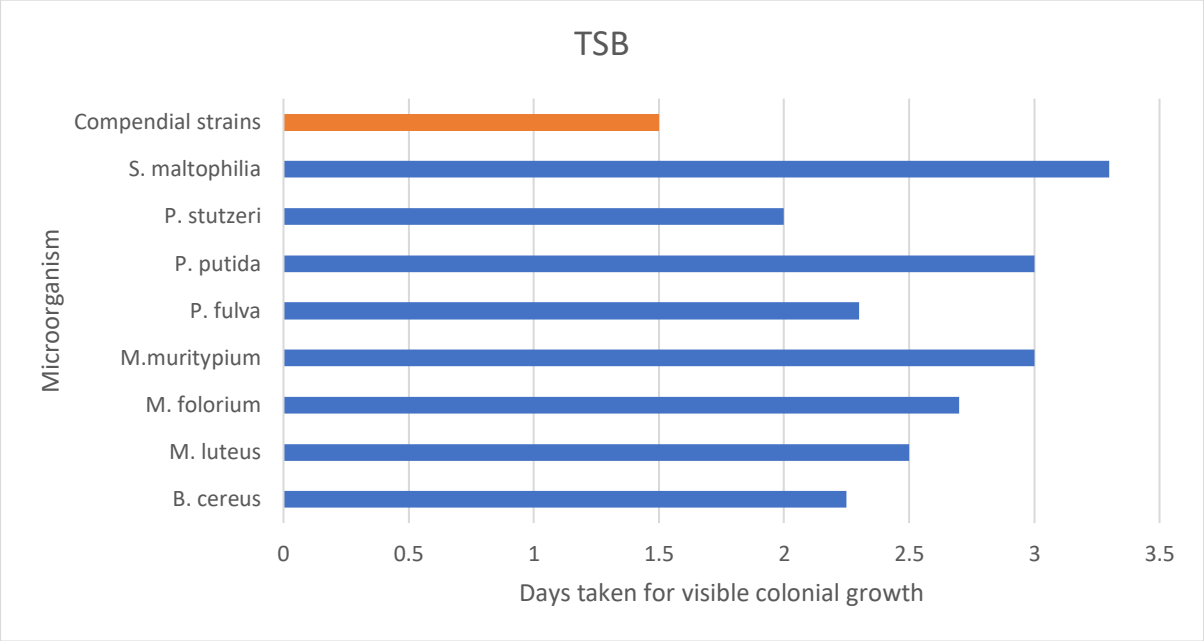

Figure 6: Growth promotion data for TSB

Comparison

Table 11 displays the outcome of the tests on the culture media for environmental isolate (means) compared with compendial strain (means), noting the differences. Data is additionally provided for the different taxonomic groups of environmental isolates.

Table 11: Summary of data across each culture media type

Medium	**9 cm TSA**	**9 cm TSA (with neutraliser)**	**TSA contact plates**	**R2A agar**	**FTB**	**TSB**
Mean environmental isolate (days)	1.9	1.9	1.8	1.8	2.6	2.6
Mean compendial strain (days)	1.3	1.5	1.0	1.0	1.0	1.5
Difference between mean environmental isolate and mean compendial strain (days)	0.6	0.4	0.8	0.8	1.6	1.1
Mean GPC isolate (days)	1.7	2.0	2.1	2.0	2.0	2.5
Mean GPR isolate (days)	1.5	2.0	3.0	Not tested	5.0	3.0
Mean GPSR isolate (days)	1.7	1.6	1.5	1.6	1.6	2.3
Mean GNR isolate (days)	1.5	2.1	1.6	2.1	2.1	2.7

The data shows that, in each case, the time required for growth for the average (mean) environmental isolate is for longer compared with the average time (mean) required for a compendial strain to grow (range of this difference is between 0.6 to 1.6 days, or what can be rounded to one half day to two days for practical purposes). With different types of environmental isolates, by morphology, no clear pattern emerged. Gram-positive rods took slightly longer to grow across three types of media (TSA contact plates, TSB and FTB) and Gram-negative rods took longer to grow for two different media types (9 cm TSA containing a neutraliser and R2A). These patterns are displayed in Figure 7.

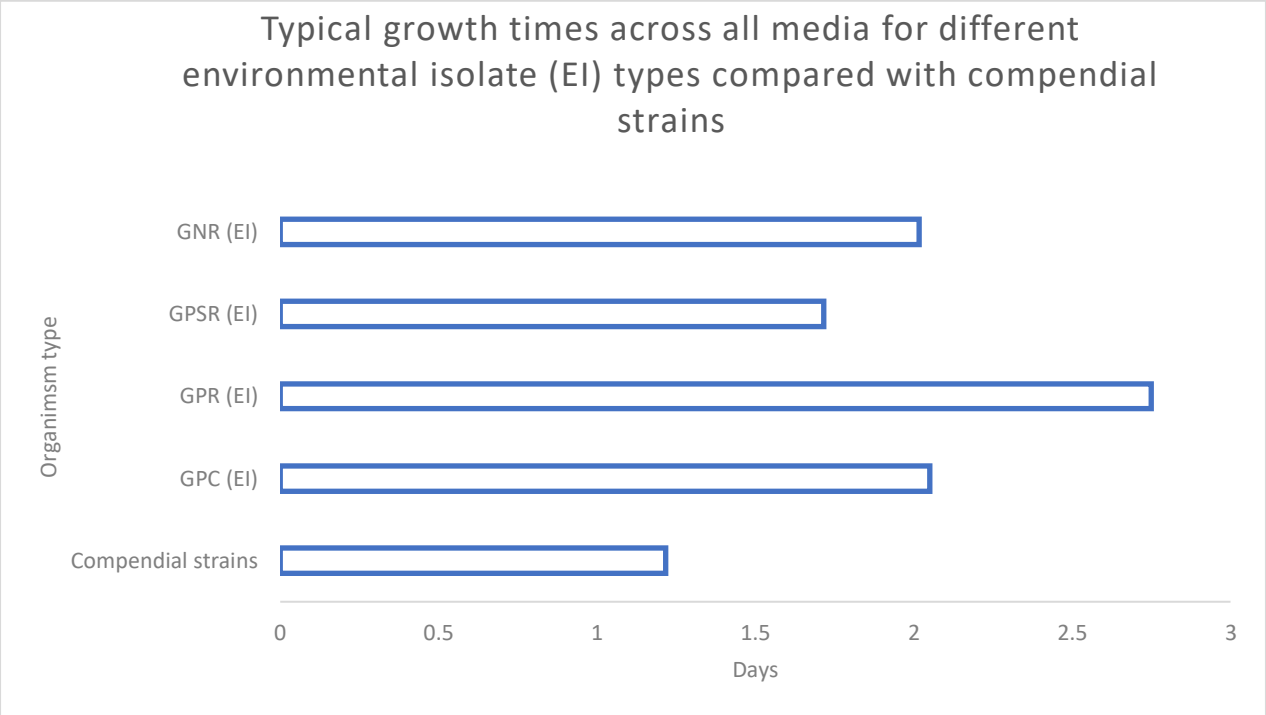

Figure 7: Variations in growth times for environmental isolates divided by morphological group

With different media types, growth times for media challenged with environmental isolates were generally longer for a broth medium compared with plate media. This is illustrated graphically in Figure 8.

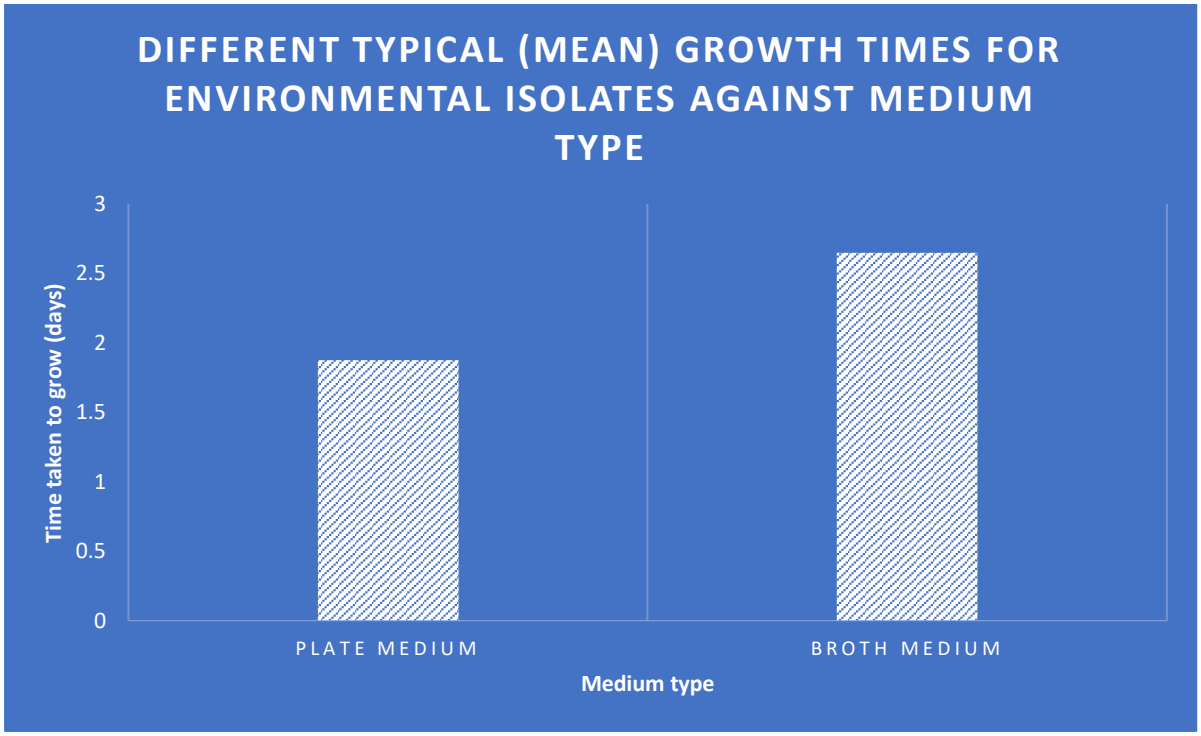

Figure 8: Comparative growth rates for environmental isolates challenged against solid media (plates) and liquid media (broth)

With environmental isolates taking up to 2 days longer to grow compared with compendial strains, it is reasonable to consider whether any organism would have exceeded the maximum incubation time for the test. For this analysis, presented in Table 12, the organism that took the longest time to grow for a specific medium is examined against the typical pharmacopeial tests that the medium is typically used for.

Table 12: Comparison of the slowest growing environmental isolates against maximum test incubation times

Culture medium	**Time taken for the slowest environmental isolate to grow**		**Primary test**	**Recommended test incubation time**	**Incubation time exceeded by the slowest growing microorganism?**
	Species	**Time taken**			
9cm TSA (no neutraliser)	*P. fulva*	3 days	Microbial Limits Test	5 days	No
9cm TSA (with neutraliser)	*S. maltophilia*	3 days	Environmental monitoring	7 days	No
TSA contact plates	*S. maltophilia*	4 days	Environmental monitoring	7 days	No
R2A agar	*P. fulva*	3 days	Pharmaceutical grade water	5 days	No
TSB	*S. maltophilia*	5 days	Sterility Test	14 days	No
FTB	*C. lipophitopharium*	6 days	Sterility Test	14 days	No

The information presented in Table 12 indicates that while the growth rates of some environmental isolates were longer than compendial strains, the times taken to grow did not exceed the maximum incubation times for the primary tests. Therefore, adjustments may need to be made for growth acceptance criteria when using environmental isolates if laboratory procedures have these set at a shorter time than the time stated in the compendia (as they generally are for media release). However, there is no suggestion, based on the data, that pharmacopeia recommended incubation times would need to be extended.

Discussion

It has become commonplace to use environmental isolates for the release of microbiological culture media and for undertaking method suitability testing in relation to most compendial microbiological assays [7–10]. This has been partly driven by a desire to expand or revise the microbial test panel and partly by regulators. Underlying this trajectory is the assumption that organisms in the pharmaceutical plant will have either adapted to survive on limited nutrients or may have been damaged in some way through exposure to an antimicrobial agent, such as a disinfectant. It is also presupposed that the adaptation responses are retained. It then follows that if environmental isolates grow then this somehow gives greater confidence about the suitability or robustness of the test.

The data compiled in this paper suggests that there is, in general, a slightly longer time period required for environmental isolates to grow. This was for up to two days on average and for up to four days for the slowest growing organism (*C. lipophitopharium* in FTB). While this observation is noteworthy, there was no case of the pharmacopeia incubation time or by good test practices (as with the case of media used for environmental monitoring) being exceeded. However, facilities that have adopted leaner approaches to sample incubation, such as by reducing environmental monitoring incubation times, may need to consider the data presented here and make adjustments should their own facility isolates be shown to grow at a rate slower than their test incubation times. By slower growth rate, what is most probably occurring is an extended cell lag phase before exponential growth begins [11 12]. Within this slower pattern, the growth rates for plate media and broth media showed some variation.

The data compiled indicates that growth on plate media (solid agar) is more rapid compared with broth media. One reason for slower growth rates in broth may relate to variations with oxygen. Liquid broth provides varying oxygen levels and the oxygen available decreases as the depth of the broth increases. It is possible that the slower growing organisms had more exacting aerotolerances [13]. With plate media, the use of agar as the solidifying agent did not appear to result in an inhibition of growth compared with broth media [14].

The data also finds that the organisms that tend to grow more slowly are Gram-negative bacteria (this was seen for each type of medium, with the exception of FTB). The slower-growing Gram-negative bacteria were ‘pseudomonad’ type organisms. This may suggest that the stressors from within a water system (such as osmotic pressure or nutrient starvation) require a more prolonged adaptive response by these more sedentary bacteria when they are transferred onto standard laboratory culture media. Beyond this species-specific observation, Gram-positive bacteria grew slightly more slowly than Gram-negatives. This may point to the nature of different environmental stressors rather than a property of cell morphology [15].

Moreover, the 'Gram-positive' and 'Gram-negative' differentiation is too broad given that cellular structural entities can be quite different between species [16]. These considerations, supporting the retention of wildtype characteristics, cannot be answered here, although they present interesting areas for further inquiry.

It should be noted that not all researchers support the continuation of wildtype characteristics following the first subculture onto laboratory media (finding that most microorganisms normalise rapidly) [17] and conversely it is far easier to instead 'domesticate' an organism [18]. Yet, the data presented here shows a difference in growth rates (albeit a small one), which might suggest some retention of wildtype properties.

Based on their being some retention of different phenotypic properties, this leads on to the question 'for how long might wildtype characteristics be retained?' The stress adaptive response is unlikely to be permanent and at some point the environmental isolate will not be significantly phenotypically different from any other laboratory strain (all compendial strains were once 'environmental isolates', albeit from decades ago). This cannot be answered here. However, to guard against a marked or irreversible loss of aggressive behaviour that will probably occur through successive subcultures, it is good practice that isolates are changed regularly. In terms of how regular this should be, this represents another area for further research. The isolates used in this paper ranged between one and six months old at the time of use. Alternatively, it may be possible to limit the adaptive response to nutrient rich laboratory media by maintaining the environmental isolates in a stressed condition. For example, where isolates are to be used for the preservative efficacy test the challenge organisms could be kept, or spend a defined period in, unpreserved product or isolates from water systems held in water. Even here there are difficult questions to answer. Should, for example, waterborne bacteria be held in water? If so, water of what type of water purity? For how long? Under what level of osmolarity and so on.

In terms of study limitations, such work as presented here will inevitably be biased through the types of culture media and organisms used (although an attempt has been made to be representative). Another limitation is with cell heterogeneity. While some wildtype characteristics appear to be retained it is unlikely these characteristics have been afforded to all cells which leads to the flaw of being self-selecting in terms of subpopulations. The method treated all environmental isolates as a standard culture, with the only attempt made to preserve the 'wildtype' characteristics being seeking to minimise the serial subculture of the isolates. Therefore, given it is unknown to what extent the wildtype characteristics are preserved and the degree to which colonial selection occurs in terms of preferably selecting cells that have adapted to nutrient rich laboratory media [19], bias may have crept in. Hence, a further area for future study could be with ensuring that culture maintenance process sustains the cultural characteristics of "wild" plant isolates [20]. The difficulty here is with the absence of any consensus in terms of how to achieve this and the possibility that different techniques would be required for different organisms isolated from different locales given the above discussion about different stressors.

The extent that the obtained results are generalisable will depend on similar work being undertaken with other organisms; seeking to replicate the work at other facilities, using

different isolates and culture media; and perhaps with attempting to subject microbial populations to different forms of ‘stress’.

In conclusion, the research presented here suggests that microbiologists should expect environmental isolates to sometimes take slightly longer to grow on media compared with type cultures. Variations are dependent upon origin, organism, and culture media. In most cases, this growth will be well within the maximum incubation time for the test. For example, given that the sterility test incubation time is 14 days, type cultures tend to grow within 1 or 2 days and environmental isolates within 1 and 6 days; in both cases, the time required for visible growth is well within the required incubation time. Yet for other media this may not be the case. Should one or more environmental isolates be found to take longer than the test incubation time, and the environmental isolate is found to be relevant to the test and the prolonged time found to be reproducible, then a justification can be considered for extending the test time.

These are important points for pharmaceutical microbiologists to consider given that the regulatory driver for the inclusion of environmental isolates within a test panel shows no sign of abating. As example, the draft EU GMP Annex (March 2020 edition) recommends using environmental isolates for media fill growth promotion testing, in terms of what are referred to as ‘local isolates’ [21]. In addition, the current PIC/S guidance for sterility testing [22] mentions ‘environmental or fastidious organisms’ if alternative culture media are selected. This leaves the use of environmental isolates firmly on the agenda for reasons of regulatory jurisprudence. What can be drawn from this paper to support this practice is with ensuring that the growth-based acceptance criteria selected are based on science and are suitable for the intended application.

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