International Journal of Research Publication and Reviews

Journal homepage: www.ijrpr.com ISSN 2582-7421

Unlocking the Pandora's Box of Uncultivable Microbes: The "iChip" As A Gateway to Antimicrobial Drug Discovery

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DOI: https://doi.org/10.55248/gengpi.4.1223.123533

A B S T R A C T

A wide range of microorganisms make up the microbial world, the majority of which have not yet been studied or grown. Thus, the term "microbial dark matter". Modifying artificial nutrient medium only results in a marginal increase in cultivability. So, using naturally occurring combinations of growth factors is an alternate method of cultivating species whose requirements are unknown. By putting cells extracted from various environmental samples into diffusion chambers and subsequently reintroducing them into the natural environment for incubation, this technique cultivates microorganisms. Microorganisms can be grown and separated in axenic culture in a single step by resizing the chambers and putting only one to several cells inside each one. It has been demonstrated that using this cultivation platform—also known as the "iChip" or "isolation chip"—uncultivable bacteria can be successfully isolated. Teixobactin and Clovibactin are novel antibiotics that were extracted from organisms isolated using the iChip. Despite the fact that these novel compounds are still in the earliest phases and cannot be guaranteed to reach the market, the iChip's use ought to contribute to the discovery of additional potentially useful novel antibiotics.

Keywords: iChip, Isolation Chip, Uncultivable Microbes, Diffusion Chamber, Teixobactin, Clovibactin

1. Introduction

Microorganisms are the most diverse and numerous cellular life forms on Earth and play a critical role in biogeochemical cycles [1]. The diversity of bacteria found in nature is far greater than that which can be cultured in a lab, with only 0.1–1% of them having been cultivated, which hinders the greater understanding of the microbial world [2, 3]. In the past, the majority of our knowledge about microbiology came from data obtained only from cultivated organisms, limiting physiologic and genomic information to pure cultures [1]. The diversity of microorganisms was initially identified from the analysis of microbial small subunit ribosomal RNA gene sequences directly obtained from environmental samples [4, 5, 6]. An "uncultivable" microorganism indicates that there are gaps in the current understanding of their biology, which creates potential and difficulties. Exploring the untapped microbial variety is highly important for both the fundamental and practical sciences and is acknowledged as a major obstacle in the field of microbiology at present [7, 8, 9]. Exploring microbial "dark matter" can come in various approaches including simulated environments, co-culture, and host-associated environments [2]. Since bacterial resistance is becoming a major concern for human health, medical scientists around the world are faced with the problem of finding novel antibiotics that have innovative chemical scaffolds and antibacterial mechanisms for use in therapy.

An innovation was developed to cultivate microbial dark matter directly in diffusion chambers. In this approach, the bacteria are contained in a semipermeable chamber, allowing nutrition and growth factors from the surrounding environment to enter but preventing the cells from passing through the membrane barrier. A microscopic inspection of the chambers showed that microcolonies of bacteria were growing within them, most of which could be separated and re-inoculated into new chambers [2, 10]. It's been shown that this method produces a higher number of novel bacteria than conventional petri-dish techniques [11].

2. Methods

This article review uses the National Institutes of Health, Academia, ResearchGate, and Google Scholar databases to recover systematic reviews and experimental results on the use of isolation chips (iChip) for cultivating microorganisms and their application in antimicrobial discovery. The studies that were found by search algorithms were evaluated by the researchers to determine if they met the inclusion requirements for this article. A thorough review was conducted in order to demonstrate the possible application of isolation chips in the investigation of a significant proportion of the microbial population and their relevance in drug discovery.

3. Uncultivable Microbes

The terms “unculturable” or “uncultivable” represent microorganisms that have not yet been cultured in vitro using synthetic medium. It does not imply that they are "not culturable," but rather that no one has tried to grow them. Therefore, these are microorganisms that are not yet cultured [12].

3.1 Origin

Unculturable microbes were first observed in 1898, when Austrian microbiologist Heinrich Winterberg noticed that there were fewer colonies on the petri dish than in his samples [13]. Successive experiments have shown the same results, showing the disparity between the quantity of microorganisms that can be grown on standard laboratory media and the vast number that can be seen under a microscope. Today, it's referred to as the Great Plate Count Anomaly (Figure 1) [14]. Several explanations were hypothesized for this. It was suggested that the microorganisms that failed to be cultured were dead cells. However, it was found to be metabolically active, but it does not replicate on laboratory media [2].

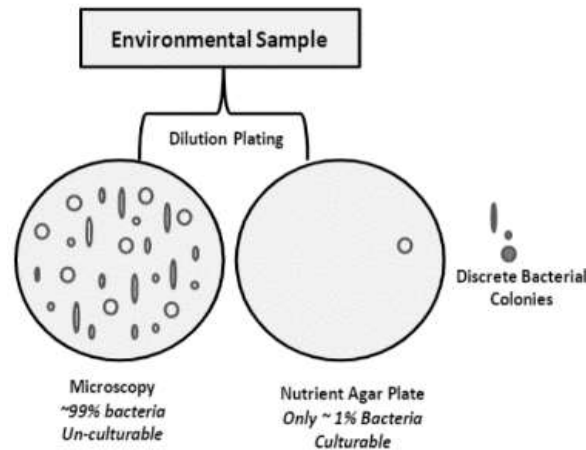

Fig. 1 – The Great Plate Count Anomaly. Adopted from Harwani (2013) [19]

Phylogenetically relevant markers such as the 16S rRNA gene sequences suggest evidence of bacterial taxa that are not culturable in synthetic media [2,15]. The corresponding sequence to the 16S rRNA gene of the candidate phylum TM7 was found to be prominent in different environments, including water, soil, and the human microbiome. TM7 is among the many broadly distributed phylum that were identified as unculturable, which suggests the diversity of microbial dark matter [3, 15–17].

3.2 The Challenge in Cultivating these Microbes

The inherent complexity of these microorganisms and their environments makes the cultivation of unculturable microorganisms challenging in a number of ways. Appropriate growth media and incubation conditions are the critical factors in microbial recovery and cultivation. Mimicking the ecological habitat, specifically its pH, temperature, salinity, nutrients, and moisture, are among the considerations [18].

Unculturable bacteria have a wide variety. Some are resistant to culture using synthetic media, some have fastidious growth requirements, and some need specific physical conditions to survive, such as pH, temperature, and oxygen levels [19, 20]. A few categories of unculturable bacteria include obligatory symbiotic and parasitic bacteria, which proliferate under conditions set by the host but not on artificial media, and viable but non-culturable bacteria, which are viable in the natural environment but unable to divide in regularly used growth mediums [19, 21]. Marine bacteria are also known for being unculturable due to the existence of dormant cells, low growth rates, poor colony development, the need for metabolites produced by other microorganisms, and inadequate growth conditions [22].

4. Isolation Technique of Uncultivated Microbes Using iChip

4.1 iChip Method

A technique for the isolation of unculturable microbes is through the use of an in-situ cultivation model. Samples from soil or seawater are taken and suspended in liquid agar in order to isolate microorganisms (Figure 2A). To make sure that each through hole receives one cell, the mixture is diluted. The cells in the iChip became immobilized and solidified due to the liquid agar (Figure 2B). Subsequently, to stop cell migration, 0.03 µm-pore-sized polycarbonate membranes are put on both sides of the center plate. To seal the system and keep the membrane in place, cover plates with corresponding through-holes are fastened in place (Figure 2C). The entire system is meant to keep cells from escaping while permitting the dispersion of external elements [11, 23].

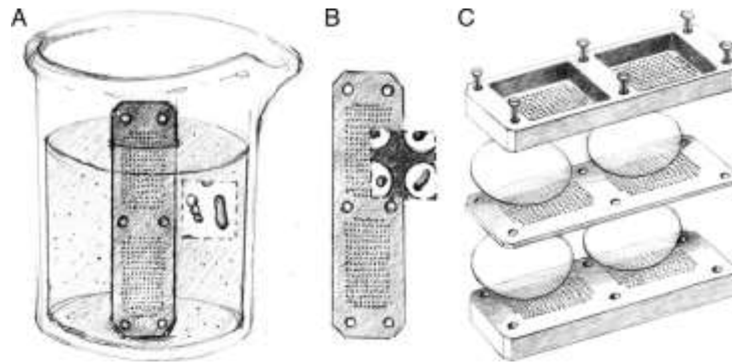

Fig. 2 – Design of an Isolation Chip. Adopted from Nichols et al. (2010) [11]

In the experiment conducted by Kaeberlein et al. (2002), microorganisms were cultivated using traditional petri dishes and the iChip technique from soil samples and seawater. A greater quantity of colony-forming cells was seen in the iChips when the microbial species was identified by microscopy and 16S rRNA gene analysis. There was virtually no overlap between the isolated species in the cells generated by the iChip and the colonies developed on petri dishes [10]. This was supported by another study by Nichols et al. (2010), wherein the colony counts in petri dishes were roughly five times lower, exhibiting statistically significant disparities compared to the recoveries obtained from either the iChip or diffusion chambers [11].

4.2 Modified Diffusion Chamber

The original concept of the iChip was developed by Kaeberlein et al. (2002). They argued that in pure culture, uncultivable microbes could develop if given the substances that make up their natural surroundings. To make these components accessible, microbes were inserted into diffusion chambers and kept in an aquarium that mimicked the natural habitat of these species. Thus, they tested this concept by utilizing diffusion chambers for cultivating these microorganisms [10]. By creating an incubation technique that closely resembles the natural environment of cells, the diffusion chamber aims to "trick" the cells into believing they are growing in their original habitat.

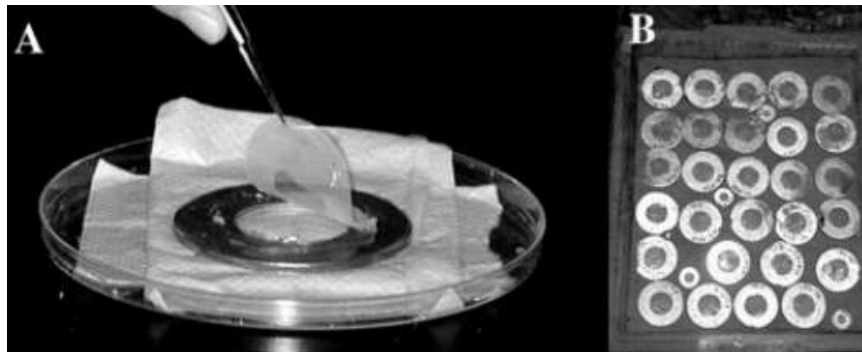

Fig. 3 - Diffusion growth chamber for in situ cultivation of environmental microorganisms. Adopted from Kaeberlein et al. (2002) [10].

The membrane stops the cell from moving while facilitating the flow of substances between the chamber and its surroundings. After attaching the first membrane to the base of the chamber, microorganism-containing agar was added, and the assembly was sealed with a second membrane (Figure 3A). These sealed chambers were placed inside a marine aquarium and placed on the sediment of a tidal flat (Figure 3B). The upper membrane, which was later filled with seawater in the aquarium, was separated from the agar by a thin layer of air. This arrangement allowed the top membrane to be removed, allowing for the study of the unaltered agar surface [10]. The diffusion chamber approach's comparatively poor throughput is one of its drawbacks. Additionally, hundreds of microcolonies from different species are produced in a single diffusion chamber, necessitating tedious separation under a microscope [24].

4.3 The Isolation Chip (iChip)

The iChip (Figure 2) is a platform for cultivation that makes it possible to isolate and grow previously uncultivated species in a single phase that was devised in order to improve the method's efficiency [25]. A collection of flat plates with many registered through-holes makes up the iChip. These plates are made from hydrophobic plastic polyoxymethylene, more popularly known as Delrin, a thermoplastic that, because of its physical and biocompatible characteristics, has been employed as a durable implant material for joint replacement parts, dental implants, and cardiac valve prostheses [26]. The middle plate, which measures 72 by 19 by 1 mm, and the two symmetrical top and bottom plates, which measure 72 by 19 by 6.5 mm each, both have multiple through-holes that are 1 mm in diameter. These through-holes are grouped in two arrays, with each array containing 192 through-holes. The bottom plate also has ridges that provide rigidity. The array is large enough to fit inside conventional membranes with a diameter of either 25 or 47 mm.

A total of four membranes are needed for the assembly. 47-mm polycarbonate membranes with pores of 0.03 µm in size were used to prevent cell migration into and out of the agar plugs. After that, the top and bottom plates are fastened and positioned, and the screws are tightened to apply pressure. The pressure effectively seals the agar plugs and individual through-holes, negating the need for adhesive. Through this process, the assembly is transformed into a combination of 384 microdiffusion chambers, with roughly one cell per through-hole. The immobilized cells receive their growth factors and nutrients by means of further in situ incubation in their original environmental habitat. After incubation, iChips are removed and properly cleaned in particle-free DNA-grade water. The number of colonies present is then able to be determined by examining the central plate with a compound or high-power dissecting microscope [11].

4.4 FACS-iChip: Single-Cell Sorting Technique

The iChip's original design was composed of two symmetric top and bottom plates and the center plate, which needed a lot of work to assemble. It also relies on single-cell sorting techniques in the cultivation of microorganisms. Methods for sorting single cells include flow cytometry, laser capture microdissection, manual cell picking, and microfluidics, aside from dilution methods [27]. A fluorescent-activated cell sorter (FACS) is a specialized tool for flow cytometry, a method that analyzes and sorts individual cells according to their chemical and physical properties. A single-cell-sorting flow cytometer has the additional capacity to physically isolate and gather individual cells for additional research or culture, in contrast to conventional flow cytometers that examine cells as they pass past a laser beam [28, 29]. When a laser strikes the cells, two types of light scattering take place: forward scatter (FSC), which is useful for sorting cell size, and side scatter (SSC), which is proportional to the intrinsic complexity of the cell. These two types of light sort the fluorescent-labeled cells [28, 29, 30]. FACS-iChip enhances the functionality of current techniques, and accurate single-cell sorting was successfully accomplished by the use of a flow cytometer [25].

5. Antibiotic Discovery

5.1 iChip Bridges Gaps on Antibiotic Discovery

The selectivity of growth media limits the number of microorganisms that may be recovered from natural samples, according to studies. Thus, in the event that microbe culture conditions can replicate the environmental circumstances derived from the sample collection, the possibility of isolating organisms that were never cultivated before increases [31, 32]. Which is why a promising method is to cultivate microorganisms in their natural habitat before isolating them for study. By facilitating the culture and investigation of previously uncultivable microorganisms, the iChip (Isolation Chip) has proven to be extremely relevant in the field of antibiotic discovery. The iChip technique, which enables the extraction of compounds from microorganisms in the environment that do not thrive in standard laboratory settings, will tremendously aid in the identification of novel antibiotics [32, 33].

5.2 Antibiotic Compounds from an Uncultured Bacterium

Teixobactin is a novel antibiotic discovered through the use of iChip technology. It exhibits a unique mechanism of action against gram-positive bacteria. It is produced by a soil bacterium called *Eleftheria terrae*, belonging to a new genus related to *Aquabacteria*. *E. terrae* was described as an "uncultivable" bacterium that cannot be cultured through conventional methods [33–36]. Teixobactin was found in an active fraction that was partly purified and taken from the culture supernatant of *E. terrae*. It is a distinct depsipeptide containing enduracididine, methyl phenylalanine, and four D-amino acids. Through homology searches and genome sequencing of E. terrae, the biosynthesis route of teixobactin was discovered [33, 34].

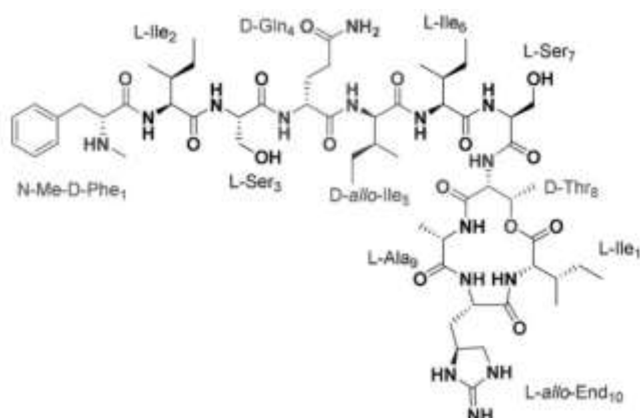

Fig. 4 - Chemical structure of Teixobactin. Adopted from Guo et al. (2018) [35].

Teixobactin is a depsipeptide that consists of 11 amino acid residues. It has the characteristic of seven L-aa and four D-aa residues (Figure 4) [34, 35]. Narrow bactericidal activity was observed in native Teixobactin against S. aureus, M. tuberculosis, methicillin-resistant S. aureus (MRSA), as well as vancomycin-resistant Enterococci (VRE) [33, 34]. It was also found to be extremely active against C. difficile [36]. Teixobactin-resistant mutants of S. aureus and M. tuberculosis were not produced on media with a low dose of Teixobactin [35]. The mechanism of action of Teixobactin is unique compared

to other antibiotics. It works by disrupting bacterial cell wall synthesis by inhibiting lipid II (a peptidoglycan precursor) and lipid III (a teichoic acid precursor). This leads to bacterial cell death via autolysis due to the disruption of the biosynthesis of peptidoglycan, a structural component of the bacterial cell wall responsible for its integrity and survival [33, 36–37]. Gram-negative bacteria have an outer membrane that contains lipopolysaccharide, which makes it impossible for Teixobactin to permeate. It was also reported to have no activity against E. coli, possibly due to its impermeability or efflux [33, 36].

Clovibactin is another novel antibiotic that was isolated using the iChip from a colony identical to *E. terrae*. This isolate was referred to as *E. terrae ssp. carolina* as it came from sandy soil in North Carolina [38]. Clovibactin was found to have activity against Gram-positive pathogens such as methicillin-resistant *S. aureus*, daptomycin-resistant, and vancomycin-intermediate-resistant *S. aureus* (VISA) strains, as well as vancomycin-resistant *E. faecalis* and *E. faecium* [38, 39]. Similar to Teixobactin, Clovibactin was found to have no antibiotic effect against *E. coli* [38].

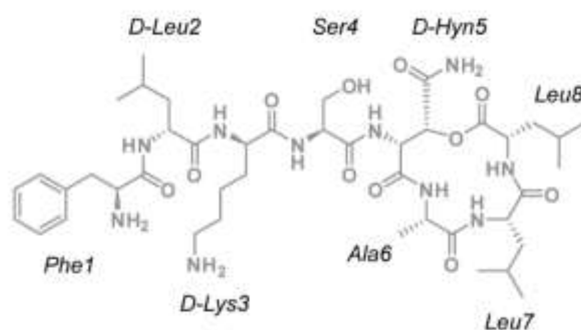

Fig. 5 - Chemical structure of Clovibactin. Adopted from Shukla et al., (2023) [38].

Two D-amino acids at the N-terminus and D-3-hydroxyasparagine in the depsi-cycle define the chemical structure of Clovibactin. The molecular scaffold of Clovibactin has some similarities to Teixobactin. Certain differences exist in the shorter linear N-terminus, in which Clovibactin has four residues while Teixobactin has seven [38, 40]. Clovibactin acts by selectively binding to pyrophosphate groups of the components of the bacterial cell wall, gripping around it like a cage through the formation of supramolecular structures that enclose the target and cause further damage [40]. The novel antibiotic was reported to have strong antimicrobial activity and low cytotoxicity. When compared to vancomycin, it was found to be more effective in killing S. aureus. Clovibactin can induce strong bacterial cell lysis that is greater than Teixobactin [38]. It has also been reported to have successfully treated mice in preclinical studies [40].

6. Discussion

The Isolation Chip technology appears to be a useful tool for efficiently isolating uncultivable microorganisms. Bacterial cells in the iChip system are easily segregated from one another, and their close proximity to the surrounding environment promotes their growth. Its design makes it easier for bacteria to incubate in their native habitat [24]. The conflict between rapidly developing species, which frequently outcompete slower or rarer species on traditional culture plates, presents difficulty when cultivating new bacteria. However, each bacterial cell in iChips occupies one of the numerous through holes, which increases the chances that these slower-growing or rarer species will develop successfully. However, it is important to note that iChip has limitations. For example, not all bacteria that grow well in iChip are suitable for further development on synthetic media, and subculturing is subsequently carried out in a conventional laboratory setting [41]. iChip is continuously being modified to enhance further not just the number of cells cultivated but also to accommodate the needs of specific microorganisms, like the recent study on the use of iChip for cultivating thermo-tolerant microorganisms from a hot spring [42].

7. Conclusion

The iChip enables the culture of bacteria that were previously difficult or impossible to grow in laboratory environments. Numerous of these uncultivable microorganisms might have unique bioactive substances with possible antibacterial qualities. Because standard cultivation techniques frequently fall short of producing a significant number of environmental microbial species in the laboratory, iChip's significance stems from its potential to overcome these limitations. Through increasing the variety of cultivated microbes, iChip aids in developing a larger microbiological library. Finding new compounds with antibacterial properties against a variety of diseases depends on this diversity. Antibiotics are among the many naturally occurring products that come from the microbial world. By making exploring this diversity of microbes easier, iChip raises the possibility of discovering novel compounds that could be turned into potent antimicrobial medications.

Finding new classes of antimicrobial chemicals is crucial since antibiotic resistance is a developing global concern. The ability of iChip to identify previously unidentified microorganisms and their bioactive components may help in the development of medications that effectively combat resistant strains. Microbes from various environments, including unexplored ones, can be cultivated using iChip. This technique might result in the identification of distinct substances with antibacterial qualities in specific environments.

Acknowledgements

The completion of this journal review is in large part due to the assistance and guidance offered by our adviser in this subject, the Drug Discovery and Development Laboratory, Professor Jacqueline Padilla. We were able to complete this review piece thanks in large part to their guidance. Everyone earnestly participated in the open exchange of ideas, constructive discussions, and mutual support that characterized our collaborative work environment. Furthermore, this work represents a collective effort, and we are truly grateful to everyone who played a role in bringing it to fruition.

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