

## Application of iChip to Grow “Uncultivable” Microorganisms and its Impact on Antibiotic Discovery

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**ABSTRACT - Purpose.** Antibiotics have revolutionized modern medicine, allowing significant progress in healthcare and improvement in life expectancy. Development of antibiotic resistance by pathogenic bacteria is a natural phenomenon; however, the rate of antibiotic resistance emergence is increasing at an alarming rate, due to indiscriminate use of antibiotics in healthcare, agriculture and even everyday products. Traditionally, antibiotic discovery has been conducted by screening extracts of microorganisms for antimicrobial activity. However, this conventional source has been over-used to such an extent that it poses the risk of “running out” of new antibiotics. Aiming to increase access to a greater diversity of microorganisms, a new cultivation method with an *in situ* approach called iChip has been designed. The iChip has already isolated many novel organisms, as well as Teixobactin, a novel antibiotic with significant potency against Gram-positive bacteria.

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### INTRODUCTION

Antibiotics are products of evolutionary selection at the microorganism level. These biochemical entities have existed in the natural world long before Alexander Fleming (1881-1955) had his chance encounter with a petri dish of bacterial colonies that had been killed by mold contamination in 1928. This was the birth of penicillin, derived from the *Penicillium* genus of Ascomycetous fungi, which was rightly called a miracle drug. The duo of Howard Florey, a pathologist, and Ernst Chain, a biochemist, further developed this bactericidal substance to increase production efficiency and were able to carry out *in vivo* studies (1).

Around 1941, penicillin was being produced in large quantities for clinical studies and the results were very promising due to the low doses required for therapeutic action. The plan was to use penicillin to protect soldiers against infections from battlefield injuries, and harnessing the full power of penicillin required scaling up production (2). Dr. Florey and his assistant, Dr. Heatley, came to the US to search for new sources of penicillin. Their joint effort with the Department of Agriculture led to a moldy cantaloupe and the mold, *Penicillin chrysogenum* gave 200 times more penicillin than any source that had been tested before (3). The

discovery of penicillin and its benefits to humankind commenced the search for other antibiotics present in the natural world. In 1945, Florey and Chain shared a Nobel Prize with Alexander Fleming for the discovery of penicillin (3). Thus, the “Golden Age” of antibiotics started, a period from 1950 to 1960, when half of the antibiotics commonly used today were discovered (4).

Antibiotics are the pillars of modern medicine, and have made a significant impact on the life expectancy and quality of life, which divides the history of medicine to pre- and post-antibiotic eras (5). The word “antibiotic” refers to a chemical substance of microbial origin that inhibits growth or obstructs metabolic pathways and are used therapeutically to overcome invading infections caused by pathogens (6). Its origins are from microorganisms themselves, driven by the force of natural selection to compete for resources with other microorganisms in the microbiome (6). It is a wonderful example of evolutionary selection arming microorganisms with chemical weapons and how this stockpile has been used by humankind as a tool against disease causing microbes (5).

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## HUMANS AND MICROORGANISMS

Humans have evolved from single cell ancestors and as a species, have grown amongst a world teeming with microorganisms. From scalding volcanic vents in deep oceans to radioactive surroundings, microorganisms not only occupy the outside world but also exist in our internal organs, such as the GI tract (7,8). Microbiota refers to "the ecological community of commensal, symbiotic, and pathogenic microorganisms that share our body space." (9) Many of these internal microorganisms play an essential role in digesting certain foods, assisting in the production of vitamins B & K, and protecting the intestinal mucosa by combating other aggressive microorganisms (10). The long held notion of humans being sterile at birth is also being questioned. Aagaard et. al. have found that the placenta has its own microbiome, composed of nonpathogenic commensal microbiota that closely resembles the microbiome of the oral cavity (11). Although it is not known whether the placental bacteria can reach the developing fetus nevertheless, once bacteria colonize the microbiota they mature along with us and play a key role in ensuring proper digestive function (12). Recent mice studies have shown that transplantation of gut microorganisms from an obese mouse to a normal mouse results in the obesity of the recipient as well (13).

There are, however, other microorganisms that impact human health negatively and are collectively termed "pathogenic microorganisms" (or simply pathogens). These are microorganisms that cause a variety of diseases ranging from mild ENT (ear, nose and throat) infections, to life-threatening meningitis and pneumonia. Along with pathogenic bacteria, other microorganisms such as viruses, fungi and protozoans can be disease-causing agents. For this article, we will focus on pathogenic bacteria, which are the main targets of antibiotic therapy.

Bacteria lack a nuclear membrane enclosing their genetic material and fall under the Prokaryotic domain. From the medical aspect, it is important to be able to classify or identify pathogens for proper diagnosis and treatment. In 1884, Danish scientist Hans Christian Gram, developed a staining procedure that differentiated two types of bacteria based on the structural differences in their cell walls (14). Gram-positive bacteria retain the primary

crystal violet dye due to the presence of a thick peptidoglycan layer. On the other hand, the dye on the cell membrane of Gram-negative bacteria is washed away with alcohol or acetone due to its relatively simple lipopolysaccharide composition. The Gram stain might seem like an arbitrary criterion used to differentiate bacteria and although there are Gram-indeterminate species such as *M. tuberculosis*, the Gram stain does distinguish between two fundamentally different kinds of bacterial cell wall that determines the overall effectiveness of antibiotics on these two classes of bacteria. The distinctive feature of Gram-negative bacteria is the presence of a unique double membrane surrounding each bacterial cell (15). This outer membrane is selectively permeable thereby preventing certain drugs and antibiotics from penetrating the cell. This first line of defense coupled with an active efflux system, which pumps antibiotics out makes Gram-negative bacteria more resistant to antibiotics than Gram-positive bacteria (16,17). Certain pathogens such as *Pseudomonas aeruginosa* utilize this efflux mechanism to create resistance against fluoroquinolone, a broad-spectrum antibiotic used to treat a range of Gram-negative infections. Another mechanism of resistance against fluoroquinolone is alteration of bacterial targets through gene mutations (18).

## ANTIBIOTIC RESISTANCE.

Resistance to the therapeutic effect of antibiotics is a growing problem with serious implications to human health. It occurs when an antibiotic loses its ability to effectively control or inhibit the growth of invading bacteria. So, the bacteria continue to multiply even when therapeutic levels of the antibiotic are present. This is a natural phenomenon where selection pressure to survive drives the invading pathogens to activate or even devise a mechanism to nullify the antibiotic effect (19). Due to the unregulated use of antibiotics in agriculture, overuse in medicine, and abundance in everyday products, we expose microorganisms in the environment to a variety of antibiotics (20).

There are a number of other ways that bacteria develop resistance to antibiotics. Bacteria can be naturally resistant to certain antibiotics (i.e. they carry genes for resistance; also called "innate resistance"), acquire resistance from another bacterium, or become resistant due to a genetic

mutation. In terms of genetic variability, bacteria are relatively flexible with the ability to alter or bypass ligands/mechanisms targeted by antibiotics, rendering the antibiotic useless. Additionally, bacteria are capable of transferring genes through conjugation (21). This process involves a bacterium transferring its genetic material, which might include genes encoding antibiotic resistance. This transfer usually occurs within the same species but genetic interchange can also happen between organisms belonging to the same genus, making even more species of microbes resistant to antibiotics (22). Bacteriophages can assimilate portions of bacterial DNA that might contain antibiotic resistance genes into its own genome. The bacteriophage can transfer the resistance genes into any bacteria that it infects. This process is called transduction and has been utilized by researchers to endow bacterial cells with genes of interest. Moreover, the exceptional growth rate of these microorganisms allows them to evolve and adapt rapidly to new environmental conditions (23).

Antibiotics are extremely important to the health care system, critical in preventing post-operative infection. Without antibiotics, modern medicine could not have progressed to its present state. But we now face a new challenge: the overuse of antibiotics which leads to rise of highly resistant bacteria. A 2013 report from the Center for Disease Control (CDC) on antibiotic resistance threats in the US shows that 50% of all the antibiotics prescribed for people are not needed or not optimally effective as prescribed. It is a dilemma faced by physicians, when patients display symptoms of an infection caused by an indeterminate pathogen. Should the physician wait for diagnostic test results that may take a few days? Or would prescribing an antibiotic be more prudent? Faster diagnostic capability would greatly decrease antibiotic prescriptions that are given in a "just-in-case" scenario. Identification of the invading pathogen would further help in selecting between a broad-spectrum antibiotic and a specific/narrow spectrum antibiotic. In hospitals, immunosuppressed patients are more susceptible to infections and, thus, often require the aid of antimicrobials. The extensive use of antimicrobials and close contact among vulnerable patients creates a conducive environment for the spread of antimicrobial-resistant germs (24,25). Patient misuse of antibiotics has also contributed to antibiotic resistance (26). It is of utmost importance

to follow the prescribed antibiotic prescription. If the full course is not completed, a some population of pathogens can survive the incomplete antibiotic treatment, have the opportunity to become resistance against the prescribed antibiotic and require stronger antibiotics in the future (27).

Antibiotic overuse in agriculture, especially in livestock, is also a cause for concern. The meat industry has transformed immensely over the decades with large corporations taking over farms and applying an unethical business model to the industry. There are 9 billion animals produced for food in the U.S. every year (28), the majority of which are raised in concentrated animal-feeding operations (CAFOs) and given regular doses of antibiotics. It is logical to think that livestock need to be treated with antibiotics when there is an infection; however, 80% of those antibiotics are being used non-therapeutically as metaphylaxis or growth promotion (29).

An accidental discovery in 1948 by scientists at Lederle Laboratories showed that the animals receiving sub-therapeutic doses of antibiotic in their feed seemed to grow faster than others (30). Mixing antibiotics in animal feed complemented the goals of CAFOs, which is to make their animals grow faster and to prevent diseases/infections that may reduce production. By putting antibiotics in the animal feed, we have developed a misconception that livestock can be raised in deplorable, unsanitary, and unhealthy conditions. For a while, the industry continued adding penicillins and tetracyclines to their animal feed, previously known as growth promotion, now termed feed efficiency. However, this method of adding antibiotics to animal feed is being re-examined because of its contribution to antibiotic resistance. There are risks associated with adding antibiotics to animal feed for extended periods of time. Eventually, the animals begin to retain the antibiotic-resistant strains of bacteria and can be transmitted to other animals (28). People who work on farms and in slaughterhouses are at risk of contracting these resistant strains (31). Transmission can also occur when undercooked or raw meat from animals containing resistant bacteria is consumed. Although most bacteria do not survive the cooking process, there are certain bacterial species that can withstand the heat during this process (28).

Superbugs are strains of bacteria that are resistant to several types of antibiotics. According

to the CDC, each year these superbugs infect more than 2 million people nationwide, and kill at least 23,000 patients in the US. On a global scale, the superbugs cause 700,000 deaths annually and is predicted to rise to 10 million in 2050 (29). In the past, the most dangerous superbugs were confined to health care settings; however, today, superbugs are no longer limited to only hospitals. Some strains may be found in the community, infecting even healthy individuals (32). Effective antibiotics entering the market cannot compete with the rapidly evolving pace of bacteria. The CDC is pushing hospitals to develop antibiotic stewardship programs that educates the staff and ensures that antibiotics are only used when necessary. Antibiotics should be prescribed only when needed, and the proper antibiotic should be used for the specific type of bacteria (20). Rapid diagnostic testing that reveals results within hours could help to ensure that antibiotics are used properly (33). The general public also needs to be educated in the use of antibiotics and the contribution to rise of superbugs when antibiotics are not used prudently. There needs to be an overhaul of the current antibiotic market to conserve the potency of current antibiotics because superbugs do not follow geographical boundaries nor discriminate between people. When our antibiotics of last resort are rendered useless by an unyielding pathogen, it will be too late to change our habits.

### **ANTIBIOTIC DISCOVERY & DEVELOPMENT**

The drug development process is complex, time-consuming, and expensive. According to the World Health Organization, it can take 12 years to discover and develop a new medicine. This venture typically costs more than \$1.5 billion to complete all of the research and development necessary before the drug can be marketed (34). The emergence of antibiotic resistance hovers on the horizon, as a top public health threat, with discovery and development of new antibiotics becoming an increasingly daunting task. The Waksman platform collapsed around the 70's, when the traditional methods for screening cultures of soil-derived organisms for activity against other microorganisms yielded the same compounds over and over again (35). As the demand for the discovery of new antibiotics increased, several

discovery approaches such as genomics, high-tech chemical approaches, and high-throughput screening were launched in the 1990s. But the number of companies researching antibiotics has decreased significantly since then and has a direct impact on the number of antibiotics in development. With factors such as high attrition rate in clinical trials and unattractive economic prospect surrounding antibiotics, many pharmaceutical companies have ventured to the greener pastures of lifestyle drugs that provide a favorable return on their investment.

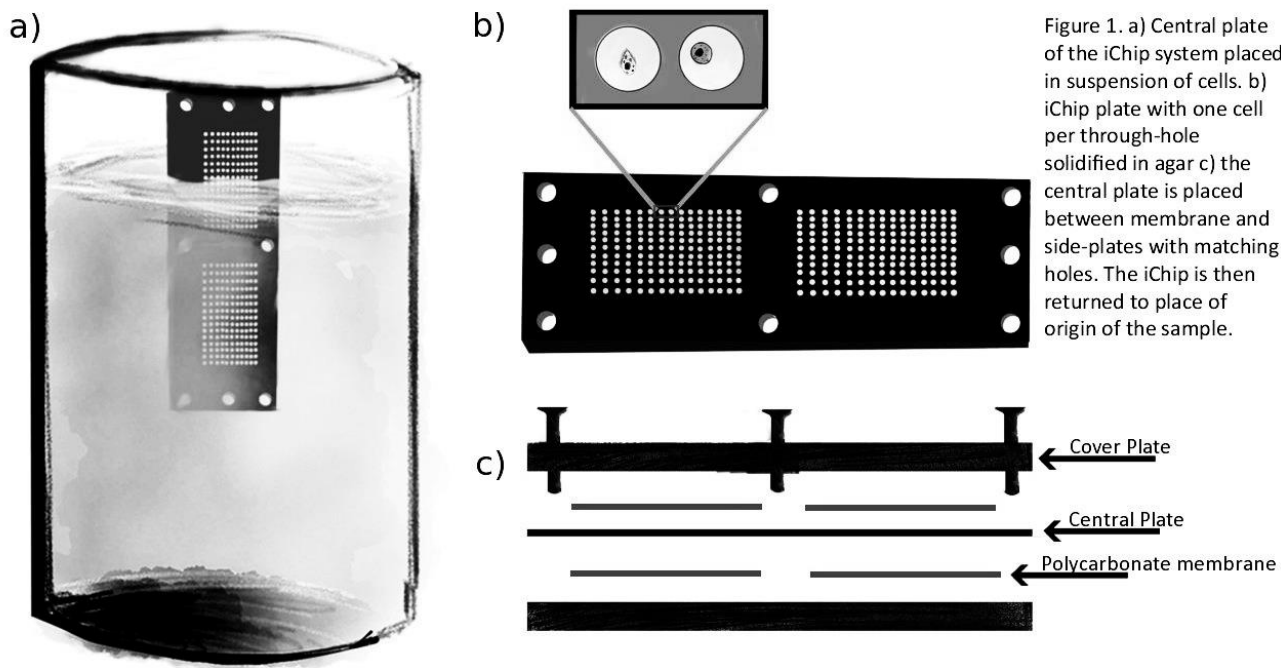
Unlike drugs for chronic conditions, antibiotics have shorter regimens and new antibiotics are hidden away as a measure of last resort, only to be used when the available antibiotics become ineffective. This causes the pharmaceutical companies to lose valuable time when they can gain a return on their investment before generics arrive in the market. Regulators, policy makers, and companies are working together to create incentives for antibiotic research by streamlining the approval process and rewarding pharmaceutical companies by extending patent exclusivity. In 2012, Congress passed into law the Generating Antibiotic Incentives Now (GAIN) Act (34), which extends drug-patent exclusivity for an additional five years, requires the FDA to provide updated trail guidance, and expedites the FDA-approval process. A new intravenous antibacterial drug, Dalvance was approved in May 2014 after receiving expedited review and being designated as a Qualified Infectious Disease Product (QIDP) under the GAIN Act (36). As of September 2014, FDA has granted 59 QIDP designations for 39 different unique molecules (37). Additionally, there has been a proposal put forward by a UK government-appointed review team, where companies that already have the "highest priority antibiotics" in their pipelines would receive a "lump-sum" payment thereby delinking profitability from sales volumes.

Our arsenal of antibiotics that can effectively treat bacterial infections is dwindling due to the appearance of resistant bacteria (38). Approaches such as creating optimized versions of existing antibiotics and designing synthetic entities for activity against microorganisms have been used, but without the discovery of a reliable lead/target pair, the chances of acquiring novel antibiotics does not seem promising. Natural products have been a

major source of antibiotics that work effectively, since they have been molded by evolution to work on natural targets (5). They are naturally designed to penetrate the membrane of target bacteria and have the ability to specifically bind to intracellular targets. So, exploiting the natural diversity of microorganisms is one of the most viable methods to discover novel chemicals that might yield new antibiotics to combat resistant pathogens. But throughout the history of microbiology, we have only been able to culture a small fraction of the diverse microbiological population for our own purposes, including discovery of antibiotics. An overwhelming majority of microbial species has not been explored since they do not grow in synthetic media *in vitro*. This has hindered our capability to fully exploit the natural microbiological population as a source of antibiotics.

## ICHIP TECHNIQUE

Currently, microbiological cultures are grown in solid agar-based growth media or in a liquid nutrient medium. After introduction of starter bacteria into culture they are grown according to their nutritional requirements, temperature requirements, CO<sub>2</sub>/O<sub>2</sub> optimal concentration, etc. However, many bacteria are fastidious, with very complex nutritional/environmental requirements and culturing such bacteria is challenging (39,40). The idea of using synthetic media is still the foundation of microbial recovery and propagation. Moreover, microbiological techniques have not changed over the years compared to the rapid progress seen in other sub-fields of biology, such as genetics and molecular biology.



Since microorganisms have adapted to thrive in their own specific environment, the core idea of creating an artificial environment might not be the most practical approach for microbial isolation. This is apparent when only a fraction of the microorganisms from a sample can be cultured in a laboratory setting, compared to the high range of

diversity shown by rRNA and metagenomic studies of the same sample (41). Petri-dishes and incubators have been the staple methods of microbiology, but to actually explore the microbial diversity, a drastic change in our strategy of isolation to an *in situ* model might be necessary. A new isolation chip (iChip) has been developed by Epstein et al. to

grow currently “uncultivable” microorganisms and access novel bioactive molecules that might be developed as therapeutic antibiotics (Figure 1) (42). The iChip applies an *in situ* cultivation model to isolate such inaccessible microorganisms. The iChip utilizes a system of hundreds of miniature diffusion chambers, each loaded with a single cell. The diffusion system allows the cells on the iChip interact with naturally occurring nutrients and environmental factors (42). This technique has been demonstrated to yield a higher number of novel microorganisms compared to traditional petri-dish methods (50% vs. 1%) (43).

The iChips are made of a central hydrophobic plastic poloxymethylene (POM) plate with an array of holes 1mm in diameter. POM, developed in 1959 by DuPont as Delrin®, is a thermoplastic which has been used as a long-term implant material in cardiac valve prostheses, dental implants or joint replacement components due to its biocompatible properties as well as physical characteristics (44). For the isolation of microorganisms, samples are collected from soil or seawater and suspended in liquid agar (Figure 1a). The suspension is diluted to ensure that one cell is placed per through-hole. The liquid agar solidifies and immobilizes the cells in the iChip (Figure 1b). Then, polycarbonate membranes (pore size of 0.03µm) are applied to both sides of the central plate to prevent cell migration. Cover plates with matching through-holes are then screwed to seal the system and hold the membrane in place. The whole system is designed to prevent cell from escaping; however, it allows diffusion of environmental factors. In their experiment, Epstein et al. cultured microorganisms from seawater and soil samples using the iChip system as well as traditional petri-dishes (45). The iChips were then returned to the environment from where the sample originated for incubation. Using microscopy and gene analysis of 16S rRNA to identify the microbial species, a higher number of colony forming cells were observed in the iChips (42). Cells grown in the iChip were different from the colonies that were grown in petri-dishes with essentially no overlap between the species isolated.

The iChip was successful in isolation of novel species of bacteria; however, the problem of successfully transferring them or sub-culturing them to standard petri-dishes still exists. Previous studies by Epstein et al. on diffusion chambers, which led to the development of iChip, have shown

success with *in vitro* domestication. The process of *in vitro* domestication refers to the sub-culturing of bacterial isolates, which typically take multiple transfers through the diffusion chamber before the isolates sustainably grow in standard cultures (46). This means that after isolation of “uncultivable” microorganisms using iChip, they can be domesticated for further exploration of antimicrobial potential (42). Using this two-step model of i) iChip isolation and then ii) domestication to standard culture, we could potentially achieve microbial recovery at an order of magnitude compared to the level using standard cultivation techniques. This allows access to a significant number of new species that cannot be isolated and grown by traditional *in vitro* studies (45). The iChip is also a practical and robust device. The POM can handle temperatures up to 170°C and can be autoclaved for sterilization (44). The thermoplastic structure has low water absorption and is resistant to mechanical abrasion. Moreover, the assembly and disassembly of an iChip takes under 5 minutes making it usable for massive *in situ* cultivation of environmental microorganisms (47).

### TEIXOBACTIN: A NOVEL ANTIBIOTIC

With the iChip, we can access “uncultivable” bacteria, discover novel species, and screen them for potential antibiotic activity. Among the 10,000 extracts derived from previously uncultured bacteria by iChip, a promising novel antibiotic Teixobactin was discovered (5). The source of teixobactin was a new species of  $\beta$ -proteobacteria, *Eleftheria terrae*, which belongs to a new genus related to Aquabacteria (45). Although aquabacteria are not known to produce antibiotics, teixobactin showed potent bactericidal activity against Gram-positive pathogens including multidrug-resistant strains (MDR) (47). Teixobactin was also highly effective against *Mycobacterium tuberculosis*, a pathogen against which there is a current and urgent unmet medical need especially in the developing countries (48–51). On a global scale, tuberculosis represents the second leading cause of death from infectious diseases (52)

From a structural viewpoint (Figure 2), teixobactin is a depsipeptide with a molecular mass of 1,242 Daltons (5). Examination of *S. aureus* cultures treated with teixobactin, and current antibiotics, vancomycin and oxacillin showed

similar inhibitory effect (Figure 3a-b). Teixobactin treatment resulted in dose dependent accumulation of the soluble cell wall precursor UDP-MurNac-pentapeptide, which was also seen in vancomycin-treated control cells. Vancomycin blocks a membrane-associated step of peptidoglycan biosynthesis resulting in cell death (53–55), and teixobactin appears to inhibit the same synthesis pathway by forming a complex with the precursors of peptidoglycan (lipid II) and also teichoic acids (lipid III) in the cell wall of Gram-positive bacteria (45). Vancomycin binds with terminal D-Ala-D-Ala moieties of peptidoglycan precursors and was once thought to be “resistance-proof”. Early studies on vancomycin in the 1950’s showed that vancomycin had ototoxic and nephrotoxic effects but purer preparations, retested in the 1970’s showed little or no toxic effects. With methicillin resistance increasing in staphylococci, vancomycin use accelerated worldwide in the 1980’s (56,57).

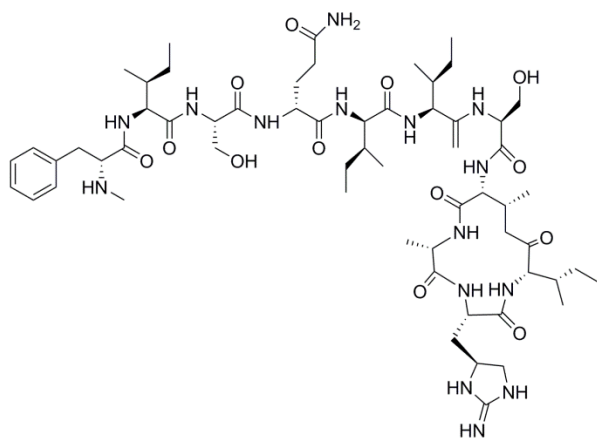


Figure 2. Structure of Teixobactin

Vancomycin resistant Gram-positive *Staphylococcus aureus* were eventually observed. The resistant strains produced enzymes that replaced the terminal D-Ala residue with D-lactate (D-Lac) or D-serine (D-Ser) and removed high affinity targets of vancomycin rendering the antibiotic ineffective (58–60). The mechanism of teixobactin, which targets invariable domains on the precursors, might be a bigger hurdle to resistance development. *E. terrae*, the producer of

teixobactin, is a Gram-negative bacteria and does not require any significant defensive measures other than the lipopolysaccharide cell layer to keep teixobactin out of the cell. So, resistance will need to originate from some other sources. But microorganisms have had millions of years to develop resistance to teixobactin, and the possibility of resistance genes already existing in nature is plausible. Even mutational resistance might arise *in vivo* after prolonged use of teixobactin.

*In vitro* toxicity tests of teixobactin with mammalian NIH/3T3 and HepG2 cells showed that there was no hemolytic activity or any DNA binding at 100 µg/ml. An animal efficacy study was also performed in a mouse septicemia model infected intraperitoneally with methicillin-resistant *S. aureus* (MRSA) at a dose that leads to 90% death (47). One hour post-infection, teixobactin was introduced through IV administration at variable single doses (1- 20 mg/kg). The results of the septicemia model were very encouraging with a PD<sub>50</sub> (protective dose at which half of the animals survive) of 0.2 mg/kg compared to the 2.75 mg/kg PD<sub>50</sub> of vancomycin. Intentional efforts to raise teixobactin-resistant *S. aureus* or *M. tuberculosis* by exposing the pathogens to sub-MIC (Minimum inhibitory concentration) levels of teixobactin for a period of 25 days failed to produce resistant mutants *in vitro* (Figure 3d) (5). Teixobactin had excellent bactericidal activity against *S. aureus*, was superior to vancomycin in killing late exponential phase populations, and retained bactericidal activity against vancomycin intermediate *S. aureus* (VISA) (5).

In summary, teixobactin stands out as a promising candidate to be developed for therapeutic use due to its effectiveness against Gram-positive bacteria, low toxicity profile, absence of resistance and positive results in mice studies but the main challenge of clinical trials with humans and regulatory hurdles still remain. Novobiotic pharmaceutical currently owns the license on teixobactin was awarded two NIH NIAID SBIR grants in January of 2015. One of the grants is a Phase II grant for the “Preclinical Development of Teixobactin, a New Antibiotic” (61).

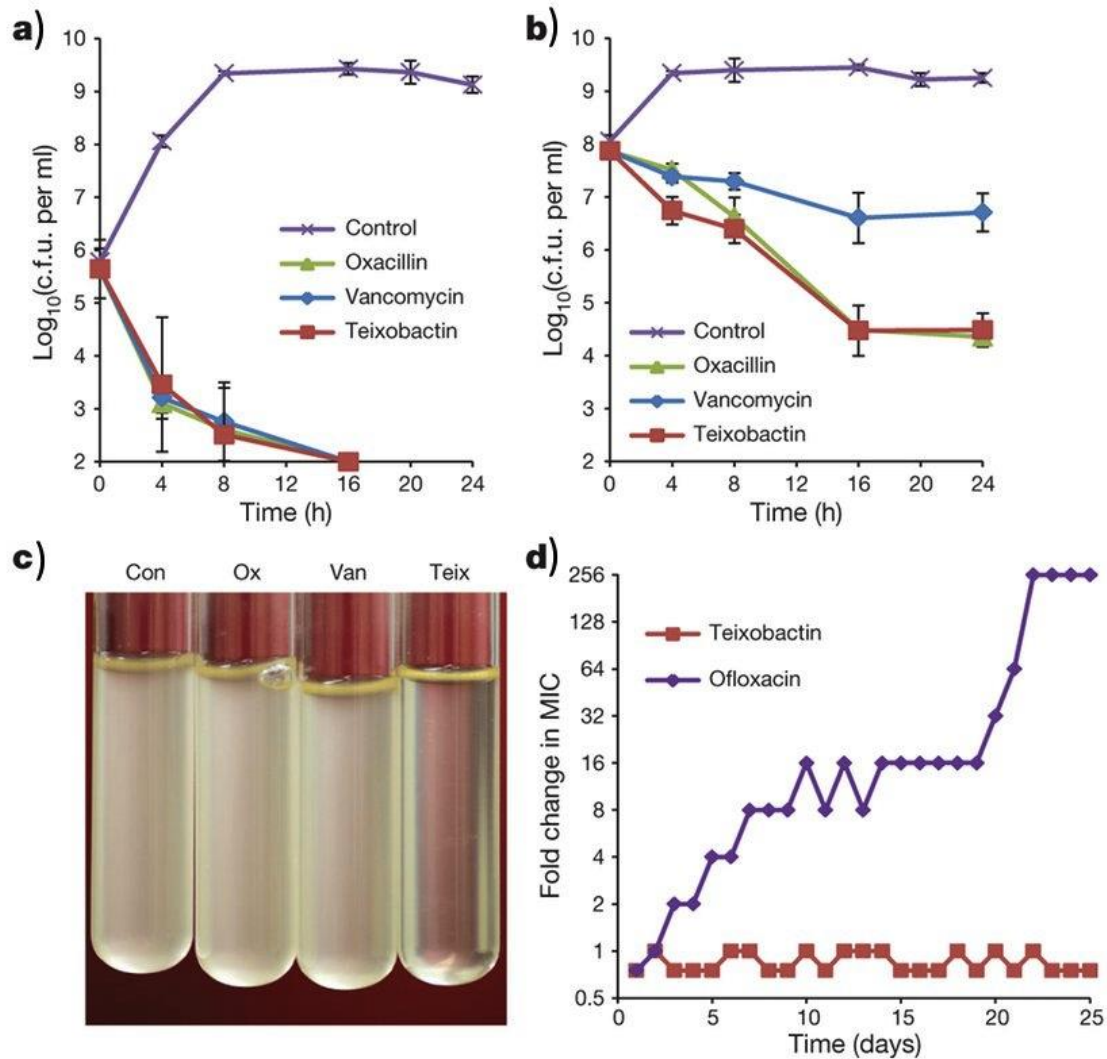


Figure 3. a) Early phase *S. aureus* and b) Late phase *S. aureus* challenged with antibiotics. c) Teixobactin treatment resulted in lysis of bacterial colony in liquid media broth d) Resistance acquisition during serial passaging in the presence of sub-MIC levels of antimicrobials

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## SUMMARY

Antibiotics are indispensable tools but have been used recklessly throughout their history. Alexander Fleming, in his Nobel speech stressed judicious use of Penicillin to prevent resistance:

*“Then there is the danger that the ignorant man may easily under-dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.”*

- Alexander Fleming  
Nobel lecture, December 11, 1945

Contrary to Fleming’s advice, sub-therapeutic doses of antibiotics have been administered to livestock in the guise of growth promotion. Many patients are still unaware of the consequences that occur when they do not follow proper antibiotic therapy. These factors contribute to the rapidly increasing resistance against many antibiotics. Unfortunately, with the unattractive economic

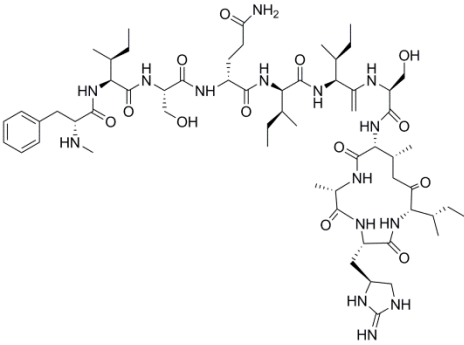
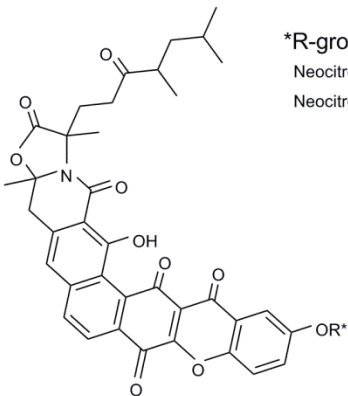
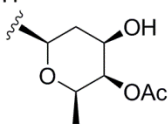
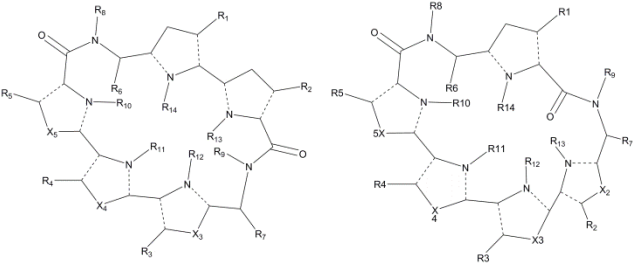


prospects, investment in discovery of new antibiotics has dried up. “Running out” of effective antibiotics is a terrifying thought; however, it seems more like a daunting possibility. Preventing this scenario will require judicious use of existing antibiotics and revitalization of the dwindling investment in antibiotic discovery. The GAIN Act is a step in the right direction to incentivize pharmaceutical companies to develop antibiotics through patent extension and expedited approval process. As of December, 2014 there were 37

antibiotics, with the potential to treat serious or life-threatening infections, are in clinical trials. At best, chances are that only 1 in 5 candidates that enter human trails become approved for patients (62).

During the Golden Age of antibiotics, almost all antibiotics were of bacterial or fungal origin but within a few decades all “cultivable” species of microorganisms had been over-mined for bactericidal compounds. Using the iChip, discovery of many novel, previously “uncultivable” microbial species has been possible. It is estimated that

**Table 1.** Potential antibiotics derived from iChip and diffusion chamber isolates

Antibiotic	Derived from	Potential applications	Structure
Teixobactin	<i>Eleftheria terrae</i>		
Neocitreamicin I Neocitreamicin II	<i>Nocardia</i> species strain G0655	Gram positive microbes including Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) and vancomycin-resistant enterococci (VRE)	 <p>*R-group Neocitreamicin I = H Neocitreamicin II = </p>
Novo10	<i>Oerskovia pauro-metabola</i>	Ongoing tests for antimicrobial and anti-neoplastic function.	

currently we can only cultivate 1% of the microbial diversity, which means that the remaining 99% has never been studied for any bioactive molecules. The iChip provides a highly successful platform for microbial isolation and expands the pool of potential microorganisms that can be the source of new antimicrobials, which is critical in our arms race against superbugs. For example *E. terrae*, a new species of related to aquabacteria was discovered through the iChip technique and extracts from *E. terrae* yielded Teixobactin, a novel antibiotic with high effectiveness against Gram-positive bacteria. But iChip is not able to recover all of the microbial species from a sample. Isolation of a novel species by iChip is scarcely enough for advanced studies and consequent *in vitro* domestication reduces the microbial recovery rate to 10%, which is still an order of magnitude higher than the recovery rate using traditional methods. In addition to future antibiotics that may result from novel species derived from iChip, there may be other beneficial ways in the pharmaceutical, biofuel, and renewable chemical production field where we could utilize new species of bacteria.

Exploiting the population of uncultured bacteria is likely to revive the Waksman platform of antibiotic discovery. Along with Teixobactin, the *in situ* isolation approach has helped discover other novel antibiotics such as Neocitreamicin I and II. With the help of the diffusion chamber, an earlier prototype of iChip, a novel *Nocardia* strain, the producer of neocitreamicins was isolated from a soil sample of Falmouth, Massachusetts. The neocitreamicins showed *in vitro* activity against Gram-positive bacteria including MRSA and VRE (63). Novobiotic pharmaceuticals also holds patent for Novo10, an antibiotic candidate with potential anti-neoplastic properties. Novo10 is derived from *Oerskovia paurometabola* a Gram positive anaerobe, isolated from soil sample collected in Gloucester, Massachusetts using a diffusion chamber (64). The iChip and its prototype, the diffusion chamber, have discovered many novel species and bioactive molecules. Teixobactin, neocitreamicins and Novo10 are the best candidates among many others found in isolate extracts for antibiotic activity. The main goal of iChip is to increase microbial recovery rate, which will lead to the discovery of novel species that were considered “uncultivable”. This hidden diversity of microorganisms gives us better chances of finding

novel and natural antibiotics. But the challenge of iChip being accepted into isolation/culturing practices still remains. The iChip’s portability, robustness and simplified use allows the possibility of microbial isolation from diverse environments. Over the years metagenomics has been utilized to discover microbial communities in a culture-independent analysis (65). In addition to finding microbial community composition, metagenomics can be applied as a targeted approach to screen functional DNA fragments or global gene expression. This “shotgun” approach of finding novel microbes among the “uncultivable” bacteria can now be highly precise by using single cell genomics on iChip isolates. Teixobactin will probably receive Qualified Infectious Disease Product (QIDP) designation under the GAIN Act, 21 U.S.C § 355 (f)(1)(c) provisioned for multi-drug resistant tuberculosis (66). But there will still be years of challenges, intense research and clinical trials before teixobactin appears as an approved, commercial product

## REFERENCES

1. Abraham EP, Chain E, Fletcher CM, Gardner AD, Heatley NG, Jennings MA et al. Further observations on Penicillin. *Lancet* 1941;**238**:177–189.
2. Aldridge S, Sturchio JL. *The discovery and development of penicillin. 1928-1945*. American Chemical Society 1999<http://www.acs.org/content/dam/acsorg/education/whatischemistry/landmarks/flemingpenicillin/the-discovery-and-development-of-penicillin-commemorative-booklet.pdf> (accessed 1 May 2015).
3. Ligon BL. Sir Alexander Fleming: Scottish researcher who discovered penicillin. *Semin Pediatr Infect Dis* 2004;**15**:58–64.
4. Singh SB. Confronting the challenges of discovery of novel antibacterial agents. *Bioorg Med Chem Lett* 2014;**24**:3683–3689.
5. Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP et al. A new antibiotic kills pathogens without detectable resistance. *Nature* 2015;**517**:455–459.
6. Waksman SA. What Is an Antibiotic or an Antibiotic Substance? on JSTOR. *Mycologia*, 1947;**39**:565–569.
7. Jebbar M, Franzetti B, Girard E, Oger P. Microbial diversity and adaptation to high hydrostatic pressure in deep-sea hydrothermal vents prokaryotes. *Extremophiles* Published Online First: 23 June 2015. doi:10.1007/s00792-015-0760-3

8. Munteanu A-C, Uivarosi V, Andries A. Recent progress in understanding the molecular mechanisms of radioresistance in *Deinococcus* bacteria. *Extremophiles* Published Online First: 4 June 2015. doi:10.1007/s00792-015-0759-9
9. Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA et al. The NIH Human Microbiome Project. *Genome Res* 2009;**19**:2317–2323.
10. Zhang Y-J, Li S, Gan R-Y, Zhou T, Xu D-P, Li H-B. Impacts of Gut Bacteria on Human Health and Diseases. *Int J Mol Sci*; **16**:7493–7519.
11. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med* 2014;**6**:237ra65.
12. Schwartz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C et al. Microbiota and SCFA in Lean and Overweight Healthy Subjects. *Obesity* 2010;**18**:190–195.
13. Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* 2008;**3**:213–223.
14. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Discov* 2007;**6**:29–40.
15. Wang J, Kodali S, Lee SH, Galgoci A, Painter R, Dorso K et al. Discovery of platencin, a dual FabF and FabH inhibitor with in vivo antibiotic properties. *Proc Natl Acad Sci U S A* 2007;**104**:7612–7616.
16. Poole K. Efflux-Mediated Resistance to Fluoroquinolones in Gram-Negative Bacteria. *Antimicrob Agents Chemother* 2000;**44**:2233–2241.
17. Lomovskaya O, Lee A, Hoshino K, Ishida H, Mistry A, Warren MS et al. Use of a Genetic Approach To Evaluate the Consequences of Inhibition of Efflux Pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1999;**43**:1340–1346.
18. Wolfson JS, Hooper DC. Bacterial Resistance to Quinolones: Mechanisms and Clinical Importance. *Clin Infect Dis* 1989;**11**:S960–S968.
19. Sanchez G V, Roberts RM, Albert AP, Johnson DD, Hicks LA. Effects of knowledge, attitudes, and practices of primary care providers on antibiotic selection, United States. *Emerg Infect Dis* 2014;**20**:2041–2047.
20. Barriere SL. Clinical, economic and societal impact of antibiotic resistance. *Expert Opin Pharmacother* 2015;**16**:151–153.
21. Naik MM, Dubey SK. Lead resistant bacteria: lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. *Ecotoxicol Environ Saf* 2013;**98**:1–7.
22. Mazel D, Davies J. Antibiotic resistance in microbes. *Cell Mol Life Sci* 1999;**56**:742–754.
23. Ghodhbane H, Elaidi S, Sabatier J-M, Achour S, Benhmida J, Regaya I. Bacteriocins Active Against Multi-Resistant Gram Negative Bacteria Implicated in Nosocomial Infections. *Infect Disord Drug Targets* 2014.
24. Nambudiri VE. More Than Skin Deep—The Costs of Antibiotic Overuse. *JAMA Intern Med* 2014;**174**:1724.
25. Cantón R, Horcajada JP, Oliver A, Garbajosa PR, Vila J. Inappropriate use of antibiotics in hospitals: the complex relationship between antibiotic use and antimicrobial resistance. *Enferm Infecc Microbiol Clin* 2013;**31 Suppl 4**:3–11.
26. Zayegh I, Charrois TL, Hughes J, Hoti K. Antibiotic repeat prescriptions: are patients not re-filling them properly? *J Pharm policy Pract* 2014;**7**:17.
27. Zayegh I, Charrois TL, Hughes J, Hoti K. Antibiotic repeat prescriptions: are patients not re-filling them properly? *J Pharm policy Pract* 2014;**7**:17.
28. Wallinga D, Burch DGS. Does adding routine antibiotics to animal feed pose a serious risk to human health? *BMJ* 2013;**347**:f4214.
29. O’Neill J, Hala A, Knox J, Hall W, McDonnell A, Truscott-Reid N et al. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. 2015 [http://amr-review.org/sites/default/files/AMR\\_Review\\_Paper - Tackling a crisis for the health and wealth of nations\\_1.pdf](http://amr-review.org/sites/default/files/AMR_Review_Paper_-_Tackling_a_crisis_for_the_health_and_wealth_of_nations_1.pdf) (accessed 29 Jun 2015).
30. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001;**65**:232–260 ; second page, table of contents.
31. Amachawadi RG, Scott HM, Aperce C, Vinasco J, Drouillard JS, Nagaraja TG. Effects of in-feed copper and tylosin supplementations on copper and antimicrobial resistance in faecal enterococci of feedlot cattle. *J Appl Microbiol* Published Online First: March 2015. doi:10.1111/jam.12790
32. Paterson DL. The challenge of treating superbugs. *Semin Respir Crit Care Med* 2015;**36**:1–2.
33. Le Dorze M, Gault N, Foucrier A, Ruppé E, Mourvillier B, Woerther PL et al. Performance and impact of a rapid method combining mass spectrometry and direct antimicrobial susceptibility testing on treatment adequacy of patients with ventilator-associated pneumonia. *Clin Microbiol Infect* Published Online First: December 2014. doi:10.1016/j.cmi.2014.12.007
34. Harbarth S, Theuretzbacher U, Hackett J. Antibiotic research and development: business as usual? *J Antimicrob Chemother* Published Online First: February 2015. doi:10.1093/jac/dkv020
35. Lewis K. Antibiotics: Recover the lost art of drug discovery. *Nature* 2012;**485**:439–440.

36. Commissioner O of the. FDA approves Dalvance to treat skin infections. <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm398724.htm> (accessed 15 May2015).
37. Woodcock J. 21st century cures: examining ways to combat antibiotic resistance and foster new drug development. 2014.<http://docs.house.gov/meetings/IF/IF14/20140919/102692/HHRG-113-IF14-Wstate-WoodcockJ-20140919.pdf> (accessed 15 May2015).
38. Martínez JL, Baquero F. Emergence and spread of antibiotic resistance: setting a parameter space. *Ups J Med Sci* 2014;**119**:68–77.
39. Hoover WH, Stokes SR. Balancing carbohydrates and proteins for optimum rumen microbial yield. *J Dairy Sci* 1991;**74**:3630–3644.
40. Price PB, Sowers T. Temperature dependence of metabolic rates for microbial growth, maintenance, and survival. *Proc Natl Acad Sci U S A* 2004;**101**:4631–4636.
41. Rappé MS, Giovannoni SJ. The uncultured microbial majority. *Annu Rev Microbiol* 2003;**57**:369–394.
42. Arias CA, Murray BE. A new antibiotic and the evolution of resistance. *N Engl J Med* 2015;**372**:1168–1170.
43. Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A et al. Use of iChip for high-throughput in situ cultivation of ‘uncultivable’ microbial species. *Appl Environ Microbiol* 2010;**76**:2445–2450.
44. Penick KJ, Solchaga LA, Berilla JA, Welter JF. Performance of polyoxymethylene plastic (POM) as a component of a tissue engineering bioreactor. *J Biomed Mater Res A* 2005;**75**:168–174.
45. Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A et al. Use of iChip for high-throughput in situ cultivation of ‘uncultivable’ microbial species. *Appl Environ Microbiol* 2010;**76**:2445–2450.
46. Kaeberlein T, Lewis K, Epstein SS. Isolating ‘uncultivable’ microorganisms in pure culture in a simulated natural environment. *Science* 2002;**296**:1127–1129.
47. Hunter P. Antibiotic discovery goes underground: The discovery of teixobactin could revitalise the search for new antibiotics based on the novel method the researchers used to identify the compound. *EMBO Rep* Published Online First: April 2015. doi:10.15252/embr.201540385
48. Baddeley A, Dean A, Dias HM, Falzon D, Floyd K, Baena IG et al. Global Tuberculosis Report 2014. 2014[http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf) (accessed 1 May2015).
49. Muniyandi M, Ramachandran R. Socioeconomic inequalities of tuberculosis in India. *Expert Opin Pharmacother* 2008;**9**:1623–1628.
50. Lönnroth K, Castro KG, Chakaya JM, Chauhan LS, Floyd K, Glaziou P et al. Tuberculosis control and elimination 2010–50: cure, care, and social development. *Lancet* 2010;**375**:1814–1829.
51. Spence DP, Hotchkiss J, Williams CS, Davies PD. Tuberculosis and poverty. *BMJ* 1993;**307**:759–761.
52. Frieden TR, Brudney KF, Harries AD. Global tuberculosis: perspectives, prospects, and priorities. *JAMA* 2014;**312**:1393–1394.
53. Nieto M, Perkins HR. Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. *Biochem J* 1971;**123**:789–803.
54. Sheldrick GM, Jones PG, Kennard O, Williams DH, Smith GA. Structure of vancomycin and its complex with acetyl-D-alanyl-D-alanine. *Nature* 1978;**271**:223–225.
55. Liu J, Volk KJ, Lee MS, Pucci M, Handwerker S. Binding Studies of Vancomycin to the Cytoplasmic Peptidoglycan Precursors By Affinity Capillary Electrophoresis. *Anal Chem* 1994;**66**:2412–2416.
56. Moellering RC. Vancomycin: a 50-year reassessment. *Clin Infect Dis* 2006;**42 Suppl 1**:S3–S4.
57. Griffith RS. Introduction to vancomycin. *Rev Infect Dis*;**3 suppl**:S200–S204.
58. Courvalin P. Vancomycin resistance in Gram-positive cocci. *Clin Infect Dis* 2006;**42 Suppl 1**:S25–S34.
59. Levine DP. Vancomycin: a history. *Clin Infect Dis* 2006;**42 Suppl 1**:S5–S12.
60. Zaffiri L, Gardner J, Toledo-Pereyra LH. History of antibiotics: from fluoroquinolones to daptomycin (Part 2). *J Invest Surg* 2013;**26**:167–179.
61. Press Releases — Novobiotic Pharmaceuticals. 2015.<http://www.novobiotic.com/news/> (accessed 18 May2015).
62. Hay M, Thomas DW, Craighead JL, Economides C, Rosenthal J. Clinical development success rates for investigational drugs. *Nat Biotechnol* 2014;**32**:40–51.
63. Peoples AJ, Zhang Q, Millett WP, Rothfeder MT, Pescatore BC, Madden AA et al. Neocitreamicins I and II, Novel Antibiotics with Activity against Methicillin-Resistant *Staphylococcus aureus* and Vancomycin-Resistant Enterococci. *J Antibiot (Tokyo)* 2008;**61**:457–463.
64. Stackebrandt E, Breyman S, Steiner U, Prauser H, Weiss N, Schumann P. Re-evaluation of the status of the genus *Oerskovia*, reclassification of *Promicromonospora enterophila* (Jäger et al. 1983) as *Oerskovia enterophila* comb. nov. and description of *Oerskovia jenensis* sp. nov. and *Oerskovia paurometabola* sp. nov. *Int J Syst Evol Microbiol*

- 2002;**52**:1105–1111.
65. Schloss PD, Handelsman J. Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. *Genome Biol* 2005;**6**:229.
66. Food and Drug Administration Safety and

Innovation Act. United States: Congress 2012  
<http://www.gpo.gov/fdsys/pkg/PLAW-112publ144/pdf/PLAW-112publ144.pdf> (accessed 18 May2015).