Exploiting the toolbox of directed evolution to combat the antimicrobial resistance pandemic

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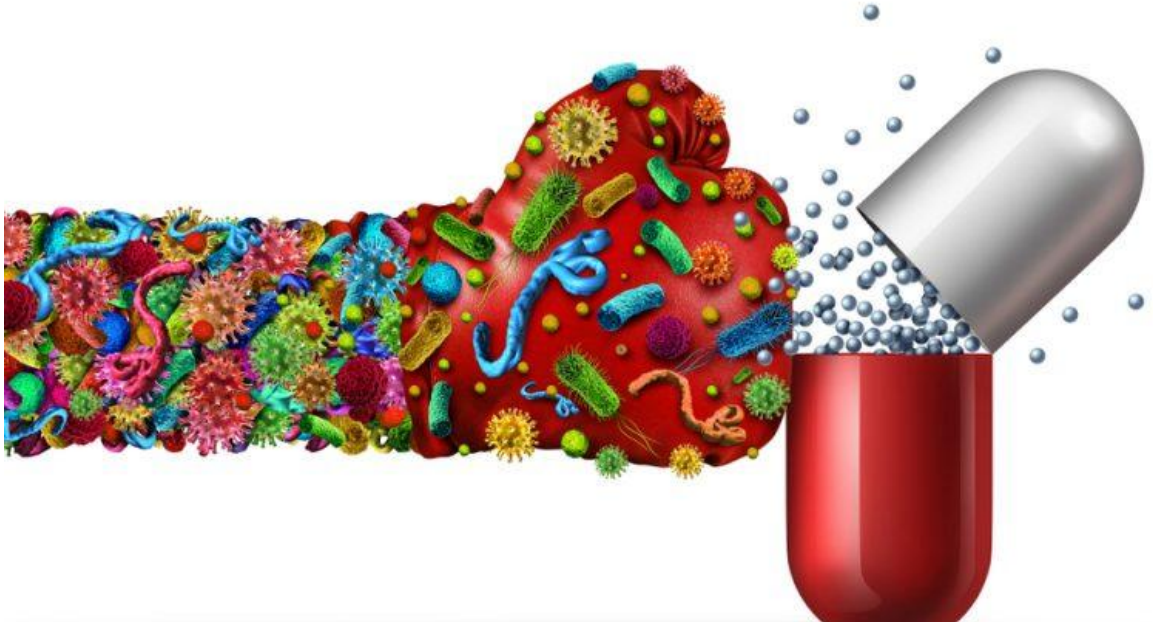
Bachelor's Thesis



Summary

In this review it was explored how the concept of directed evolution can be used to combat the antimicrobial resistance crisis with special focus on antifungals. Directed evolution is the targeted artificial evolution of a specific DNA segment. *In vitro* mutagenesis techniques have been the most dominant method of generating mutant libraries and evolving antimicrobials. They are easily applicable, but do have certain disadvantages such as being labor and time intensive, limited size of target DNA that can be mutagenized and some methods are biased towards certain mutations. *In vivo* mutagenesis methods are conceptually more difficult, but solve most of the issues *in vitro* mutagenesis has. Even though its large potential, empirical evidence of antimicrobials evolved using *in vivo* mutagenesis is still minimal. As of now, the screening step is the bottleneck and determines throughput. A phage based evolution method is fully continuous eliminating intermediary screening steps and thus increasing throughput. A screening method of an experimental evolution set-up might also allow for continuous screening and increased throughput. Directed evolution can also be used to resensitize resistant pathogens to certain antimicrobials and to increase the lifetime of current and future drugs. Apart from the evolution of antimicrobials, directed evolution methods have also been developed to mapp (genomic) resistance profiles and fitness landscapes of antimicrobials. This gives us a better understanding of the evolution of resistance and lets us predict the chance resistance will occur to a certain drug. All in all, directed evolution provides us with a large and diverse toolbox to combat the antimicrobial resistance crisis.

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1. Introduction

It has been known for a while that antimicrobial resistance (AMR) is a serious problem and that it continues to grow larger (WHO, 2014; WHO 2020). In 2016 it was estimated that each year 700.000 people die due to drug resistance in pathogenic bacterial strains, HIV & malaria (O'Neill, 2016). This number is most likely higher, because of poor registration in many underdeveloped countries. Moreover, the estimate excludes deaths due to drug resistant fungal (and some viral) pathogens. If treatment procedures stay as they currently are, it is estimated that by 2050 each year ten million people are at risk of death and 100 trillion dollars of economic output is lost, because of AMR (O'Neill, 2016). A lot of attention is directed towards (multi) drug resistant pathogenic bacteria, but the problem of antifungal resistance - even though a serious problem as well - is less recognized (Nature Microbiology, 2017; Jugessur & Denning, 2016). Every year 1.6 million people die due to fungal infections (note: not only drug resistant fungi) (LIFE, 2017). Especially for immunocompromised patients, fungal infections can be life threatening, but fungal infections in healthy people are increasingly occurring (Nature Microbiology, 2017). Pathogenic fungi not only pose a threat for human welfare, but also for many economic activities. It is estimated that worldwide 20% of crop yields each year are lost due to fungal infections and another 10% postharvest (Fisher et al., 2018). Antifungals are also applied as coatings and preservatives for a wide range of materials. The azole class is the most used antifungal both for animal and human treatment as well as crop protection. However, due to its widespread usage and the high genomic plasticity and reproduction rate of fungi, global azole resistance is increasingly becoming a problem (Fisher et al., 2018). Resistance to other less extensively used antifungals has also increasingly been reported (Fisher et al., 2018). Multidrug resistance has been reported in *Candida Glabrata* and *Candida Aureus* (Nature Microbiology, 2017; Chowdhary &Meis, 2020). Filamentous fungi such as *Aspergillus terreus* and *Scedosporium spp.* that intrinsically exhibit resistance against many antifungals increasingly become a serious problem (Fisher et al., 2018) . A large number of plant pathogens such as *Zymoseptoria tritici* (wheat pathogen), *Mycosphaerella fijiensis* (banana pathogen) and the cereal powdery mildew fungus *Blumeria graminis* are resistant to a large range of antifungals (Fisher et al., 2018).

There are four main mechanisms by which microorganisms develop resistance to antimicrobials: 1) decreasing intracellular drug concentrations by the upregulation of efflux pumps, 2) down- or upregulation of the target, 3) alteration of target site by mutations, 3) metabolic modifications and adaptations to the effect of the drug (Vandeputte et al, 2012; Péman et al, 2020). These can occur on their own or in combinations. These resistance mechanisms can develop, because the antimicrobial drug stays the same while the targeted pathogen evolves. Between 1950 and 1980 , also known as the 'golden era' a lot of new antimicrobial compounds were discovered. However, since the 1980s the discovery of new antibiotics has dwindled dramatically (O'Neill, 2016). Evolution is not inherently a bad thing. It can and has also been used to our advantage, creating enzymes or proteins with improved or novel functions and bacterial/fungal strains with increased biomolecule production capacities (Yuan et al., 2015). In the past decade a lot of work has been done in the field of labory controlled evolution (Morrison et al., 2020; Packer & Liu., 2015). This knowledge can be deployed to evolve antimicrobials to circumvent AMR.

An experimental technique that can be used to evolve proteins (and possibly other compounds) is 'directed evolution'. Directed evolution is composed of the random mutagenesis of a specific gene or group of genes and the screening for improved variants (Packer & Liu., 2015). Directed evolution explores the chemical sequence space of proteins and this can potentially result in antimicrobials with altered or new properties that circumvent AMR. Directed evolution can also be used the other way around: evolving pathogens to become resistant to antimicrobials (that are in development). This way, mechanisms of drug resistance can be understood and the chance of resistance occurring against new antimicrobials can be estimated.

The questions that this review will address are the following:

How can evolution - specifically directed evolution - be used in a controlled laboratory environment to understand bacterial resistance evolutionary pathways in pathogens and to improve or change antimicrobial compounds?

Do these principles and methods also work to understand antifungal resistance and to develop new antifungals?

First, a brief description will be given of the different types of antifungals, their *modi operandi* and biosynthesis. This is useful to know, since some types of antifungals may be more suitable for evolution experiments than others. Then different methods of inducing and following evolution are discussed, which can be used to discover altered or improved antimicrobial compounds. Thirdly, resensitization of drug resistant pathogens will be discussed and the role evolution experiments can play therein. Lastly, a directed evolution method will be described that can be used to understand evolutionary pathways of pathogens that become resistant to a certain antimicrobial and to construct genomic resistance profiles for antimicrobials (in development).

2. Antifungal classification

Antifungals are compounds - either naturally or chemically synthesized - that kill (fungicide) or inhibit the growth (fungistatic) of target fungal species. In order for an antifungal - or any antimicrobial - to be the subject of directed evolution, the antifungal needs to be produced by an organism. There are roughly two types of naturally produced antifungals: (small) metabolic compounds and peptides/proteins (Selitrennikoff, 2001, Vandeputte et al., 2012). The first group of antifungals is the end product of different enzymes working together. These enzymes are often grouped together in biosynthetic gene clusters. The second group of antifungals are (partly) directly genetically encoded. This makes it easier for directed evolution to evolve antifungal proteins. Individual genes in a multi-enzyme pathway or biosynthetic gene cluster can be evolved with directed evolution, but often mutations in multiple genes need to occur simultaneously in order for an improved compound to emerge (Nyerges et al., 2018). As will be described later, new directed evolution methods have been developed that should make the evolution of multi-enzyme pathways and biosynthetic gene clusters more doable.

Since fungi are eukaryotes, they have a lot of similarities with animal and plant cells, which makes it harder for drugs to selectively target pathogenic fungal cells. A distinctive feature of fungal cells is the (composition of the) cell wall. Therefore, most antifungals focus on disrupting the cell wall or inhibiting its synthesis (Cortés et al., 2019).

There are four classes of antifungals that are used for the treatment of fungal infections in animals: azoles, polyenes, pyrimidine analogs & echinocandins (Fisher et al., 2018). Of these types of drugs polyenes and echinocandins are naturally produced and can thus be the subject of (directed) evolution experiments (Vandeputte et al., 2012). Polyenes are cyclic amphiphilic organic molecules and disrupt fungal cell membrane by the sequestration of ergosterol and subsequent pore formation (Vandeputte et al., 2012). The efflux of ions leads to the death of the fungal cell (Houšť et al, 2020). Polyenes are naturally produced by *Streptomyces* bacteria through a special gene cluster encoding for polyketide synthases, cytochrome P450-dependent enzymes, ABC transporters and enzymes that synthesize and facilitate the binding of mycosamine moieties. Echinocandins are cyclic lipo-hexapeptides and are non-ribosomally produced by several fungal species (Emri et al., 2013). They inhibit (1-3)-β-glucan synthase responsible for the build up of the fungal cell wall. Biosynthetic gene clusters of echinocandins encode among other gene products for non-ribosomal peptide synthases, different oxygenases and fatty-acyl-AMP ligases (Emri et al., 2013).

For the treatment of fungal infections in plants seven main classes of antifungals are used: azoles, morpholines, strobilurins, benzimidazoles, succinate dehydrogenase inhibitors and anilinopyrimidines and inorganic fungicides (Fisher et al., 2018). Of these compounds only strobilurins are naturally produced and can be the subject of (directed) evolution experiments. Strobilurins are benzene or pyrimidine derivatives of methacrylic acid produced by basidiomycete fungi and kill fungal cells by inhibiting electron transfer in mitochondria (Nofiani et al., 2018). The biosynthetic gene cluster responsible for the production of strobilurins include a polyketide synthase, oxygenases and methyltransferases (Nofiani et al., 2018).

There are also proteins that have antifungal activity. These antifungal proteins include PR-1 proteins, 1,3-β-glucanases, chitinases, chitin-binding proteins, thaumatin-like proteins, defensins, cyclophilin-like protein, glycine/histidine-rich proteins, ribosome-inactivating proteins (RIPs), lipid-transfer proteins (LTPs), killer proteins and protease inhibitors (Selitrennikoff, 2001).They exhibit different killing mechanisms such as the inhibition of cell wall synthesis, cell wall polymer degradation, pore formation in the cell membrane, damage to ribosome subunits, inhibition of DNA synthesis and inhibition of the cell cycle (Selitrennikoff, 2001). Various antifungal proteins -

especially those that don't target the fungal cell wall - are to a certain extent toxic to the animals (or plants), due to similarity between host and fungal pathogen (Cortés et al., 2019). They may therefore not be suitable for clinical applications.

In the next section different directed evolution methods and strategies will be discussed.

2. Directed evolution methods using *in vitro* mutagenesis

2.1 Error-prone PCR

One widely applied method to introduce random mutations in a designated gene (region) *in vitro* is error-prone PCR (Packer & Liu., 2015). This PCR method makes use of a low-fidelity polymerase to increase the rate of mutations during DNA replication. The rate of mutations per given number of basepairs can be tuned by means of regulating manganese and dGTP concentrations (Packer & Liu., 2015). After multiple rounds of PCR a mutant library is generated, which can be cloned into an expression host to screen for 'beneficial' mutations. This method can be applied to generate antimicrobials with improved function. In a study random mutagenesis and subsequently error-prone PCR were used to generate phage lytic enzyme mutants (PlyGBS) that expressed improved lytic activity against group B streptococci (GBS) (Cheng and Fischetti, 2006). Phage lytic enzymes (PLEs) kill target bacteria cells by digesting their cell wall leading to osmotic lysis of the cells (more info on PLEs in the next paragraph). PlyGBS can be used to kill GBS that are resistant to antibiotics. PlyGBS however did show a relatively low lytic activity compared with other bacteriophage lysins. After directed evolution using error-prone PCR two mutant PlyGBS were isolated that showed 18-fold and 28-fold increased *in vitro* killing activity compared to the wild type. Analysis in mice models also showed improved killing activity *in vivo*. Other researchers used error-prone PCR to evolve the endolysin PlyC (Heselpoth & Nelson, 2012). They showed an increase of thermostability of the endolysin PlyC after just one round of mutagenesis, which led to a killing activity post heat treatment that was twice as high compared to wild type PlyC. Another study performed error-prone PCR to target N-acyl-homoserine lactonase (Gurevich et al., 2021). This enzyme digests N-acyl-homoserine lactones (AHLs) that are used by many gram negative bacteria (including plant & human pathogens) for quorum sensing, which is a gene regulatory mechanism dependent on cell density. They isolated a mutant that showed the same activity at a temperature eight degrees higher.

Error-prone PCR is a relatively easy applicable method to generate mutant libraries. However, error-prone PCR does show a bias towards mutations at certain positions (Nyerges et al., 2018). Therefore there is no random distribution of mutations, which may mean only a portion of the potential fitness landscape of antimicrobials is explored.

2.2 DNA shuffling

Another technique that is often used in combination with random mutagenesis is genome shuffling (Packer & Liu., 2015). Here DNA regions that are homologous to each other can be swapped creating new genomic combinations (Biot-pelletier & Martin, 2014). Some of these combinations can lead to beneficial gene variants. Using this principle, the production (Luo et al., 2012; Wang et al., 2014)) or activity (Han et al., 2017; Shi et al., 2018) of antimicrobials can be substantially increased. Genome shuffling can be used to engineer and improve phage lytic enzymes (PLEs) in particular (São-José, 2018). PLEs that digest the bacterial cell wall from the outside - necessary for phage infection - are called virion-associated lysins (VALs). PLEs that digest the bacterial cell wall from the inside - necessary for phage particles release - are called endolysins (São-José, 2018). PLEs contain one or more catalytic domains (CDs) that are responsible for the cleavage of peptidoglycan. PLEs can also contain a cell wall binding domain (CBD), responsible for the anchorage of the PLE to a specific cell wall component. These domains facilitate highly specific killing spectrums of PLEs, which is a therapeutic benefit since it limits off target killing of host cells and commensal bacteria (São-José, 2018). Given the highly modular structure of PLEs genome shuffling of CDs and CBDs from different phages can be done to engineer PLEs with improved therapeutic properties. Yang and coworkers used domain shuffling of chemically synthesized CDs and CBDs to generate a library of chimeolysins and developed a system for rapid screening of the lytic activity of these mutants (Yang et al., 2015). Using three distinct CBDs and seven distinct CD random combinations of CBS and CDs were cloned into an expression plasmid. A previously constructed lysin ClyN was cloned into a separate plasmid and was used to facilitate the screening process. Both plasmid were inserted into *E.coli* and the cells were subsequently plated onto agar plates overlaid with a target

bacterial strain. Upon the addition of IPTG, inside lysis of the host cells by ClyN was induced and the engineered chimeolysins spilled into the environment. Clearance zones of the target strain were measured to determine the outside lytic activity of the chimeolysins. Multiple mutants were isolated that showed killing activity, of which a mutant named ClyR performed best. ClyR displayed solid *in vitro* as well as *in vivo* lytic activity against multiple streptococci species, indicating that ClyR is a promising candidate for streptococcal infections.

2.3 Site-directed mutagenesis

Directed evolution can not only be used to improve antimicrobial proteins or peptides itself, but also enzymes responsible for the production of antimicrobial compounds. Enzymes can often very specifically catalyze certain reactions and therefore can make antimicrobials that are currently impossible to synthesize chemically. Enzymes of which extensive knowledge about structure and function is known, can be the subject of directed evolution experiments to engineer these enzymes in such a way that they catalyze reactions necessary for *in vitro* drug synthesis (Walsh, 2001). In many cases (groups of) important amino acids for functionality are known, which allows for a more focused mutagenesis approach such as site-saturation mutagenesis (SSM). In SSM a random mutant library is made in which a limited number of amino acid positions are substituted with all possible amino acids (Siloto & Weselake, 2012). The cytochrome enzyme P450 that natively hydroxylates C-H bonds in complex molecules has been the subject of different engineering projects. Researchers showed that P450 can be evolved to perform other types of reactions such as carbene and nitrene transfers (Brandenberg et al., 2017). Subsequently, using site-saturation mutagenesis and whole-cell catalyst screening in 96-well plates they evolved P450 to selectively amidate certain C-H bonds in carbonyl nitrene precursor molecules. Different P450 mutants were isolated that produced different lactams (β,γ & δ). This shows that directed evolution can be used to engineer enzymes with highly specific catalytic functions (Cho et al., 2019).

A downside to *in vitro* mutagenesis is that it is laborious; it costs a lot of time and quite a lot of intervention steps are required from the researcher. Furthermore, the evolution of multiple genes at the same time is difficult. Directed evolution using *in vivo* mutagenesis solves part of these problems.

3. Directed evolution methods using *in vivo* mutagenesis

3.1 Orthogonal DNA replication

Random mutagenesis *in vivo* methods such as mutagenic compounds, uv-light or mutator strains (e.g. *E.coli* XL1-Red**)** have the large downside that mutations occur genome-wide (Packer & Liu., 2015). Eventually deleterious mutations in essential genes occur, limiting the length span of evolution experiments. Because techniques for *in vivo* directed mutagenesis are conceptually complex, *in vitro* mutagenesis was the preferred method for a long time (Packer & Liu., 2015). However, in the last decade *in vivo* directed mutagenesis techniques have been developed. The first that shall be discussed is orthogonal DNA replication. This system makes use of a polymerase that (preferably) replicates only a certain (region of a) plasmid.

One of the earlier orthogonal DNA replication strategies was developed in *E.coli* and used an error-prone variant of DNA repair polymerase Pol I (Fabret, 2001). This polymerase displays preference for the replication of certain regions in plasmids. In one such region the gene of interest was cloned, which was replicated with an elevated mutagenesis rate. Optimization of this strategy resulted in a mutation frequency of 0.81 kb^{-1} $generation^{-1}$. Camps and coworkers used this technique to evolve TEM-1-lactamase and generated TEM-1-lactamase mutants that were able to hydrolyse the antibiotic aztreonam (Camps et al., 2003). They confirmed mutations leading to resistance found in clinical isolates and also discovered a new mutation that led to increased aztreonam resistance. However, a drawback of this orthogonal DNA replication system is that it is not truly orthogonal, since the mutagenic Pol1 still replicates some portions of the chromosomal DNA and thus introduces mutations there. Moreover, the target region that can be mutagenized is limited in size(~700bp).

More recently, researchers developed a strategy for orthogonal DNA replication (OrthoRep) in the eukaryote *S.cerevisiae* (Ravikumar et al., 2014). For this, they used the linear plasmid from *Kluyveromyces lactis* (pGKL1), which is exclusively replicated by a specific polymerase: TP-DNAP1. Unlike the polymerase Pol1 in *E.coli*, TP-DNAP1 only replicates pGKL1. Multiple TP-DNAP1 mutants were generated with different mutagenesis rates, the highest being 0.01 kb^{-1} $generation^{-1}$ (Ravikumar et al., 2018). This is 100.000 times the genomic mutation rate, which was not increased itself. It was found that OrthoRep maintains high mutagenesis rates for at least 90 generations, but in evolution experiments high mutagenesis rates were found for over 300 generations. This can be explained by the fact that the gene of the polymerase is located on the host genome, which is not subjected to the imposed mutagenesis. Another advantage of OrthoRep is that pGKL1 can encode for 22 kb of DNA, enabling the evolution of single genes up to multi-gene pathways. Using this strategy Ravikumar and coworkers, evolved dihydrofolate reductase (DHFR) to become resistant to the anti-malarial drug pyrimethamine. Mutational trajectories and fitness peaks in the landscape of DHFR-mediated drug resistance were found that had not been described before. This shows that OrthoRep can be used to obtain extensive knowledge on resistance development against antimicrobial drugs. Besides knowledge on resistance development, OrthoRep can provide a targeted approach to generate mutant libraries of single or multiple antimicrobial biosynthetic genes.

3.2 Ty1-mediated mutagenesis

Another study made an *in vivo* directed mutagenesis system for yeast using its native long terminal repeat retrotransposable element called Tyl (Crook at al., 2016). Certain studies have shown that heterologous gene expression in the Tyl transposon is possible (Curcio & Garfinkel, 1991; Boeke et al., 1988). Crook and coworkers used this knowledge to engineer a Tyl transposon with an inducible galactose promoter and a 'cargo' gene in an optimized *S.cerevisiae* BY4741 strain. When grown in a galactose containing media Tyl is transcribed and subsequently converted back to cDNA by a reverse transcriptase. This transcriptase is inherently mutagenic and introduces mutations with a frequency of 0.15 kb^{-1} transposon $replication^{-1}$ uniformly along the length of the transposon, thereby generating a cargo gene mutant library. The cDNA is then incorporated into the genomic DNA after which a new cycle of transposition and thus mutagenesis can occur. In this way large and diverse mutant libraries can be synthesized (1.6×10^7 distinct mutants/L). Transposition rates were maintained for cargo genes of up to 5 kb, indicating this method can be used for the direct evolution of large genes and even multi-gene pathways. They also measured transposition rates in other yeast strains (*S. cerevisiae* CEN.PK2 & *Kluyveromyces lactis*), which were only slightly lower than for the optimized *S.cerevisiae* BY4741 strain. This shows potential for the broad application in eukaryotes that have LTR retrotransposon activity.

Furthermore, the researchers showed that with Tyl-mediated mutagenesis beneficial mutants were found faster than with error-prone PCR mediated mutagenesis. The quality of mutants was also better for Tyl-mediated mutagenesis. They did three different evolution experiments to show this directed evolution method can successfully generate improved protein function. Using Tyl-mediated mutagenesis they successfully evolved a decarboxylase enzyme (Ura3p) to have increased substrate specificity. In another evolution experiment they isolated a beneficial mutant of transcription factor (Spt15p) that led to increased butanol tolerance and improved growth. For both Ura3p and Spt15p two iterative rounds of evolution were necessary, in between which the best performing mutants were cloned into a fresh strain to avoid strain adaptation. Lastly, they evolved the multi-enzyme pathway of xylose catabolism. After one week of evolution they isolated three different mutants that showed increased growth rates and shorter lag phases in xylose media.

It was thus shown that Ty1-mediated mutagenesis is suitable for the directed evolution of single genes and biosynthetic gene clusters in yeast. This directed evolution method could also be deployed to evolve antimicrobial compounds. For screening of beneficial mutations of Ura3p and Spt15p selection pressure was caused by increasing concentrations of a toxic compound. To avoid concurrent strain adaptation the mutant genes had to be cloned into fresh strains after a round of evolution. Screening for beneficial mutations of the xylose multi-enzyme pathway was done on agar plates containing xylose as the only carbon source and growth assays of promising colonies were done in 96-well plates. Screening for beneficial mutants of antimicrobial genes can be done on agar plates overlaid with a resistant or sensitive pathogen. Another way of finding improved mutants is by applying selection pressure on the producer strain by co-culturing the producer strain and a target pathogen strain under antagonistic pressure (Bull et al., 2015). This means the producer strain can only grow (or stay alive) if it kills the target strain. This method of selection will be further discussed in the section on experimental evolution. As the Tyl system was used to increase the host tolerance to butanol, the Tyl system could likely also be used to explore mutations in putative resistance

genes that make the host strain (more) resistant to a certain antibiotic. The concept of the prediction of resistance genomic profiles will also be further explored in a later section.

As mentioned earlier, concurrent strain adaptation can happen besides the generation of mutations. Improved growth could be due to the strain being better adapted to the imposing selection pressure, not caused by the occurrence of mutations (in the target loci) . This is the case for evolution experiments in which selection pressure is caused by a toxic or otherwise harmful component. Another point of concern is that multiple rounds of transposition could eventually lead to the incorporation of transposons in some regions of the genomic DNA that harm the fitness of the host. This would make it harder to select for colonies/cells that have beneficial mutations. Therefore the durability of the evolution experiments might be limited.

3.3 Retron-mediated mutagenesis

Another *in vivo* directed evolution method that was developed uses retrons to mutagenize a specific DNA segment (Simon et al., 2018). Retrons encode for a guide RNA (msr), a target (msd) and a reverse transcriptase. After transcription the mRNA of the reverse transcriptase is cleaved and translated. The guide and target RNA form hairpin structures and are linked together. The transcriptase reverts the target RNA back into DNA, after which the target RNA is hydrolysed and replaced by its DNA variant. A specific region on the target sequence is homologous to a region in the genomic DNA and can edit this region so it becomes identical to its own sequence. Simon and coworkers optimized this retron system in *E.coli* and let the retron be transcribed by an error-prone polymerase. The error-prone polymerase introduces mutations along the length of the retron. Furthermore, the retrons' reverse transcriptase that is inherently mutagenic provides a second mechanism for mutagenesis leading to a 190-fold increase in mutation frequency (0.001 kb^{-1} $generation^{-1}$) compared to background cellular mutation rates. Mutations in the target sequence subsequently lead to mutations in its homologous gene region (~30 bp) in the host genome. This is a small region that can be targeted compared to other *in vivo* mutagenesis methods. However, it is likely that combining retrons with different targeting sequences can target and mutagenize larger DNA loci by letting the targeting sequences partially overlap.

3.4 Experimental evolution of antibiotics

Although directed evolution is suitable for the modification of the direct function of specific proteins and peptides, it is difficult to evolve (small) non-peptide antimicrobials (Wollein Waldetoft et al., 2019). These metabolic compounds are often produced by complex interactions between different biosynthetic gene clusters. Mutations generally have to occur in multiple loci in order for an improved antimicrobial compound to be produced. Moreover, certain microorganisms have 'silent' or cryptic biosynthetic pathways that when turned on could produce new types of antibiotics. Genes and gene clusters that encode for these pathways are susceptible to evolution, due to the fact that their organization allows for higher frequencies of rearrangements and mutations (Wollein Waldetoft et al., 2019). Many natural antibiotics also consist of a stable core unit (scaffold) and side groups that have the potential to be modified. These things suggest that there is a huge exploration space for antimicrobials. The advantage of experimental evolution is its enormous exploration capacity and the fact that the potential antibiotic space can be explored in an unbiased manner.

Charusanti and coworkers showed that an adaptive evolution set-up can work to let a bacterial strain produce an antimicrobial compound that its wild-type strain does not produce. Streptomyces clavuligerus was put in a coculture with methicillin-resistant Staphylococcus aureus for serial passages on agar plates for four months. In this time frame a silent pathway in S.clavuligerus was turned on to produce holomycin that successfully inhibited the growth of S.aureus, providing empirical evidence that experimental evolution can work in a realistic time frame.

In the experimental evolution set-up Wollein Waldetoft and coworkers propose, a producer organism co-evolves under antagonistic pressure with a target organism that has resistance to the producers' antimicrobial (Wollein Waldetoft et al., 2019). This antagonistic relationship can be competition over a limited resource for example. The selection pressure must be such that in order for the producer strain to survive, it would need to evolve and adapt its killing mechanism. The producer organism should have a large potential for biosynthetic compound development. Actinobacteria that greatly contributed in the 'golden era' to the discovery of many antibiotics would be suitable producers. Marine gamma- and deltaproteobacteria could potentially also be used as producers, since they exhibit a rich spectrum of compounds with antimicrobial activity (Timmermans et al., 2017). For fungal producers, the genera

Trichoderma and Aspergillus show a lot of potential due to the fact that they have numerous cryptic antimicrobial biosynthetic gene clusters (Mukherjee et al., 2012). The target strain should be fluorescently labelled. Increased killing activity by the producer strain is thus signalled by a decrease in fluorescence. In order to avoid counter evolution of the target strain the producer strain is evolved in serial passages. To accomplish this the target strain can be engineered to have a higher heat sensitivity than the producer strain, which allows the target strain to be killed at a temperature at which the producer stays alive. After heat treatment and mixing of the culture an aliquot is used for the next cycle with 'fresh' target bacteria.

Genetic variety is a prerequisite for evolution and methods to increase genetic variation will speed up the evolutionary process. Mutagens or mutator strains could be used to enrich the genetic variation. The researchers describe that screening does not require handpicking colonies with (increased) killing activity. Rather, selection happens by the growth advantage that producers cells with advantageous mutations have over producer cells that lack these mutations. This growth advantage may be small, but over several passages could still be effective in selecting producer cells with beneficial mutations. This allows for larger through-put, compared to evolution experiments with traditional screening methods.

There are a few hurdles that experimental evolution might have. In order for the producer strain not to kill itself, it must be resistant to the antimicrobials it produces. It might be the case that the target organism deploys the same resistance mechanism as the producer organism. The evolution of compounds that circumvent this resistance may also kill the producer. Therefore, both mutations in the biosynthetic genes and in the resistance mechanism of the producer strain most occur. This could increase the time frame of developing a new antimicrobial. If this is the case, it would be favorable to select a producer strain with another resistance mechanism than the target strain. Another problem lies in the fact that secreted antimicrobials are public goods. A producer cell that has (a) favorable mutation(s) - which leads it to express a new or improved antimicrobial compound - might not have a fitness advantage, because other cells that don't have these mutations also benefit from the new compound that is excreted. It is therefore difficult to select for producer cells that exhibit advantageous evolutionary development. A solution to this problem would be to restrict the movement of cells and compounds by a structured medium. Because population growth occurs through division of cells, you would get spatially segregated clusters of cells that belong to the same 'mother' cell. Each cluster of cells would have nearly an identical genomic composition and thus produce the same compounds. The diffusion of compounds that are excreted into the environment is also restricted. This allows for the coupling of genotype and growth advantage and thus enables screening of improved mutants (Wollein Waldetoft et al., 2019).

Echinocandin resistance is mostly caused by mutations in the FKS genes encoding for (1-3)-β-glucan synthase (Cowen et al., 2014). Since echinocandin production is regulated by a complex biosynthetic gene cluster, experimental evolution might be the preferred method to evolve echinocandins. Evolution experiments could be done with an echinocandin resistant target strain and a echinocandin producing host strain. This could lead to altered echinocandins with renewed abilities to bind to mutated variants of (1-3)-β-glucan synthase.

Producer strains often have the ability to produce different antimicrobials. If the goal of the evolution experiment is to discover new antimicrobial compounds, antimicrobials that already have been discovered and are dominantly expressed by the producer can interfere with the screening for or purification of these new compounds. It might therefore be desirable to silence genes responsible for production of these 'known' antimicrobials. In a study actinomycete streptothricin and streptomycin knockout strains were made using the CRISPR-CAS9 system (Culp et al., 2019). Co-culturing of these knockout strains with *E.coli* tester strains showed several knock-out strains retained killing activity. Using bioactivity-guided purification on strains with the highest killing activity the researchers were able to identify multiple rare or unknown variants of antibiotics. These results indicate that this strategy can be used in experimental evolution to guide the evolutionary process towards new or rare antimicrobial compounds.

3.5 Evolution of Bacteriophages

Besides traditional antimicrobials, bacteriophages can be deployed to treat bacterial infections. Bacteriophages often have specific host species that they infect and subsequently kill, which makes them interesting for individual focused

treatment. They propagate at the site of infection and only in the presence of their target cells, which means low dosages and treatment frequencies are needed (Heselpoth, 2018; São-José, 2018).

Besides these therapeutic benefits, phages have a high reproduction rate, which allows for relatively fast evolution of potential therapeutic phages. Following a similar approach as the experimental evolution of antimicrobial producing strains, phages can be put in co-culture with a pathogen of interest. Cebriá-Mendoza and coworkers showed that after a 20 serial passage evolution experiment of *Mycobacterium smegmatis bacteriophage* in co-culture with its host *M.smegmatis*, the phage's infectivity was increased significantly compared to the founder phage (Cebriá-Mendoza et al., 2019). In this study, the research did not mutagenize the phage genomes prior to the evolution experiment. This could be donel, either with mutagenic compounds, Uv-radiation or by error-prone PCR. This could speed up the evolutionary process.

The fast evolution of bacteriophages enables effective and adaptive treatment of bacterial infections. Moreover, there is evidence to believe that some bacteriophages can also be used to treat fungal infections (Górski et al., 2019). Apart from bacteriophages, there are also phages that infect fungi, so called mycophages (Tiwari et al., 2014). Evolution experiments could be performed on these phages in order to improve their fungal infectivity and therapeutic properties.

Other techniques

Other methods for *in vivo* directed evolution worth mentioning include a variety of CRISPR-Cas9 mediated techniques. Advantage of these methods is that they can easily be adapted across different target strains and can introduce mutations genome-wide with a high specificity. Some methods require double strand breaks which is a downside due to its toxicity. Another limitation is the fact that only relatively small segments can be mutated (<300 bp), though by using multiple guide RNAs this can technically be overcome (Wang et al., 2019). Another *in vivo* directed evolution technique developed for *E.coli* called eMutaT7, uses a cytidine deaminase fused to an orthogonal RNA polymerase (T7 RNA polymerase). Genes of interest can specifically be targeted by upstream T7-promoters. Strong points of eMutaT7 are the relatively high mutagenesis rate of ~0.094 mutations kb^{-1}, the fact that multiple genes under control of a T7 promoter can be mutagenized and the controllable mutation rate via arabinose concentration. A limitation of this method is the narrow mutational spectrum of the deaminase, which only introduces C→T and G→A mutations. However the researchers propose that other DNA-modifying enzymes can be linked to T7 RNA polymerase which would increase mutation types (Park & Kim, 2021).

Throughput limitation

Directed evolution using *in vivo* mutagenesis is less laborious for the mutagenesis part, but for the screening part often intervention of the researcher is still needed. The selection or screening methods often determine the throughput quantity (Packer & Liu., 2015). Screening methods screen for certain phenotypes that are linked to favorable genotypes. For antimicrobials the screened phenotype is the extent of killing activity of expression strains. High-throughput screening methods mostly happen with liquid cultures. This is difficult to perform for antimicrobials, due to the problem of public goods as explained earlier. This limits the way in which screening assays can be done, because cells need to be spatially separated in order to avoid the public goods problem. Mutant libraries in expression hosts are spatially separated either on agar plates or in liquid wells and screened/selected for (increased) killing of the target pathogen. This has a limited throughput capacity (10^2-10^4) (Packer & Liu., 2015). Ongoing developments of automatization of these screening processes help widen this bottleneck. Directed *in vivo* mutagenesis methods could also be combined with the selection method described in the experimental evolution section. This would increase the throughput capacity substantially. A third way to overcome this hurdle is by a directed evolution set-up that is fully continuous.

4. Continuous directed evolution *in vivo*

4.1 Phage-assisted continuous evolution

A *in vivo* directed evolution technique that is fully continuous is phage-assisted continuous evolution (PACE) (Esvelt et al., 2011). PACE links the 'improvement' of a certain protein to the fitness of an infectious bacteriophage that contains the gene of the protein to be improved. PACE has been used to evolve polymerases, proteases and genome-editing proteins. PACE can also be used to evolve and improve protein-protein binding interactions (Morisson et al., 2020). The evolution experiments take place in a vessel with a fixed volume, also called 'lagoon'. In the lagoon *E.coli* cells (host cells) and bacteriophages (M13) are present. There is a constant drainage of the culture media and a constant supply of new culture media containing only *E.coli* cells. The reproduction rate of the *E.coli* cells is slower than the dilution rate, so that there are constantly new cells. The reproduction rate of the phages is faster than the dilution rate. Since there is no supply of new phages, the phages have to adequately propagate in order to keep being present in the lagoon. In order for the phages (M13) to propagate they need to infect the *E.coli* cells for which they need protein III (pIII). However the phage vector lacks the gene (gIII) that encodes for this protein. This gIII is inserted into another vector also 'accessory plasmid' and is present in the *E.coli* host cells (Esvelt et al., 2011).

The phages do contain a gene for the protein to be evolved (i.e. protein. X) whose activity is linked to transcription of the pIII gene. In a study a variant of PACE to evolve protein-protein binding interaction was optimized (Badran et al., 2016). This strategy is facilitated by the two-hybrid-system. In this system a RNA polymerase is coupled to protein X. In order to bind to the target promoter - in this case the promoter of gIII - and start transcription, protein X needs to bind to a 'bait' protein. This 'bait' protein is covalently linked to a DNA binding protein that binds a DNA region upstream of the target promoter. The binding of protein X to this 'bait protein is the subject of directed evolution in PACE. At the beginning of the PACE experiment there is low or no binding affinity between the two proteins. Certain mutations need to be introduced in the gene of protein X, in order for protein X to be able to bind to the bait protein. In addition to the accessory plasmid, the *E.coli cells* have a plasmid with genes encoding for mutagenesis proteins, such as error-prone polymerase subunits, with an inducible promoter. Due to the fact that host cells are continuously replaced, mutations will only accumulate in the phage DNA vectors (including gene X). Once mutations have been introduced in gene X which lead to better binding affinity between protein X and the bait protein, transcription of the pIII gene can be induced. Phages with these favorable mutations will be in possession of pIII and can propagate at a much faster rate than phages who don't have these mutations. This leads to the wash-out of phages with no favorable mutations, while phages with favorable mutations stay longer present in the lagoon. In a relatively short time frame this can lead to the evolution of a desired protein X mutant. To avoid instant wash-out of all the phages in the beginning the procedure can be designed to allow phage propagation without selection pressure in the first few hours. This allows for evolutionary drift. When subsequently selective pressure is put on the phages there is a higher chance that initially not all the phages are washed-out.

An example of the successful continuous evolution of PACE is the modification of *Bacillus thuringiensis* endotoxin (Bt toxin) (Badran et al., 2016). Bt toxin is an insecticidal protein that binds to receptors on the insect midgut cells. This interaction leads to pore formation in the cell membrane and eventually cell death. Crops have been engineered to produce this toxin to protect them from insect gluttony. However, resistance against this Bt has been developed in certain insect species. Resistance against Bt toxin occurs when receptors that bind to the specific Bt toxin are downregulated or modified such that they no longer bind to the toxin. One of such insects that shows resistance is the cabbage looper *Trichoplusia ni* (*T.ni*). Badran and coworkers showed *T.ni* that was resistant to bt toxin Cry1Ac could be made sensitive to the toxin again by PACE continuous evolution of Cry1Ac to bind to a cadherin-like receptor TnCAD. The PACE experiments resulted in a modified Cry1Ac protein that could bind to the TnCAD receptor and induce pore formation. The evolved Cry1Ac protein also showed killing activity on species closely related to *T.ni*.
This variant of PACE might be especially useful for the evolution of killer toxins. Killer toxins are small glycosylated proteins produced by ascomycete yeast that kill sensitive fungal and bacterial cells in a two-step mechanism. First, they bind to a receptor on the cell wall. Once internalized they can kill the cell by the inhibition of cell wall synthesis, inhibiting of DNA synthesis, arresting of the cell cycle or pore formation (Selitrennikoff, 2001). Just as resistance occurred for Bt toxin by downregulation of the receptor it binds to, resistance could occur for killer toxins. PACE could be used to evolve the binding domain of the killer toxins to bind to other (similar) receptors and in this way circumvent the resistance mechanism.

The advantage of PACE is that it is not laborious and time consuming. During the PACE procedure no intervention of the scientist is needed in the evolution process. Another advantage is that PACE enables a high mutation frequency, 2.3 kb^{-1} viral $generation^{-1}$ (Morisson et al., 2020). This leads to relatively short time frames in which successful evolution results can be achieved: one day to several weeks. Furthermore, because mutations only accumulate in the phage vector the persistence of host cells is not a problem as it is in other *in vivo* directed evolution. Lastly, different PACE experiments can also be done parallel to each other, evolving different proteins at the same time.

A downside of the PACE system is that it allows only for the directed evolution of a single protein. Of course many PACE experiments, each evolving a different protein, can be done parallel, but that still selects for improved mutants of single protein antimicrobial killing systems. A second disadvantage is the fact that PACE evolution experiments are limited to the *E.coli* cytoplasm. Furthermore, effort prior to the evolution experiment must be made in order to link pIII transcription to the evolution of the protein of interest. Lastly, PACE requires specialized equipment such as a chemostat or turbidostat, which may not be available in the average laboratory.

5. Resensitization of drug resistant Pathogens

5.1 Peptide-conjugated phosphorodiamidate morpholino oligomers

There is not only effort made to develop improved or new antimicrobials, but also to resensitise drug resistant pathogens to currently used antibiotics. One such effort makes use of Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs). PPMOs are synthetic analogs of RNA (or DNA) and can be designed to be complementary to specific mRNA sequences. When they bind a mRNA molecule they inhibit its translation and thus silence the gene from which it was transcribed. This can also be accomplished with small RNA molecules (miRNAs). An advantage of PPMOs over synthesized short RNA molecules, is that the latter can be broken down by RNAse hydrolases while the former can't be broken down by these enzymes. PPMOs can therefore stay present in cells for a longer period. The delivery of a PPMO is facilitated by a membrane penetrating peptide that is conjugated to it. They can be delivered both in prokaryotic and eukaryotic cells, enabling a wide range of pathogens that can be targeted. PPMOs can be used in two different ways. First they can be designed to bind to mRNAs of essential gene(s) of the pathogen. Silencing of this/these gene(s) lead(s) to the death of these pathogens. Often PPMOs are designed to silence certain resistance genes of microbial pathogens, which leads to increased antibiotic sensitivity. The PPMO itself doesn't kill the pathogen, but the antibiotic does (Cansizoglu and Toprak, 2017). In a study a PPMO was designed to silence the ArcA gene encoding for a protein domain of the ArcAB-TOL efflux pump. The PPMO reduced the minimum growth inhibition concentration (MIC) of multiple antibiotics by a factor of 50. *E. coli, Klebsiella pneumoniae* and *Salmonella enterica* could be killed by using antibiotic concentrations that are normally below the MIC concentrations (Cansizoglu and Toprak, 2017). In another study PPMOs were successfully deployed to inhibit growth of the multi-drug resistant *P. aeruginosa in vitro*. Furthermore, in mice models the burden of *P. aeruginosa* lung infections was reduced heavily (Moustafa et al., 2021).
Fungal genes encoding for ABC and MFS efflux systems could be a target for PPMOs. Azoles inhibit lanosterol 14a-demethylase (Erg11) an enzyme in the ergosterol biosynthetic pathway. Increased azole resistance can occur through the overexpression of Erg11 increasing target abundance (Cowen et al., 2014). A PPMO could be designed to suppress the translation of Erg11 and so restore azole's effectiveness. PPMOs that target genes encoding for enzymes necessary for the build up of the fungal cell wall and membrane in itself might kill fungal cells without additional antimicrobial drug treatment.

An advantage of PPMOs is that the nucleotide sequences can easily be redesigned, which allows for co-evolution of PPMOs with mutations in resistance genes. PPMO can also be designed in such a way that it specifically targets the cells of certain pathogens. This would avoid unwanted off-target killing of host cells and commensal bacteria (Cansizoglu and Toprak, 2017). Furthermore, PPMOs could also be developed for a drug prior to the occurence of resistance. This can be done by predicting mutation profiles in putative resistance genes leading to resistance against the drug. In the next chapter a resistance prediction method will be discussed.

Resistance against PPMOs themselves can develop when the cell membrane of a pathogen cell becomes less permeable for the PPMO. Cansizoglu and Toprak observed that changing the peptide conjugated to the PMO did reverse this effect. PPMOs doses need to be of high concentration in order to silence their target gene(s) (Cansizoglu and Toprak, 2017) .

5.2 Bacteriophage causing genetic trade-off

Another way to resensitize drug resistant pathogens is by targeting the mechanism of resistance directly and causing a genetic trade-off. Chan and coworkers identified a bacteriophage OMKO1, belonging to the virus family *Myoviridae*, of which it was found that it can infect *Pseudomonas aeruginosa* by binding to a surface exposed channel of its multi-drug efflux (Mex) system (Chan et al., 2016). *P. aeruginosa* is a multidrug resistant opportunistic pathogen caused for a large part by its Mex system. The researchers observed that *P. aeruginosa* evolving resistance to OMKO1, led to the increased sensitivity to certain antibiotics. The greater the resistance to an antibiotic depended on the efflux system the greater the effect was. These results were found for clinical strains as well as environmental strains of *P. aeruginosa*. Application of the two different approaches would eventually lead to ineffective treatment, but combined seems a promising long term treatment strategy. This strategy could be used to treat infections caused by MDR pathogens and to slow the process of resistance occurring in other pathogens. Directed evolution experiments could be done on bacteriophages or mycophages to engineer phages that selectively bind to efflux proteins of bacterial or fungal pathogens. This way this strategy could be applied to a wide range of pathogens and in combination with antimicrobials of which is known that resistance is efflux mediated such as for the azole class (Cannon et al., 2009). An advantage over chemical efflux pump inhibitors is that phages can be evolved to circumvent the incidence of resistance through site alteration of the binding-site.

Niimi and coworkers engineered *S.cerevisiae* to hyper-express heterologous efflux pump proteins (Niimi et al., 2004). Efflux pump genes are encoded on a plasmid pABC3 with a highly active promoter. This transcription cassette integrates into the host genome via homologous recombination. They further knocked out the genes encoding for native efflux pump proteins to suppress endogenous efflux activity. Using this system they successfully cloned and expressed efflux pumps of several pathogenic fungi. This system would provide an efficient and controllable platform for the directed evolution experiments of efflux pump binding phages.

6. Understanding and predicting resistance development

New antimicrobials need to be developed and are being developed. In order to develop antimicrobials that are efficient for a long time it is also necessary to consider the evolution of resistance against these compounds. For some drugs a single point mutation may lead to increased resistance levels in pathogens, while other antimicrobials require several mutations (in different genes) in pathogens in order for it to be less effective. It will thus be easier to develop resistance to the former group of antimicrobials, than for the latter group. However, predicting resistance is not an easy task. First off, there can be a lot of mechanisms working together to generate resistance. Second, resistance to the same antibiotic works differently in different pathogens. Lastly, there is a large pool of potential antibiotic agents to be tested (Nyerges et al., 2018).

6.1 Directed evolution with random genomic mutations

A method that was recently developed to predict the chance of resistance occurring is called directed evolution with random genomic mutations (DIvERGE) (Nyerges et al., 2018). In DIvERGE random mutations are introduced into a DNA segment of interest by using an oligonucleotide pool with overlapping oligonucleotides that are homologous to the DNA loci. The oligos are made using a soft randomization protocol, which depending on the spiking ratio has a certain preference (%) to the WT sequence (Hermes et al., 1989). Tuning the spiking ratio up or down respectively introduces more or less random mutations. Increasing the mutation rate too much decreases the similarity of the oligonucleotides with the WT sequences and thus will lead to less effective incorporation into the genomic DNA of the host cell. A spiking ratio of 2% was found to be optimal by Nyerges and coworkers. After the oligo pool is synthesized the oligonucleotides are delivered into competent cells. Because of the high similarity of the oligonucleotides with the DNA segment, the oligonucleotides are incorporated into the genomic DNA. This introduces mutations in the DNA loci. In different host cells different mutations are incorporated, which leads to genetic diversity. To incorporate the

oligos they used an optimized version of multiplex automated genome engineering (MAGE). In MAGE single stranded oligonucleotides can be incorporated into the bacterial genome (Wang et al., 2009). However, a lot of modifications in the genome have to be made beforehand and off-target mutations occur frequently because the methyl mismatch repair system needs to be inactivated. The researchers improved this recombineering method to address these issues and called it pORTMAGE. pORTMAGE allowed for efficient recombineering without off-target mutations and the requirement of prior modification. This method can also be used over a wide range of bacterial species.

A variant of MAGE was also developed (eMAGE) to allow for multiplex automated genome engineering in *S.cerevisiae* (Barbieri et al., 2017). Previously other genomic engineering methods in eukaryotes would require double strand breaks (DBS). This has a couple of downsides if the objective is to perform multisite editing or to have single base pair mutation resolution. First, DBS are cytotoxic and introducing DBS at multiple sites will lead to the death of many targeted cells. When single base pair modifications are introduced into eukaryotic genomes by DBS, there is a high chance that additional insertions or deletions will occur. This also limits the possibility of modifying multiple target loci in the genome. Barbieri and coworkers modified the MAGE system by incorporating synthetic single-stranded DNA oligodeoxynucleo-tides (ssODNs) complementary to the lagging strand at the replication fork. This circumvents the issue of DBS and allows for multiplex recombineering and single base pair mutation resolution. Due to the fact that this system targets conserved mechanisms across eukaryotes, it can also be relatively easily adapted to work in a wide range of eukaryotes.

One characteristic of DIvERGE is the uniformity of the frequency of mutation positions, due to the soft randomization protocol and an overlapping oligo pool design. The DIvERGE cycle can be done again, which enables multiple rounds of mutagenesis and selection. This will increase the coverage of possible mutations. Different oligonucleotides that partially overlap each other can span large DNA regions. It was found that they can successfully introduce mutagenesis in large DNA loci, enabling the evolution of multi-enzyme pathways. Moreover, every region in the host genome can be targeted, which enables genome-wide evolution.
Another advantage is that using a soft randomization protocol to create mutation libraries is cost effective, since there is no need for pre-designed oligonucleotide pool libraries. Moreover, the researchers showed that DIvERGE experiments can achieve positive results in one day, which is time efficient compared to other methods. Lastly, DIvERGE is applicable to a variety of host species without the need for prior genomic modification allowing the studying of resistance evolution in different pathogens.

DIvERGE also has some limitations. It requires knowledge about the gene(s) that are responsible for the occurrence of resistance to a specific drug. When this knowledge is not available it should be obtained first if DIvERGE wants to be performed. As DIvERGE focusses on genomic loci, it cannot detect resistance gene(s) on plasmids. Since horizontal gene transfer is an important source of resistance genes on plasmids spreading in bacterial populations, doing DIvERGE doesn't cover the complete area of evolving resistance.

Nyerges and coworkers used DIvERGE in bacteria to unravel resistance genotypes against the antibiotics trimethoprim and ciprofloxacin (Nyerges et al., 2018). Numerous mutations causing resistance were not known previously. The performed DIvERGE to evolve resistant pathogens to the antibiotic gepotidacin that is in clinical phase 2 trials. Unlike other studies that reported no emergence of resistance to gepotidacin, Nyerges and coworkers observed a 557-fold increase in resistance to gepotidacin in *E.coli* mutants after a single round of DIvERGE. This shows the potential of DIvERGE to unveil resistance genomic profiles in pathogens and to identify resistance proof antibiotics in the early stage of development.

DIvERGE could also be used in bacterial and fungal strains to evolve antimicrobials. Instead of targeting putative antimicrobial resistance genes in pathogenic bacteria, (parts of) antimicrobial biosynthetic gene clusters or genes encoding for antimicrobial protein (complexes) can be targeted in producer model organisms.

Concluding remarks

In this report numerous ways how directed evolution has and can be exploited to evolve antimicrobials have been discussed. As of yet, most directed evolution experiments geared towards this end, use *in vitro* mutagenesis techniques such as error-prone PCR or site-saturation mutagenesis. These methods are well established and relatively easy to perform, hence their widespread application. *In vitro* mutagenesis techniques can mostly be used to perform evolution experiments at the protein level. Antimicrobials that have been successfully evolved include N-acyl-homoserine lactonase and the endolysins PlyGBS and PlyC. When a lot is known about a protein's structure and function, focused techniques such as saturation mutagenesis can be used to evolve these proteins for complex synthesis functions. One such protein is the enzyme cytochrome P450, which was successfully evolved to synthesize a variety of lactams including β-lactam.

However, *in vitro* mutagenesis has its disadvantages. It is labor and time intensive, in some cases biased towards certain mutations and only realistically applicable to the protein level. *In vivo* mutagenesis techniques have been developed that solve these issues. They eliminate some of the cloning steps needed in *in vitro* mutagenesis and are suitable for a more continuous evolution set-up. Furthermore, depending on the method *in vivo* directed evolution experiments can be done at different levels: single proteins, multi-enzyme pathways and genome wide. Difficulties such as low mutagenesis rates and target specificity have been improved substantially over the last decade. A potential limitation of *in vivo* directed evolution methods is that they are often restricted to a certain expression strain. Methods like PACE and retron-mediated mutagenesis are designed for bacterial systems. In the case of retron-mediated mutagenesis experiments are also restricted to the evolution of bacterial proteins. Others can be adapted to also work in fungal models such as DIvERGE. Yet others are designed specifically for fungal hosts such as OrthoRep and Ty1-mediated mutagenesis. To the best of my knowledge no studies have been reported about *in vivo* directed evolution used to evolve antimicrobials with improved or altered function apart from the successful evolution of bt-toxin Cry1Ac. The realization of this large potential thus has yet to be realized.

A large challenge for the efficient evolution of antimicrobials is high-throughput screening. The complex phenotype to genotype linkage and the fact that antimicrobials are public goods makes high-throughput screening of mutant expression hosts difficult. In PACE this issue is solved, but this system also has its own limitations. The screening method of the experimental evolution set-up based on continuous selection pressure in serial passages and spatial separation in a semi-liquid media could be used in conjunction with *in vivo* mutagenesis techniques to increase throughput. Experimental evolution can also be suitable to evolve non-peptide (small) antimicrobials.

Directed evolution methods have also been used to unveil genomic resistance profiles of pathogens against certain antibiotics of which DIvERGE looks the most promising. This is important for the understanding of the evolution of resistance and to predict the chance resistance will occur to antimicrobials that are still in development. Knowledge thereon could be used to introduce antimicrobials in the market with lower chances of resistance occurring. Lastly directed evolution can be used to resensitize resistant pathogens for traditional antimicrobials and to suppress the occurrence of resistance to current and future antimicrobials.

To conclude, this review has shown that the concept directed evolution can be exploited in numerous and different ways to combat antimicrobial resistance. A lot of these strategies can also be applied to combat antifungal resistance. Now these methods need to be put into practice to actualize their potential.

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