Improving on Nature's Defenses: Optimization & High Throughput Screening of Antimicrobial Peptides

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Abstract: Antimicrobial peptides (AMPs) are ubiquitous in nature where they play important roles in host defense and microbial control. Despite their natural origin, antimicrobial spectrum and potency, the lead peptide candidates that so far have entered pharmaceutical development have all been further optimized by rational or semi-rational approaches.

In recent years, several high throughput screening (HTS) systems have been developed to specifically address optimization of AMPs. These include a range of computational *in silico* systems and cell-based *in vivo* systems.

The *in silico*-based screening systems comprise several computational methods such as Quantitative Structure/Activity Relationships (QSAR) as well as simulation methods mimicking peptide/membrane interactions. The *in vivo*-based systems can be divided in *cis*-acting and *trans*-acting screening systems. The *cis*-acting pre-screens, where the AMP exerts its antimicrobial effect on the producing cell, allow screening of millions or even billions of lead candidates for their basic antimicrobial or membrane-perturbating activity. The *trans*-acting screens, where the AMP is secreted or actively liberated from the producing cell and interacts with cells different from the producing cell, allow for screening under more complex and application-relevant conditions.

This review describes the application of HTS systems employed for AMPs and lists advantages as well as limitations of these systems.

Keywords: Antimicrobial peptide (AMP), high throughput screening (HTS), suicide expression system (SES), *trans*-acting peptide system (TAPS), Novispirin, Plectasin, quantitative structure-activity relationship (QSAR).

INTRODUCTION

Antimicrobial Peptides (AMPs) or Host Defense peptides (HDPs) are a recently discovered group of antimicrobial agents with new modes of action [1,2]. They are widely distributed in animals, plants and microbes and are among the most ancient host defense factors. In microbes, they eliminate competing microorganisms occupying the same ecological niches. In higher organisms, they constitute part of the innate immune system aiding the regulation of endogenous microbial flora and the response against invading and pathogenic microorganisms.

In general, the peptides range in size from 6 amino acids up to around 60. To date, more than 500 different AMPs have been isolated which can be divided into several classes based on bioactivity, structural features and/or amino acid (aa) composition [3]. The simplest structures are small elongated α-helical peptides. Other AMPs fold into looped structures, β-sheets or form compact and rigid, disulfide-bridged tertiary structures.

Most AMPs are cationic and amphipathic - features that promote interaction with the negatively charged bacterial and fungal membrane. They work primarily by compromising the membrane of the target organism. When analyzed at the molecular level, several different mechanisms of membrane disruption have been shown to exist. However, for most AMPs, the overall outcome is membrane disruption and/or cell lysis [4-6]. In addition, several amphipathic AMPs have been shown or hypothesized to interact with other cellular components in addition to the membrane [7-9]

The selectivity towards microbial membranes is mediated by membrane composition, membrane charge, trans-membrane potential and lipid polarity [10]. The outer layer of microbial membranes is populated with negatively charged phospholipids, whereas the outer layer of plant and animal membranes primarily is composed of neutral lipids. In addition to the differences in polarity, the specific types and ratios of phospholipids are different between microbes and those of higher organisms, allowing for discrimination between cell types.

It has proven extremely difficult to spontaneously induce resistance to AMPs in sensitive target organisms. This is a reflection of the unique nature of the molecular target. However, a few bacterial pathogens have evolved countermeasures to reduce the effectiveness of AMPs through a reduction of the negative charge of the bacterial membrane or cell wall [11-15], changes in membrane fluidity, active efflux of the AMPs [16,17], production of proteases to inactivate the AMPs [18-21] or downregulation of AMP expression [22]. Many of these reported 'resistance genes' are genetic loci, that when deleted or mutated lead to an *increase* in sensitivity [10].

A number of other microorganisms, e.g. *Burkholderia*, *Serratia*, *Providencia* and *Proteus* are inherently insensitive to AMPs due to specific compositions of their membrane and cell wall [23,24].

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AMPs from multicellular organisms often encompass other biological activities in addition to their antimicrobial properties. Some AMPs, *e.g.* LL37 and α-defensins have been shown to attract specific subsets of peripheral T cells, monocytes and neutrophils [25]. LL37 also possesses angiogenic properties [26]. Other peptides, such as PR-39, which was isolated from pig intestine, have been shown to inhibit neutrophilic NADPH oxidase activity [27], to modulate neutrophil and leukocyte recruitment [28], and to increase syndecan expression [29], thereby potentially promoting wound repair.

From a therapeutic standpoint the AMPs have several properties that make them attractive biopharmaceutical candidates:

- Microbicidal activity
- Rapid onset of action killing most bacteria in minutes
- Low mammalian cell toxicity
- Few observed resistance mechanisms
- Potential for sequestering lipopolysaccharide (LPS) and/or lipothechoic acid (LTA)
- Synergistic effect with other classes of antibiotics
- Stimulation of the immune system.

A few AMPs, Bacitracin, polymyxins and gramicidins have been used for decades as efficient topical antibiotics, and the lantibiotic-type AMP Nisin, has been used extensively in food preservation. However, the commercial exploitation of *gene-encoded* AMPs has been slow so far. There are several reasons for this including lack of oral availability and limited systemic potential for highly charged peptides, high production cost and hesitance to consider lead research candidates that act on cell membranes as opposed to a strictly defined molecular target. Nevertheless, the emergence of multi-resistant pathogenic bacteria and the corresponding demand for antibiotics with novel modes of actions, combined with an improved understanding of the universal nature of AMPs, have renewed interest in this unique group of antimicrobial agents. At this point, more than 14 companies predominantly located in North America and Europe have or have had an active clinical development program for AMPs [30].

Key to the early stages of any pharmaceutical discovery and development program is the identification and optimization of lead candidates. This process involves lead class identification followed by lead compound optimization. The path taken in this phase depends on the specific technology platform available. An essential feature for lead identification and optimization is that AMPs are gene-encoded; this has allowed many of the molecular and biotechnology tools originally developed for other proteins and polypeptides to be utilized for AMPs and to complement a chemical approach.

Due to the multitude of structurally different AMPs a number of complementary methodologies are available for HTS of peptide libraries, each optimally suited for a given class of molecules or a specific step in the pathway of lead optimization. For example, the chemical or genetic systems employed for optimizing small 18-aa linear broad-spectrum AMPs are different from the ones optimizing 40-residue peptides with multiple disulfide bonds and selective antimicrobial activity.

The AMPs found in nature have been evolutionarily optimized to function in specific physiological or ecological niches, *e.g.* tears, saliva, mucosal barrier or the phagosomes of macrophages, in response to specific microbial challenges. However, AMPs have not been optimized by evolution to optimally function in complex therapeutic conditions such as burn wounds, chronic ulcers or cystic fibrosis. To overcome these limitations, a number of proprietary technologies for generating and screening molecular diversity through HTS have been developed. These screening systems have been designed to avoid or, in some special cases, directly take advantage of the obvious dilemma of expressing potent and broadly active antimicrobials in microorganisms.

This review has been limited to comprise only HTS technologies applied to AMPs, *e.g.* peptides that primarily target the microbial cell wall and membrane. A number of other related technologies have been developed to identify peptides that target intracellular proteins or other essential cellular structures, but these will not be described in further detail. Also, this review will not address the rapidly expanding field of synthetic chemical peptide libraries as this will be covered elsewhere in this issue.

The HTS technologies included here have been divided into three areas:

- *in silico* (virtual screening and computational methods)
- *in vivo* (cell based)
- *in vitro* (test tube)

The *in silico* tools take advantage of biophysical characteristics as well as computational tools and algorithms, while the *in vitro* systems described couple transcription and translation. The cell-based, *in vivo* systems include *cis*-acting screening systems where the antimicrobial effect is directed towards the producing cell, as well as *trans*-acting screening systems where the AMP is interacting with target cells different from the producing cells.

1. *IN SILICO* / VIRTUAL SCREENING

When employing natural screening systems, one is confined to combinations of the 20 naturally occurring amino acids. This means that randomly mutating one position gives rise to 20 different molecular combinations to screen; randomly mutating two positions theoretically gives rise to 400 different combinations to screen, and so forth. A 20-aa peptide theoretically would then give rise to 20^{20}, or $\sim 10^{26}$ possible combinations, while a random library of a 40-aa peptide theoretically contains 20^{40} or $\sim 10^{52}$ combinations. Given only 10^9 - 10^{12} combinations can be screened in cell based systems and in theory 10^{15} in certain cell-free systems, there is obviously a need for methods that reduce the sequence space to be screened. This reduction in sequence space can come about in many, often complementary ways as described below.

To guide lead optimization high-resolution X-ray crystallography or nuclear magnetic resonance (NMR) structures are required. Knowledge of the spatial location of specific residues as well as that of the conformation and direction of the side chains is used in connection with computer-aided design tools to further facilitate rational/semi-rational optimization of the lead backbones.

A desirable goal is to develop a computational or *in silico* system capable of automatically evaluating very large compound libraries, thereby limiting the vast sequence space to be screened by more traditional methods. Virtual Screening (VS) encompasses a variety of computational techniques that allow researchers to reduce a huge virtual library to a more manageable size and by performing screens *in silico*, faster and less expensively than traditional HTS methods [31].

There are many ways in which VS can be carried out, based on similarity and substructure searching methods [32], pharmacophore matching or 3D-shape matching [33,34] and docking methods when the structure of the molecular target is known [35]. These computational methods may provide the key to limit the number of compounds to be evaluated by experimental HTS.

VS is currently the most widely used method for compound selection in the pharmaceutical industry and there are several excellent reviews about the applications of VS in traditional drug discovery [36,37].

In the AMP field VS has only been applied to the simplest AMPs, the amphipathic α-helices. Most of the described VS studies adopt a three-stage approach to the design of new AMPs. The first step is to generate a number of physicochemical variables (molecular descriptors) consistent with and relevant to the structural information and the specific objectives of the study. The second step is to identify a relationship between these biophysical parameters and the biological data and then to construct a predictive model. The third step is to run this model through a library of AMPs displaying a broad range of biological activity. Finally, the AMPs that the model predicts to be most potent are produced and evaluated experimentally (Fig. **1**).

The sequence and the structure of AMPs can be very diverse; nevertheless almost all α-helical AMPs have a fundamental biophysical principle. The peptide structure often is amphipathic with the polar amino acids aligned along one side and the hydrophobic residues along the opposite side of the helical peptide [38]. The positive charged amino acid side-chains promotes attraction and binding of peptide to the negatively charged lipid bilayers while the hydrophobic amino acids favor insertion and permeability of the microbial membrane [2].

Based on the amphipathicity of AMPs, methodologies have been developed to represent the spatial segregation of hydrophilic and hydrophobic amino acid, like the helical wheel projection [39]. Measure of the overall amphipathicity of helical sequences based on hydrophobic index scales have been proposed [40-42]. A measure of membrane-interaction potential which considers three-dimensional geometries has also been proposed. The method, called the depth-weighted inserted hydrophobicity (DWIH), was based on the sequestration of hydrophobic residues within a hydrophobic compartment, such as that produced by a membrane bilayer [43]. Recently a new consensus hydrophobicity scale has been proposed [44] based on the widest possible set of literature-based scales, normalized and filtered by different methods, and extrapolated to non-proteinogenic amino acids by correlating experimental descriptors of hydrophobicity with their new scale.

Many structure/activity relationship (SAR) studies have been performed on AMPs and structural variations of α-helical AMPs generally lead to simultaneous variations in the physicochemical properties that define them. This complicates attempts to determine their individual contribution to antimicrobial potency and specificity. Nevertheless a consensus seems to indicate that length, sequence, charge, overall hydrophobicity, amphipathicity and the respective widths of the hydrophobic and

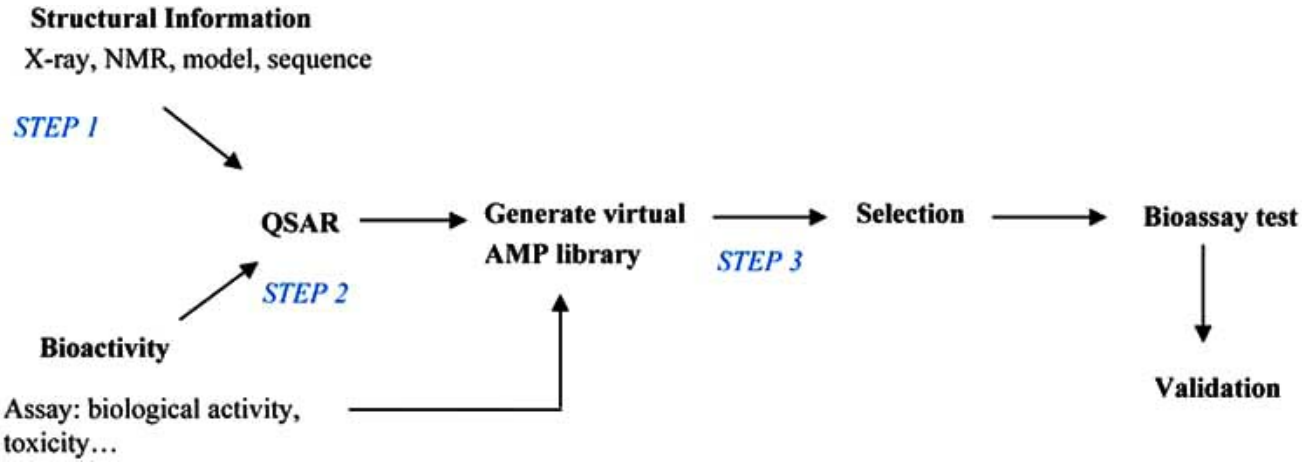

Fig. (1). Virtual Screening approach for designing new AMPs.

Step 1 is to generate a number of molecular descriptors related to the structure. Step 2 is to build a quantitative predictive model (QSAR). Step 3 is to test the predictive model using a number of *in silico* selected peptides.

hydrophilic faces of the helix, can influence the potency and spectrum of activity of α-helical AMPs. These parameters are intimately related, and modifications aimed at altering one can result in significant changes to one or more of the others [1,45]. Based on these considerations, a comparative sequence analysis of over 150 linear AMPs, ranging from 16 to 40 residues in length, has revealed common motifs and features, and significant positional conservation in terms of residue types. A template has been derived and used with success to propose potent new AMPs, reducing the sequence and design complexity [45]. Similar consensus principles were proposed for the application to more complex structures, *e.g.* β-defensins, where alignment of a number of structurally similar molecules can help to define the sequence space preserving structural integrity and bioactivity [46].

QSAR and Selection Algorithms

Another well-known strategy for VS is based on quantitative structure-activity relationship (QSAR) methods. QSAR analyses are routinely used in agrochemistry, ecotoxicology and environmental microbiology and are considered state-of-the-art methods for prediction of new substrates with a required activity in drug design [47]. It is of relevance to understand how modifications in peptide sequences may lead to better performing drugs and such understanding is possible through mathematical models such as QSAR, expressing how alterations in peptide sequence relate to changes in biological performance.

To establish a QSAR model, the sequence or preferably the molecular structure of the peptide must be defined, described by diverse physicochemical parameters and related to the biological activity. Basically, the structural and chemical variation in a series of compounds can be translated into a set of physicochemical variables, also called descriptors. The diversity of these descriptors is large and is reported in the literature [48]. They can be related to experimental data such as molecular weight, partition coefficient, HPLC retention times, but also to 2D or 3D structure when the structure of a compound is known. Topological, geometrical, electrostatic, partial surface area, thermodynamic and physichochemical surface properties such as molecular electrostatic field or hydrogen bonding field, are among the most commonly used. The specific nature of the physicochemical variables is important for the quality and reliability of the QSAR model and knowledge-based selection of parameters that are suitable for specific applications is an important task.

A development of particular interest in this field is the use of theoretical amino acid "z scores" based on the work by Hellberg *et al.* [49]. These "z scores" are obtained by principal component analysis (PCA) of 29 experimental descriptors for each amino acid such as molecular weight, logP, pKa and ^{13}C NMR-shifts. PCA is a multivariate projection method that allows extraction and display of the variation in a data matrix. The projection of these descriptors on 3 axes reflect their hydrophobicity (or polarity), steric and electronic properties. In this approach, peptides are described with "z scores" correlated to the biological effect using multiple regression methods, such as partial least squares (PLS) [50] to generate a QSAR model. The development of "z scores" has proven to be useful for modeling a number of biological effects of small peptides. The same approach has also been expanded to a larger set of non-coded amino acids and additional descriptive parameters [51]. Recently a study of modified antibacterial lactoferricins has shown the value of these amino descriptors to develop a predictive QSAR model utilized to design novel and more active peptides [52]. The main drawback of this method is that it is essentially restricted to peptides of same length and, as the length of a peptide increases, so does the number of "z-scores" and the complexity of the property model (3 "z scores" per amino acids). The main advantage is that only the amino acid sequence and not the defined molecular structure is needed.

A more complex study based also on amino acid descriptors has been performed by Mee *et al.* [53] using an algorithm based on D-optimal design. D-optimal design is a method that allows the selection of a set of representative compounds according to the information spanned in the property space considered [54]. Combined with a combinatorial search algorithm, the prediction of potent compound analogs is possible [53]. In this work 1104 parameters from three sets of numerical descriptors (z scores, molecular interaction field, molecular properties) were generated to describe a 15-residue Cecropin/Melittin peptide hybrid (CAMEL 0). From an initial set of 20^{15} possible 15-mer peptide 60 peptide analogs were selected using a D-optimal design algorithm. Based on these data, a PLS model was generated and utilized to test 39 peptide analogs selected with a combinatorial search algorithm. In this study, the combinatorial search algorithm uses the PLS model as "fitness" function to go through a large set of peptides analogs, where all possible single residue mutants are evaluated. A peptide analog with 7-point mutations was reported to have an overall better activity than the original peptide.

Another strategy has focused on the combination of non-linear modeling methods such as Neural Networks to predict molecular properties with a QSAR model and Genetic Algorithms (GA) [55] to design optimized peptides [56]. GA is inspired by population genetics, and has been used in this study to select peptides with a better activity using a PLS model as "fitness function". Compared to a combinatorial search algorithm described above, GA performs a more rapid random selection. Comparison of the GA with other standard optimization techniques like Monte Carlo (MC) optimization has proven GA to be more efficient with 90 out 100 peptides in the final population predicted to be more active.

At Novozymes, we have developed our own strategy for prediction and optimization of new AMPs based on a 3D-QSAR methodology [57]. We worked with an 18 amino acid α-helical peptide called Novispirin G10, designed by Lehrer and co-workers [58]. From a NMR structure of the parent Novispirin molecule, the 3D structures of 58 Novispirin analogs with 1, 2 or 3 mutations were modeled. The antimicrobial potency as defined by the degree of inhibition in the Suicide Expression System (SES) was determined for these analogs. All the structures were modeled in a defined mixture of solvent water/trifluoroethanol (TFE). TFE is widely used for the

study of peptides in solution because NMR and conformational dynamics studies show that many peptides adopt an α-helical conformation in presence of this organic co-solvent [59]. As the molecules originating from the TFE/buffer system represent an artifact when describing the membrane-bound structure of the peptides, only the peptide-part of the modeled structures was kept for the QSAR analysis [60]. Three sets of descriptors were generated from the 3D peptide structures, principally based on partial surface area and quantification of hydrophobic/hydrophilic regions. Among them, VolSurf descriptors were particularly interesting. VolSurf is a computational procedure to produce and to explore the physicochemical property space of a molecule starting from 3D maps of interaction energies between the molecule and chemical probes. In our study, we used the H2O (hydrophilic interaction), DRY (hydrophobic interaction), N1 (amide nitrogen atom), $O^{::}$ (carboxy oxygen atom sp2) and O^{-} (phenolate oxygen sp2) probe to describe interactions of peptides with membrane in a water environment [61].

Then, the information present in 3D grid maps is compressed into a few 2D numerical descriptors which are simpler to understand and to interpret. Examples are the part of the molecular envelope accessible by solvent water and by hydrophobic molecules as well as the amphiphilic moment. So, according to the specific mutations, the structural variations can be quantitatively represented. The advantage of these descriptors is they are related to the 3D structure of the peptides and they can be used directly to generate a QSAR model. The drawback is that most often only modeled structures are employed.

All possible single residue mutants were tested with this QSAR model. Structural modeling and molecular dynamics optimization of all 360 (18 times 20) different mutants was performed with an automatic procedure. The 3D-QSAR algorithm then proposed the specific mutations that were predicted to increase Novispirin activity. From these predictions, 16 Novispirin analogs with a better activity compared to the parent Novispirin, were synthesized and 11 of these agreed with the predictions. This study has shown that a reduction of one positive charge per peptide does not necessarily reduce its activity. However, the position of the introduced hydrophobic amino acid does affect the activity of the peptide and interactions with the membrane. It confirms that a balance between charge and hydrophobicity is important for this class of α-helical AMPs. Similar relationships have been observed with other AMPs [62,63].

Simulation Methods

Simulation methods have been investigated to explore the interactions of AMPs with lipid bilayers and to understand the effects of such interactions on the conformational dynamics of the peptides.

MC [64] and Molecular Dynamics (MD) [65] approaches have been used in the simulation of membrane perturbation and/or pore formation by peptides. In particular Caerin, Dermaseptin, Cecropin P and Magainin were studied in detail [66].

In a MC simulation, random changes in the position/orientation of a peptide helix are made relative to a (mean field) bilayer, and then accepted/rejected on the basis of the change in the overall energy of the system. This method has the advantage of enabling an efficient search of a large number of peptide orientations but does not provide a temporal sequence of events, *i.e.* there is no time axis. MD simulations employ numerical methods to integrate classical equations of motion, yielding the coordinates of all atoms in a system as a function of time. Due to lack of sufficient computational capacity the time accessible to such simulation remains short (up to approx. 10 nanoseconds) and it is often uncertain whether an optimal peptide-bilayer interaction has been reached.

Recently MD simulations covering 30 nanoseconds of both a natural and a synthetic truncated Dermaseptin S3 in the presence of a zwitterionic lipid bilayer have been performed and have yielded results consistent with proposed mechanisms for the lytic activity of AMPs [67].

These simulations have complemented biophysical studies and suggested valuable insights into how AMPs interact with membranes. However, to provide a complete atomic description of the mechanisms of a multiple AMP molecules with a lipid membrane remains a major computational challenge. These simulations are still extremely costly in term of computer time, and simulation methods will not be part of Virtual Screening for AMPs in the near future.

2. *IN VIVO* SCREENING SYSTEMS

Trans-Acting Screening Systems Using Insensitive Hosts

The simplest conceivable HTS setup employs an organism insensitive to the AMP in question. The literature contains several examples of heterologous expression and secretion of fully matured and active AMPs in such diverse organisms as *S. cerevisiae* [68-70], *P. pastoris* [71,72], *E. coli* [73] as well as other organisms [74-76]. These papers have established setups compatible with HTS systems, but do not report on their use as such.

Ideally, the screening host should be identical to the production host, as it is often observed that mutants that can be expressed in one host are only poorly expressed in others. The reasons for this are generally not well understood. Bakers yeast, *S. cerevisiae*, is a particularly well suited screening host for several reasons; it is amenable to extensive genetic manipulation, allows for secretion of correctly folded peptides containing disulfide bridges and allows for construction of large libraries in excess of 10^9. Furthermore, *S. cerevisiae* has a reasonably weak protease profile minimizing degradation of the produced peptide.

Recently, a fungal defensin, named Plectasin, was identified by Novozymes [77]. Plectasin was isolated from the saprophytic ascomycete fungus *Pseudoplectania nigrella* using transposon-assisted signal trapping [78]. Sequencing of the isolated cDNA revealed a gene encoding a 23-aa signal sequence, a 32-aa pro-region, and a structural gene of 40 aa. Sequence analysis and homology alignment indicated that the molecule had homology to the arthropod and mollusk defensin family of AMPs. The characteristic Cys-pattern was conserved as well as several other regions of the peptide. Purified Plectasin has shown potent cidal activity against a

range of Gram-positive bacteria with minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) as low as 0.4 μg/mL.

In an attempt to further optimize and broaden the activity of Plectasin against other bacterial species, a HTS campaign was initiated. As mature and fully functional Plectasin can be produced in *S. cerevisiae*, an expression vector was constructed, where the cDNA encoding the mature part of Plectasin was fused to the alpha-leader from *S. cerevisiae* and introduced into various constitutive or inducible yeast expression vectors. The endogenous pro-region of Plectasin was not utilized in this setup and a galactose-inducible expression system was used. This system takes advantage of the GAL1 promoter which is repressed by glucose and activated by galactose. An advantage of this system for HTS is the use of dual carbon sources allowing initial biomass accumulation without expression of the protein of interest. After consumption of the glucose, the *GAL1* promoter is de-repressed by the depletion of glucose and induced by galactose. The temporal expression profile can be optimized by adjusting the absolute amount and ratio between galactose and glucose.

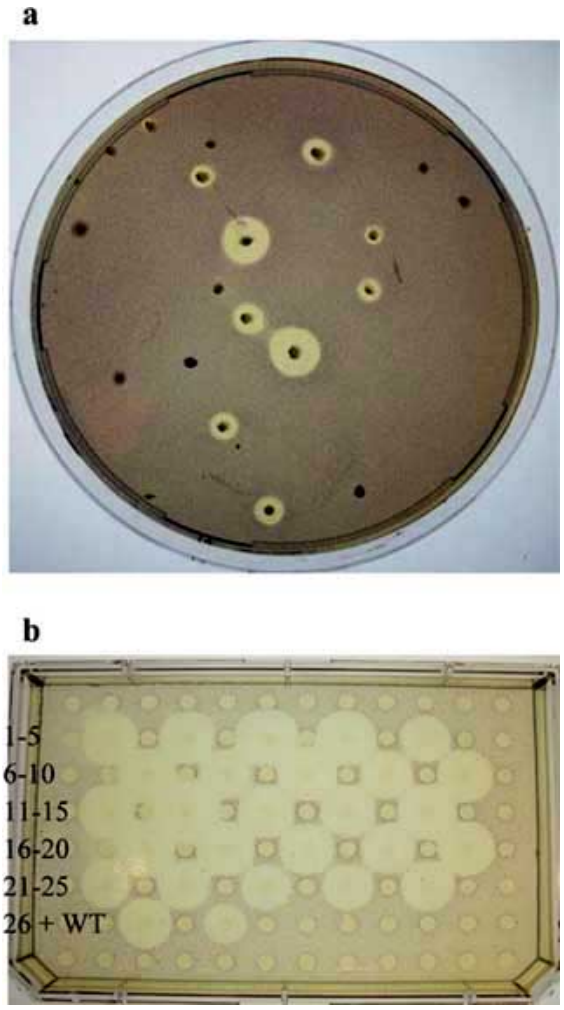

Fig. (2). HTS for Plectasin variants with improved activity. a. Primary plate screen of a mutant Plectasin library. Target organism is *Staphylococcus carnosus*. b. RDA of 26 selected mutants with increased bioactivity compared to wild type (wt). Equal aliquots (20 microliters) of induced culture supernatants were added to a MHB agar plate containing the target organism *Staphylococcus carnosus*.

Several different screening setups have been evaluated in both liquid and solid formats. In one screening setup, a random error-prone PCR library containing ~10^7 Plectasin variants was constructed and transformed into *S. cerevisiae* strain JG169. An appropriate dilution of the yeast library was added to SC agar media (1.5% galactose, 0.5% glucose) and cast into trays. The thickness of this layer was limited to a few millimeters to achieve a horizontal alignment of the embedded yeast cells. After this first layer was solidified, another 'insulating' layer of agar media (SC media, 1.5% galactose, 0.5% glucose) was added. After 2 days of incubation, the yeast cells had grown to a reasonable size and were overlaid with the target organism, *Staphylococcus carnosus*. After another day of incubation, yeast colonies giving rise to clearing zones were apparent and could be picked for re-testing and further analysis. Fig. (**2a**) shows an example of a primary screen. Though rather elaborate, the setup described above provides a very sensitive method that preserves the integrity of the individual yeast colonies.

The most potent clones from the primary screen were re-isolated and their activities confirmed in a more controllable and sensitive Radial Diffusion Assay (RDA) format (Fig. **2b**). As the clearing zone is a product of potency, amount and diffusion constant of a given AMP variant, the expression level of each improved candidate will have to be normalized.

The throughput of the above setup is dependent on the level of automation; however, both liquid and solid screening formats can be extensively automated using workstations for liquid handling and/or robotic colony-pickers.

Cis-Acting Systems Using Sensitive Hosts

Another system for HTS is one where both genotype and phenotype are logically and physically coupled, *e.g.* a system where the peptide is exerting its antimicrobial activity on the producing cell – a *cis*-acting system. The major advantage of such systems is the potential for selection - as opposed to screening - of the most potent candidates. Several related *cis*-acting systems described in literature and in patent applications will be discussed below.

As essential feature of these systems is a tightly controlled promoter system allowing for conditional expression of the AMP, thereby preventing growth-inhibition or killing of the host under conditions where AMP synthesis is not induced.

The first system was described by Taguchi *et al.* in 1994 and termed the *in vivo* monitoring system [79]. In this pioneer paper, the structure-function relationship of Apidaecin, an antimicrobial peptide discovered in lymph fluid of the honeybee, was analyzed using random mutagenesis. Their *in vivo* monitoring system was based on *E. coli* host cells sensitive to the AMP produced under controlled transcriptional regulation. To avoid proteolytic degradation, the gene encoding Apidaecin was fused to the secretory protease inhibitor, SSI. To mimic an outer

membrane interaction, the SSI-Apidaecin fusion was secreted to the periplasmic space. In this setup it was shown that the degree of growth inhibition of the host cells was dependent on the concentration of the transcriptional inducer, isopropyl-β-D-thiogalactopyranoside (IPTG). Thus, higher levels of IPTG resulted in increased levels of Apidaecin and hence a larger degree of growth inhibition. Subsequently, a mutant with an antimicrobial activity lower than wild type was identified and the decrease in activity validated after purification.

Later on, Taguchi *et al.* used the same system to study a second antimicrobial peptide, Thanatin, to elucidate the versatility of the *in vivo* functional mapping methodology [80]. Thanatin is a 21-residue inducible defense peptide found in the hemipteran insect *Podisus maculiventris*. A library of Thanatin mutants was generated using error-prone PCR and transformed into *E. coli* cells. The primary screen correlated colony size on solid media with antimicrobial activity of various Thanatin-derivatives and colonies larger than wild type were identified. The selected clones were analyzed in a secondary screening by monitoring the growth curves in liquid medium to estimate more precisely their antimicrobial activity. This method allowed identification of several Thanatin variants with decreased antimicrobial activity. Further *in vitro* activity assays of purified variants showed that the Minimal Inhibitory Concentration (MIC) values of the peptides were in accordance with the *in vivo* assay, thus validating the screening approach.

A similar system was developed by Choi *et al.* to screen and analyze SARs of AMPs expressed in *E. coli*. These authors also used relative colony-size as a primary screening parameter to identify new variants [81]. Their assay was based on the pET-expression system, where an IPTG-inducible T7 RNA polymerase conditionally expressed the AMPs by a T7-specific promoter [82] (Novagen brand, EMD Biosciences, Inc, Madison, USA). To validate the method, a partially randomized library encoding amphipathic α-helical peptides of 20 amino acids corresponding to the Shiva-1

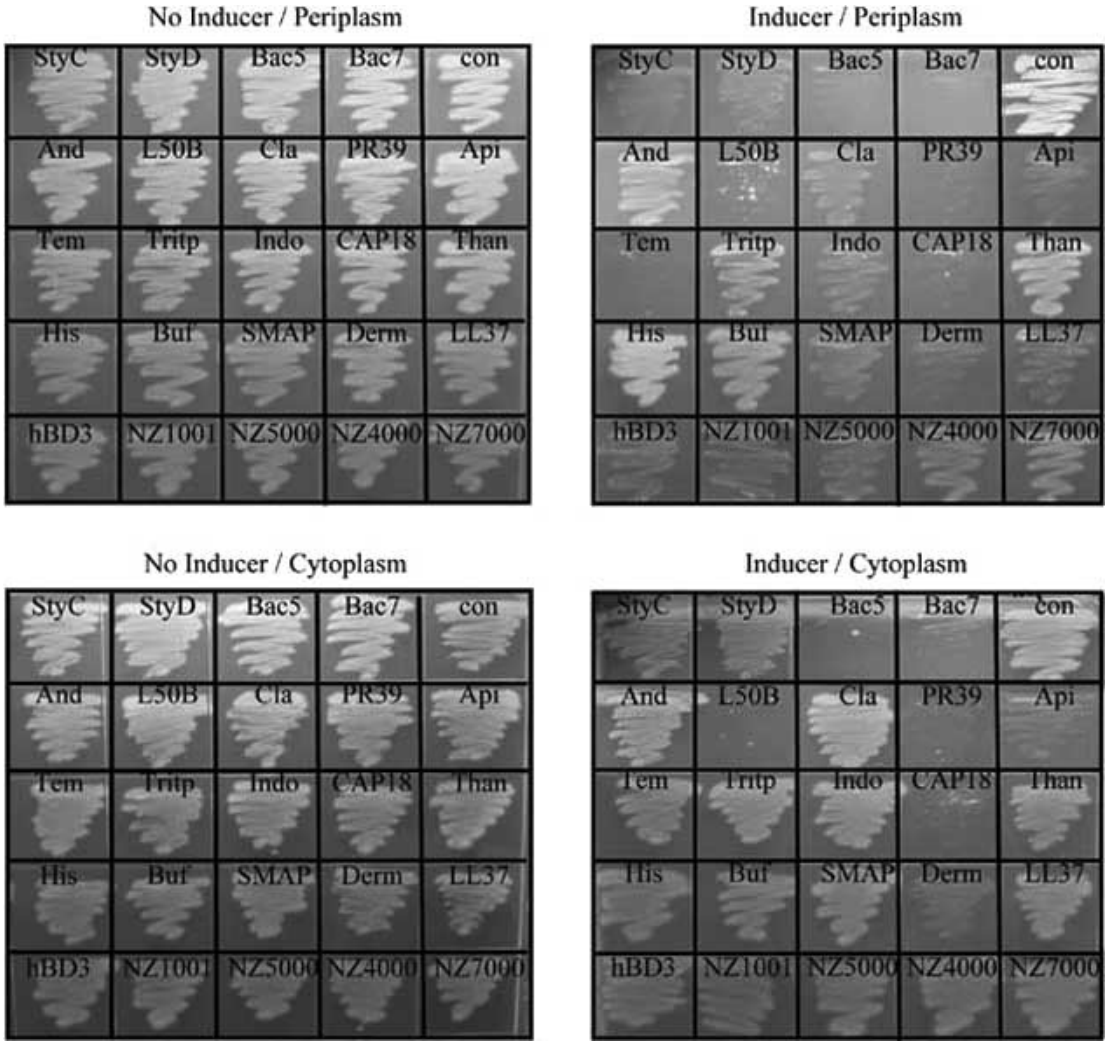

Fig. (3). Growth inhibition assay of *E. coli* on solid media.

A collection of AMPs were tested in the SES with periplasmic or cytoplasmic expression (Fig **3** top and bottom, respectively). Growth inhibitory effect of the different AMPs was analyzed on plates without and with the presence of inducer (0.1%). Results show low inhibitory effect under non-inducing conditions (left side). However, at full induction levels of peptide synthesis (right side), several clones become strongly growth inhibited while some others are partially inhibited or not inhibited at all.

peptide was introduced into *E. coli* [83]. Screening of the active clones was then performed by selecting the slow-growing colonies from plates containing IPTG. Increased activities of the selected clones compared to Shiva-1 were further confirmed by growth inhibition assays of liquid cultures in the presence of IPTG. Although the precise inhibitory mechanism of the selected peptides remains unknown, the method developed in this study showed that selection of new active AMPs can be achieved.

Recently, Walker *et al.* [84] published an *in vivo* study of conditionally expressed bioactive peptides in the cytoplasm of *E. coli*. In their approach a randomized oligonucleotide library encoding peptides up to 20 amino acid in size was screened in order to isolate potential antibiotic peptides based on their capability of inhibiting cell growth. Sequence analysis of the isolated clones showed that two of the most potent inhibitors were peptide-protein chimeras due to deletions and mutations in the plasmid. Thus, the fusion partner could serve as a stabilizing motif for the peptides. Moreover, four out of ten of the most active inhibitory peptides contained one or more proline residues either at or very near the C-terminal end of the peptide which could act to prevent degradation by peptidases present in the cytoplasm. Together, these results suggest that the presence of a stabilizing motif is important for the bioactivity of small peptides.

Suicide Expression System

At Novozymes, we have developed a proprietary *cis*-acting screening system, The Suicide Expression System (SES) [85]. The SES is based on various inducible promoter systems allowing for conditional expression of AMPs in *E. coli*. Two different versions of the SES exist; in one version the AMP is directed to the periplasmic space of *E. coli* via a signal sequence where the AMP molecules are allowed to interact with the inner or outer membrane. In the other version the AMP remains in the cytoplasm. These two different screening set-ups could, in principle, help discriminate between AMPs predominantly interacting with

Table 1. AMPs Tested in the Cytoplasmic and Periplasmic Version of the SES

AMP		Origin	Growth inhibition cytoplasm/periplasm	Ref
StyC	StyelinC	tunicate (*Styela clava*)	- / +	[111]
StyD	StyelinD	tunicate (*Styela clava*)	- / +	[111]
Bac5	Bac5	bovine leukocytes (*Bos taurus*)	+ / +	[112]
Bac7	Bac7	bovine leukocytes (*Bos taurus*)	+ / +	[112]
And	Andropin	insect hemolymph (*Drosophila melanogaster*)	- / +	[113]
L50B	Enterocin L50B	*Enterococcus faecalis*	+ / +	[110]
Cla	ClavaninA	tunicate (*Styela clava*)	- / +	[114]
PR39	PR-39	porcine leukocytes	+ / +	[115]
Api	Apidaecin	insect hemolymph, honeybee	+ / +	[116]
Temp	Temporin B	frog skin (*Rana temporaria*)	- / +	[117]
Tritp	Tritrpticin	porcine neutrophils	- / +	[118]
Indo	Indolicidin	bovine neutrophils	- / +	[119]
CAP18	CAP18	rabbit neutrophils	+ / +	[120]
Than	Thanatin	insect hemolymph	- / -	[121]
His5	Histatin 5	human saliva	- / -	[122]
Buf	Buforin II	Asian toad (*Bufo bufo garagrizans*)	- / -	[123]
SMAP	SMAP29	sheep leucocytes (*Ovis aries*)	- / +	[124]
Derm	Dermaseptin	frog skin	+ / +	[125]
LL-37	LL-37	human neutrophils	- / +	[126]
hBD3	Defensin	human epithelia	- / +	[127]
NZ1001		Artificial	- / +	[128]
NZ4000		Artificial	- / +	
NZ5000		Artificial	- / +	
NZ7000		Artificial	- / +	

the bacterial membranes and AMPs also targeting intracellular components. This is purely speculative and has not been solidly backed by experimental evidence. Both the periplasmic and cytoplasmic versions are *cis*-acting systems as the AMP only interacts with the producing cell.

A crucial feature of this system is that AMPs are not stabilized by fusion partners, as it would impede the activity of some AMPs and could give rise to both false-positive and -negative candidates. However, small unstructured peptides are notoriously unstable in bacteria, and the expression level of individual AMP have to be precisely adjusted to counteract differences in stability, potency and activity in the presence of molecules and other cellular constituents. The amount of AMP synthesized and hence the degree of inhibition is positively regulated at the level of transcription. This is an essential feature that allows for a fine tuning of the AMP level and hence gives a cyclic nature to the selection scheme. Using successive rounds of selection with lower and lower amounts of inducer can allow identification of more and more potent AMPs.

To validate the SES a broad collection of AMPs was tested. These AMPs included peptides of different origin, from different structural classes and with different spectrum of activities. Growth inhibition was tested on solid and liquid media and the AMPs were targeted either to the cytoplasm or to the periplasm. As seen in Fig. (**3**), the majority of the AMPs inhibited cell growth most effectively in the periplasm. However, AMPs like PR-39, Enterocin L50B, Bac7 and Bac5, some of which have confirmed intracellular targets, inhibited growth equally well in both the periplasm and the cytoplasm. In contrast, Histatin 5, Buforin II and Thanatin showed no significant degree of inhibition. A summary of the results are listed in Table **1**.

Bactericidal AMPs so far tested in the SES do not kill the producing cell but only inhibit its growth. This might appear odd at first, but can be explained by the combination of i) a reversible compromization of the membrane, ii) the lability and hence short lifetime of AMPs in cellular settings, iii) the rapid decrease in the proton-motive force and hence energy available for further synthesis of AMPs. Other antibiotic peptides or proteins with irreversible mechanisms of action, *e.g.* the gyrase inhibitor CcdB or a lysozyme fragment have been tested in the SES and do indeed kill the producing cell and only in the expected compartment, *i.e.* intracellularly and periplasmically, respectively (data not shown).

The rationale for developing the SES was to facilitate identification of more potent AMP variants of specific classes as well as new AMP classes. Therefore, we have tested and implemented a number of different selection and screening schemes. One selection setup enriches for cells expressing potent AMP by taking advantage of the ability of β-lactam antibiotics to kill only growing cells. Since cells in the SES indeed are growth inhibited, induction conditions can be tuned in a way such that only bacteria expressing the most potent peptide variants will become growth inhibited. When ampicillin is added to this pool of bacteria expressing the variant library, non-inhibited bacteria expressing less potent AMPs will continue to grow and thus be killed by the ampicillin. If this library is plated out after a few hours of incubation, the bacteria expressing potent peptides can be enriched up to 800 fold (data not shown).

Another screening setup is based on robotics to manipulate large number of samples by handling cellular cultures in microwell plates. The different steps of a HTS procedure using the SES have been illustrated in Fig. (**4**). An AMP library is first introduced into an *E. coli* host to generate large numbers of transformants. Then, a robot picks the individual bacterial colonies from solid media and arrays them on 96 or 384-microwell plates as individual cultures.

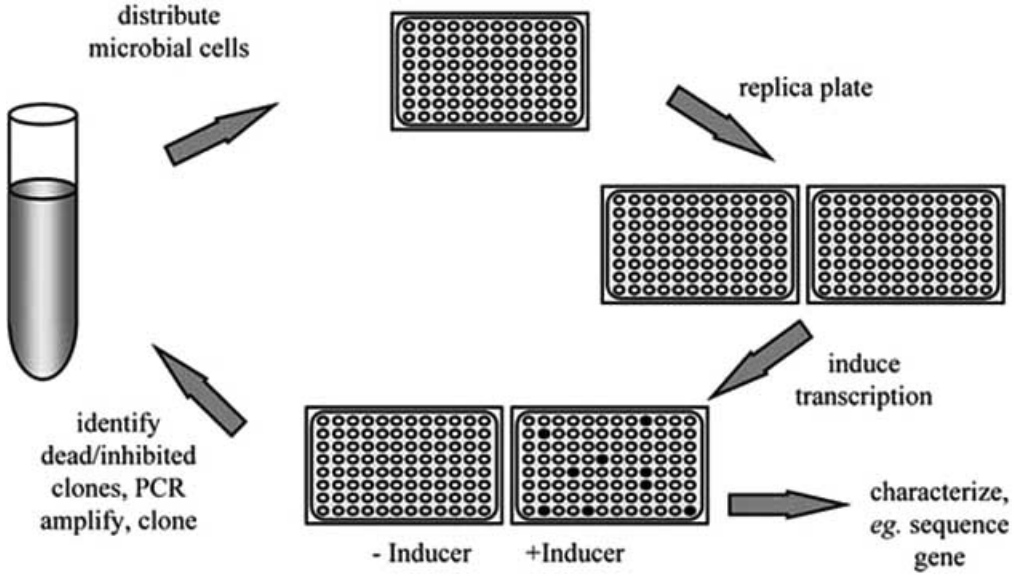

Fig. (4). Identification of modified AMPs using the SES.

AMP libraries are generated and transformed into *E. coli* host cells. The following steps rely on robotic manipulation of the samples including picking of the colonies and screening for growth sensitivity upon induction. Finally, DNA sequencing and characterization of the genes encoding AMPs with increased antimicrobial activity is performed. Selected clones can go through the cycle several times to further improve activity.

These are then robotically replica-plated into non-inducing and inducing liquid media. Clones encoding potent peptide variants with enhanced growth inhibition are isolated and enter a secondary screening carried out using automated microtiter plate optical density (OD) readers in order to eliminate false positives and to quantify the potency of growth inhibition. Growth rates of the samples are followed simultaneously in the presence and absence of inducer and the percentage of growth inhibition are calculated for each clone. Finally, sequence analysis of the variants passing the secondary screening reveals the identity of the mutations leading to increased antimicrobial activity compared to the parent molecule.

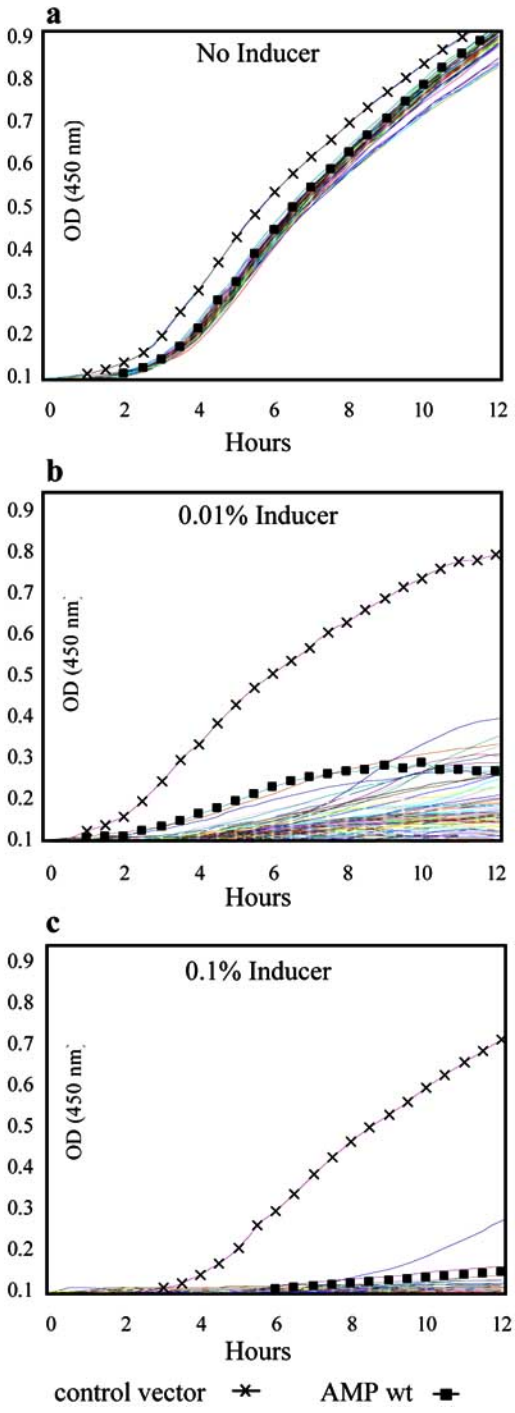

Fig. (5). Growth curves of *E. coli* upon expression of AMP variants under different inducing conditions.

Growth curves of selected clones encoding different AMP variants were monitored by spectrophotometer and analyzed for inducer-dependent growth inhibition. Fig. **5a** corresponds to the growth curves obtained when no inducer was added to the media, whereas Fig. **5b** and **5c** correspond to curves obtained when adding 0.01% and 0.1% inducer, respectively. OD_{450} optical density at 450 nm.

An example of a secondary screening comparing growth curves obtained from different *E. coli* cultures isolated from a first round of a SES screening is shown in Fig. (**5**). Cells expressing a control peptide and cells expressing the parent AMPs are included as controls. Cells were grown in the absence (Fig. **5a**) or in the presence of 0.01% or 0.1% of inducer (Fig. **5b** and **5c,** respectively). The results illustrate the correlation between the level of inducer and growth inhibition. In this way, cultures can be grouped according to their growth inhibition efficiency and clones with improved inhibitory effect can be identified. Also, it is evident that a lowering of the level of inducer, and hence level of AMP inhibition, allows for a continued selection of AMP variants with increased potencies.

Most of the clones identified during the primary screen appear more potent in the secondary screen as well. However, a few false-positive less potent clones appear and will be discarded after the secondary screening.

To validate and further prioritize the improved candidates, they are produced and purified, and the MICs against the host, as well as a range of other relevant microorganisms, are determined under conditions compatible with National Committee for Clinical Laboratory Standards (NCCLS). Table (**2**) shows an example of improved MIC values for selected peptides.

Trans-Acting Screening Systems Using Sensitive Hosts

In addition to the SES, we have developed a novel assay termed TAPS that allows the identification of new or improved genes encoding peptides that can kill or inhibit the growth of target cells [86]. The TAPS method, an acronym for *Trans* Acting Peptide System, is based on a sensitive host producing a peptide followed by screening for its activity in *trans* against an indicator strain. The advantage of such a "trans" system is that the AMP can be conditionally expressed to high levels in a host that can be genetically manipulated, *e.g*. the Gram-negative bacterium *E. coli* and subsequently its antimicrobial activity can be evaluated against different microbes including Gram-negative and Gram-positive bacteria or fungi. Additionally, TAPS offers the possibility of producing correctly folded AMPs containing their native disulfide bonds in the host cells, thereby retaining their antimicrobial activities.

The TAPS approach requires that expression of the peptide is under control of an inducible promoter with tight regulation as the host cells are sensitive to the peptide they are producing. Secondly, the produced peptide has to be released to the media such that it can interact with the target organism. Different approaches can be taken to achieve these

Table 2. Antimicrobial Activities of SES-Selected AMPs. NZ1001 is the Parent Molecule and NZ1001-1 to NZ1001-8 are Derivatives of NZ1001. The MICs (Minimal Inhibitory Concentration, μg/ml) are Determined Under NCCLS-Compliant Conditions

	NZ1001	NZ1001-1	NZ1001-2	NZ1001-3	NZ1001-4	NZ1001-5	NZ1001-6	NZ1001-7	NZ1001-8
M. luteus (ATCC9341)	12	1.5	1.5	4	3	1.5	2	2	4
S. aureus (ATCC29737)	≥ 128	6	4	8	12	8	16	4	6
S. epidermidis (DSM1798)	48	2	3	3	8	3	6	≤ 1	1.5
E. faecalis (DSM2570)	≥ 128	16	8	16	24	48	64	32	24
P. aeruginosa (ATCC27857)	64	32	48	64	64	24	48	8	32
K. pneumoniae (ATCC10031)	2	1.5	2	4	3	≤ 1	≤ 1	≤ 1	≤ 1
S. choleraesuis (DSM9220)	12	4	6	16	6	3	4	2	3
E. coli TOP10 (laboratory strain)	16	6	2	2	3	≤ 1	≤ 1	≤ 1	6

requirements depending on the nature of the peptide and the type of target organism.

TAPS screening can be carried out either on solid or in liquid media. Fig. (**6**) illustrates the TAPS method performed on solid media. First, a plasmid library is introduced into *E. coli* host cells. It is important that the transformants are cultivated on the surface of a cellulose acetate filter placed on media without inducer to avoid expression of the AMP and hence growth inhibition. In the next step, the filter containing the colonies is transferred to culture media containing inducer to permit peptide synthesis. Depending on the nature of the peptide, the filter containing the host cells can be submitted to different treatments to enhance the release of the peptide from the host cells. Subsequently, the target strain is overlaid onto the plate and allowed to grow for a specific period of time. Finally, visual inspection of the host cells capable of reducing the proliferation of the target cells is performed and the nucleotide sequence encoding for the AMP is recovered from the host cells and sequenced to uniquely identify the corresponding peptide.

To validate the performance of TAPS on a solid format we analyzed the ability of a disulfide bridged peptide named AntiFungal Peptide (AFP) isolated and characterized in our laboratory [87] to inhibit growth of the fungus *Fusarium longipes*. Although AFP possesses no activity against Gram-negative bacteria, expression of AFP in *E. coli* origami host cells (Novagen brand, EMD Biosciences, Inc, Madison, USA) produces an inhibitory effect on cell growth (data not shown). The *E. coli* origami strain harbors mutations in both the thioredoxin reductase (*trxB*) and the glutathione reductase (*gor*) genes. Consequently, the activity of such enzymes in reducing the environment in the bacterial cytoplasm is diminished or abolished thereby allowing disulfide bond formation in the cytoplasm [88,89]. First, *E. coli* origami cells were transformed either with the control vector, pNZ1, or with the construct pNZ1-AFP to express AFP in the cytoplasm. Two bacterial colonies containing each of the constructs were allowed to grow on the surface of an acetate filter placed on solid culture media. From this step, the filter was treated as illustrated in fig. (**6**). As shown in Fig. (**7**), the pink *Fusarium longipes* mycelium is uniformly distributed throughout the top layer of the

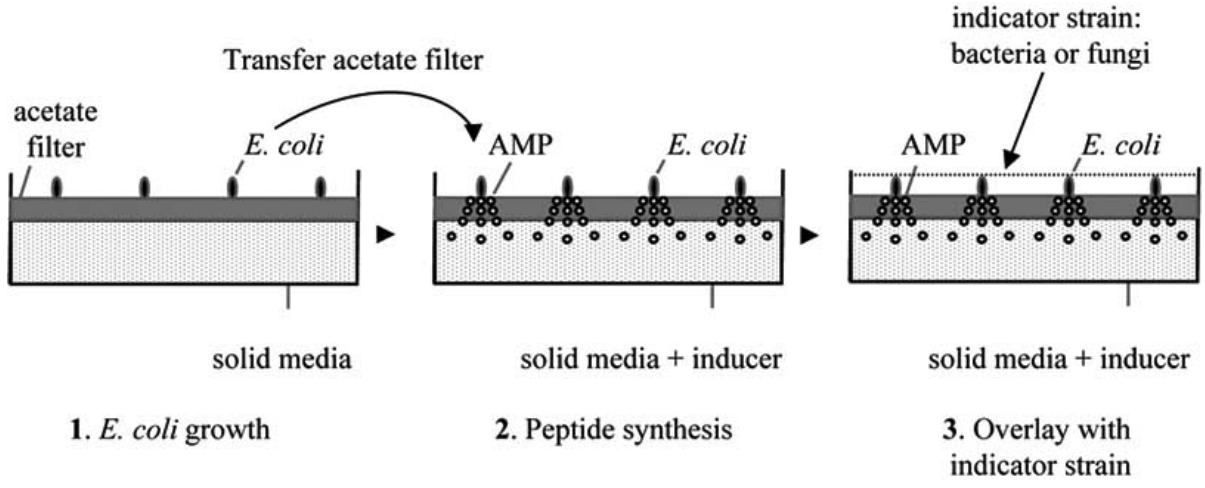

Fig. (6). Trans-Acting Peptide System (TAPS).

This method is based on having sensitive host cells expressing an AMP followed by detecting its activity in *trans* against an indicator strain, which can either be fungi or bacteria.

plate, except where *E. coli* origami cells express AFP. In contrast, no clearing zones are detected on top of the cells transformed with the control vector, pNZ1. These results indicate that *E. coli* origami cells produce an active peptide capable of killing or inhibiting growth of the fungus *Fusarium longipes.*

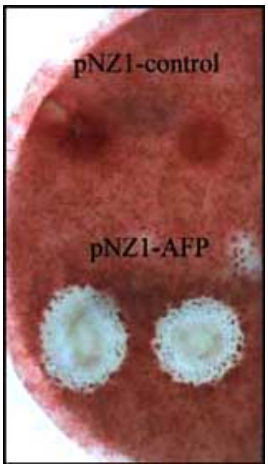

Fig. (7). TAPS assay in solid format.

E. coli origami cells expressing AFP in the cytoplasm inhibit the growth of the fungus *Fusarium longipes*.

As mentioned above, the TAPS screening can also be performed using liquid media. This procedure requires the use of robotics to analyze large numbers of clones (Fig. **8**). In this system, independent colonies are inoculated into 96 or 384-well plates by a robot and cultured. These are then replicated robotically and grown to exponential phase until the inducer is added to trigger peptide synthesis. The next step consists of lysing the cells such that the peptide is released to the media and an aliquot of the lysed culture is used to perform an activity test against a desired target organism. Different activity tests can be performed, such as RDA where an aliquot of the hydrolyzed culture is added to the agarose media inoculated with the target strain [90]. An example of a RDA against the indicator strain *Staphylococcus carnosus* obtained from a screening plate is presented in Fig. (**8**) where clearing zones corresponding to clones exhibiting antimicrobial activity of plectasin can easily be identified [77].

3. *IN VITRO* SYSTEMS

Recent advances in cell-free protein synthesis systems such as RNA-peptide fusion display [91,92] ribosome display [93-96], and *in vitro* transcription/translation have been developed [97-100]. Such systems have in common the capacity to synthesize proteins at high speed and overcome the problem of cell toxicity.

Additionally, *in vitro* systems have the advantage of being compatible with HTS methods [100-103]. In this respect, much effort has been invested in the development of new approaches to improve such technology. Recent *in vitro* strategies exploiting the virtues of PCR and *E. coli*-coupled transcription/translation have been developed by Rungpragayphan and coworkers providing a novel system

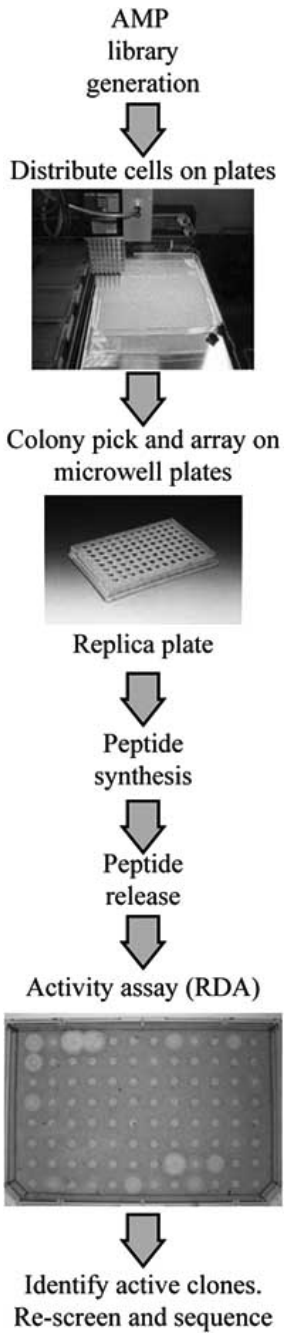

Fig. (8). TAPS; HTS identification of modified AMPs using a liquid medium setup.

AMP libraries are constructed and transformed into *E. coli* host cells. Colonies are robotically picked and arrayed into microtiter plates followed by peptide synthesis and release. Active clones are identified by a RDA and the mutations determined by DNA sequencing.

for high throughput (HTP) construction and screening of protein libraries. The strategy was termed SIMPLEX (Single- Molecule-PCR-Linked *in vitro* Expression) and has been used for construction and screening of combinatorial protein libraries in an array format [101]. Although the *E. coli* system appears very powerful, the wheat germ cell-free system developed in Y. Endo's laboratory also shows great potential [100]. To maximize the yield and throughput of

the system, Endo *et al.* and Sawasaki *et al.* designed an optimal expression vector for large-scale protein production and developed a new strategy termed the split-primer method to construct PCR-generated DNAs for high throughput production of many proteins in parallel [102,103].

A very elegant technology that uses water-in-oil emulsions to compartmentalize *in vitro* transcription /translation of single genes was developed by Tawfik and Griffiths [104]. Such systems use man-made compartments to link genotype and phenotype at the molecular level. The gene, the protein it encodes, and the products of the activity of this protein all remain within the same compartment. This technology was further improved by adding a new step consisting on re-emulsification of water-in-oil emulsions. The result was the formation of water-in-oil-in-water emulsions containing an external aqueous phase through which droplets containing fluorescent markers could be isolated by fluorescence-activated cell sorting (FACS). This technology opens a wide potential in the area of HTS for molecular evolution [105].

Despite that *in vitro* systems have shown promising results, little has been reported on their application to AMP studies. One of the reasons could be due to the mode of action of AMPs. Some AMPs may have the capacity to bind DNA or RNA and consequently cause inhibition of the whole protein synthesis machinery. For example it has been described that Buforin II has strong affinity for DNA and RNA [7], PR-39 arrests DNA synthesis [106], and defensins are able to break single-strand DNA [107]. Thus, *in vitro* transcription/ translation of such types of peptides could in principle be problematic. Moreover, many cationic peptides exhibit high affinity for LPS which is the main component of the outer membrane of Gram-negative bacteria [108,109]. In such cases *E. coli in vitro* transcription/translation systems would be inadequate for synthesis of cationic peptides if LPS is present in the extracts.

Although *in vitro* systems might have these limitations, it has been shown that *in vitro*-synthesized enterocin L50A (EntL50A) and L50B (EntL50B) possess antimicrobial activity, with EntL50A being the most active. Furthermore, the two bacteriocins exerted a synergistic effect when combined together [110]. These interesting results show that cell-free protein synthesis is a powerful tool for synthesizing active AMPs.

Since AMPs have the potential of being toxic to the cells expressing them, *in vitro* transcription/translation systems, either from *E. coli* or wheat germ extracts, should be considered as a powerful tool in combination with HTS to express and select peptide variants with improved activity.

CONCLUSIONS

Peptides will constitute a large part of future therapeutics. This is due to the ease of generating and screening large peptide libraries, their chemical manipulability, biocompatibility and relatively simple commercial production.

Obviously, there is a desire in the pharmaceutical industry to screen large compound libraries to best identify lead candidates. In essence, this is a numbers game, and a preferred strategy would be to combine the various disciplines including *in silico*, *in vivo* and *in vitro* approaches.

Despite the development of new computational drug design technologies, their use in the peptide field is still marginal. However, the reviewed papers serve to illustrate that computer-aided VS can provide a rapid and powerful tool for sequence space elimination and hence more productive discovery and optimization of new bioactive AMPs. Improved accuracy of the methods, faster algorithms and faster computers will certainly further promote the process. QSAR approaches seem particularly relevant for virtual library screening as it take into account available structural information for AMPs and, combined with a genetic or a combinatorial search algorithm, can predict the efficiency of various analogs independent of peptide length.

The future exploration of *in vivo* and *in vitro* HTS approaches will take several directions relying on robotics and automation, miniaturization from 96 to 384 or 1536 microtiter plate formats, compartmentalization as well as screening on single cells. Specific to the *cis*-acting systems like SES, implementation of selection strategies analogous to the described ampicillin approach and FACS sorting will greatly reduce the candidates to be characterized in more elaborate screens.

An important aspect is the correlation of the various screening formats to the true *in vivo* scenario where animals or humans are used to determine efficacy. The simplest pre-screens like the *cis*-acting SES are rather inflexible and cannot easily accommodate conditions mimicking true *in vivo* situations. Other screening formats, *e.g. trans*-acting secretion in *S. cerevisiae,* are compatible with conditions that represent simple therapeutic settings such as blood, bronchoalveolar lavage or wound fluid.

It will be a future challenge to bridge various disciplines and to broaden the screens to include complementary functionalities such as immunogenicity, anti-inflammatory activity, systemic stability and oral availability as required for specific therapeutic areas.

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ABBREVIATIONS

AMP = Antimicrobial peptide

GA = Genetic algorithm

HDP = Host defense peptide

HTS = High throughput screening

IPTG = Isopropyl-β-D-thiogalactopyranoside

LPS = Lipopolysachharide

LTA	=	Lipotechoic acid
MBC	=	Minimal bactericidal concentration
MC	=	Monte Carlo
MD	=	Molecular Dynamic simulation
MIC	=	Minimal inhibitory concentration
NCCLS	=	National Committee for Clinical Laboratory Standards
PCA	=	Principal component analysis
PLS	=	Partial Least Squares
QSAR	=	Quantitative structure/activity relationship
RDA	=	Radial diffusion assay
SAR	=	Structure/activity relationship
SES	=	Suicide expression system
TAPS	=	*Trans*-acting peptide system
TFE	=	Trifluoroethanol
VS	=	Virtual screening
WT	=	Wildtype

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