RESEARCH ARTICLE

Streptomyces as Overexpression System for Heterologous Production of an Antimicrobial Peptide

Marisol Roldán-Tapia^a, Jozef Anné^b, Ana Gisela Reyes^c, Ulises Carrasco^{2a}, Cesar Millán-Pacheco^d, Javier Barrios-González^{3a} and Armando Mejía^{*4a}

^aDepartment of Biotechnology, Universidad Autónoma Metropolitana-Iztapalapa, México DF, México, San Rafael Atlixco 186, Col. Vicentina. C.P. 09340; ^bRega Institute, Katholieke Universiteit Leuven, Laboratory of Molecular Bacteriology, Minderbroedersstraat 10, B-3000 Leuven, Belgium; ^cConacyt-CIBNOR, Centro de Investigaciones Biológicas del Noroeste, La Paz, BCS, México; ^dFacultad de Farmacia, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, CP 62209, Cuernavaca, Morelos, México

Abstract: Defense mechanisms of plants against phytopathogens include cationic antimicrobial peptides (CAPs). The broad-spectrum activity of these peptides has been evaluated previously against different phytopathogens. Their lack of toxicity for plants and animals is a promising feature for pest control. Although, some attempts have been made previously in order to increase their heterologous expressions, the employed strategies have so far proven to be ineffective. Low production yield and elevated costs are the obstacles to overcome. In this study, a strategy for CAP overexpression is presented based on the construction of an expression cassette for *Streptomyces lividans* TK24. This system contains elements that allow the increase of the efficiency of the peptide's expression. The main elements included in this cassette are the sequences of the promoter and signal peptide from a subtilisin inhibitor gene of *Streptomyces venezuelae*. This allows the efficient secretion of the peptide to the growth medium, thereby simplifying its recovery and avoiding its toxic effect on the producing organism. The results obtained demonstrate the system's efficiency by achieving a peptide concentration of 11.61 mg/ml. This represents at least a 10-fold increase compared to previously established strategies.

ARTICLE HISTORY

Received: July 15, 2016
Revised: November 29, 2016
Accepted: February 1, 2017

DOI: 10.2174/0929866524666170208154327

Keywords: Heterologous production, antimicrobial peptide, *Streptomyces lividans*, secreted peptide.

INTRODUCTION

Recently, the importance of antimicrobial peptides in plant disease control has been widely recognized. They could be used in several fields of application, and large quantities of antimicrobial peptides would be required. However, their production is very expensive; this is why a suitable production method, alternative to traditional chemical synthesis is necessary.

Production of recombinant antimicrobial peptides in prokaryotic systems has demonstrated the viability of this approach. Nevertheless, expression of antimicrobial peptides in *Escherichia coli* is potentially limited due to their toxicity to host cells and susceptibility to proteolytic degradation, although this can be reduced using a fusion protein approach [1]. As an alternative, we describe a successful antimicrobial peptide production system in *S. lividans* which showed to be

effective for the secretion of large quantities of cationic antimicrobial peptides.

Antimicrobial peptides, particularly those of the cationic type (CAPs), affect the plasma membrane of the target organism by inducing both destabilization and stress [2]. Mammalian cells are not affected by these peptides as their membranes contain cholesterol (up to 25%), a predominance of phosphatidylcholine lipids, and an essentially neutral (zwitterionic) outer leaflet [3], which interfere with CAP activity. This selectivity can be exploited in many applications [4, 5]. Additionally, there is a low probability that the exposed microorganisms will develop resistance against CAPs. Unfortunately, although they could be used in several fields of application, including agriculture, their production costs are elevated as the most effective approach so far is by chemical synthesis. In addition, there are heterologous expression strategies using *Escherichia coli* as host [6]. Examples of this approach include the production of cecropin [7, 8], moricin [9], human defensins [10], magainin [11, 12] and concatemers, such as buforin II. In some of these strategies, an attempt has been made to prevent CAP lethality by using recombinant protein technology in order to mask the peptide's intrinsic antimicrobial activity [13]. There are other

*Address correspondence to this author at Department of Biotechnology, Universidad Autónoma Metropolitana San Rafael Atlixco 186, Vicentina, Iztapalapa., P.O. Box: 09340. Ciudad de México, México;
Tel: ++52(55)58046438; Fax: +52(55)58044712;
E-mail: ama@xanum.uam.mx

systems, such as transgenic mouse erythrocytes for magainin production and recombinant hybrid production, such as the cecropin and magainin peptide in *Pichia pastoris* [14]. Unfortunately, these strategies have limitations such as low yield [7, 9] and proteolysis of the fusion protein that masks its toxic properties [15]. Additionally, when these strategies are employed, the cytoplasmic accumulation of the expressed proteins in the form of inclusion bodies is frequently observed [16], inducing activity inhibition and rendering the purification process difficult. Consequently, this is reflected in low yield values [17].

Therefore, as a solution to the difficulties for heterologous expression of CAP we demonstrate efficient production by *Streptomyces lividans*

2. MATERIALS AND METHODS

2.1. Peptide Modelling and Simulations

For the construction of the CAP, Glu was substituted by Ala at the 14th position of the original sequence (Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Glu-Ala). The initial model was built using iTasser server [18, 19]. The model was immersed in a cubic box of water TIP3P with a concentration of KCl 0.15 M using charmm-gui web server (<http://www.charmm-gui.org>) [20]. All molecular dynamics simulations were performed using the GROMACS 5.0.5 [21, 22] software using charmm 36 parameters [23] with the gromacs inputs as suggested by charmm-gui server. The simulations were run by 100 ns at 37°C, 1 atm of pressure and periodic boundary conditions.

2.2. Strains, Plasmids Constructions, Media and Growth Conditions

The following phytopathogens were used in this research: *Collectotrichum* sp, *Fusarium oxysporum*, *Fusarium solani*, *Nigrospora oryzae*, *Pseudomonas aureofaciens*, *Pseudomonas syringae* pv. tomato, *Ralstonia solanacearum*, *Xanthomonas axonopodis* pv. Citri and *Bacillus subtilis*. All of them were provided by Química Agronómica de México, S. de R.L. M.I. Culture media preparation were performed according to [24] and [25].

2.3. DNA Manipulations

Molecular biology techniques were performed according to [24] and [25]. Restriction endonucleases and DNA modifying enzymes were provided by Invitrogen and Promega. PCR reactions were carried out using the primers reported by Van Dessel, 2003 [28] employing the GoTaq Flexi DNA polymerase.

2.4. CAP Selection

The standard peptide was synthesized by Thermo Fisher Scientific de México (Invitrogen) with a 50% purity and it was stored at -20°C.

The selected CAP is a broad-spectrum cationic peptide that has been reported as non-toxic for plants and animals. Its activity was evaluated through serial dilutions (1×10^{-1} to

Table 1. Strains and plasmids used.

Strain	Genotype	Reference
<i>Escherichia coli</i> DH5 α	<i>F' endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZAM15 Δ(lacZYA-argF)U169, hsdR17(r_K^-), λ^-</i>	Grant, 1990 [26]
<i>Streptomyces lividans</i> TK24	<i>str-6</i> SLP2- SLP3-	Kieser, 2000 [27]
Plasmid	Characteristics	Reference
pIJ486b	Vector from pIJ10, like pIJ487 but with opposite orientation of the fragment <i>Bgl</i> II containing the cloning sites.	Kieser, 2000 [27]

1×10^{-6}) and performing bioassays on different pathogens by assessing optical density (OD) at 600 nm [29]. Liquid LB medium was used to culture these bacteria and the incubations were carried out at 37°C. Results were expressed as the inhibition percentage of bacterial growth mediated by the peptide's activity (Figure 3).

2.5. Design of Expression Cassette

For the construction of the cassette, preferential codons were used for *S. lividans* with G and C at the third position [30]. The peptide of interest was fused to the *vs*i promoter and signal sequence (*vs*i-ss) of the subtilisin inhibitor from *Streptomyces venezuelae* CBS762.70, which is a signal peptide with a proven high secretion efficiency [31, 32]. The *vs*i promoter displays a superior transcription initiation of 1.5 and 1.9-fold compared to the *aph* and *ermEp** promoters, respectively [33].

The cloning vector used was pIJ486, which is derived from the pIJ101 plasmid including a transcription terminator sequence and a thiostrepton resistance marker.

2.6. Minigene Amplification and Cloning in *Streptomyces lividans* TK24

Based on the cassette designed in silico, the subcloned minigene was synthesized in the PIDT-smart vector which was amplified by transforming *Escherichia coli* DH5 α through electroporation. The fragment was digested using the *Bam*HI and *Hind*III restriction enzymes and cloned into the pIJ486 vector to finally be used to transform protoplasts of *S. lividans* TK24 [24].

2.7. *S. lividans* TK24 Protoplast Transformation

Biomass was obtained from a 48 h preculture of *S. lividans* TK24 grown in YEME medium for 48 h and incubated at 30 °C and 150 rpm. A flask containing 250 ml of S medium, was added 50 ml of glucose solution (66.6 mg/ml) and 12.64 ml of glycine 20%. Then was inoculated with 5ml of fragmented mycelium, which was incubated at 30°C and

150 rpm for 24 h. Protoplasts were obtained using a technique previously described by Hopwood [24].

Four plates were prepared containing R₂ medium described by Hopwood [24], supplemented with CuSO₄·5H₂O (2mM-1/1000) and CaCl₂·2H₂O 36.8% (1/100). 10 µl of ligation mixture and 500 µl of PEG6000 35 % (v/v) were added to a tube containing 200 µl of a protoplast suspension (OD600 with a value close to 1). The protoplast suspension and ligation mixture were mixed and incubated at a room temperature (30 °C) for 24 h. Each R₂-plate was inoculated with 150 µl of this transformation mixture. Plates were subsequently incubated at 30 °C for 24 h in order to allow protoplast regeneration. After this period, 50 µg/ml thiostrepton was added and dispersed within the plate. The transformants were allowed to grow for an additional 24 h [8].

2.8. Transformant Selection by Colony PCR

In order to verify that the expression cassette was present in the transformants, colonies were disrupted and their DNA taken as template. A PCR was performed using the conditions and primers reported by Van Dessel, 2003 [28] PCR results were corroborated by automated sequencing. Bioassays were carried out to verify the production of the peptide and its secretion to the growth medium.

2.9. Activity Assays on Bacteria

To rule out that the observed activity originated from a secondary metabolite from *S. lividans* but not the peptide, an ultrafiltration step was performed. Biomass was retrieved from a six day-old culture using quick filtration. The supernatant was collected in Amicon® ultrafiltration units with a regenerated cellulose membrane for proteins lower than 3.0 kDa.

Activity bioassays were carried out using both fractions. As reported by Berghé and Vlietinck (2005) [34], LB plates were inoculated with a spore suspension of the indicator microorganism (*B. subtilis* WT or a phytopathogen) (1x10⁶ CFU). Wells were made and 100 µl of the filtered supernatant was added at different time points during the incubation. Negative (liquid medium) and positive (synthetic peptide 48 µM) controls were also included. Incubation was carried out over 16 h.

2.10. Peptide Identification

The use of Tricine-SDS-PAGE 16% gels with 6M urea is recommended for the separation of peptides within the 1-100 kDa range with a good resolution [35], AB-6 stock acrylamide and loading buffer B were used, and 0.2 µg of peptide. The electrophoretic run was carried out over 120 min at 100 Volts and the proteins were stained with Coomassie blue. Gels were scanned and the images were analyzed in a photo document system (Gel Doc™ EZ, Bio-Rad) in order to generate a densitometric profile of the bands.

Experiments were performed in triplicate and repeated three times. The standard deviation was always lower than 2%.

3. RESULTS

The simulation of the helix showed that the peptide is stable along 100 ns at 37°C. The simulation showed that there exists a region particularly stable between residues 3 to 11 (FKCRRWQWRMKLEA). The backbone RMSD of all the peptide along the time (calculated each ns) was of 2.49 +/- 0.52 Å and for those residues 3 to 11 was of 0.62 +/- 0.4 Å. Figure 1 shows the changes that occurred on two snapshots of the simulations.

Based on these results, we assumed that the peptide might conserve the helical conformation along time so the peptide was chemically synthesized and its activity was assessed observing a broad spectrum against phytopathogenic bacteria and fungi (Table 2).

Figure 1. Initial model (C-score of -0.01 and 0.71+/-0.11 of TM.) on white, structure after 77 ns with the higher backbone RMSD over the residues 3-11 of 2.1 Å (middle gray) and final structure after 100 ns with a backbone RMSD over the same residues of 0.58 Å (darker gray).

Table 2. Activity of the peptide. Growth inhibition percentage (%) (optical density at 600 nm).

Microorganism	Synthetic peptide	Produced peptide	Taxon
<i>Collectotrichum sp.</i>	5.83%	5.36%	Fungi
<i>Fusarium oxysporum</i>	6.31%	5.81%	Fungi
<i>Fusarium solani</i>	7.00%	6.24%	Fungi
<i>Nigrospora oryzae</i>	57.84%	52.21%	Fungi
<i>Pseudomonas aureofaciens</i>	67.42%	62.03%	Bacteria
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	19.29%	17.75%	Bacteria
<i>Ralstonia solanacearum</i>	12.48%	11.18%	Bacteria
<i>Xanthomonas axonopodis</i> pv. <i>citri</i>	79.52%	73.06%	Bacteria

3.1. CAP Production System

By using preferential codons for *S. lividans* TK24 and taking into account the considerations previously described in the Methods section, an expression cassette was developed as shown in Fig 2.

```

5' GGATCCGGTTATACCATACCGTTTCGTCATATGCTGGACAGGCGCCACGGA
ACCGCTCCCAACCAACCCCTGAGCTGCCCGAACGAGCGTGCGGTACCGCTCACC
GGAACGCCACATCCGGAAATCGACCATCCGGATGGCACTCACTCTCCGCACCG
GCAAGACTCCTCACCAGTCACCAACCGCATCGATCGAAGGAGAGTTTACCA
TGCGTCGCACCCCTCAAGGCCGTGGGAGCAGCCGCGCGCGGCCACCTGCGTC
CTCGCCGCGACGGCAGGCACCGCGCAGGCCGAGGCTTCAAGTGCCGCGGTG
GCAGTGGCGGATGAAGAAGCTGGCCGCTAGAGCTT 3'

```

Figure 2. Expression cassette for *S. lividans* TK24. Restriction site, promoter, Shine-Dalgarno sequence, start codon, signal peptide, peptide of interest, modified amino acid, stop codon.

From the subcloning into pIDT-Smart, this expression cassette was cloned into the pIJ486 vector to finally transform protoplasts of *S. lividans* TK24. The transformation was verified by colony PCR, using primers complementary to the multiple cloning site within the pIJ486 vector for amplification of the fragments as reported by Van Dessel, 2003 [28].

DNA sequencing was performed using the purified material obtained from the bands in the agarose gel. Results showed 100% identity between the amplified region and the oligonucleotide sequence of the designed expression cassette.

3.2. Activity Assays on Bacteria

Peptide production was evaluated in a Petri dish using the cover bioassay. On the surface of these bioassay dishes, a halo of inhibition was observed, resulting from the secreted peptide by the transformant. It was verified that no activity was obtained from the parental strain.

Figure 3. Cover bioassay. Left: Parental strain with *B. subtilis*, Center: Transformant with *B. subtilis*, Right: Transformant with the *Xanthomonas* phytopathogen.

Figure 4. Diffusion bioassay. Line A, Concentration curve of the synthetic peptide (0.0132, 0.0099, 0.0066 and 0.0033 mg/μl); Lines B and C, supernatant activity after six days of fermentation (200, 180, 150, 100 μl); Line D, activity of the fraction above 3 kDa; Line E, growth of the fraction lower than 3 kDa and Line F, activities of the supernatants from the parental strain.

The results suggest that CAP is actively secreted into the culture medium because of the signal peptide, as previously demonstrated by other studies in which it was secreted through the Sec pathway [31, 32]. In order to verify this, a cell culture fractionation was performed on a six day-old fermentation in which the biomass was removed through quick filtration. The supernatant was collected in Amicon® ultrafiltration units of 3.0 kDa, obtaining two fractions: one superior to 3 kDa and another one below this value. Considering that the peptide has an average mass of 3.51 kDa, it should be located in the fraction superior to 3.0 kDa.

The Figure 4 shows the inhibition of the indicator microorganism with the fraction superior to 3.0 kDa. This effect was absent in the fraction lower than 3.0 kDa.

Performing different diffusion bioassays, a peptide standard curve was constructed and the quantification of the produced peptide was achieved, obtaining a concentration of 11.6 g/l.

In addition, the effect of the peptide on the producer microorganism, *S. lividans*, was also measured. The results showed a slight negative effect on growth in the diffusion bioassay. These results suggest this sensitivity could be the reason why the peptide production stopped in the transformant strain of *S. lividans*.

3.3. Peptide Identification

In order to identify the peptide, Tricine-SDS-PAGE gels were prepared for fragment separation through vertical electrophoresis. The separation range in these gels is 1-100 kDa.

The acquired images of the Tricine-SDS-PAGE gels were analyzed in a photo documentation system (Gel Doc™ EZ Bio-Rad) in order to generate a densitometric profile of the bands. Thus, a second quantification of the produced peptide was carried out, obtaining a result of 11.61 mg/ml. Therefore, the quantification results obtained from the bioassay were corroborated.

DISCUSSION

During the last years, considerable efforts were made in order to maximize the yield and quality of the proteins produced in heterologous systems [17]. This is even more critical in the case of peptides or proteins that are potentially

Figure 5. Tricine-SDS-PAGE. 4% stacking gel, 10% spacing gel and 16% separating gel with 6M urea and stained with Coomassie blue. Lane 1: molecular weight marker; lanes 2- 5: synthetic peptide; lanes 6-10: samples from the transformant growth media.

toxic for the producing organism. Thus, the aim of this study was to achieve the overexpression of a CAP. The strategy employed was the design of a specific system using a selected CAP with known properties regarding its amino acid sequence, size, stoichiometric charge, reliability parameters such as the C-score, structural similarity parameters (RMSD) and topological measurements (TM-score). The presence of hydrophobic and positively-charged amino acids are important for CAP activity and it has been reported that the substitution of a glutamic acid for alanine frequently results in improved activity [36, 37]. Taking this into account, it was confirmed that by modifying the amino acid sequence, specifically substituting Glu for Ala in the 14th position, results in enhanced toxicity. This offers a reliable and stable model.

Regarding the production strain, (*Streptomyces lividans*) it does not possess a periplasmic space. Thus, the produced proteins are secreted into the growth medium, simplifying their folding, purification and proper activity. This also contributes to prevent its intracellular accumulation that normally has toxic effects on the host.

During the construction of the expression cassette the use of preferential codons in *Streptomyces* [30, 38], was considered. The promoter of the parental strain was exchanged for the *S. venezuelae* *vsj* sequence that, according to previous reports, has displayed a high efficiency [31-33]. The replicative vector pIJ486 was selected, which includes a transcription terminator sequence [39] and resistance to thiostrepton as a selection marker. The Shine-Dalgarno sequence was located [40]. The start codon was ATG in order to provide a correct RNA translation and stability.

Finally, the secretion pathway of the produced peptide was considered. Two of these have been described in *Streptomyces* sp: the Sec and Tat pathways. In this study, the former was selected for peptide excretion. The proteins in this pathway contain a signal peptide that is eventually removed by a Type I signal peptidase (Spase). These are essential for cell viability and their elimination leads to the accumulation of precursor proteins in cell membranes, and subsequently to cell death [41, 42]. *S. lividans* possesses four Type I SPases: W, X, Y, Z [43-45]. This pathway might be the underlying cause of the system's efficiency regarding CAP secretion, cell death evasion and the prevention of the toxic effect on

the host microorganism, in comparison to other expression systems previously reported [7-9, 46].

Regarding the secreted cationic peptide, it displayed a broad-spectrum activity against Gram-positive and Gram-negative bacteria as well as fungi. The effect is variable depending on the affinity of the membrane towards the peptide, its lipid composition and its physicochemical properties. According to several authors, peptide concentration is also an important factor related to its effectiveness when acting on the membrane of the target microorganism [47-51]. The effect was less pronounced on fungi, probably because of the decreased interaction between the peptide and their cell membrane [12, 52, 53].

Using the described system for CAP production, it is possible to produce efficiently, an interesting metabolite to handle phytopathogens in agriculture at low costs. Additionally, its application could be used in other fields such as medical, food industry, stockbreeding, etc.

CONFLICT OF INTEREST

Authors thank to the following sources of founding: Marisol Roldán-Tapia, UAM-I, CONACYT (no. 332832); Armando Mejía, UAM-I; Jozef Anné, Rega Institute, KUL; Ana Gisela Reyes, UAM-I; Ulises Carrasco, UAM-I; Cesar Millán-Pacheco, UAM-I; Javier Barrios-González, UAM-I

ACKNOWLEDGEMENTS

We thank Alejandra Serrato Díaz from Divisional Laboratory of Molecular Biology (UAM) for her technical help. Authors also gratefully acknowledges to the UAM Super Computer Center (UAM-Iztapalapa) Aitzaloe and Yoltla.

REFERENCES

- [1] Yalin Y., Z.T., Da T., Jun Z., Jiarong W., Jianhua W., *High-level production of a candidacidal peptide lactoferrampin in Escherichia coli by fusion expression*. J. Biotech, **2009**. 139(4): p. 326-331.
- [2] Marcos *et al.*, M.A., Pérez-Payá E, Misra S, López -García Belén, *Identification and Rational Design of Novel Antimicrobial Peptides for Plant Protection*. Annu Rev Phytopathol., 2008. **46**: p. 273-301.
- [3] Verkleij *et al.*, Z.R., Roelofs B, Comfurius P, Kastelijn D and Van Deenen L. , *The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy*. Biochim Biophys Acta., 1973. **323**: p. 178-193.
- [4] Sahl, P.a., *The co-evolution of host cationic antimicrobial peptides and microbial resistance*. Nat. Rev. Microbiol. , 2006. **4**: p. 529-536.
- [5] Yount, Y.a., *Mechanisms of antimicrobial peptide action and resistance*. Pharmacol Rev., 2003. **55**: p. 27-55.
- [6] Li., *Recombinant production of antimicrobial peptides in Escherichia coli: a review*. Protein expr purif., 2011. **80**(2): p. 260-267.
- [7] Andersons, D.E., A.; Josephson, S.; Hansson, L.; Steiner, H. , *Biologically active and amidated cecropin produced in a baculovirus expression system from a fusion construct containing the antibody-binding part of protein A*. Biochem J 1991. **280**: p. 219-224.
- [8] Callaway *et al.*, L.J., Haselbeck B, Baltaian M, Bonnesen SP, Weickmann J, Wilcox G, Lei SP, *Modification of the C terminus of cecropin is essential for broad-spectrum antimicrobial activity*. Antimicrob Agents Chemother., 1993. **37**: p. 1614-1619.
- [9] Hara, S.Y., M. , *Production in Escherichia coli of moricin, a novel type antibacterial peptide from the silkworm, Bombyx mori*. Biochem Biophys Res Commun. , 1996. **220**(3): p. 664-669.

- [10] Xu *et al.*, W.F., Peng L, Fang X, Cen P Expression of human beta-defensin-2 with multiple joined genes in *Escherichia coli*. Appl Biochem Biotechnol., 2005. **120**(1): p. 1-13.
- [11] Lee *et al.*, H.S., Kim SC., Expression of an antimicrobial peptide magainin by a promotor inversion system. J Microbiol Biotechnol., 1998. **8**: p. 34-41.
- [12] Sharma *et al.*, K.-C.A., White SP, Dhanjal NK, Huang W, Paulhiac C, Friedman EJ, Manjula BN, Kumar R, High-efficiency synthesis of human alpha-endorphin and magainin in the erythrocytes of transgenic mice: a production system for therapeutic peptides. . Proc Natl Acad Sci USA, 1994. **91**(20): p. 9337-9341.
- [13] Lee *et al.*, M.I., Park CB, Kim SC Acidic peptide-mediated expression of the antimicrobial peptide buforin II as tandem repeats in *Escherichia coli*. Protein Expr Purif. , 1998. **12**: p. 53-60.
- [14] Fengliang *et al.*, X.X., Liexi Wang, Wenqing Zhang, Dexiang Gu, Expression of recombinant hybrid peptide cecropinA(1-8)-magainin2(1-12) in *Pichia pastoris*: Purification and characterization. Protein Expr Purif., 2006. **50**: p. 147-156.
- [15] Piers *et al.*, B.M., Hancock RE Recombinant DNA procedures for producing small antimicrobial cationic peptides in bacteria. Gene, 1993. **134**: p. 7-13.
- [16] Pimienta *et al.*, F.R., Sánchez JC, Vallín C, Secretion of human interferon alpha 2b by *Streptomyces lividans*. Appl Microbiol Biotechnol., 2002. **58**: p. 189-194.
- [17] Anné, J.M., B.; Van Impe, J.; Van Mellaert, L., Recombinant protein production and streptomycetes. J. Biotech., 2012. **158**(4): p. 159-167.
- [18] Roy, A.K., A.; Zhang, Y. , I-TASSER: a unified platform for automated protein structure and function prediction. Nature Protocols, , 2010. **5**(4): p. 725-738. .
- [19] Zhang, Y., I-TASSER server for protein 3D structure prediction. . BMC Bioinformatics, , 2008. **9**(11): p. 40.
- [20] Jo, S.K., T.; Iyer, V. G.; Im, W., CHARMM-GUI: a web-based graphical user interface for CHARMM. . Journal of Computational Chemistry, 2008. **29**(11): p. 1859-1865. .
- [21] Van Der Spoel, D.L., E.; Hess, B.; Groenhof, G.; Mark, A. E.; Berendsen, H. J. C, GROMACS: fast, flexible, and free. . Journal of Computational Chemistry, 2005. **26**(16): p. 1701-1718.
- [22] Abraham, M.J.M., T.; Schulz, R.; Páll, S.; Smith, J. C.; Hess, B.; Lindahl, E, GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX, 2015. **1-2**: p. 19-25. .
- [23] MacKerell, J., A. D.; Banavali, N.; Foloppe, N. , Development and current status of the CHARMM force field for nucleic acids. Biopolymers, 2001. **56**: p. 257-65.
- [24] Hopwood *et al.*, B.M., Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM, Schrempf H., Genetic manipulation of *Streptomyces*: A laboratory manual. 1985, John Innes Foundation.: Norwich, CT.
- [25] Sambrook *et al.*, F.E., Maniatis T Molecular cloning: A laboratory manual in Cold Spring Harbor Laboratory. 1989: Cold Spring Harbor, N.Y.
- [26] Grant., *E.coli* Genotypes. 1990.
- [27] Kieser *et al.*, T., Bib, M., Buttner, M., Chater, K., Hopwood, D., Practical *Streptomyces* Genetics. , ed. T.J.I. Foundation. 2000, Norwich.
- [28] Van Dessel *et al.*, V.M.L., Geukens N, Anné J, Improved PCR-based method for the direct screening of *Streptomyces* transformants J Microbiol Methods, 2003. **53**(3): p. 401-403.
- [29] Stevens *et al.*, S.B., Klapes NA, Klaenhammer TR, Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. App Environ Microbiol., 1991. **57**: p. 3613-3615.
- [30] Wright, F.B., MJ. , Codon usage in the G+C-rich *Streptomyces* genome. . Gene, , 1992. , **113**: p. 55-65.
- [31] Lammertyn *et al.*, D.S., Schacht S, Van Mellaert L, Anné J Influence of charge variation in the *Streptomyces venezuelae* alpha-amylase signal peptide on heterologous protein production by *Streptomyces lividans*. Appl Microbiol Biotechnol., 1998. **49**(424-430).
- [32] Lammertyn *et al.*, V.M.L., Schacht S, Dillen C, Sablon E, Van Broekhoven A, Evaluation of a novel subtilisin inhibitor gene and mutant derivatives for the expression and secretion of mouse tumor necrosis factor alpha by *Streptomyces lividans*. Appl Environ Microbiol., 1997. **63**: p. 1808-1813.
- [33] Van Mellaert *et al.*, L.E., Schacht S, Proost P, Van Damme J, Wroblewski B, Molecular characterization of a novel subtilisin inhibitor protein produced by *Streptomyces venezuelae* CBS762.70. DNA Seq., 1998. **9**: p. 19-30.
- [34] Bergher, P., Antiviral botanicals in herbal medicine. Med. Herb., 2005. **14**: p. 1-12.
- [35] Schägger., Protocol: Tricine-SDS-PAGE. Nat Protoc., 2006. **1**: p. 16 - 22
- [36] Strøm *et al.*, S.W., Svendsen JS, Rekdal Ø, Increased antibacterial activity of 15-residue murine lactoferricin derivatives. J Pept Res., 2001. **57**: p. 127-139.
- [37] Strøm *et al.*, S.J., Rekdal Ø, Antibacterial activity of 15-residue lactoferricin derivatives. . J Pept Res., 2000. **56**: p. 265-274.
- [38] Ueda *et al.*, T.S., Nishiyama KI, Kumagai I, Miura KI Effect of a rare leucine codon, TTA, on expression of a foreign gene in *Streptomyces lividans*. Biochim Biophys Acta., 1993. **1172**: p. 262-266.
- [39] Pulido, D.J., A. , Optimization of gene expression in *Streptomyces lividans* by a transcription terminator. Nucleic Acids Res. , 1987. , **15**(10), : p. 4227-4240. .
- [40] Strohl., Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. . Nucl Acids Res., 1992. **20**(5): p. 961-974.
- [41] Dalbey *et al.*, L.M., Bron S y van Dijk JM The chemistry and enzymology of the type I signal peptidases. Protein Sci. , 1997. **6**: p. 1129-1138.
- [42] Koshland *et al.*, S.R., Botstein D Diverse effects of mutations in the signal sequence on the secretion of beta-lactamase in *Salmonella typhimurium*. Cell, 1982. **30**: p. 903-914.
- [43] Parro, V.M., R.P. , A new signal peptidase gene from *Streptomyces lividans* TK21. . DNA Seq. , 1998. , **9**: p. 71-77. .
- [44] Parro *et al.*, S.S., Anne J, Mellado RP Four genes encoding different type I signal peptidases are organized in a cluster in *Streptomyces lividans* TK21. Microbiology, 1999. **145**: p. 2255-2263.
- [45] Schacht *et al.*, V.M.L., Lammertyn E, Tjalsma H, van Dijk JM, Bron S., The Sip (Sli) gene of *Streptomyces lividans* TK24 specifies an unusual signal peptidase with a putative C-terminal transmembrane anchor. . DNA Seq., 1998. **9**: p. 79-88.
- [46] Parachin *et al.*, M.K., Viana AA, Dias SC, Franco OL. , Expression systems for heterologous production of antimicrobial peptides. Peptides, 2012. **38**(2): p. 446-456.
- [47] Hwang, Action of antimicrobial peptides: two-state model. Biochemistry 2000. **39**: p. 1-6.
- [48] Matsuzaki, Magainins as paradigm for the mode of action of pore forming polypeptides. Biochim Biophys Acta, 1998. **1376**.
- [49] Matsuzaki *et al.*, S.K., Fshibe N Relation ship of membrane curvature to the formation of pores by magainin 2. Biochemistry, 1998. **37**: p. 11856-11863.
- [50] Nes, N.-M.a., Ribosomally synthesized antimicrobial peptides: Their function, structure, biogenesis and mechanism of action. Arch Microbiol., 1997. **167**(2-3): p. 67-77.
- [51] Shai, Mode of action of membrane active antimicrobial peptides. Pept Sci., 2002. **66**(4): p. 236-248.
- [52] Hancock, R.E.W., Lehrer, R. , Cationic peptides: a new source of antibiotics. . Trends in Biotechnol. , 1998. , **16**: p. 82-88. .
- [53] Nakashima *et al.*, M.Y.a.T.T., Actinomycetes as host cells for production of recombinant proteins. Microb cell Fact, 2005. **4**(1): p. 7.