

METHODS IN GENE ENGINEERING

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Summary

This article highlights some of the molecular biological methods that are currently used or contemplated for engineering genes and expressing recombinant proteins. Some PCR-based methods have been applied in studying structure-function relationships of the *Bacillus thuringiensis* insecticidal proteins called delta-endotoxins. These approaches include splicing by overlap extension, site-directed mutagenesis *via* amplification of an entire plasmid and dideoxy-based direct DNA sequencing that are powerful ways of recombining, modifying and verifying nucleotide sequences, respectively.

Here also describes gene expression strategies for producing nonfusion and fusion proteins in *E. coli*. Selecting the optimal expression system for heterologous genes can be crucial in obtaining biologically active recombinant proteins. Nonfusion proteins can be highly produced by placing a strong regulated promoter and an efficient ribosome-binding site upstream of the cloned genes.

Fusion proteins can be constructed using a variety of different affinity tag fusion systems that have been used successfully for purification of different fusion proteins. Western blotting has proven to be a very useful immunoassay method for detection of a

specific protein in a complex sample.

1. Introduction

Currently, there are many methods for engineering genes that are too numerous to cover here, but a synopsis of some of the major uses and applications are presented to tie together the concepts developed thus far. This article describes some particular gene engineering strategies that have been used to manipulate the delta-endotoxin genes from *Bacillus thuringiensis* (Bt), a family of Gram-positive sporulating soil bacteria that synthesise parasporal crystalline inclusions with larvicidal activity [see also – *Biopesticide production*].

The *Bt* delta-endotoxins were re-classified by Crickmore and his colleagues in 1998 as crystalline (Cry) and/or cytolytic (Cyt) proteins that exist as inactive protoxins found within inclusion bodies which require alkaline solubilisation and proteolytic activation in the insect larval midgut. It has been proposed that after activation by gut proteases, the active toxins kill the susceptible larvae *via* a two-step receptor mediated mechanism, in which the initial toxin-receptor interaction is followed by membrane insertion of the toxins to form transmembrane leakage pores. These pores cause the target midgut epithelial cells to swell and lyse by colloid-osmotic lysis, resulting in extensive damage to the midgut and eventually larval death. However, the precise mechanism of action of the *Bt* toxins is still not completely understood, although knowledge of how these insecticidal proteins work at the molecular level has increased substantially over the last decade.

To date, the three-dimensional structures of several different Cry delta-endotoxins, Cry1Aa, Cry2Aa, Cry3Aa, Cry4Aa and Cry4Ba, have been elucidated by X-ray crystallography. All the structures display a high degree of overall structural similarity and are composed of three structurally distinct domains. It is apparent that the N-terminal domain, a seven-helix bundle (six amphipathic helices around a central core helix), is clearly equipped for membrane insertion and pore formation. This suggestion has been supported by various studies demonstrating that the isolated helical fragment from different Cry toxins is responsible for pore-forming activity.

2. Gene Engineering with Polymerase Chain Reaction

In order to work out the structure and function of a gene, sufficient amounts of the gene of interest must be available for analysis. Apart from standard DNA cloning methods, a relatively new, cell free technique known as *the polymerase chain reaction* (PCR) as originally described by Mullis and his colleagues in 1986 [see also – *Physical methods applied to biotechnology*] allows a selected gene to be amplified into multiple, identical copies. The PCR method is now proving to be one of the most important technical breakthroughs that has many applications in recombinant DNA research since the development of gene cloning itself. This technique can sometimes bypass the lengthy and laborious steps required for DNA cloning [see also – *Genetic manipulation of bacteria; – Conventional Plant Breeding for higher yield and Pest Resistance*] in living organisms. It has the advantages that it is comparatively fast and is achievable even though there are only a few copies of the starting DNA sequence. The only requirement

is that the nucleotide sequences at the borders of the chosen DNA region must be known, thus limiting this method to the study of a gene that has already been characterised in part by molecular cloning methods.

2.1 Principles and Limitations of PCR

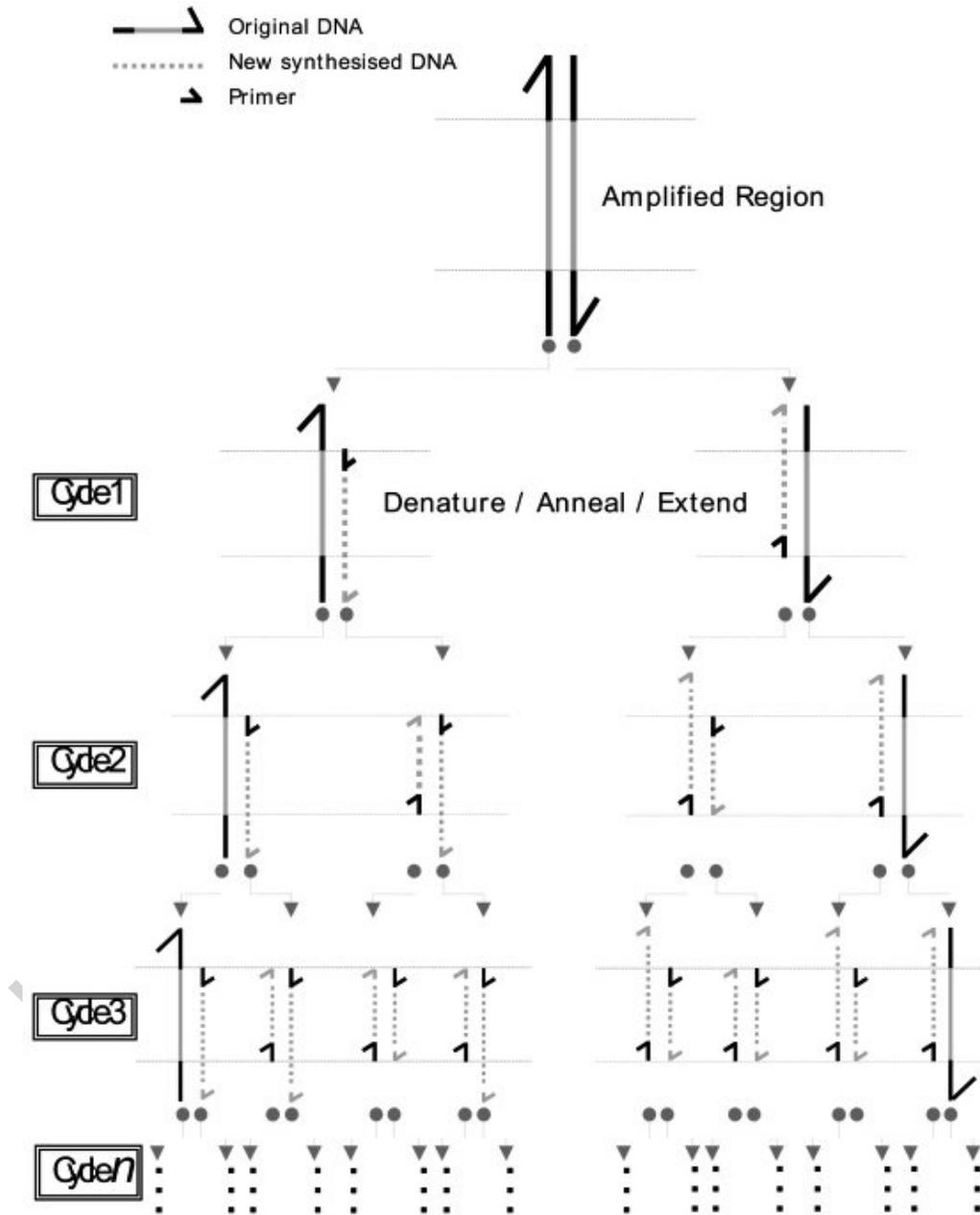


Figure 1: Amplification of target DNA by PCR. The diagram shows the steps involved in the first few cycles of a PCR. The original DNA template is denatured by high temperature. Then the temperature is reduced to allow specific primers to anneal to the target sequence for new DNA synthesis. The first PCR cycle is characterised by a product of indeterminate length while a subsequent cycle produces discrete a short fragment which accumulates exponentially with each round of amplification.

In the PCR process, a specific DNA fragment can be selectively enriched by more than a hundred thousand times relative to the original DNA template. The theoretical basis of this process is diagrammatically outlined in Figure 1. There are three nucleotide segments: the segment of double-stranded DNA template and two single-stranded oligonucleotide primers. In addition, there is a heat-stable DNA polymerase as well as appropriate deoxynucleotide triphosphates (dNTPs). The specific PCR primers are chemically synthesised, such that they are complementary to DNA flanking the sequence of interest. Both primers are designed to anneal to opposite strands of the DNA template, with their 3'-ends pointing towards each other. A basic concept is that to start DNA synthesis, DNA polymerase always requires a primer to which it can add nucleotides. Since the directionality of polymerase movement along DNA, the synthesis can proceed in only one direction from the primer.

The key steps of PCR are

- denaturation of a double-stranded DNA template by heating samples to an elevated temperature above its melting point,
- annealing of pairs of specific primers to the single-stranded template at a reduced temperature (dependent on the melting temperature of the primers used), and
- extension by DNA polymerase from the 3'-end of each primer to copy the template molecule.

The first cycle of this three-step process results in new DNA strands of indeterminate length whilst another cycle of this synthesis generates two discrete single-stranded products that are exactly the length between the primer ends.

The amount of these products doubles with every subsequent cycle, accumulating exponentially so that more than one million copies of the discrete template DNA are made at the end of 20 cycles.

However, the exponential phase of PCR production does not continue indefinitely and after about 20 cycles most PCR reactions enter linear and then plateau phases. Reagent concentrations become limiting, and high concentrations of PCR products tend to result in re-annealing rather than new primer binding.

The length of the target DNA between primers is one limiting factor, generally the longer the sequence the less efficient the PCR. Also, the sequence of the target DNA itself can affect the efficiency. Too much secondary structure in GC rich areas of the denatured strands can prevent DNA polymerase from reading the template. Additionally, the amplification can be hindered by the existence of complementarity between the primers themselves, causing an artefact known as primer-dimer.

Since the PCR amplification is very sensitive, a frequently quoted limitation of this method is its susceptibility to contamination by other DNA from any air-borne cellular debris or contaminated reagents. Therefore, it is prudent to carry out PCR experiments in separate areas of the laboratory, using dedicated equipment and multiple controls with each reaction.

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Bibliography

Angsuthanasombat C., Chungjatupornchai W., Kertbundit S., Luxananil P., Settasatian C., Wilairat P. and Panyim S. (1987) Cloning and expression of 130-kd mosquito-larvicidal delta-endotoxin gene of *Bacillus thuringiensis* var. *israelensis* in *Escherichia coli*. *Mol. Gen. Genet.* 208, 384-389. [This represents an example of over-expression of a biologically active protein that can be simply assayed for its larvicidal activity using a host-cell extract or whole cells expressing toxins].

Boonserm P., Min M., Angsuthanasombat C. and Lescar J. (2006) Structure of the functional form of the mosquito-larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. *J. Bacteriol.* 188, 3391-3401. [This is the most recent study for determination of three-dimensional structure of the *Bacillus thuringiensis* delta-endotoxin by X-ray crystallography].

Boonserm P., Pornwiroon P., Katzenmeier G., Panyim S. and Angsuthanasombat C. (2004) Optimised expression in *Escherichia coli* and purification of the functional form of the *Bacillus thuringiensis* Cry4Aa δ -endotoxin. *Protein Express. Purif.* 35, 397-403. [This is an example of gene engineering strategies via PCR-based splicing by overlap extension that attempts to improve the expression of the *Bacillus thuringiensis* delta-endotoxin in *Escherichia coli*].

Chen E. T. (1994) The efficiency of automated DNA sequencing. *In Automated DNA sequencing and analysis* (ed. M.D. Adams *et al.*), pp. 3-10. Academic Press, London, UK. [This provides a good introduction to the essential features of automated DNA sequencing].

Crickmore N., Zeigler D. R., Feitelson J., Schepf E., Van Rie J., Lereclus D., Baum J. and Dean D. H. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62, 807-813. [This is the latest revision of the nomenclature for the insecticidal crystal proteins (delta-endotoxins) from *Bacillus thuringiensis*].

Horton M. (1993) *In vitro* recombination and mutagenesis of DNA: SOEing together tailor-made genes. *In Methods in molecular biology*, vol 15 (ed. B. A. White), pp. 251-261. Humana Press, Totowa, USA. [This describes an original concept of PCR-based splicing by overlap extension].

Kanintronkul Y., Sramala I., Katzenmeier G., Panyim S. and Angsuthanasombat C. (2003) Specific mutations within the α 4- α 5 loop of the *Bacillus thuringiensis* Cry4B toxin reveal a crucial role of Asn-166 and Tyr-170. *Mol. Biotechnol.* 24, 11-19 [This study employing PCR-based site-directed mutagenesis to demonstrate certain residues within the α 4- α 5 loop are crucial for larvicidal activity of the *Bacillus thuringiensis* toxin].

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685. [This is an original work describing separation of proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis primarily based on its molecular mass].

Likitvivanavong S., Katzenmeier G. and Angsuthanasombat C. (2006) Asn183 in α 5 is essential for oligomerisation and toxicity of the *Bacillus thuringiensis* Cry4Ba toxin. *Arch. Biochem. Biophys.* 445, 46-55. [This represents a recent study employing PCR-based site-directed mutagenesis to identify a critical residue for toxin oligomerisation and toxicity of the *Bacillus thuringiensis* larvicidal protein].

Li J., Carroll J. and Ellar D. J. (1991) Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. *Nature* 353, 815-821. [This work provides the first three-dimensional

structure of the *Bacillus thuringiensis* delta-endotoxin by X-ray crystallography].

Mullis K. B., Faloona F., Scharf S. J., Saiki R. K., Horn G. T. and Erlich H. A. (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* 51, 263-273. [This is an excellent paper giving experimental proof of principle of enzymatic amplification of DNA in vitro].

Puntheeranurak T., Leetacheewa S., Katzenmeier G., Krittanai C., Panyim S. and Angsuthanasombat C. (2001) Expression and biochemical characterisation of the *Bacillus thuringiensis* Cry4B $\alpha 1$ - $\alpha 5$ pore-forming fragment. *J. Biochem. Mol. Biol.* 34, 293-298. [This is an example of over-expression of a heterologous protein from a cloned gene in *Escherichia coli* that leads to the formation of inclusion bodies which can be conveniently purified by centrifugation, subsequently unfolded and refolded into a correctly folded-active form].

Puntheeranurak T., Uawithya P., Potvin L., Angsuthanasombat C. and Schwartz J. L. (2004) Ion channels formed in planar lipid bilayers by the diptheran-specific Cry4Ba *Bacillus thuringiensis* and its $\alpha 1$ - $\alpha 5$ fragment. *Mol. Membr. Biol.* 21, 67-74. [This study demonstrates that the isolated helical $\alpha 1$ - $\alpha 5$ fragment is a functional channel former like the full length Cry4Ba toxin]

Sanger F., Nicklen S. and Coulson A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* 74, 5463-5467. [This study describes the principle of the dideoxy termination method for DNA sequencing that is one of the most influential scientific works ever published].

Towbin H., Staehelin T. and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* 76, 4350-4354. [This is an excellent work giving experimental procedure and its implications of Western blot analysis].

Biographical Sketch

Dr. Chanan Angsuthanasombat was born on February 5, 1961 in Phatthalung Province, Thailand. He received his B.Sc. in chemistry from Chiang Mai University, Thailand in 1984 and then pursued his M.Sc. in biochemistry from Mahidol University, Thailand in 1986 under a scholarship of Mahidol University. He was then recruited as a staff member of the Centre for Molecular Genetics and Genetic Engineering, Mahidol University. In 1991, he was awarded a British Council Scholarship for his Ph.D. studies in molecular biology at the Department of Biochemistry, University of Cambridge, UK. Since his return from UK, he has continued to work on molecular mechanisms of actions of *Bacillus thuringiensis* mosquito-larvicidal proteins. His achievements have been recognised in Thailand as demonstrated by the 1994 Young Scientist Award from the Foundation for Promotion of Science and Technology under the Patronage of His Majesty the King. He was the recipient of Research Career Development Award in 1996, and Thailand Research Fund Fellowship in 1994, 1997 and 2000. He is currently an associate professor at the Institute of Molecular Biology and Genetics, Mahidol University.