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Synthesis of the N-terminal of the Ice Nucleation Protein Gene of *Pseudomonas syringae* by Assembly PCR

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Abstract: The construction of a synthetic gene coding for the N-terminal of the ice nucleation protein of *Pseudomonas syringae* using assembly polymerase chain reaction was described. The *Inak-n* gene was assembled from 34 overlapping oligonucleotides in a single step and amplified by PCR using specific cloning primers. Expression of this gene in *E. coli*, in the form of a fusion protein, was confirmed by Western blotting. This synthetic gene would be a useful tool for the display of antigens on the surface of bacterial cells.

Key words: Cell surface display, gene synthesis, site-directed mutagenesis, gene expression

INTRODUCTION

The Ice Nucleation Protein (INP) encoding genes known generally as *Inak*, is present in ice nucleation active bacteria such as *Pseudomonas syringae, Pseudomonas fluorescens* and *Erwinia herbicola*. The *Inak* gene encodes a unique outer membrane-associated protein, the INP which is able to accelerate ice crystal formation in supercooled water at temperatures of -2 to -10°C[1,2] .

INP is a monomeric protein composed of more than 1,200 amino acid residues with a deduced molecular mass of 118 kDa[3] and three distinct domains: (I) an N-terminal domain with 175 amino acids, which is hydrophobic and function as the membrane anchor (ii) a central Cylindrical Repeating Domain (CRD), 48-residue long, which is not essential for membrane anchoring but has a catalytic role in the formation of ice crystals and (iii) a C-terminal domain of 49 amino acids that is hydrophilic and extracellular[4-7]. INP is attached to the surface of the cell membrane via a glycosyl-phosphatidylinositol (GPI) anchor, which is unique in prokaryotes since previously only eukaryotic proteins were known to posses GPI anchors[4,8,9]. Unlike the eukaryotic GPI anchor however, the N- and C- termini of the INP protein are free. This enable foreign proteins to be fused to either or both of these regions and be displayed on the surface of the host cell[10-12].

This feature of INP would be useful in the development of live multivalent bacterial vaccines. In this study a gene synthesis method developed by Stemmer *et al.*[13], a variant of the Polymerase Chain Reaction (PCR) technique, was used to construct a synthetic *Inak* gene of the N-terminus of the ice nucleation protein of *Pseudomonas syringae* to be used as a surface display system. This method, also known as assembly PCR, involves generating overlapping oligonucleotides which, when assembled, form the template for the gene of interest. The oligonucleotides are then repetitively extended by PCR, to assemble the full-length gene in a single step. Assembly PCR was used in this work to enable the construction of the gene containing the combined preferred codon usage frequencies of *E. coli* and *S. typhi* which are to be used as the hosts for expression in this (*E. coli*) and future (*S. typhi*) studies.

MATERIALS AND METHODS

Assembly PCR: Gene synthesis by assembly PCR consists of four stages: oligonucleotide design and synthesis, gene assembly, gene amplification and cloning. The strategy of assembly and amplification is summarized in Fig. 1.

The ice nucleation protein N-terminus gene, designated as *Inak-n* (633 bp in length) was designed

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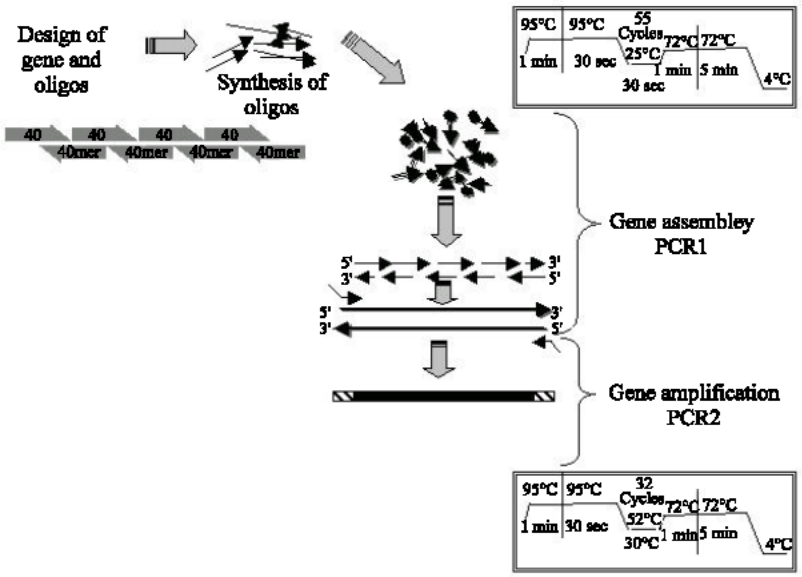

Fig. 1: The strategy for synthesis of the synthetic *Inak*-n gene by assembly PCR. The gene was assembled from a total of 34 oligonucleotides

Table 1: Dilutions of the oligo mixture used in assembly PCR

Code	Dilution (v:v)	Concentration of mixture (μM)	Final concentration in PCR reaction mixture (μM)
D_0	0	250	10
D_1	1:5	50	2
D_2	1:10	25	1

based on the amino acid sequence of the corresponding wild type *Inak* gene (GenBank accession number: AF013159). Thirty four short oligonucleotides (maximum 40 mer) were designed (17 for each upper and lower strands which completely cover the whole sequence) using the OLIGO 5 (National Bioscience) and MacDNAsis Ver. 3.2 (Hitachi Software Engineering Co. Ltd) softwares in such a way that complementary oligonucleotides will overlap by about 20 nucleotides.

A master mix of oligos was prepared containing a final concentration of 250 μM oligos in sterile Milli-Q® water with each oligo at equimolar ratio. The neat (D_0) or diluted (D_1 and D_2, Table 1) oligo mixture were used as templates in the assembly PCR procedure.

Gene assembly was carried out as described by Stemmer *et al*.[13] with some modifications: 1 μL of an oligo mixture was added to 25 μL PCR reaction mixture, 2.5 μL (10X) PCR buffer; 2.5 μL (2 mM) dNTP mixture; 4 mL (25 mM) $MgCl_2$; 0.2 μL of 5 U μL^{-1} of Taq DNA polymerase, sterile water to 25 μL and subjected to the assembly reaction. Parameters for the assembly reaction using the GeneAmp PCR system 9700 were as follows: 1 cycle at 95°C for 1 min; 55 cycles at 95°C for 30 sec., 52°C for 30 sec and 72°C for 1 min and 1 cycle at 72°C for 5 min to complete the elongation of PCR products.

In the amplification stage of the process a pair of specific primers (forward primer, 5'-CCTGGAATTCATGACTCTCGACAAG-3';reverseprimer, 5'-GACAGGATCCAATTAGATCACTGTG-3') wereused to amplify the correct construct from the mixture of DNA fragments produced in the first assembly stage. The recognition sequences for *Eco*RI and *Bam*HI restriction enzyme were incorporated into the forward and reverse primers, respectively. The PCR reaction was carried out using 5 μL of the PCR product from the assembly stage as template in a total volume of 50 μL and containing the following components: 4 U of Expand High Fidelity PCR enzyme mix (Roche), 0.5 μM of each primer, 4 mM of $MgCl_2$ and 200 μM dNTP mixture. An annealing temperature of 52°C was used with 32 cycles of PCR. The denaturing and elongation temperatures as well as the holding times at each temperature were the same as that described for the assembly stage PCR.

Cloning and sequencing: Two microliter of the PCR product was cloned into pCR®2.1-TOPO® vector (Invitrogen, USA), according to the manufacture's instructions (Fig. 2A). The cloning mixture was then used to transform *E. coli* Top 10 competent cells as described by Sambrook and Russell[14] and selected on LB plates containing 100 mg μL^{-1} ampicillin and X-gal. White transformants were screened by PCR (using the same PCR primers mentioned earlier) to determine the presence of the insert. Briefly, each selected white transformant was touched gently with a sterile, fresh toothpick or yellow

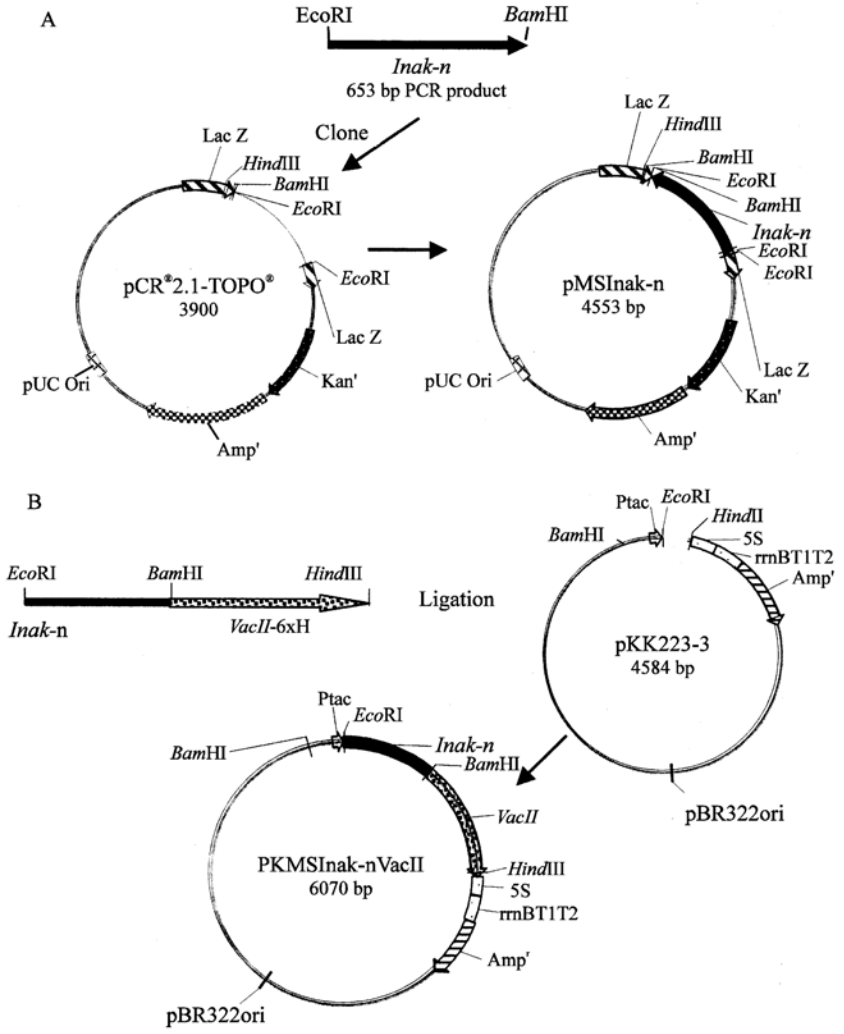

Fig. 2: Cloning and construction of the expression plasmid. Panel A shows cloning of *Inak*-n PCR product into PCR® 2.1- TOPO® to create plasmid pMSInak-n. Panel B shows the construction of the expression plasmid pKMSInak-nVacII

pipette tip. The toothpick or pipette tip was then touched onto a fresh agar plate (in duplicate) containing 100 mg μL^{-1} ampicillin and incubated overnight at 37°C. The same toothpick or pipette tip was also dipped into a PCR reaction mixture for a few seconds. Amplification was performed using 1 mM of each primer and the same PCR parameters as described above and plasmids were isolated using the Mini-Prep Plasmid Isolation Kit (Qiagen) from transformants which gave positive results by PCR. To confirm the presence of the insert, approximately 500 ng of the recombinant plasmid DNA were digested using *Eco*RI and *Eco*RI and *Bam*HI, respectively. A recombinant plasmid showing the expected results was selected, designated as pMSInak-n and the insert was sequenced.

Site-directed mutagenesis: A mutation introduced during the assembly and amplification reactions were corrected by PCR-based site-directed mutagenesis using the QuikChange® XL Site Directed Mutagenesis Kit (Stratagene). One microliter of plasmid pMSInak-n (approximately 100 ng μL^{-1}) was added to a PCR mixture containing 5 µL of 10x *Pfu* DNA polymerase buffer, 8 µL of 4 mM $MgCl_2$, 2.5 µL of each 1 µM of mutant forward and mutant reverse primers, respectively and 1 µL of 12.5 mM dNTPs. The reaction mixture was made up to a final volume of 50 µL with sterile Milli-Q® water. A hot start step was performed at 94°C for 4 min followed by the addition of 1 µL of 2.5 U μL^{-1} *Pfu* DNA polymerase. PCR was performed using the following conditions: segment 1: 1 cycle at 94°C for 1 min, segment 2: 12 cycles of 94°C for

1 min, 55°C for 2 min, 72°C for 4 min, segment 3: 1 cycle at 72°C for 10 min. A low number of cycles was used to reduce the chances of random mutations occurring during the reaction. The PCR product was incubated for 2 min at 37°C.

To remove the parental plasmid, the PCR products were treated with 1 μL of *Dpn*I (10 U μL^{-1}) and an additional 0.5 μL of *Pfu* DNA polymerase was added to the reaction mixture. The mixture was then incubated at 37°C for 45 min which results in the digestion of the parental plasmid DNA, followed by incubating at 72°C for an additional 35 min to allow completion of synthesis. Aliquots of the mixture were then used to transform competent *E. coli* Top 10 cells and several transformants were selected. Plasmids were extracted from selected transformants and their inserts were sequenced.

Expression in *E. coli*: The *Inak*-n synthetic gene was excised from pMSInak-n by digestion with *Eco*RI and *Bam*HI. A previously constructed synthetic mycobacterial gene called VacII containing a *Bam*HI and a *Hin*dIII restriction site at the 5' and 3' end, respectively, was then ligated to *Inak*-n. This procedure resulted in the fusion of VacII downstream of the *Inak*-n gene. VacII also contained a 6 x histidine (6xHis) motif to facilitate the detection of the fusion protein. The *Inak*-n::VacII fusion gene was then cloned downstream of the *tac* promoter in the pKK223-3 expression vector (Fig. 2B). The recombinant plasmid was designated as pKMSInak-nVacII.

Growth and induction of expression were carried out as described by Lee *et al.*[12] as follows: A single colony of *E. coli* XL1-Blue carrying the plasmid pKMSInak-nVacII, designated as r-*E. coli* XB, was inoculated into 10 mL LB media containing 100 mg μL^{-1} ampicillin and incubated overnight at 37°C with shaking at 200 rpm. Five hundred microliter each of the overnight culture was used to seed culture tubes containing pre-warmed 10 mL LB medium (with 100 mg μL^{-1} ampicillin) which was then incubated with shaking (200 rpm) until the cultures reached an OD_{600} of 0.6. Expression of the fusion protein was induced by the addition of 0.5 or 1 mM of IPTG into the culture tubes and incubation was continued. One millilitre samples were then collected from each tube at various time intervals (2, 4 and 6 h) after the addition of IPTG. *E. coli* XL1-Blue transformed with non-recombinant pKK223-3 was used as negative control and processed as described above.

Western blotting: Proteins were prepared and fractionated by SDS-PAGE on 12% polyacrylamide gels according to the method of Laemmli[15] and were transferred to nitrocellulose membranes (Hybond™-C extra nitrocellulose membrane; Amersham) in transfer buffer containing 48 mM Tris-base (pH 8.3), 39 mM glycine, 20% (v/v) methanol. The membranes were blocked with 3% (w/v) skimmed milk in Tris-buffered saline containing 0.05% Tween 20. The blocked membranes were probed with Ni-NTA^{AP} conjugate (Qiagen) and detected with the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) according to the manufacturer's instructions.

RESULTS

Construction of the *Inak*-n synthetic gene by assembly PCR: The products of this assembly step were analyzed using a 0.8% agarose gel electrophoresis. The results show the expected smear when D_1 and D_2 were used as templates which spanned the regions around the expected size of the synthetic gene of 653 bp (633 bp of the gene plus restriction sites and other flanking sequence) (Fig. 3, lane 3 and 4, respectively), whereas the use of D_0 as template resulted in a thick smear at the bottom of the gel (Fig. 3, lane 2). D_1 gave the best result as the smear centered on the required size of 653 bp.

The PCR product obtained at the assembly step using oligo mixture D_1 was chosen as template for the amplification process using the cloning primers. The results of the PCR were analyzed by gel electrophoresis using a 0.8% agarose gel which revealed the presence of the expected 653 bp band of the synthetic *Inak*-n gene (Fig. 4, lane 2 and 3). This gene which contains the N-terminus of the ice nucleation protein was then cloned into the pCR®2.1-TOPO® vector.

Plasmids from transformants which gave the expected product by PCR screening were also analysed

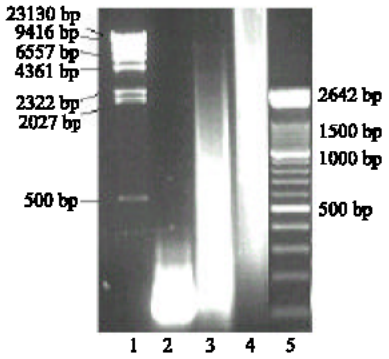

Fig. 3: Analytical agarose gel electrophoresis of assembly PCR. Lane 1: λ DNA *Hin*dIII marker. Lane 2: D_0 as template. Lane 3: D_1 as template. Lane 4: D_2 as template. Lane 5: 100 bp DNA ladder

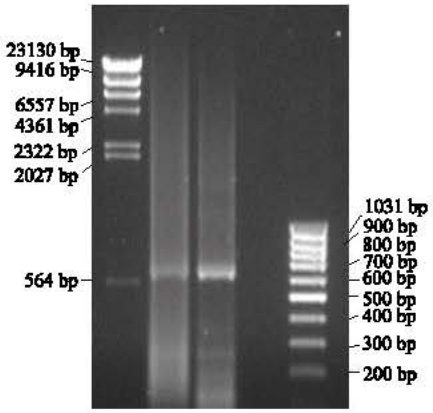

Fig. 4: Analytical agarose gel electrophoresis of the product of the second PCR reaction (amplification step). Lane 1: λ DNA *Hin*dIII marker. Lanes 2 and 3: Product of PCR using different concentrations of template. Lane 4: control (no DNA template). Lane 5: 100 bp DNA ladder

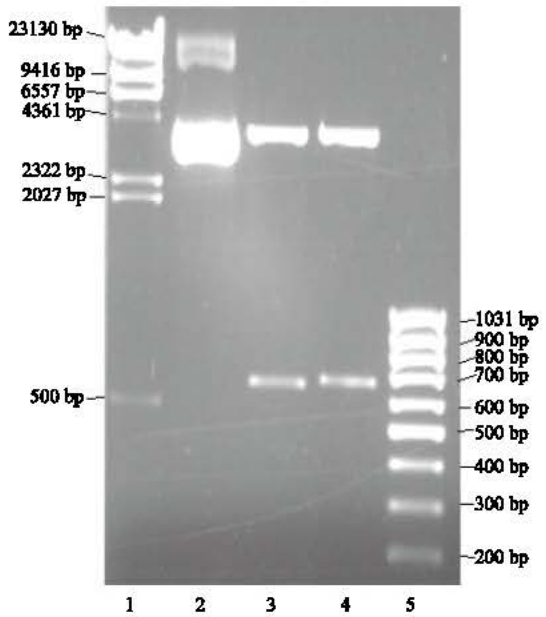

Fig. 5: Restriction enzyme digestion of plasmid pMSInak-n. Lane 1: λ DNA *Hin*dIII marker. Lane 2: Uncut plasmid. Lane 3: Plasmid digested with *Eco*RI and *Bam*HI. Lane 4: Plasmid digested with *Eco*RI. Lane 5: 100 bp DNA ladder

by restriction enzyme digestion. Agarose gel electrophoresis of a recombinant plasmid digested with *Eco*RI and *Bam*HI (double digest) and *Eco*RI (single digest) showed the presence of inserts with the expected sizes of 653 bp (Fig. 5, lane 3) and 690 bp (Fig. 5, lane 4), respectively. This clone was designated as pMSInak-n and chosen for further studies.

To confirm the success of the cloning procedure, the insert in pMSInak-n was sequenced (BioBasic Inc., Canada) using M13 forward and reverse primers. Sequence analysis of the synthetic gene showed that two types of mutations have occurred during construction of the gene; a single base (G) addition between position 293 and 294 (with reference to the designed sequence) and a T to C single base change at position 622 (Fig. 6). The first mutation will result in a frame shift which must be repaired whereas the second is silent which will not be repaired. Sequencing of three different clones identified different mutations distributed randomly, suggesting that the oligos were not the source of these errors and were most probably introduced during the assembly PCR step.

Site-directed mutagenesis: To overcome the problem of the insertional mutation, site-directed mutagenesis was performed to remove the additional G base. After this procedure, plasmid DNA was extracted from transformants and the presence of the *Inak*-n gene was determined by PCR and digestion with *Eco*RI, both of which gave the expected results (results not shown). Sequence analysis of the repaired sequence confirmed the successful removal of the additional base (Fig. 6).

Creation of pKMSInak-nVacII and expression of the fused gene in *E. coli*: The synthetic *Inak*-n and VacII genes were successfully used to create the expression plasmid pKMSInak-nVacII. Western blotting of an *E. coli* clone carrying pKMSInak-nVacII showed the expression of the *Inak*-n::VacII fusion protein with the presence of a band of the expected size of approximately 52 kDa (Fig. 7, lane 4-8).

DISCUSSION

In this study the technique of assembly PCR was used for the construction of a 653 bp synthetic gene encoding the N-terminus of INP (*Inak*-n) of *P. syringae*. Assembly PCR is rapid and relatively simple allowing synthesis of a gene in a single step, followed by amplification of the gene using specific primers. Genes of up to 4 kb in size have been successfully synthesized using this method[13,16-18]. This method also allows the synthetic gene to be designed with the codon usage preferred by the host bacteria which may improve expression[19].

Although the initial step of gene assembly resulted in a range of products which appeared as a smear as shown by gel electrophoresis, the final gene amplification

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Designed    CCTGGAATTCATGACTCTCGACAAGGCGTTGGTGCTGCGTACCTGTGCAAATAACATGGC 60
Assembled   CCTGGAATTCATGACTCTCGACAAGGCGTTGGTGCTGCGTACCTGTGCAAATAACATGGC
Repaired    CCTGGAATTCATGACTCTCGACAAGGCGTTGGTGCTGCGTACCTGTGCAAATAACATGGC

Designed    CGATCACTGCGGCCTTATATGGCCCGCGTCCGGCACGGTGGAATCCAGATACTGGCAGTC 120
Assembled   CGATCACTGCGGCCTTATATGGCCCGCGTCCGGCACGGTGGAATCCAGATACTGGCAGTC
Repaired    CGATCACTGCGGCCTTATATGGCCCGCGTCCGGCACGGTGGAATCCAGATACTGGCAGTC

Designed    AACCAGGCGGCATGAGAATGGTCTGGTCGGTTTACTGTGGGGCGCTGGAACCAGCGCTTT 180
Assembled   AACCAGGCGGCATGAGAATGGTCTGGTCGGTTTACTGTGGGGCGCTGGAACCAGCGCTTT
Repaired    AACCAGGCGGCATGAGAATGGTCTGGTCGGTTTACTGTGGGGCGCTGGAACCAGCGCTTT

Designed    TCTAAGCGTGCATGCCGATGCTCGATGGATTGTCTGTGAAGTTGCCGTTGCAGACATCAT 240
Assembled   TCTAAGCGTGCATGCCGATGCTCGATGGATTGTCTGTGAAGTTGCCGTTGCAGACATCAT
Repaired    TCTAAGCGTGCATGCCGATGCTCGATGGATTGTCTGTGAAGTTGCCGTTGCAGACATCAT

Designed    CAGTCTGGAAGAGCCGGGAATGGTCAAGTTTCCGCGGGCCGAGGTGGTTCATG-TCGGCG 299
Assembled   CAGTCTGGAAGAGCCGGGAATGGTCAAGTTTCCGCGGGCCGAGGTGGTTCATGGTCGGCG
Repaired    CAGTCTGGAAGAGCCGGGAATGGTCAAGTTTCCGCGGGCCGAGGTGGTTCATG↑TCGGCG

Designed    ACAGGATCAGCGCGTCACACTTCATTTCGGCACGTCAGGCCGACCCTGCGTCAACGTCAA 359
Assembled   ACAGGATCAGCGCGTCACACTTCATTTCGGCACGTCAGGCCGACCCTGCGTCAACGTCAA
Repaired    ACAGGATCAGCGCGTCACACTTCATTTCGGCACGTCAGGCCGACCCTGCGTCAACGTCAA

Designed    CGTCAACGTCAACGTCAACGTTAACGCCAATGCCTACGGCCATACCCACGCCCATGCCTG 419
Assembled   CGTCAACGTCAACGTCAACGTTAACGCCAATGCCTACGGCCATACCCACGCCCATGCCTG
Repaired    CGTCAACGTCAACGTCAACGTTAACGCCAATGCCTACGGCCATACCCACGCCCATGCCTG

Designed    CGGTAGCAAGTGTCACGTTACCGGTGGCCGAACAGGCCCGTCATGAAGTGTTCGATGTCG 479
Assembled   CGGTAGCAAGTGTCACGTTACCGGTGGCCGAACAGGCCCGTCATGAAGTGTTCGATGTCG
Repaired    CGGTAGCAAGTGTCACGTTACCGGTGGCCGAACAGGCCCGTCATGAAGTGTTCGATGTCG

Designed    CGTCGGTCAGCGCGGCTGCCGCCCCAGTAAACACCCTGCCGGTGACGACGCCGCAGAATT 539
Assembled   CGTCGGTCAGCGCGGCTGCCGCCCCAGTAAACACCCTGCCGGTGACGACGCCGCAGAATT
Repaired    CGTCGGTCAGCGCGGCTGCCGCCCCAGTAAACACCCTGCCGGTGACGACGCCGCAGAATT

Designed    TGCAGACCGCCACTTACGGCAGCACGTTGAGTGGCGACAATCACAGTCGTCTGATTGCCG 599
Assembled   TGCAGACCGCCACTTACGGCAGCACGTTGAGTGGCGACAATCACAGTCGTCTGATTGCCG
Repaired    TGCAGACCGCCACTTACGGCAGCACGTTGAGTGGCGACAATCACAGTCGTCTGATTGCCG

Designed    GTTATGGCAGTAACGAGACCGCTGGCAACCACAGTGATCTAATTGGATCCTGTC 653
Assembled   GTTATGGCAGTAACGAGACCGCCGGCAACCACAGTGATCTAATTGGATCCTGTC
Repaired    GTTATGGCAGTAACGAGACCGCCGGCAACCACAGTGATCTAATTGGATCCTGTC
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Fig. 6: Multiple alignment of the designed *Inak*-n gene sequence (upper strand) with the assembled sequences before (middle strand) and after site-directed mutagenesis (lower strand). The arrow shows the repair site

reaction was able to select the correct fragment and gave rise to a single band of the correct size as shown by gel electrophoresis.

While this method is simple in principle, in practice numerous complications can lead to problems in the synthesis. One potential problem of this technique is the occurrence of base errors during synthesis. The frequency of nucleotide errors depends either on the quality of the oligonucleotides, or the fidelity of the polymerase. Stemmer *et al.*[13] found three point mutations when synthesizing the b-Lactamase gene (0.9 kb), even when both *Taq* and *Pfu* DNA polymerases were used to ensure yield and fidelity through the proofreading 3'-5' exonuclease activity of *Pfu.* In another example, Withers-Martinez *et al.*[17] observed nine point mutations during the synthesis of a 2.1 kb gene. Mutations were also found in our synthesized gene although the Expand High Fidelity system was used to reduce the introduction of base errors in the PCR products. These mutations were likely to have been introduced randomly during gene synthesis and not from errors in the oligos themselves because sequence analysis of the synthetic gene from different clones showed different mutations at different sites.

These errors could be overcome by screening large number of transformants for a clone with an error-free insert but this would be time and labour consuming. In this study several clones were analyzed but all showed varying numbers of errors. A clone with the fewest number of errors was chosen for repair by PCR-based site-directed mutagenesis since it requires only simple *in vitro* manipulation of the entire plasmid.

Expression of the synthetic gene in *E. coli* XL1-Blue was studied using Western blot and Ni-NTA^{AP} conjugate

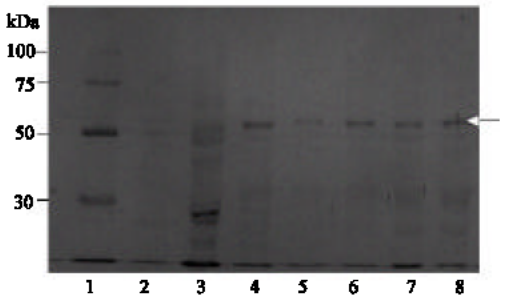

Fig. 7: Western blot analysis of *Inak*-n::*VacII* protein expression in *E. coli* XL1-Blue 2, 4 or 6 hrs after induction with 0.5 mM or 1.0 mM of IPTG. Lane 1: 6xHis molecular weight ladder. Lane 2: *E. coli* XL1-Blue carrying non-recombinant pKK223-3 (0.5 mM IPTG/2 h). Lane 3: r-*E. coli* XB (0.5 mM IPTG/2 h). Lane 4: r-*E. coli* XB (1.0 mM IPTG/2 h). Lane 5: r-*E. coli* XB (0.5 mM IPTG/4 h). Lane 6: r-*E. coli* XB (1.0 mM IPTG/4 h). Lane 7: r-*E. coli* XB (0.5 mM IPTG/6 h). Lane 8: r-*E. coli* XB (1.0 mM IPTG/6 h). The membrane was probed with Ni-NTA^{AP} conjugate (Qiagen)

to detect the 6xHis motif in the *Inak*-n:*VacII* fusion protein. The results show successful expression of this synthetic gene as shown in Fig. 7.

Work is now underway to study the expression of this synthetic gene in the typhoid vaccine strain *S. typhi* Ty21a as a second host system with the objective of developing this organism as a potential live vaccine for tuberculosis and various other diseases.

REFERENCES

1. Wolber, P.K., C.A. Deininger, M.W. Southworth, J. Vandekerckhove, M. van Montagu and G.J. Warren, 1986. Identification and purification of a bacterial ice nucleation protein. Proc. Natl. Acad. Sci. USA., 83: 7256-7260.
2. Turner, M.A., F. Arellano and L.M. Kozloff, 1991. Components of ice nucleation structures of bacteria. J. Bacteriol., 173: 6515-6527.
3. von Heijne, G., 1984. Analysis of the distribution of charged residues in the N-terminal region of signal sequences: Implications for protein export in prokaryotic and eukaryotic cells. EMBO. J., 3: 2315-2318.
4. Kozloff, L.M., M.A. Turner and F. Arellano, 1991. Formation of bacterial membrane ice-nucleating lipoglycoprotein complexes. J. Bacteriol., 173: 6528-6536.
5. Nemecek-Marshall, M., R. LaDuca and R. Fall, 1993. High-level expression of ice nuclei in a *Pseudomonas syringae* strain is induced by nutrient limitation and low temperature. J. Bacteriol., 175: 4062-4070.
6. Wolber, P.K., 1993. Bacterial ice nucleation. Adv. Microb. Physiol., 34: 203-237.
7. Kawahara, H., 2002. The structure and function of ice crystal-controlling proteins from bacteria. J. Biosci. Bioeng., 94: 492-496.
8. Ferguson, M.A. and A.F. Williams, 1988. Cell surface anchoring of protein via glycosyl-phosphatidylinositol structures. Annu. Rev. Biochem., 57: 285-320.
9. Cornelis, P., 2000. Expression genes in different *Escherichia coli* compartments. Curr. Opin. Biotech., 11: 450-454.
10. Jung, H.C., J.H. Park, S.H. Park, J.M. Lebeault, J.G. Pan, 1998. Expression of carboxymethylcellulase on the surface of *Escherichia coli* using *Pseudomonas syringae* ice nucleation protein. Enzyme Microb. Technol., 22: 348-354.
11. Kim, E.J. and S.K. Yoo, 1998. Cell surface display of CD8 ecto domain on *Escherichia coli* using ice nucleation protein. Biotechnol. Technol., 12: 197-201.
12. Lee, J.S., K.S. Shin, J.G. Pan and C.J. Kim, 2000. Surface-displayed viral antigens on *Salmonella* carrier vaccine. Natl. Biotechnol., 18: 645-648.
13. Stemmer, W.P., A. Crameri, K.D. Ha, T.M. Brennan and H.L. Heyneker, 1995. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. Gene, 164: 49-53.
14. Sambrook, J. and D.W. Russel, 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.
15. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
16. Baedeker, M. and G.E. Schulz, 1999. Overexpression of a designed 2.2 Kb gene of eukaryotic phenylalanine ammonia-lyase in *Escherichia coli*. FEBS lett., 457: 57-60.
17. Withers-Martinez, C., E.P. Carpenter, F. Hackett, B. Ely, M. Sajid, M. Grainger and M.J. Blackman, 1999. PCR-based gene synthesis as an efficient approach for expression of the A+T-rich malaria genome. Protein Eng., 12: 1113-1120.
18. Lin, Y., G. Cheng, X. Wang and T.G. Clark, 2002. The use of synthetic genes for the expression of ciliated proteins in heterologous system. Gene, 288: 85-94.
19. Norazmi, M.N., Z.F. Zainuddin, R. Suppian and J.W. Dale, 1999. The use of assembly polymerase chain reaction for cloning of a malarial epitope into *Mycobacterium smegmais* importance of overcoming codon bias. Biotechnol.Technol., 13: 485-489.