

CHAPTER 3

Construction of ArgRWT- and ArgRNV- biotinylated peptide fusion protein

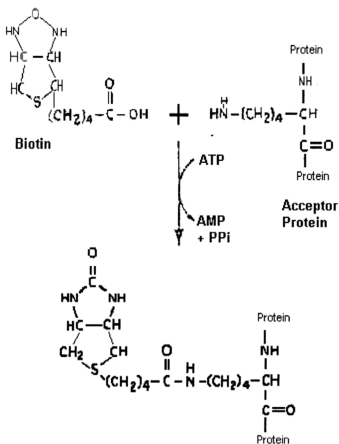
CHAPTER 3

Construction of ArgRWT- and ArgRNV-biotinylated peptide fusion protein

3.1 Introduction

This chapter describes the construction of fusion genes that will produce chimeric protein of ArgR. Full length ArgRWT and full length ArgRNV were separately linked to a biotinylated peptide containing 126 amino acids residues (approximately 13 kDa). The biotinylated peptide was separately fused to the N-terminus of ArgRWT and ArgRNV. The fusion protein derivatives of ArgRWT and ArgRNV produced elongated hexameric ArgR proteins with an approximate size of 180 kDa (30 kDa x 6).

The biotinylated peptide is part of the PinPoint™ Xa-3 plasmid DNA segment (Figure 2.1, Chapter 2). The peptide carries a single biotin molecule on a lysine residue. Biotin (Figure 3.1) is a coenzyme (vitamin H) synthesized by plants, most bacteria, and some fungi which occurs primarily in a protein-bound state within the cells. Biotin is bound to specific proteins via an amide linkage between the biotin carboxyl group and a lysine amino group (Figure 3.1; Cronan, 1990; Samols *et al.*, 1988). This biotin moiety is accessible to two proteins that strongly bind biotin which are avidin and streptavidin: resin containing either molecule will capture biotinylated proteins, thus facilitating purification of the proteins such as recombinant fusion proteins. *E. coli* produce a single endogenous biotinylated peptide protein, the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase, while other bacteria contain one to three biotinylated peptide



Structure of Protein linked to Biotin



Figure 3.1. Biotin, biotinylated protein, and the biotin ligase reaction. Biotin is attached to acceptor proteins by biotin ligase in a two step reaction given in the lower part of the figure. Biotin is first converted to biotinoyl-AMP which then reacts with the amino group of a specific lysine residue of the acceptor protein. The biotinoyl lysine moiety is called biocytin (Cronan, 1990).

proteins (Fall, 1979). *E. coli* BCCP in its native confirmation does not bind to avidin, rendering the downstream affinity purification highly specific for the recombinant fusion protein.

3.2 Preparation of cloning vector and DNA insert

Construction of recombinant DNA was accomplished by subcloning the gene of interest into the cloning vector to create an in-frame fusion (the C-terminus of the biotinylated peptide with the N-terminus of *argRWT* and *argRNV*, separately). Plasmids pAM204 and pAM401, *argRWT* and *argRNV*, respectively (Merican, 1995) were used as the source of the DNA insert. It is important to note that plasmids pAM204 and pAM401 which contain the *argRWT* gene and the *argRNV* gene, respectively, had the amino terminus of ArgRWT and ArgRNV modified by site directed mutagenesis which introduced a *Bam*HI site (see Figure 3.7; Merican, 1995). The mutagenesis resulted in the substitution of the arginine residue at position two to glycine (R2 to G) [ArgR sequence: ATG (met) *GGA* (Gly) *TCC* (Ser) in pAM204]. The *argR* gene containing the base substitution (*argRWTR2G*, *argRNVTR2G*) produces a phenotypically ArgR⁺ protein in *in vivo* *cer*-mediated recombination assay (Merican, 1995).

3.2.1 Isolation of plasmid DNA

Plasmids PinPoint™ Xa-3 (cloning vector), pAM204 and pAM401 were isolated from DS956 using alkaline lysis method in small scale, medium scale, and large scale preparation. The isolated plasmids were electrophoresed on a

0.8% agarose gel to confirm their size (Figure 3.2). Since DS956 lacks the wild-type *argR* gene, plasmid multimerization were observed (Figures 3.2 and 3.3).

During this study, I noticed that plasmids pAM204 and pAM401 tend to be lost during storage. This may be because plasmid multimers accumulate clonally within the culture, creating a subpopulation of cells with a significantly increased rate of plasmid loss and leading to copy number depression (Summers, 1998). Furthermore, from my observation, plasmid pAM204 was always isolated in a much higher amount than pAM401.

3.2.2 Purification of plasmid DNA

Isolation of plasmids using CsCl-EtBr density gradient centrifugation was used to obtain high purity plasmid DNAs. By using CsCl-EtBr density gradient centrifugation method, most unwanted substances like chromosomal DNA, linear plasmid DNA, protein, and RNA will be separated from circular plasmid DNA (Sambrook *et al.*, 1989). Thus, the circular plasmid DNA can be recovered easily and with high purity. The concentration and purity of the plasmids are summarized in Table 3.1. Agarose gel electrophoresis of purified plasmids showed that most contaminants (especially RNAs), had been removed from the circular plasmid DNAs (Figure 3.4). It is very important to have pure plasmid DNAs to prevent contamination of the plasmids from unwanted substances, that can inhibit the enzymatic reactions such as restriction endonucleases digestion and ligation of DNA fragments.

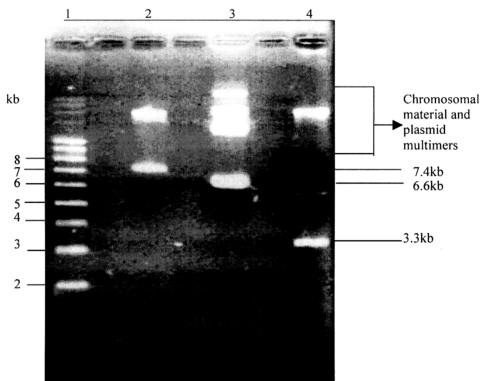


Figure 3.2. Isolation of plasmid DNA using alkaline lysis method. Lane 1: 2-10 kb supercoiled DNA size marker (the size of supercoiled markers are indicated on the left). Lanes 2, 3 and 4: plasmids pAM401, pAM204, and PinPoint™ Xa-3, respectively. The sizes of the plasmids are indicated on the right.

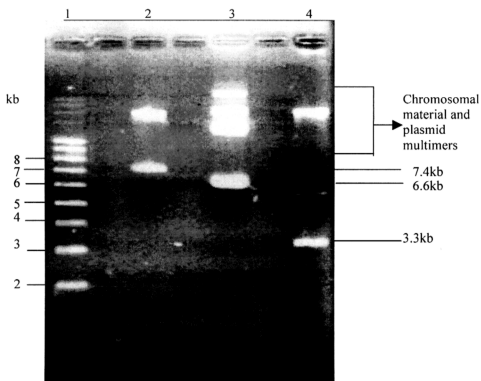


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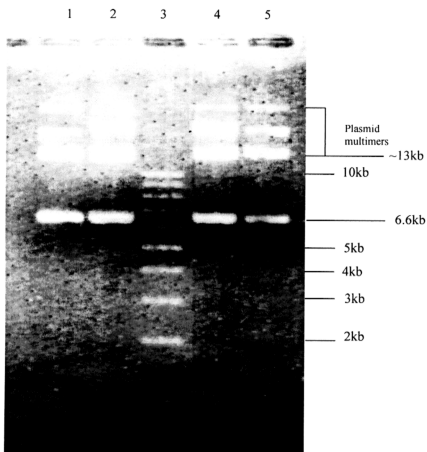


Figure 3.3. Multimerization of multicopy plasmid. All lanes (except lane 3): Plasmid pAM204. Plasmid pAM204 (6.6kb) exhibited plasmid multimerization. Lane 3: 2-10 kb supercoiled DNA size marker (the size of supercoiled markers are indicated on the right).

Table 3.1. Concentration and purity of plasmid DNAs used in this study.

| No | Plasmid DNA | DNA concentration (OD ₂₆₀) (µg/ml) | DNA purity (OD ₂₈₀ /OD ₂₆₀) |
|----|----------------|---|---|
| 1 | PinPoint™ Xa-3 | 2543.33 | 0.72 |
| 2 | pAM401 | 976.67 | 0.36 |
| 3 | pAM204 | 9590.30 | 0.52 |

Optical density (OD) of the DNA solution was measured at 260 nm, 230 nm, and 280 nm using a Beckman DU 7500 UV/VIS spectrophotometer.

Note : Pure preparations of DNA have OD₂₈₀/ OD₂₆₀ value of 0.8.

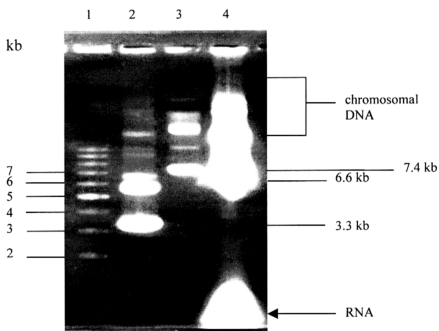


Figure 3.4. Purification of plasmid DNA using CsCl-EtBr density gradient centrifugation. Lane 1: 2-10kb supercoiled DNA size marker. Lanes 2 and 3: purified plasmids (PinPoint™ Xa-3, 3.3 kb and pAM401, 7.4 kb) using CsCl-EtBr density gradient. Contaminants mostly RNA were removed from plasmid DNA through CsCl-EtBr density gradient. High purity of plasmid DNA is absolutely necessary for some DNA analysis and manipulation such as DNA sequencing and restriction endonuclease. Lane 4: pAM204 (6.6 kb). The contaminants such as chromosomal DNA and RNA still remain in the DNA solution as indicated.

3.2.3 Digestions of plasmid DNA

Plasmid PinPoint Xa-3 was digested with *Bam*HI restriction endonuclease. Calf intestinal alkaline phosphatase (CIAP) treatment was carried out to prevent recircularization of linearized plasmid vector by removing the 5' terminal phosphate groups. Both plasmids containing the genes of interest, pAM204 and pAM401 were also digested with *Bam*HI. All *Bam*HI-digested plasmids were electrophoresed on a 0.8% agarose gel (Figure 3.5).

Plasmids PinPoint™ Xa-3 Vector has a single *Bam*HI restriction site at position 404 bp, producing linearized plasmid fragment with the size of 3.3 kb (Figure 3.5). Plasmid pAM204 with the size of 6.6 kb has two *Bam*HI restriction sites. Therefore, when the plasmid was digested with *Bam*HI, two DNA fragments were obtained; a small fragment of 0.8 kb and a large fragment of 5.8 kb. Plasmid pAM401 with the size 7.7 kb also has two *Bam*HI restriction sites, producing a small DNA fragment of 0.8 kb and a large DNA fragment of 6.9 kb upon digestion with *Bam*HI (Figure 3.5). The 0.8 kb DNA fragment contained the *argRWT* or *argRNV* gene. The digested fragments of interest for both insert and vector were recovered using the NucleoSpin® Extraction Kit (Clontech) and were electrophoresed on a 0.8% agarose gel. Figure 3.6 showed the recovery of DNA from agarose gel using the kit.

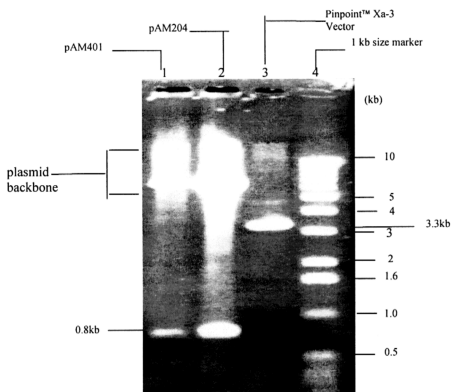


Figure 3.5. Fragments of *Bam*HI-digested plasmids DNA. Lanes 1 and 2: *Bam*HI-digested fragment of plasmids pAM204 and pAM401 released a 0.8kb DNA fragment containing the *argRWT* and *argRNV* genes, respectively. Lane 3: *Bam*HI-digested fragment of plasmid Pinpoint™ Xa-3 Vector (3.3 kb linear plasmid). Lane 4: 1 kb DNA ladder (lane 4) with the corresponding sizes are indicated on the right.

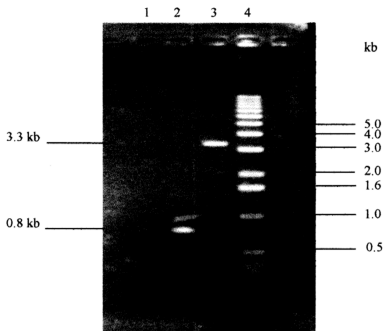


Figure 3.6. Purification of *Bam*HI-digested plasmids DNA fragments from agarose gel. Purification of digested plasmids from agarose gel using NucleoSpin® purification kit (Clontech). Lanes 1 and 2: The 0.8 kb *Bam*HI-digested fragment of pAM401 and pAM204. *Bam*HI-digested fragment of plasmid Pinpoint™ Xa-3 Vector (lane 3) in the linear form (3.3kb). 1 kb DNA ladder (lane 4) with the corresponding sizes are indicated on the right. The 0.8 kb *Bam*HI-digested fragment of pAM401 is not clearly visible because of the low concentration of the plasmid.

3.3 Construction of ArgRWT-biotinylated peptide fusion protein

The plasmid pAM204 used in this study is a derivative of pSelect-1 (Promega) carrying ampicillin and tetracycline resistant markers (Merican, 1995). Circular plasmid map of pAM204 is shown in Figure 3.7.

3.3.1 Subcloning of *argRWT* gene into PinPoint Xa-3 cloning vector

The 0.8 kb *Bam*HI-digested DNA fragment of plasmid pAM204 containing *argRWT* gene was subcloned into the *Bam*HI-digested plasmid PinPoint™ Vector Xa-3 containing the biotinylated peptide DNA sequence. The ligation reaction was incubated at 16°C overnight. The ligated DNA fragments were introduced into *E. coli* cells through CaCl₂ transformation to allow the DNA to replicate. The transformed cells were grown on LB (+ ampicillin) agar to select for recombinant cells from non-recombinant cells.

3.3.2 Analysis of *argRWT*-biotinylated peptide fusion recombinant DNA

Transformants were patched onto LB agar plates containing ampicillin (50µg/ml final concentration) and were screened for recombinant DNA using single colony lysis technique (SCFSB method) or phenol-chloroform-isopropanol extraction method (Figure 3.8). Figure 3.8 shows that two different types of recombinant plasmids containing *argRWT* gene have been constructed (due to the orientation of the *argRWT* gene). The 0.8 kb DNA fragment containing the *argRWT* gene can ligate to the *Bam*HI-digested PinPoint™ Xa-3 in two different orientations. The two types of recombinant plasmids have the same size, which is 4.1 kb. It can also be observed that the intensity of the recombinant DNA bands

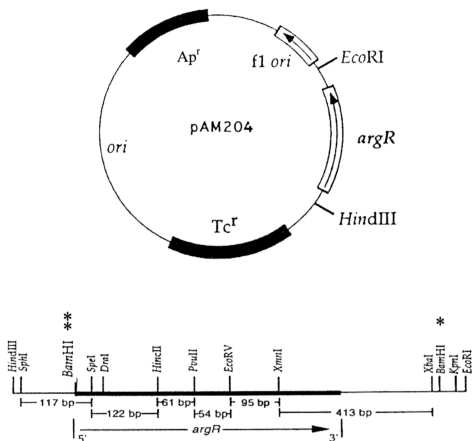


Figure 3.7. Map of plasmid pAM204 containing *argRWT* gene.
Upper Panel: Circle map of pAM204, a derivative of plasmid pSelect-1 (Promega) carrying *Ap^r* and *Tc^r* resistance markers. The *argRWT* gene is located between *EcoRI* and *HindIII* restriction sites. **Lower Panel:** Linear map of plasmid pAM204. The *argRWT* gene is indicated by the thick black line (adopted from Merican, 1995). Asterisks (*) indicate the two *BamHI* restriction site on pAM204. Double asterisks (**) indicates the *BamHI* restriction site that was introduced by site directed mutagenesis (Merican, 1995).

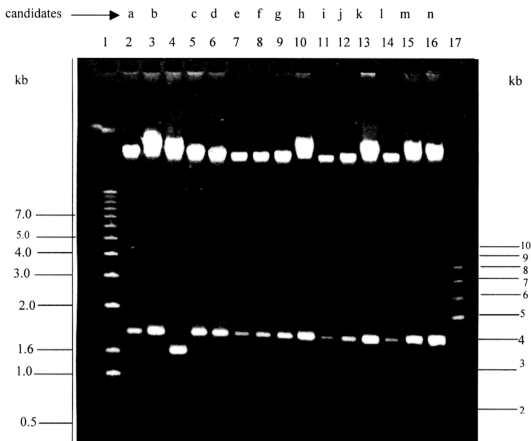


Figure 3.8. Screening of recombinant plasmid containing *argRWT*-biotinylated peptide fusion. Two types of recombinant plasmids were obtained from subcloning of the 0.8 kb *Bam*HI-digested fragment of pAM204 (*argRWT*) into 3.3 kb *Bam*HI-digested PinPoint™ Xa-3 Vector (the predicted size of the recombinant plasmid is 4.1 kb; see also Figure 3.13 and Figure 3.14). Lane 4 : recircularized vector DNA. Note that the intensity of the DNA bands in lanes 2, 5, 6, 7, 8, 9, 9, 10, 11, 12, and 14 are much less than that of DNA bands in lanes 3, 13, 15, 16. Recombinant candidates (b, g, i) were chosen for further analysis. Lane 1: 1 kb DNA size marker (marker size are indicated on the left), and lane 17: 2-10 kb supercoiled DNA size marker (marker sizes are indicated on the right).

varies (compare lanes 3, 4, 13, 15, and 16 with lanes 2, 5, 6, 7, 8, 9, 10, 11, 12, and 14) indicating differences in plasmid copy number in the cells. The plasmid in lane 14 is the religated vector. The different copy numbers of the recombinant plasmids gave us information that the two recombinant plasmids have different characteristics. Candidates b, g, and i were chosen for further analysis. Restriction endonuclease digestion pattern of candidates b, g, and i is presented in subsection 3.5.1.

In the beginning of this study, I faced difficulty in obtaining the correct recombinant plasmid because most of the time the *argRWT* gene was inserted into the cloning vector in the wrong orientation or the plasmid vector recircularized during ligation. I found that a sufficient amount and a correct proportional ratio of the DNA insert and vector are very important to produce the recombinant plasmid and to prevent recircularization of the linearized plasmid vector (Figure 3.9). I also found that purity of DNA also determines the outcome of successful DNA ligation to obtain recombinant plasmids.

3.4 Construction of ArgRNV-biotinylated peptide fusion protein

The plasmid pAM401 is a derivative of pMAL-c2 (Research Biolabs) carrying ampicillin resistant marker (Merican, 1995). Circular map of pAM401 is shown in Figure 3.10.

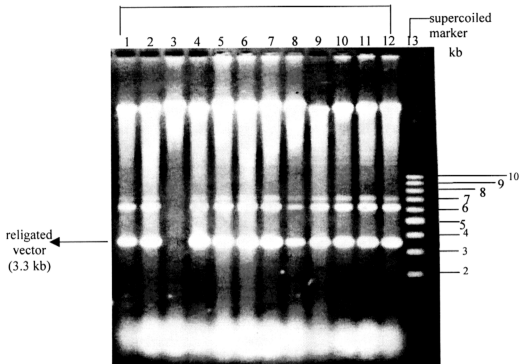


Figure 3.9. Recircularization of plasmid vector. Lane 1-12 showed the recircularization of linearized plasmid vector (3.3 kb) as indicated with the arrow (←). Recircularization of linearized plasmid vector may be due to insufficient amount of plasmid insert. Ligation reaction required much more DNA insert to prevent recircularization of linearized plasmid vector, especially when the DNA fragments were digested with *Bam*HI that produces cohesive termini. Lane 13: supercoiled DNA markers (the size of supercoiled markers are indicated on the right).

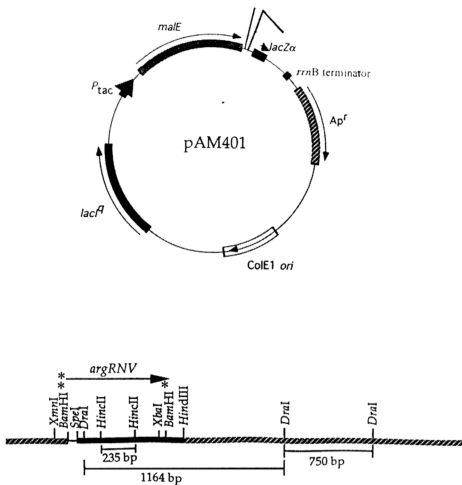


Figure 3.10. Map of plasmid pAM401 containing *argRNV* gene. Upper Panel: Circle map of pAM401, a derivative of the cloning vector pMal-c2 (Research Biolabs) carrying *Ap^r* resistance marker. Lower Panel: Linear map of plasmid pAM401. The *argRNV* gene is indicated by thick black line. The existence of two *HincII* restriction sites on *argRNV* gene differentiates *argRWT* with *argRNV* (adopted from Merican, 1995). Asterisks (*) indicate the two *BamHI* restriction site on pAM401. The downstream *HincII* site is present in both *argRWT* and *argRNV*, but the upstream *HincII* site was introduced by site directed mutagenesis during construction of *argRNV* (Merican, 1995). Double asterisks (**) indicates the *BamHI* restriction site that was introduced by site-directed mutagenesis.

3.4.1 Subcloning of *argRNV* gene into PinPoint Xa-3 cloning vector

The 0.8 kb *Bam*HI-digested DNA fragment of plasmid pAM401 containing *argRNV* gene (0.8 kb) was subcloned into the *Bam*HI-digested plasmid PinPoint™ Xa-3 containing the biotinylated peptide DNA sequence. The ligation reaction was incubated at 16°C overnight.

3.4.2 Analysis of *argRNV*-biotinylated peptide fusion recombinant DNA

Following CaCl_2 transformation, transformants were patched onto LB containing ampicillin (50 $\mu\text{g/ml}$ final concentration) and recombinant plasmids were screened using single colony lysis technique (SCFSB method) or phenol-chloroform-isopropanol method. The isolated plasmids were electrophoresed on a 0.8% agarose gel electrophoresis. Agarose gel electrophoresis analysis showed the presence of recombinant plasmids with the size of 4.1 kb (Figure 3.11 Panels A and B). We can also observed the difference in the intensity of the recombinant DNA bands between the recombinant plasmids in Figure 3.11 (Panel A) and the recombinant plasmids in Figure 3.11 (Panel B). The copy number of recombinant plasmid in Figure 3.11 (Panel A) may be less than the copy number of recombinant plasmid in Figure 3.11 (Panel B). Again, this observation gives us an information that the two recombinants have different characteristics, although the two recombinants were constructed from the same sources. Candidates A₁ and D₁ were chosen for further analysis.

It is of interest to also note that the copy number of recombinant plasmids containing the *argRNV* gene are much less than the copy number of

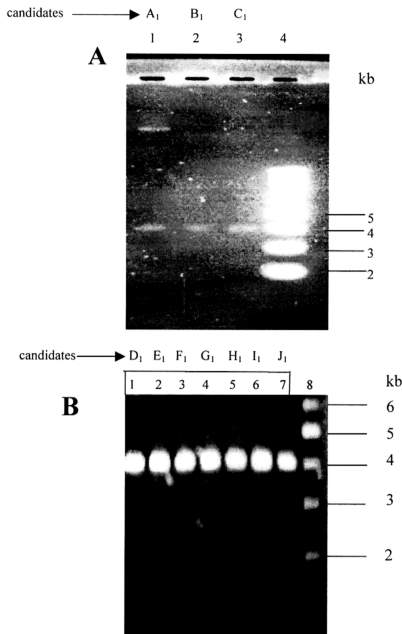


Figure 3.11. Screening of recombinant plasmid containing *argRNV*-biotinylated peptide fusion. Two types of recombinant plasmids were obtained from subcloning of 0.8 kb *Bam*HI-digested fragment of pAM401 (*argRNV*) into 3.3 kb *Bam*HI-digested fragment of PinPoint™ Xa-3 Vector (the predicted size of the recombinant plasmid is 4.1 kb). **Panel A:** Lanes 1-3 which show candidates A₁, B₁, C₁ (note the difference of DNA bands intensity between candidates A₁, B₁, C₁ and D₁, E₁, F₁, G₁, H₁, I₁, J₁). Lane 4: 2-10 kb supercoiled DNA markers (the size of the markers are on the right). **Panel B:** Lanes 1-7 which showed candidates D₁, E₁, F₁, G₁, H₁, I₁, J₁ with higher intensity. Lanes 8: 2-10 kb supercoiled DNA markers (the size of the markers are on the right).

recombinant plasmids containing *argRWT* gene. It is consistent with my earlier observation that plasmid pAM401 (carrying *argRNV* gene) has less copy number than the plasmid pAM204 (carrying *argRWT* gene; see subsection 3.2.1).

Different plasmids have different characteristics, because they have different metabolic loads, which results in different demands upon the host by the implementation of the plasmid genome. For example, if the plasmid is used to express genes whose products are toxic, then the products may interfere with the host's metabolism (Summers, 1991).

3.5 Characterization of recombinant plasmids

Since both vector and insert were digested with the same restriction enzyme, *Bam*HI, the *argRWT* or *argRNV*-containing DNA fragments can be inserted into the vector into two orientations, of which one will result in an in-frame fusion to the N-terminus of ArgRWT or ArgRNV. The two possibilities of the recombination reaction are shown in Figure 3.12 (Panels A and B). Figure 3.12 (Panel A) shows the *argRWT*-containing DNA fragment inserted in the wrong direction while Figure 3.12 (Panel B) shows the *argRWT*-containing DNA fragment inserted in the correct orientation.

3.5.1 Restriction endonuclease analysis for *argRWT* fusion derivative

The two types of recombinant plasmids will give different DNA size fragments when digested with the same restriction endonuclease (Figure 3.13). Two restriction enzymes were used to determine the orientation of the

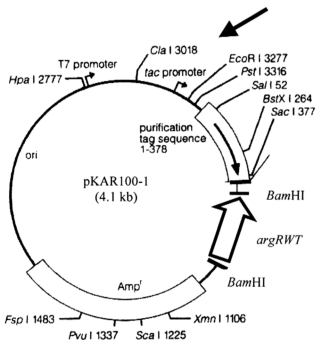
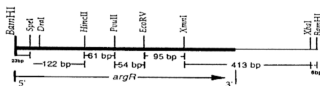
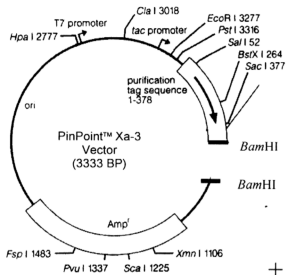


Figure 3.12A. *Bam*HI-digested fragment of pAM204 containing *argRWT* gene inserted into PinPoint™ Xa-3 cloning vector in the wrong orientation.

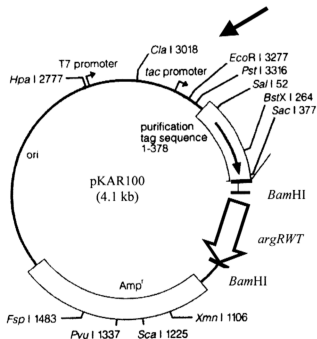
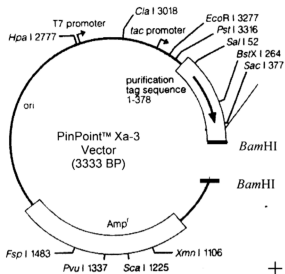


Figure 3.12B. *Bam*HI-digested fragment of pAM204 containing *argRWT* gene inserted into PinPoint™ Xa-3 cloning vector in the correct orientation.

recombinant plasmids, *Bam*HI and *Pvu*II. Figure 3.13 shows that candidates have the same size, 4.1 kb (lanes 12-14). *Pvu*II-digested candidates g and i released a small fragment of approximately 200 bp (lanes 8-11), while *Pvu*II-digested candidate b released a small fragment of approximately 600 bp (lane 2). *Bam*HI was used to ensure that the candidate recombinant plasmids carried the 0.8 kb *argRWT* containing DNA fragment. The candidates which generated *Pvu*II-digested DNA fragments of the right sizes (containing *argRWT* in the right direction) was designated pKAR100 while the candidate which generated *Pvu*II-digested DNA fragments of the wrong size (containing *argRWT* in the wrong direction) was designated pKAR100-1.

3.5.2 Restriction endonuclease analysis for *argRNV* fusion derivative

Restriction endonucleases analysis using *Pvu*II was carried out to determine the orientation of the *argRNV* gene in the recombinant plasmids (Figure 3.14). There is a slight difference in the size of DNA fragments generated upon digestion with *Pvu*II (lane 3) than the other one (lane 2). This difference is the result of different position of the *Pvu*II site of the two candidate recombinant plasmids. Candidate A₁ which generated *Pvu*II-digested DNA fragments of the right sizes (containing *argRNV* in the right direction) was designated pKAR200 while candidate D₁ which generated *Pvu*II-digested DNA fragments of the wrong sizes (containing *argRNV* in the wrong direction) was designated pKAR200-1.

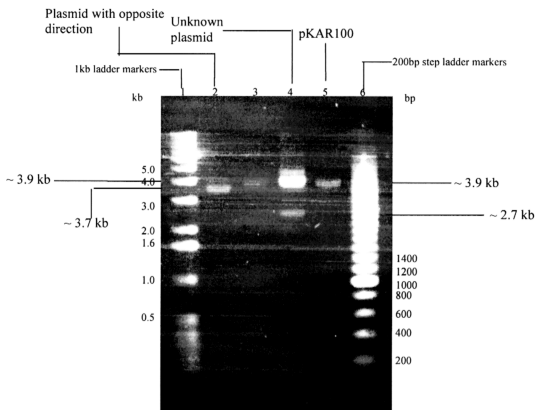


Figure 3.14. Restriction endonuclease analysis of recombinant plasmid containing *argRNV* with *PvuII* restriction enzyme. The right recombinant plasmid generated *PvuII*-digested DNA fragments, ~0.2 kb and 3.9 kb. Lane 2: candidate D₁, generated ~ 0.6 kb and 3.5 kb *PvuII*-digested DNA fragments. Lane 3: candidate A₁, generated 0.2 kb and 3.9 kb *PvuII*-digested DNA fragments 0.2 kb fragment was not detected due to its size). Lanes 1 and 6 : 1 kb ladder markers and 200 bp step ladder markers, respectively (the respective sizes of each ladder size markers are indicated).

3.5.3 DNA sequence analysis

DNA sequence analysis was performed to ensure that the recombinant plasmids created an in-frame fusion. It was indicated with reconstruction of the *Bam*HI restriction site of the sequence of both recombinant plasmids.

The *Bam*HI restriction site of plasmid pKAR100 is at nucleotide number 80 (Figure 3.15), while for plasmid pKAR200, its *Bam*HI restriction site is at nucleotide number 66 (Figure 3.16). The stop codon for plasmids pKAR100 and pKAR200 are at position 545-547 and 531-533, respectively, of the respective nucleotide sequences. DNA sequencing analysis showed not only that the direction of the insert are correct, but it also showed the creation of an in-frame fusion (the C-terminus of the biotinylated peptide with the N-terminus of ArgRWT and ArgRNV separately). It can also be seen from the amino acid sequences that the ArgRWT and ArgRNV sequence are still maintained, except for the first methionine and the second arginine of the true ArgR sequence. The first methionine of ArgR is not present and may be replaced by leucine from the biotinylated peptide while the second arginine was replaced by glycine during the creation of the fusion DNAs.

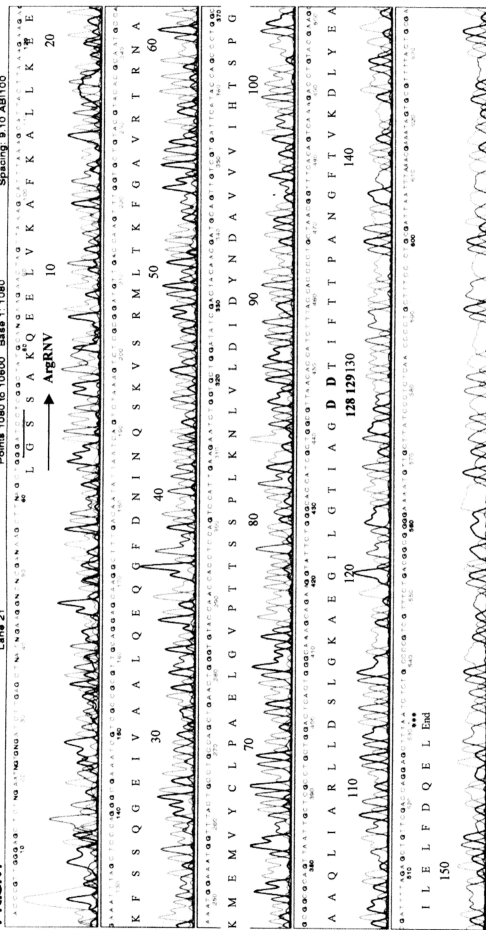


Figure 3.16. DNA sequence analysis data of recombinant plasmid pKAR200 (*argENV*-biotinylated peptide fusion). All nucleotide sequences and amino acid residues of pKAR200 are similar to the sequence in pKAR100 (*argRWT*-biotinylated peptide fusion), except for the substitution of two amino acids at residues 128 and 129 of ArgRWT (indicated by bold type) to construct ArgRNV. The reconstruction of *Bam*HI restriction site is underlined. The stop codon is indicated by triple asterisks.