CHAPTER 2

Materials and Methods

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2.1 Bacterial strains and plasmids

The bacterial strains used in this study are listed in Table 2.1. All strains were derivatives of *Escherichia coli* K-12 AB1157 (Bachman, 1972). The plasmids used and constructed in this study and their relevant properties are listed in Table 2.2. Plasmid PinPoint™ Xa-3 Vector is one of the plasmid vectors provided in the PinPoint™ Xa Protein Purification System (Promega). Plasmid PinPoint™ Xa-3 Vector circle map with reference points is shown in Figure 2.1. PinPoint™ Xa-3 is a high copy number plasmid, generally there will be 500-700 copies per cell. It has a pMB1 origin of replication in which PinPoint™ Xa-3 is not in the same incompatibility group with the reporter plasmids pCS202 or pSH10.

Table 2. 1. Bacterial Strains used in this study

Strain	Genotype	Source/Reference
DS 956	DS 941 (*xerA9 ::fol*)[a]	Flinn *et al.* (1989)
DS941	*recF*143, *lac* ZΔ*M15*, *lacI*^{q} AB1157	Summers and Sherratt (1988)

2.2 Chemicals and reagents

The major sources of chemicals are listed below (Table2.3). Sources of specialised chemicals are identified in the appropriate sections.

2.3 Bacterial growth media

Bacterial growth media are listed in Table2.4.

Table 2.2. Plasmids used in this study

Plasmids	Resistance marker	Size (kb)	Description	Source/ Reference
pAM 204	ApTc	6.6	pSelect-1 (Promega) derivative containing *argRWT* gene	Merican (1995)
pAM 401	Ap	7.4	pMal-c2 (Research Biolabs) derivative containing *argRNV* gene	Merican (1995)
Pinpoint™ Xa-3	Ap	3.3	Vector to create fusions between a cloned gene and a biotinylated peptide	Promega Corp. USA
pSH10	Km	6.4	pACYC 184-based (p15A replication origin) *cer* reporter plasmid, containing 2 copies of *cer* site in direct repeat, flanking a *lacZ* gene	Chen *et al.* (1997)
pSH11	Km	3.1	resolution product of pSH10	Chen *et al.* (1997)
pCS202	CmTc	7.6	lambda *dv*-based *cer* reporter plasmid, containing 2 directly repeated *cer* sites, flanking a Tc^r cassette, lambda *dv* replicon	Colloms *et al.* (1990)
pCS203	Cm	5.2	resolution product of pCS202	Colloms *et al.* (1990)
pKAR100	Ap	4.1	*argRWT* derivative of PinPoint Xa-3	Chapter 3
pKAR200	Ap	4.1	*argRNV* derivative of PinPoint Xa-3	Chapter 3
pKAR100-1	Ap	4.1	*argR* gene in the opposite direction of pKAR100	Chapter 3
pKAR200-1	Ap	4.1	*argR* gene in the opposite direction of pKAR200	Chapter 3

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; Km, kanamycin.

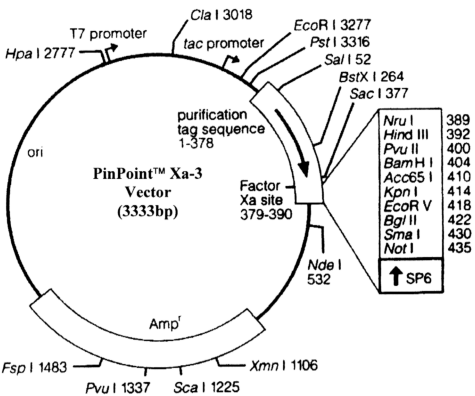

Figure 2.1. The PinPoint™ Xa-3 Vector circle map with reference points. Base#1 is the translation start site for the purification tag sequence.

	PinPoint™ Xa-3 Vector
Sequence reference points:	
a. *tac* promoter	3210-3237
b. biotin purification tag coding region	1-378
c. Factor Xa Protease recognition site	379-390
d. multiple cloning region	387-443
e. PinPoint™ Vector Sequencing Primer binding site	325-343
f. SP6 Sequencing Primer binding site	453-471
g. SP6 RNA polymerase promoter	454-470
h. T7 RNA polymerase promoter	2798-2814
i. biotinylated lysine codon	262-264
j. β-lactamase (Amp^r) coding region	921-1778

Table 2. 3. Chemicals and reagents used in this study

Chemicals	Sources
General chemicals, biochemicals, organic solvent	Sigma, BDH, Amersham/USB, Pharmacia, Bio-rad, ICN, Pierce
Media	Difco, Oxoid
X-gal, IPTG	USB
Agarose, acrylamide	Hispanagar, Bio-rad
Vitamin	Sigma
Antibiotics	USB
Enzymes	Promega, Amersham , BRL
10x restriction enzyme buffers	Promega, Amersham
5x T4 DNA ligase buffer	Promega, Amersham

Table 2.4. Bacterial growth media

Media	Materials
LB broth	tryptone, yeast extract, NaCl, distilled water
LB agar	tryptone, yeast extract, NaCl, agar, distilled water

Table 2.5. Concentrations of antibiotics

Antibiotics	Solution stock	Final concentration
Ampicillin	5 mg/ml ampicillin disodium salt in water	50μg/ml
Chloramphenicol	2.5 mg/ml chloramphenicol in absolute ethanol	25μg/ml
Kanamycin	5 mg/ml kanamycin sulfate in water	50μg/ml
Tetracycline	1.5 mg/ml tetracycline hydrochloride in 50% ethanol	10μg/ml

2.4 Growth and maintenance of bacterial culture

Except where indicated, all strains were grown at 37°C in Luria-Bertani (LB) medium (Miller, 1972). Antibiotics and other supplements were added where appropriate. Strains used were routinely maintained on appropriate plates, stored at 4°C and restreaked every 4 to 5 weeks. Bacterial strains were stored long term at -20°C (1 ml of an overnight culture was mixed with 1 ml of broth containing 50% LB broth, 20% (v/v) glycerol, and 1% (w/v) peptone). To revive strains from the –20°C stocks, inocula were streaked on LB agar and grown, prior to replating on selective media.

2.5 Amino acids, antibiotics , *lac* inducer, indicator, and vitamin

Vitamin (biotin) concentration used in liquid media was 10μM, while amino acid (L-arginine) concentration used was 1mM. The antibiotics concentration used for liquid media or selective plates are as shown in Table 2.5. All antibiotics used in this study are purchased from Amersham Life Sciences. All stocks were stored at 4°C. To prepare selective plates, appropriate volume of the stock solution was added to molten agar cooled to 40°C.

Isopropylthio-β-D-galactopyranoside (IPTG; Amersham Life Science), a lactose analogue, binds and inhibits the Lac repressor and thereby induces β-galactosidase activity. IPTG is used with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Amersham Life Science). X-gal is a chromogenic substrate, used to detect *lac* gene expression in cloning procedures to differentiate recombinants from nonrecombinants in vectors containing the *lacZ* or *lacZ* α-peptide gene (blue/white selection). The IPTG was stored at a concentration of

2.5 mg/ml in water at –20°C and added to the medium to a final concentration of 25 μg/ml. X-gal was stored at a concentration of 2.5 mg/ml in dimethylformamide (DMF) at –20°C and added to the medium to a final concentration of 25 μg/ml. IPTG was also used to induce expression of cloned genes under the control of the *lac* operator. The concentration used in growth culture was 1 mM.

2.6 Isolation and purification of covalently closed circular plasmid DNA

This protocol is as described in Sambrook *et al.* (1989).

Solutions :

Birnboim-Doly I (Solution I): 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA.

Birnboim-Doly II (Solution II): 0.2 M NaOH, 1% (w/v) SDS; made fresh prior to use.

Birnboim-Doly III (Solution III): 5 M potassium acetate; prepared by mixing equal volumes of 3 M CH_3COOK and 2 M CH_3COOH (pH 4.8).

2.6.1 Small-scale plasmid DNA preparation

One ml of overnight culture was transferred into a microcentrifuge tube (eppendorf), centrifuged at 14000 rpm for 5 min at 4°C in a microcentrifuge. The medium was discharged and the bacterial pellet was left as dry as possible. The pellet was resuspended in 100 μl of ice-cold Solution I by vigorous vortexing. 200 μl of freshly prepared Solution II was added, the tube was closed tightly and the contents mixed by inverting the tube rapidly several times (did not vortex). The tube was stored on ice and 150 μl of ice-cold Solution III was added, the tube

was closed and vortexed gently. The tube was stored on ice for 3-5 min. Cell debris, most chromosomal DNA, high molecular weight RNA, potassium, SDS, protein, and membrane complexes were removed by centrifugation (14,000 rpm, 5 min, 4°C). The supernatant containing plasmid DNA was transferred into a clean microcentrifuge tube. The DNA solution was extracted once with an equal volume of phenol, and twice with an equal volume of chloroform (the aqueous phase was taken in each case). Each step was followed by vigorous vortexing and by centrifugation (14,000 rpm, 3 min, room temperature). DNA was precipitated by adding 1/10 volume of sodium acetate and two volumes of absolute ethanol, the tube was mixed gently and stored in –20°C for 30 min. Optional: isopropanol can also be used to precipitate DNA. In this case, DNA was precipitated by adding 600 µl of isopropanol, mixed gently and stored at room temperature for at least 15 min. The DNA was collected by centrifugation (14,000 rpm, 5 min, at room temperature). The pellet was rinsed with 70 % ethanol, dried briefly and dissolved in 20 µl TE buffer (pH 8.0) containing DNase-free pancreatic RNase (20 µg/ml).

2.6.2 Midi-scale plasmid DNA preparation

Midi-scale preparations of plasmid DNA were carried out by harvesting cells from 10-20 ml of an overnight bacterial culture. The culture was centrifuged at 4000 rpm, 10 min, 4°C. The bacterial pellet was resuspended in 200 µl of Solution I. The suspension was transferred into a clean microcentrifuge tube, that was stored on ice. 400 µl of freshly prepared Solution II was added and mixed by inverting the tube several times (not vortexed). The tube was stored on ice for 3

to 5 min. 300 µl of Solution III was added and the tube was closed and then vortexed. The tube was stored on ice for 3 to 5 min. The tube was centrifuged (14,000 rpm, 5 min, 4°C). The supernatant containing plasmid DNA was transferred into a clean microcentrifuge tube and extracted once with phenol, and twice with chloroform (the aqueous phase was taken in each case). Vigorous vortex and centrifugation (14,000 rpm, 3 min, 4°C) followed each step. DNA was precipitated by ethanol or isopropanol precipitation. The DNA was collected by centrifugation (14,000 rpm, 5 min, at room temperature). The pellet was rinsed with 70 % ethanol, dried briefly and dissolved in 50 to 100 µl of TE buffer (pH 8.0) containing DNase-free pancreatic RNase (final concentration of 20 µg/ml).

2.6.3 Large-scale plasmid DNA preparation

Cells from a 200 ml overnight culture were harvested by centrifugation. The pellet was re-suspended in 5 ml of ice-cold Solution I and incubated on ice for 5 min. 10 ml of freshly made Solution II was added, mixed by gentle inversion and incubated on ice for 5 min. The tube was stored on ice for 3 to 5 min. 7.5 ml of Solution III was added and the tube was closed and then vortexed. The tube was centrifuged (10,000 rpm, 5 min, 4°C). Cell debris and most chromosomal DNA was removed by centrifugation (10,000 rpm, 15 min, 4°C). The supernatant containing the plasmid DNA was precipitated by adding 11 ml isopropanol, mixed well and incubated at room temperature for at least 15 min. The DNA was collected by centrifugation (10,000 rpm, 15 min, 25°C). The pellet was rinsed with 2 ml of 70% ethanol and centrifuged briefly. The ethanol was dried as dry as possible. The pellet was resuspended in 400 µl TE buffer.

2.6.4 Purification of plasmid DNA by Cesium chloride/Ethidium bromide (CsCl-EtBr) density gradient centrifugation

4.4 g CsCl were added and dissolved in 4ml DNA-containing TE solution obtained from the large-scale preparation (subsection 2.6.3) and was transferred into a 5 ml Quick-Seal® ultracentrifuge tube containing 400 µl of 10mg/ml ethidium bromide. The density of the resulting nucleic acid/TE/EtBr solution was 1 g/ml. 10 µl of a 1:100 dilution of Triton X-100 per ml of tube volume was added. The tube was sealed using a heat sealing device. The tube was placed in a rotor. The spacers and plugs were put over the tubes and the plugs were torqued to 120 in.lb (13.6 Nm). The rotor was placed in a Beckman XL Optima™ Series ultracentrifuge and run at 20°C, 78,000 rpm, 4 hours. After centrifugation, the tube was supported in a tube rack and exposed to ultraviolet light. Two bands were visible, a lower supercoiled plasmid band and an upper chromosomal and relaxed plasmid band. An 18-G needle was inserted into the top of tube, and the plasmid band was recovered by suction using a 3-ml syringe (inserted ~ 1 cm below the band). The plasmid DNA-containing solution was transferred into a test tube and 2 ml of deionized water was added. Ethidium bromide was removed from the plasmid sample by repeated extraction with one volume of n-butanol until the solution was colorless. The plasmid was collected by addition of two volumes of ethanol, incubation on ice for 30 min, and centrifugation (14,000 rpm, 30 min, 4°C). The plasmid was rinsed with 70% ethanol, recentrifuged (14,000 rpm, 5 min, 4°C) and the DNA pellet was dried at 37°C for 1 hour. Plasmid DNA was resuspended in 100-200 µl of TE-RNase buffer and was stored at –20°C.

Plasmid DNA isolated by all three methods described above can be used for further manipulation both *in vitro* and *in vivo*. Isolation using CsCl-EtBr density gradient centrifugation method gave high purity DNA.

2.7 *In vitro* DNA manipulation

Buffers:

1X TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA

Restriction enzyme buffers: The buffers recommended by the manufacturer of the enzymes were used.

5X T4 DNA ligase buffer (GIBCOBRL): 0.25 M Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25 % (w/v) polyethylene glycol-8000.

Phenol: All phenol used in the purification of DNA contained 0.1 % 8-hydroxyquinoline, and was buffered against 0.5 M Tris-HCl (pH 8.0).

2.7.1 Restriction endonuclease digestion of DNA

Restriction digestions were usually performed in a total volume of 10-20 µl containing 0.3 to 3 µg of DNA, 1-2 µl of 10X restriction buffer, 3 to 10 units of restriction enzyme, the final volume was made up with deionized water (in some cases, BSA was added to a final concentration of 100 µg/ml as recommended by the manufacturer). Incubation was for 1 hour at the temperature recommended by the enzyme manufacturer. The digested DNA was kept at –20°C before being used for further analysis.

2.7.2 Dephosphorylation of DNA restriction fragment

Calf intestinal alkaline phosphatase (CIAP) treatment was used to remove the 5'-terminal phosphate group from linearized cloning vector to prevent recircularisation of the vector during DNA cloning. 1.0 unit of CIAP was added directly to the restriction digestion reaction 30 min before termination of the incubation. CIAP was removed from the digested DNA by phenol extraction or by gel electrophoresis of the digested reaction mixture.

2.7.3 Phenol extraction and ethanol precipitation of DNA

The DNA solution was made up to 200 µl and extracted once with 200 µl phenol and twice with chloroform (the aqueous phase was taken in each case). Each step was followed by centrifugation (14,000 rpm, 3 min, room temperature). DNA was precipitated by adding 1/10 volume of 3M sodium acetate and two volumes of ethanol, incubation at –20 °C for 1 hour or –70 °C for 30 min. The pellet was rinsed with 70 % ethanol, dried and dissolved in 200 µl TE buffer.

2.7.4 Ligation of DNA fragments

Restriction fragments were ligated in a total volume of 20 µl, containing 1XT4 DNA ligase buffer and 2-4 units of T4 DNA ligase. For optimum condition the ratio was 2:1 insert to vector of fragments used for ligation of cohesive termini and 5:1 for ligation of blunt ends. The reaction was incubated overnight at 16 °C.

2.8 Bacterial transformation

Transformation of plasmid DNA used $CaCl_2$ treatment of cells (Cohen and Hsu, 1972; Sambrook *et al.*, 1989).

a) Preparation of competent cells

1 ml of an overnight culture was diluted 20 fold in fresh LB broth. The culture was grown at 37°C with vigorous shaking (200 rpm) up to an OD_{600} of 0.4 to 0.5 (about 3-4 hours). The cells were harvested by centrifugation (10,000 rpm, 10 min, 4°C), resuspended in 10 ml ice-cold solution of 50 mM $CaCl_2$ and harvested by centrifugation (5,000 rpm, 5 min, 4°C). The cells were resuspended in 0.4 to 1 ml ice-cold solution of 50 mM $CaCl_2$ and left on ice.

b) Transformation

A suitable volume of plasmid DNA was added to 200 µl of competent cells, mixed gently and left on ice for 15 min. The cells were heat-shocked (5 min, 37°C) and returned to ice for a further 15 min. After the addition of 200 µl prewarmed fresh LB broth, the cells were incubated at 37°C for at least 60 min and were plated on selective media.

2.9 Rapid screening of plasmid DNA

This method enables the plasmid content of a colony of cells to be observed without having to purify the plasmid DNA (Birnboim and Doly, 1979).

(a) Phenol-chloroform-isoropanol method

A single colony was picked with a sterile toothpick and spread on to agar media to give 1.5 cm square patch. After overnight incubation, cells were taken from the patch with a sterile toothpick and swirled in 40 µl of TE buffer in a 1.5

ml microcentrifuge tube. The cells were extracted with phenol-chloroform-isopropanol (25:24:1), and centrifuged (14,000 rpm, 5 min, 4°C). 15 µl of the supernatant was taken and mixed with loading dye for gel electrophoresis analysis. Plasmid DNA was analyzed by EtBr staining of the gel.

(b) SCFSB method

Buffer: 5X SCFSB: 10 % (w/v) ficoll, 5 % (w/v) SDS, 0.05 % (w/v) bromophenol blue, 0.25 % (w/v) orange G in 1X TAE. Diluted 5-fold in 1 X TAE and RNase A was added to 10 µg/ml (final concentration).

The cells were picked from the overnight patching plate with a sterile toothpick and swirled in 100 to 200 µl 1X SCFSB (single colony final sample buffer) in a 1.5 ml microcentrifuge tube. The cells were left to lyse at room temperature for 15 min and centrifuged (14,000 rpm, 15 min, 4°C). 20 µl of the supernatant was loaded directly onto an agarose gel, electrophoresed, and the plasmid DNA analyzed by EtBr staining of the gel.

2.10 DNA sequencing

DNA sequencing was carried out to determine that the insert gene was ligated to the vector correctly to create an in-frame fusion. DNA sequencing was done in the forward direction only. The DNA sequencer machine used was ABI 377 Automated DNA Sequencer (Perkin-Elmer). The sequence for the oligonucleotide sequencing primer used was 5′-CGTGACGCGGTGCAGGGCG-3′.

2.11 Gel electrophoresis of DNA

Buffers:

10X TAE: 48.4 g Tris, 3.72 $Na_2EDTA.2H_2O$, 16.4 g sodium acetate, certain volume of glacial acetic acid; giving a pH of 8.2.

Loading dye (FSB): 10% ficoll (w/v), 0.5% (w/v) SDS, 0.05% (w/v) bromophenol blue, 0.25% (w/v) orange G in 1X TAE.

Plasmid DNA was run on agarose gel in horizontal format. Agarose gel concentrations used were in the range of 0.6% to 1.0% according to the size of the fragment of interest, as described in Sambrook *et al.* (1989). Agarose gels were prepared in 1X TAE buffer. For routine DNA and restriction fragment analysis, agarose gels were run in 1X TAE buffer with the voltage between 40-70 V. FSB loading dye was added to DNA samples prior to loading on the gel.

2.11.1 Staining of DNA gel

Following electrophoresis, the gels were stained for at least 30 min in distilled water containing EtBr at a final concentration of 0.5 mg/ml and destained with two 15 min washes of distilled water. The DNA was visualized on a UV transilluminator.

2.11.2 Extraction of DNA from agarose gel

If bands were to be cut from the gel, DNA was visualized on a long-or middle-wave UV transilluminator. The band of interest was excised from the gel using a clean scapel and was placed in a 1.5 ml microcentrifuge tube. The DNA

was extracted from the gel using the NucleoSpin® Extraction Kit PT3180-2 (Clontech Laboratories) and followed the procedure given by the manufacturer. Alternatively, the DNA was run on a low melting point agarose gel and was extracted using Nucleon™GX DNA purification kit (Scotlab Bioscience) and followed the procedure given by the manufacturer.

2.12 Protein Expression and detection

Buffer:

Protein sample buffer (PSB): 50 mM Tris-HCl (pH 6.8), 5% SDS, 5% β-mercaptoethanol, 50% glycerol, 0.01% bromophenol blue.

2.12.1 Cell growth and induction

DS956 cells (harbouring the appropriate plasmids) were grown overnight at 37°C in LB medium supplemented with 10 μM biotin and 50 μg/ml ampicillin. Control cells (DS956 without plasmid) were grown in the same condition but without ampicillin. The cells were diluted 1:20 into fresh LB medium supplemented with 10 μM biotin and 50 μg/ml ampicillin and grown at 37°C (with shaking at 200 rpm) until the cells mature enough to be expressed (OD_{600} ~0.5). The cells were induced with 1 mM IPTG, supplemented with 10 μM biotin and 50 μg/ml ampicillin for five hours. Alternatively, the diluted cells were grown overnight at 37°C, and induced overnight.

2.12.2 Cell lysis

1 ml of the induced culture was taken and the cells were harvested by centrifugation (14,000 rpm, 4°C, 5 min). The cells were resuspended in 100 µl protein sample buffer (PSB). The resuspended cells were heated at 95°C for 5 min with occasional vortexing prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. This treatment will lyse the cells and coat the protein with SDS. The sample can be stored at -20°C at this point, but will require retreating with heat prior to SDS-PAGE analysis. Repeated heating of these samples, however, can result in the destruction of some proteins.

2.13 SDS-PAGE analysis

Buffers:

Laemmli running buffer (10X): 30g Tris, 144g glycine, 10g SDS, dissolved in 1 liter distilled water.

4X stacking buffer: 0.5 M Tris-HCl pH6.8, 0.4% (w/v) SDS.

4X separating buffer: 1.5 M Tris-HCl pH8.8, 0.4% (w/v) SDS.

Total cellular proteins were subjected to SDS-PAGE analysis (Laemmli, 1970). This electrophoresis system consists of separating gel (15% acrylamide) and stacking gel (4% acrylamide). Acrylamide stock solution containing a 37:1 ratio of acrylamide to bisacrylamide (BioRad) was used and 10% ammonium persulfate and TEMED (N, N, N', N',-tetramethylethylenediamine) were added prior to pouring the polyacrylamide gel, to catalyze polymerization. The composition of separating and stacking gels are listed in Tables 2.6 and 2.7.

Table 2.6 Separating gel (15% acrylamide)

Gel components	Volume
4X separating buffer	2.5 ml
Acrylamide stock solution	5.0 ml
Distilled water	2.5 ml
10% (w/v) ammonium persulfate	100 µl
TEMED	10 µl

Table 2.7 Stacking gel (4% acrylamide)

Gel components	Volume
4X stacking buffer	2.5 ml
Acrylamide stock solution	1.3 ml
Distilled water	6.2 ml
10% (w/v) ammonium persulfate	100 µl
TEMED	20 µl

Electrophoresis was performed in 1X Laemmli gel running buffer at 120 V until the dye front reached the edge of the gel. Gel staining used staining kit solution GELCODE® Blue Stain Reagent (Pierce) and followed the procedure provided by the manufacturer.

2.14 Western blotting

Western blotting analysis was used to locate the biotinylated protein. The blotting system used was a wet transfer system following the procedure as described in Towbin *et al.* (1979).

Buffer:

Transfer buffer: 25 mM Tris base, 192 mM glycine, 10% methanol, 0.1% (w/v) sodium dodecyl sulfate (SDS).

2.14.1 Preparation of the gel for protein transfer

Following SDS-PAGE electrophoresis, the gel was lifted off and notched at one of the gel corners to provide orientation to the membrane after transfer. The gel was immersed in the transfer buffer and allowed to equilibrate for at least 15 min.

2.14.2 Preparation of the transfer membrane

The membrane used for protein transfer was Immobilon PVDF membrane (Immobilon-P, Millipore). The membrane was cut to the dimension of the gel and one corner was notched or labeled to correspond to a corner of the gel. The membrane was soaked in 100% methanol for 15 seconds and transferred to a container containing Milli-Q® water for 2 min and equilibrated for at least five min in the transfer buffer.

2.14.3 Assembling of the transfer stack

A foam pad was placed on one side of the cassette holder. One sheet of filter paper was placed on top of the pad. The gel was placed on top of the filter paper and the sheet of the Immobilon-P membrane was put on top of the gel. Another sheet of filter paper was placed on top of the stack (Figure 2.2).

2.14.4 Protein transfer

The protein was transferred using a wet transfer system. The tank transfer cassette holder was closed and placed in the tank blotting apparatus (Nihon Eido) so that the cassette holder with the gel faced the cathode (-). Transfer buffer was

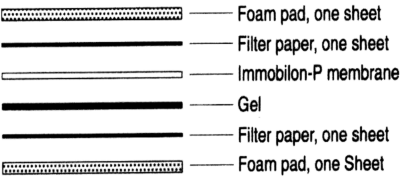

Figure 2.2. The transfer stack for a wet system in Western blotting analysis. The cassette holder with the gel is facing the cathode (-), and the cassette holder with the membrane is facing the anode (+).

added to the blotting apparatus to cover the cassette holder. The black cathode lead (-) was inserted into the cathode jack and the red anode lead (+) was inserted into the anode jack and then the anode lead and cathode lead were connected to their corresponding power outputs. The system was turned on for two hours at 250 mA to transfer the proteins to the membrane. The protein transfer was carried out at a low temperature to prevent overheating.

2.14.5 Visualization of the proteins

There are several methods that can be used to assess complete transfer of proteins to the membrane. The first one is by using Coomassie® Blue staining, which is non-reversible. The second is a transillumination method, and the third is Ponceau-S red stain. The two latter methods are reversible. The blotted membrane was immersed in Ponceau-S red stain (Sigma) for one min. The membrane was destained in Milli-Q water to the desired contrast or rinsed with 0.1 N NaOH to remove the stain completely.

2.14.6 Drying of the blotted membrane

After destaining the blotted membrane, the blot was soaked in 100% methanol for 10 seconds to drive out the water, and was placed on a piece of towel paper to let the methanol evaporated completely for approximately 15 min.

2.14.7 Detection of biotinylated protein

Protein transfer was followed by chromogenic detection to identify the protein bands of interest. The location of biotinylated proteins was indicated by the presence of dark purple bands forming in the lanes containing cellular extracts.

Buffers:

Tris-buffered saline Tween (TBST) buffer: 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20.

Blocking buffer: 2% (w/v) of nonfat dry milk (skimmed milk) in TBST buffer, alternatively: 2% (w/v) of BSA (blotting grade, Promega) in TBST buffer.

Alkaline phospatase (AP) solution: 3 µl streptavidin-AP + 15 ml TBST buffer.

AP buffer: 100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$.

NBT stock solution: 5% (w/v) of nitro blue tetrazolium chloride (NBT) in 70% dimethylformamide (stored at 4°C).

BCIP stock solution: 5% (w/v) of 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) in 100% dimethylformamide (stored at 4°C).

NBT-BCIP solution (freshly prepared): 10 ml AP buffer + 66 µl NBT stock solution (mixed first) + 33 µl BCIP stock solution.

2.14.7.1 Blocking the blotted membrane

The blotted membrane was briefly washed twice with TBST buffer and was incubated with gentle shaking for at least one hour in blocking buffer at room temperature. Alternatively, the membrane was incubated in blocking buffer overnight at 4°C without shaking. The blocking buffer was removed and the membrane was incubated in alkaline phosphatase solution for 30 min with gentle

shaking. The alkaline phosphatase solution was removed and the membrane washed with TBST buffer three times with gentle shaking. The buffer was changed every 5 min. The buffer was removed and the blot was rinsed with Milli-Q water.

2.14.7.2 Chromogenic substrate incubation

The blot was immersed in alkaline phosphatase buffer and freshly prepared NBT and BCIP solution were added to the buffer respectively (NBT solution was added first, mixed and was followed with BCIP solution). The blot was incubated for less than 15 min at room temperature (until the desired signal reached). To stop the reaction, the blot was washed with Milli-Q water. The membrane was air dried on a filter paper and was stored in a dark place. The whole procedures of Western blotting are summarized in the flow chart below (Figure 2.3).

2.15 *In vivo* Xer site-specific recombination assay

The method for *in vivo* resolution assays was as described by Colloms *et al.* (1990) and McCulloch *et al.* (1994). Reporter plasmids pCS202 or pSH10 were transformed into cells with or without plasmids expressing ArgRWT and its derivative. The cells were grown on LB agar containing the appropriate antibiotic. The transformants were patched to LB agar containing the appropriate antibiotic and the plasmids were isolated from the cells with phenol-chloroform-isopropanol extraction (25:24:1). The isolated plasmids were electrophoresed on a 0.8% agarose gel.

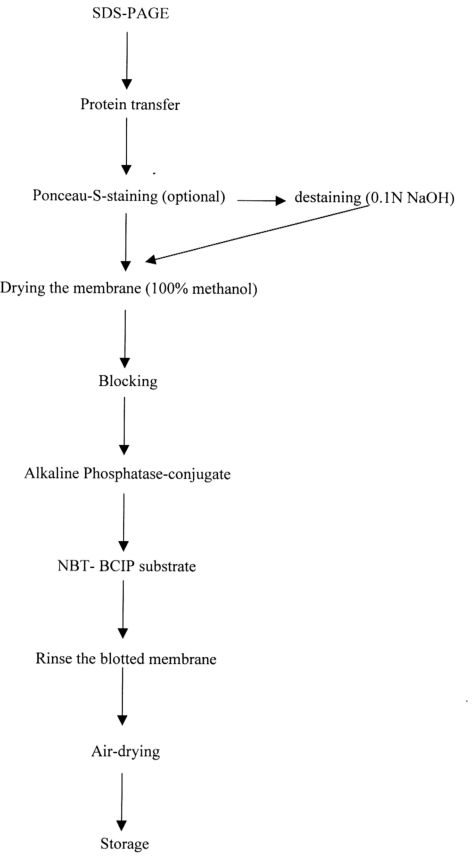

Figure 2.3. A flow chart of western blotting analysis procedures.

2.16 Protein purification

Buffer:

Cell lysis buffer: 50mM Tris-HCl (pH 7.4), 50mM NaCl, 5% glycerol.

A large scale *E. coli* culture containing the appropriate plasmid was grown, and the protein was expressed by induction with 1mM IPTG (final concentration). The cells were harvested by centrifugation (7000 rpm, 4 ^{0}C, 20 min). The pellet was dissolved in 10 ml of cell lysis buffer prior to sonication. Cell lysis was performed on ice. The cell suspension was sonicated by using 30-second pulses with a 30-second pause between pulses. The crude lysate was centrifuged to remove cellular debris (10000 rpm, 4^o C, 15 min). The cell lysate was taken for protein purification, and cellular debris were kept at cool temperature for further analysis.

The protein purification was performed by using a batch capture method with the SoftLink™ Resin (Promega). The resin was prepared for the first time by adding two-resin volumes of cell lysis buffer containing 20mM biotin and was regenerated by washing the resin with 10% acetic acid and cell lysis buffer. 500µl of resin was equilibrated in 1 ml of cell lysis buffer (in 2 ml eppendorf tube) at room temperature for 15 minutes. The buffer was discharged and the resin was mixed with 1 ml of cell lysate. The mixture was gently rocked overnight. The cell lysate was aspirated from the resin mixture and was kept for analysis. The resin mixture was rinsed with 1 ml cell lysis buffer for 10 minutes. The washing steps were performed twice. The protein was eluted by adding 200µl of cell lysis

buffer containing 20 mM biotin. The resin suspension was rocked gently overnight. The solution that contains the released fusion protein was transferred to a clean tube. The resin was kept at 4°C for analysis.

2.17 Photography

All photographs were taken using Mitsubishi Video Copy Processor (Vilber Lourmat). Some pictures of SDS-PAGE analysis were taken using Polaroid film DS-34. All photographs presented in this thesis were scanned using a UMAX Astra 610P Scanner at 300 dpi resolution.