# Elucidation of the osmoregulatory locus, *ompRZ*, in *Erwinia chrysanthemi.*

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## ABBREVIATIONS

| | |
|---|---|
| A | adeninie/adenosine |
| Amp | ampicillin |
| Asp | aspartic acid |
| ATP | adenosine 5'-triphosphate |
| bp | base pair |
| C | cytidine/cytosine |
| C-terminal | carboxy-terminal |
| ° C | degrees Celsius |
| CsCl | cesium chloride |
| DIG | digoxigenin |
| DMSO | dimethyl sulphide |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside 5'-triphosphate |
| EDTA | ethylenediaminetetra-acetic acid |
| EtBr | ethidium bromide |
| EnvZ | environmental sensor |
| EXOIII | exonuclease III |
| g | gram |
| G | guanosine |
| GCG | Genetics Computer Group |
| h | hour |
| $H_2O$ | water |

| | |
|---|---|
| Kan | kanamycin |
| kb | kilobase |
| kD | kilodalton |
| L | litre |
| LB | Luria Bertani broth |
| Lys | lysine |
| M | molar |
| min | minute |
| ml | millilitre |
| mM | millimolar |
| mol | mole |
| mRNA | messenger RNA |
| nm | nanometer |
| N-terminal | amino-terminal |
| OD | optical density |
| OMP | outer membrane protein |
| OmpR | outer membrane protein regulator |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| $Rif^r$ | rifampicin resistance |
| $Rif^s$ | rifampicin sensitive |
| RNA | ribonucleic acid |
| RNAP | ribonucleic acid polymerase |
| rpm | revolutions per minute |
| RT | room temperature |

| | |
|---|---|
| s | second |
| SDS | sodium dodecyl sulphate |
| T | thymidine |
| Taq | *Thermus aquaticus* |
| TBE | tris borate-EDTA |
| TE | tris-EDTA |
| TEMED | N, N, N', N'- tetramethylethylenediamine |
| Tris | tris(hydroxymethyl)aminomethane |
| U | units |
| UV | ultraviolet |
| V | volts |
| w/v | weight per volume |
| X-gal | 5-bromo-4-chloro-3-indolyl-β -D-galactosidase |
| α | alpha |
| β | beta |
| λ | lambda |
| μ | micro |

**Table I: Abbreviations of the standard amino acids.**

| Amino acid name | Three letter code | One letter code |
|---|---|---|
| Glycine | Gly | G |
| Alanine | Ala | A |
| Valine | Val | V |
| Leucine | Leu | L |
| Isoleucine | Ile | I |
| Methionine | Met | M |
| Proline | Pro | P |
| Phenylalanine | Phe | F |
| Tryptophan | Trp | W |
| Serine | Ser | S |
| Threonine | Thr | T |
| Asparagine | Asn | N |
| Glutamine | Gln | Q |
| Tyrosine | Tyr | Y |
| Cysteine | Cys | C |
| Lysine | Lys | K |
| Arginine | Arg | R |
| Histidine | His | H |
| Aspartic acid | Asp | D |
| Glutamic acid | Glu | E |

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Dedicated to my life giver and sustainer, the Almighty, God.

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## ABSTRACT

Bacteria are constantly faced with harsh environmental conditions to which they have to adapt. These adaptive mechanism generally involve the use of two-component sensory systems, comprising of sensor proteins interacting with their cognate response regulator proteins. To survive fluctuating environments such as osmotic conditions, certain bacterial species employ the *ompR-envZ* (*ompB*) two-component system to monitor and respond to the osmotic cue. The EnvZ protein functions as the sensor and relays information regarding changes to the external environment, to the response regulator, OmpR. OmpR, in turn, regulates the porins, OmpF and OmpC in a reciprocal manner, so that one porin predominates over the other, depending on osmotic conditions.

*Erwinia chrysanthemi*, which causes "soft rot" in a wide range of economically important crops, has been demonstrated to contain porin-like proteins similar to OmpF and OmpC. The expression of these porins was regulated in a similar manner to OmpF and OmpC with respect to medium osmolarity. Furthermore, preliminary studies have shown that changes in osmolarity affect the expression of pathogenecity genes. Evidence for an osmoregulatory system analagous to the *ompB* system of *Escherichia coli* was, therefore, sought. Primers specific for conserved regions in *ompR* were designed and used to PCR amplify a 631 bp fragment from *E. coli*. This fragment was cloned into the vector, pBluescriptSk, and end-sequenced to confirm its authenticity. The same strategy was followed, using *envZ*-specific primers to generate an *E. coli envZ* clone. Southern hybridisation analyses, using an *ompR* probe, confirmed the presence of an *ompR* homologue in *E. chrysanthemi*. An *E. chrysanthemi* genomic library was thus constructed and screened and a clone homologous to the *ompR* probe was isolated. The resulting plasmid, pRZ69, was partially characterised and determined to have both *envZ* and *ompR* homologues resident. Southern hybridisation analyses were employed to localise the *ompR* and *envZ* genes on the plasmid. A 1200 bp *Eco*RV-*Pst*I fragment containing the *ompR* homologue and a 2000 bp *Eco*RV-*Eco*RV fragment containing the *envZ* homologue, were subcloned into pBluescriptSk, generating the plasmids, pRS1 and pZS2 respectively.

DNA sequencing analyses revealed that the putative *ompB* locus of *E. chrysanthemi* (*ompRZ*) was structurally similar to the *ompB* locus of *E. coli*. The *ompR* and *envZ* genes were encoded as an operon and showed a high degree of sequence identity with that of the *ompB* locus of *E. coli* at both the nucleotide and protein level. Functional studies were performed to indicate that the *ompRZ* locus was functionally analogous to the *ompB* locus of *E. coli*. Complementation of an *E. coli ompR-envZ* mutant by the clone, pRZ69, confirmed that an *ompB* locus was cloned from *E. chrysanthemi*. Furthermore, outer membrane protein analyses showed that the introduction of pRZ69 into the mutant *E. coli* strain restored the osmoregulation of the major outer membrane proteins, OmpF and OmpC. Preliminary inactivation mutagenesis of the *ompRZ* locus was attempted, but was unsuccessful. Therefore, further studies should involve the generation of *ompR* and *envZ* mutants of *E. chrysanthemi* in order that the complex mechanisms underlying the interaction between the osmoregulatory system and the pathogenicity genes can be determined.

# CHAPTER 1

# GENERAL INTRODUCTION

# 1. ENVIRONMENTAL AWARENESS IN BACTERIA

Environmental conditions in a microbial world are often precarious. Fluctuations in nutrient and toxin levels, acidity, temperature, osmolarity and many other parameters may occur rapidly and unexpectedly. To survive, cells have to monitor these external conditions and adjust their structure, physiology and behaviour accordingly. In bacteria, these adaptive behaviours are made possible by elaborate cell sensory systems that function to optimise the bacterium's ability to survive under fluctuating conditions (Bourret *et al.*, 1991; Parkinson, 1993; Parkinson and Kofoid, 1992; Stock *et al.*, 1990). The underlying cell sensory machinery generally consist of signalling proteins that communicate by means of two distinctive domains, termed transmitters and receivers (Kofoid and Parkinson, 1988). Signals from within the cytoplasm and the environment regulate the cellular activities that result in the appropriate adaptive response, for example changes in gene expression or motility. Cell sensory systems typically respond to a wide variety of stimuli, including the presence of nitrogen (Keener and Kustu., 1988), changes in pH, osmolarity (Mizuno, 1991) and temperature, the presence of repellents and attractants, nutrient deprivation, phosphate limitation (Wanner and Wilmes-Riesenberg, 1992), plant exude and many others. This would imply that extensive families of environmental signalling proteins exist. Despite diversity among signalling proteins, common mechanisms govern their signal transducing properties. In this review these common features will be outlined, before a closer investigation of a few well-studied bacterial sensory systems will be done.



## 1.1 TWO-COMPONENT PARADIGM

A typical signal transduction system comprises two proteins, a sensor and a regulator and for this reason, the term, "two-component system" has been coined (Parkinson and Kofoid, 1992). Each of these proteins has two main functional domains. The first component, the sensor often spans the cytoplasmic membrane where it monitors some environmental parameter. The second component, the response regulator is cytoplasmically located and is typically a transcription activator. Sensors are structurally similar in bacteria, containing a C-terminal transmitter domain coupled to an N-terminal input domain (Nixon *et al.*, 1986). Generally transmitter domains are highly conserved in sequence over a length of about 200 amino acids, while input domains may vary depending on the stimulus they are specific for.

### 1.1.1 Phosphorylation activities of transmitters and receivers

Transmitters regulate the phosphorylation state of their receiver partners in two ways (Figure 1.2). One component of this regulation process involves the autokinase activity of transmitters, whereby phosphorylation groups from ATP are attached to histidine residues (Hess *et al.*, 1988; Ninfa and Bennett, 1991). This readily reversible autophosphorylation reaction forms a high-energy intermediate that serves to regulate the phosphorylation state at the site of an aspartic acid residue (Sanders *et al.*, 1989). The second component involves the apparent phosphatase activity of transmitters toward their cognate receivers (Aiba *et al.*, 1989; Igo *et al.*, 1989; Keener and Kustu, 1988; Ninfa and Magasanik, 1986). The interplay of these two transmitter activities, to regulate the flux of phosphoryl groups to and from target receivers, is dependent on specific sensory stimuli. Transmitters are presumed to function as dimers, with the catalytic site of one subunit, phosphorylating the acceptor site of the other (Swanson *et al.*, 1994; Yang and Inouye, 1991). Although very little is known about the secondary and tertiary structure of transmitters, their primary structure analyses have provided insight into their functional architecture (Parkinson and Kofoid, 1992). Sequence comparisons show that transmitters are about 240 amino acid in length with several blocks of nearly invariant residues (Figure 1.2). The histidine phosphorylation site is typically located near the N-terminal. Four blocks of residues in the carboxyl half are presumed to form the catalytic centre. Amino acid replacements at various sites within these conserved regions curtail or eliminate autokinase activity (Oosawa *et al.*, 1988; Yang and Inouye, 1991).

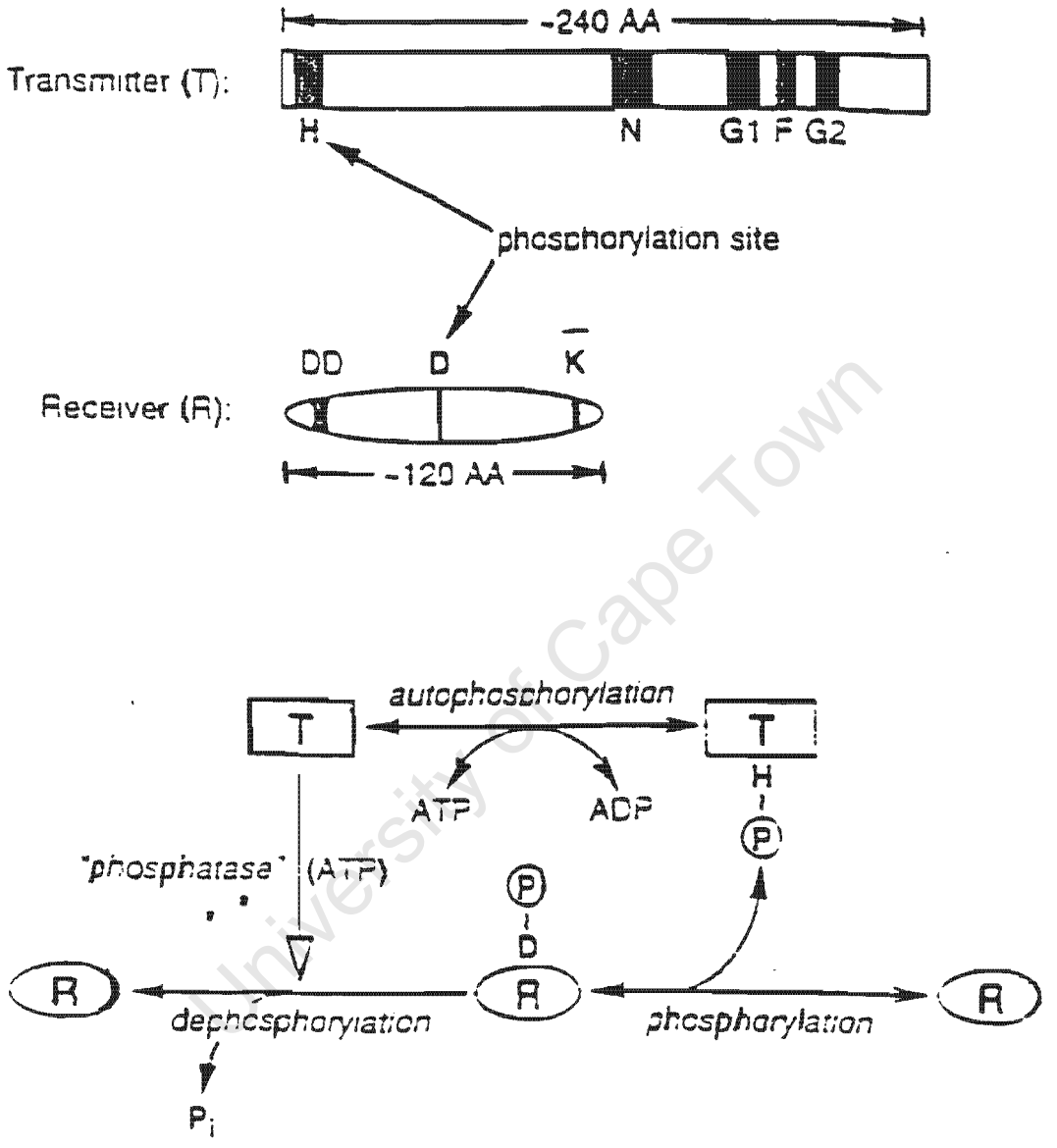





**Figure 1.2** Sequence features and phosphorylation activities of communication modules. Regions (motifs) most characteristic of transmitters or receivers are indicated by black boxes. Each motif is named for its most prominent amino acid. Details of the phosphorylation reactions are discussed in the text (Parkinson and Kofoid, 1992).

Receiver-containing proteins can phosphorylate themselves using small molecule donors such as acetylphosphate or phosphoramidate (Feng *et al.*, 1992; Lukat *et al.*, 1992). *In vivo* receivers presumably acquire their phosphate groups by catalysing phosphotransfers from cognate transmitters. Receivers also catalyse hydrolytic loss of their phosphoryl groups (Hess *et al*, 1988; Weiss and Magasanik, 1988). Transmitters can also have an apparent phosphatase activity toward their cognate receivers (Hess *et al.*, 1988; Sanders *et al.*, 1989). The phosphatase activity is ATP-dependent but nonhydrolyzable analogs serve as well, suggesting that ATP functions as a cofactor (Aiba *et al.*, 1989; Igo *et al.*, 1989; Keener and Kusta, 1988, Ninfa and Magasanik, 1986).

### 1.1.2 Transmitters

Transmitters recognise and interact with their cognate receivers in response to input sensory signals. It is assumed that these transactions involve changes in transmitter phosphorylation activities, but this has not been demonstrated explicitly. The only proven catalytic activity of transmitters is the formation of a high-energy phosphohistidine (Forst *et al.*, 1989). Receivers probably promote their own phosphorylation using these phosphohistidine transmitter intermediates as the preferred substrate (Parkinson and Kofoid, 1992). Thus, the transmitter autophosphorylation reaction represents a major control point for regulating the flow of phosphate into receivers. Several transmitters also exhibit an apparent phosphatase activity and thus provide a second point of controlling the phosphorylation state of receivers.



### 1.1.3 Structural features of transmitters

Transmitters contain short blocks of conserved regions that are arranged similarly but are variably spaced (Figure 1.2). Block H, the most variable of the five regions is located in the N-terminal half of the transmitter and includes the histidine residue, the site for autophosphorylation (Parkinson and Kofoid, 1992). The other segments are located in the C-terminal half of the transmitter. Blocks G1 and G2 resemble glycine rich portions of nucleotide-binding domains and are separated by a spacer of variable length, with block F roughly in its middle (Rosmann *et al.*, 1974). Replacements at the block H histidine abolises both

autophosphorylation and phosphatase activity (Yang and Inouye, 1993). However, mutational changes at modules around the histidine can affect either activity alone. Mutations in blocks N, G1 or G2 eliminate autokinase activity. At present the function of block F has not been determined.

### 1.1.4 Input control of transmitter activity

Input domains in sensor proteins are assumed to modulate the autokinase and phosphate activities of their associated transmitters, to control the phosphorylation state of response regulators. It is postulated that transmitters could have two autokinase states resulting from 'ON' and 'OFF' conformations (Parkinson and Kofoid, 1992). Stimuli would modulate autokinase activity by shifting the ON/OFF equilibrium (Figure 1.3). A model based on an idea by Stock *et al.* (1990) suggests that the input and output domains of sensors may undergo transitions between relaxed and tense conformations individually. When the domains are rigidly coupled a conformational change in one domain is accompanied by an opposing change in the other domain. As one domain relaxes, the other tenses. The model proposes that the 'ON' form of the transmitter corresponds to a conformationally relaxed state, whereas the stimulated input domain assume a conformationally tense state.



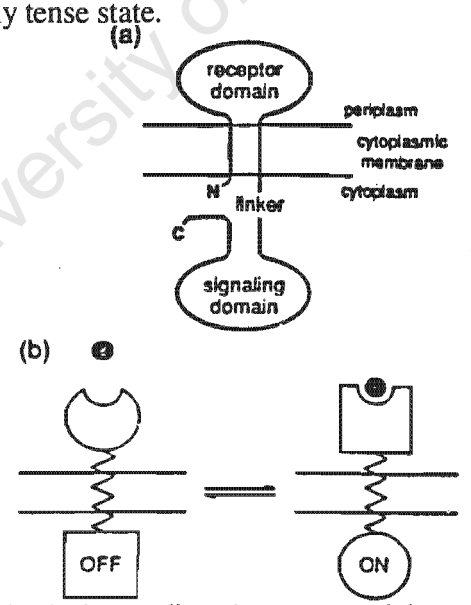



**Figure 1.3** Possible mechanical coupling between periplasm and cytoplasmic domains during transmembrane signalling. Each domain has relaxed (rounded) and tensed (squared) conformations. The black circle represents a generic stimulus (osmolarity etc). Stimulation shifts the receptor domain into the tensed state, leading to relaxation and activation of the signalling domain. ON and OFF specifically refer to the autokinase activities of transmitters in this model (Parkinson and Kofoid, 1992).

In the unstimulated form, the input domain of the transmitter is proposed to inhibit autokinase activity. Thus, the severing of the connection to the input domain should result in the activation of the transmitter. This experiment has been performed in many different sensors, including EnvZ (Aiba *et al.*, 1989; Forst *et al.*, 1987), PhoR (Makino *et al.*, 1989) and VirA (Huang *et al.*, 1990; Jin *et al.*, 1990) and in each case the liberated transmitter was associated with a substantial increase in autokinase activity.

Linker mutations could affect output activity in two different ways. Some might uncouple the two domains resulting in constitutive signalling activity, while others might lock the transmitter in the inactive state by mimicking the conformational effects of input stimuli. Linker mutations also bias signal output in two directions. A linker mutant of EnvZ behaves as though locked in the OFF state, whereas BvgS linker mutants seem to be in the ON state (Tokishita *et al.*, 1991). Mutations in the transmembrane portions of EnvZ also showed analogous signalling defects, with some mutants locked in an autokinase OFF state and others in an ON state (Russo and Silhavy, 1991; Tokishita *et al.*, 1991). Despite these findings, transmembrane signalling is still poorly understood.



### 1.1.5 Receivers

Receivers recognise and interact with their cognate transmitters, accept signals from them and as a consequence control output activity. It is assumed that these transactions involve changes in receiver phosphorylation state, although this has not been explicitly demonstrated (Parkinson and Kofoid, 1992). These reactions work to transduce information that is crucial for the survival of the cell. A molecular dissection of these reactions is outlined below.

Once a transmitter has been autophosphorylated, the transfer of phosphate from histidine to receiver is more than likely catalysed by the receiver itself rather than by the transmitter (Parkinson and Kofoid, 1992). Receivers are considered to be enzymes in their own right capable of using various phosphate sources as substrate for *in vivo* autophosphorylation. The half-lives of phosphorylated receivers vary greatly, from a few seconds for CheB to several hours for OmpR (Hess *et al.*, 1988). Receiver dephosphorylation also appears autocatalytic, because it can take place in the absence of other proteins. This dephosphorylation reaction is similar to the

autophosphorylation reaction in that it is dependent on magnesium or other divalent cations and is effectively halted on denaturation (Weiss and Magasanik, 1988).

### 1.1.6 Structure-Function Relationships

All orthodox receivers contain highly conserved aspects of primary structure and may be built along similar lines. The X-ray structure of the CheY receiver protein has been determined and has been adopted as the model receiver protein (Parkinson and Kofoid, 1992). An aspartic residue at position 57 has been shown by direct chemical analyses to be the phosphorylation site in CheY (Sanders *et al.*, 1989). Mutants with amino acid changes at this position at various receiver proteins are incapable of being phosphorylated and have no signalling activity (Bourret *et al.*, 1990; Stewart *et al.*, 1990). Thus, Asp-57 appears to be the main phosphorylation site in receiver modules.

In most response regulators the Asp-57 residue is flanked by additional acidic residues constituting the acid pocket (Parkinson and Kofoid, 1992). The acid pocket is a characteristic feature of orthodox receivers. The aspartic residue at position 13 (Asp-13) is generally present but in some receivers a glutamate residue at position 12, instead of an aspartic acid residue is present. Mutants affecting the acid pocket residues generally reduce phosphorylation ability.

There is only one other residue that is conserved to the same degree as the aspartic acid residue, a lysine at position 109 (Volz and Matsumura, 1991). Lys-109 appears to be found in all orthodox receivers and probably plays a fundamental role. It has been shown that an arginine replacement at this position results in a complete loss of function, even though the mutant protein can still be phosphorylated (Lukat *et al.*, 1991). Nuclear magnetic resonance (NMR) studies suggest that Lys-109 is repositioned in the known constitutively active CheY mutants. These findings suggest that phosphorylation, displaces the side chain of Lys-109 from the acid pocket thereby resulting in conformational changes that regulate the activity of the receiver.

In summary, it appears that the most highly conserved residues in receivers cluster together around the site of phosphorylation.

### 1.1.7 Control Over Output Activity in Response Regulators

Phosphorylation has been implicated in the control of output activity for a number of receiver subfamilies (Parkinson and Kofoid, 1992). Usually phosphorylation leads to an activation of output function but in some response regulators the unphosphorylated form may also have a functional role.

Receiver mutations that reduce phosphorylation ability usually block stimulus-induced changes in output activity. These changes often occur in the acid pocket residues. Since the activating effects of response regulator phosphorylation can be mimicked by mutational changes, phosphorylation may simply be a device for inducing conformational changes. Although little is known about how this control is achieved, it does appear that receivers have developed both positive and negative strategies for controlling associated output domains. Receivers in some response regulators seem to control their output domains through inhibition. The enzymatic activity of CheB is stimulated on removal of its receiver portion (Simms *et al.*, 1987). Similarly, the output domain of FixJ (Kahn and Ditta, 1991) is transcriptionally active, suggesting that the receiver negatively control its activity. Phosphorylation presumably activates these proteins by releasing receiver-induced inhibition. Although the evidence is circumstantial, the output domains in most response regulators appear to be under positive control. This is corroborated by experiments showing that the removal of the receiver module in OmpR does not lead to an enhancement in the DNA-binding ability of its output domain. These experiments suggest that the receiver is needed to activate output function (Tsung *et al.*, 1989) which is consistent with the conclusion that the output domain is under positive control by its associated receiver module (Brissette *et al.*, 1991).

Two general classes of mechanisms would enable receivers to exert positive or negative control over output function (Figure 1.4). Control could involve direct interaction of the receiver with the output domain, promoted through specific contacts modulated by the phosphorylation state (Figure 1.4a). Alternatively, output control might involve no direct contact between the receiver and the rest of the protein, which in principle means that any particular receiver could be functionally coupled to a variety of output domains. A simple mechanism for indirect control could be through phosphorylation-induced changes in the receiver aggregation state (Kofoid and Parkinson, 1988). Phosphorylation, for example, might cause receiver subunits to dimerize

thereby altering the function of their output domains by bringing them into closer proximity (Figure 1.4b). The presence of flexible linkers joining receiver and output domains in many response regulators implies that some movement of the two domains may be necessary for proper control, but neither direct nor indirect models make specific predictions about the importance of linkers (Parkinson and Kofoid, 1992).

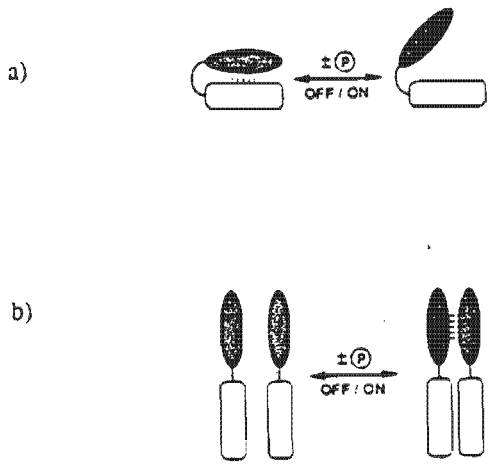



**Figure 1.4** Two general mechanisms for controlling output domain activity (ON and OFF) through changes in receiver phosphorylation state. Receivers could modulate activity by direct interaction with the output domain (top) or by mediating subunit association (bottom).
(Parkinson and Kofoid, 1992).



### 1.1.8 Transmitter-Receiver Interactions

The functionally critical step in two-component signal transduction is the interaction between the receivers and the transmitters. This interaction is proposed to involve phosphorylation and dephosphorylation reactions. The cycle of a simple circuit in which the output signal is related directly to receiver phosphorylation state is outlined in Figure 1.5.

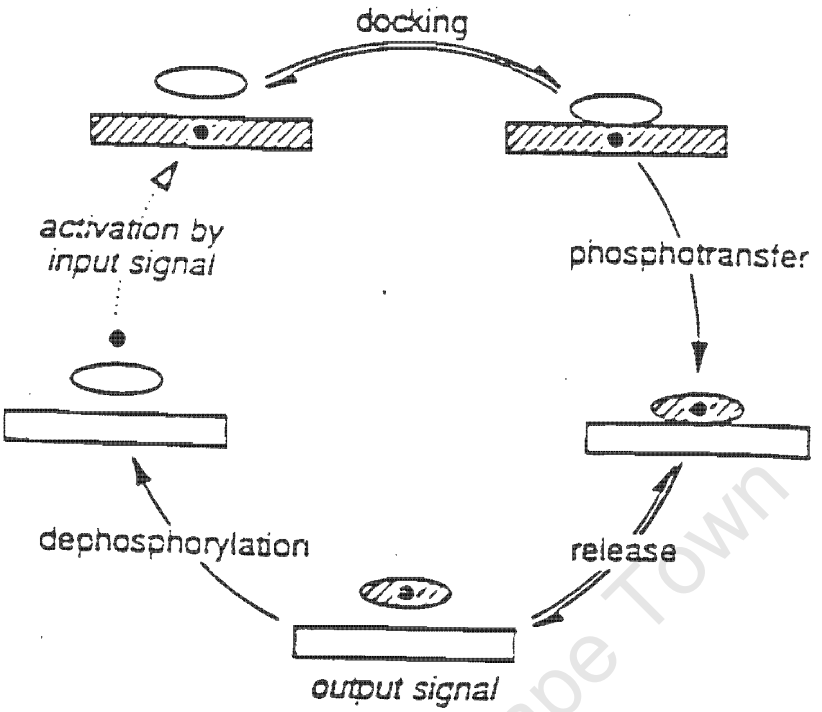



**Figure 1.5** Critical events in the transmitter-receiver cycle. Docking and releasing are controlled by reversible equilibria, whereas phosphotransfer from transmitter to receiver and subsequent dephosphorylation of the receiver may be effectively unidirectional. Hatched shading denotes modules in an activated conformation, while black circles represents phosphoryl groups (Parkinson and Kofoid, 1992).



Changes in receiver phosphorylation begin with activation and autophosphorylation of the transmitter in response to sensory input (Parkinson and Kofoid, 1992). Following this, unphosphorylated receivers associating reversibly with the activated transmitter, through specific recognition interactions. After engaging the transmitter, the receiver catalyses transfer of the phosphate from the histidine residue to its own aspartate acceptor site. Receiver phosphorylation could either result in reduced binding affinity for the dephosphorylated transmitter, thus facilitating its release and re-entry into the cycle, or lead to the production of an output signal. The changes in response regulator activity should persist until the receiver loses its phosphate, either spontaneously through its own autophosphatase activity or through transmitter-stimulated dephosphorylation.

These interactions between transmitters and receivers play a pivotal role in signal transduction schemes of a number of biological systems. This form of communication, as is characteristic of two-component systems, is found among diverse systems, including the bacterial chemotaxis system, nitrogen and phosphate regulation systems, as well as in bacterial osmoregulatory systems. A molecular dissection of these various systems will follow.

## 1.2 CHEMOTAXIS

One of the major modes of communication between a cell and its environment is by chemotaxis (taxis meaning movement). This process is normally characterised by the cell responding to chemotactic stimuli namely, attraction to some chemicals and repulsion from others (Barak and Eisenbach, 1996). Bacteria are considered to be relatively simplistic and are, therefore, the organisms in which chemotaxis has been studied most extensively. Owing to these advances bacterial chemotaxis has become one of the model systems for studying sensory signal transduction at the molecular level (Adler, 1985).

Chemotaxis in bacteria is the modulation of two swimming patterns: a run, which is smooth swimming, and a tumble, which is chaotic motion. Counterclockwise (CCW) rotation propels the cells forward, whereas clockwise (CW) rotation causes them to turn or tumble (Parkinson, 1993). When cells sense that they are heading toward attractants or away from repellents, tumbles and pauses are suppressed and the cells tend to continue on course (Macnab and Ornston, 1977). Methyl-accepting chemotaxis proteins (MCPs) and other chemoreceptors elicit chemotactic movements by regulating flagellar rotation, in response to attractant or repellent concentration (Armstrong *et al.*, 1967; Aswad and Koshland, 1975). By devising strategies for selecting mutants that are either unable to change direction or are defective in chemotaxis, six *che* (chemotaxis) genes have been identified: *cheA*, *cheB*, *cheR*, *cheW*, *cheY* and *cheZ* (Stock *et al.*, 1989). All of the proteins encoded are soluble cytoplasmic constituents that either participate directly in, or modulate signal transduction between chemoreceptors and the flagellar motor.

Three flagellar proteins appear to function at the interface between the Che proteins and the flagellar motor. These proteins are encoded by the genes, *fliG*, *fliM* and *fliN* (Parkinson *et al.*, 1983). Most bacteria detect chemotactic stimuli with chemoreceptors known as MCPs

(Hazelbauer, 1992). A family of these MCPs have been defined for *E. coli*, namely, Tsr, Tar, Tap and Trg (Stewart and Dahlquist, 1987).

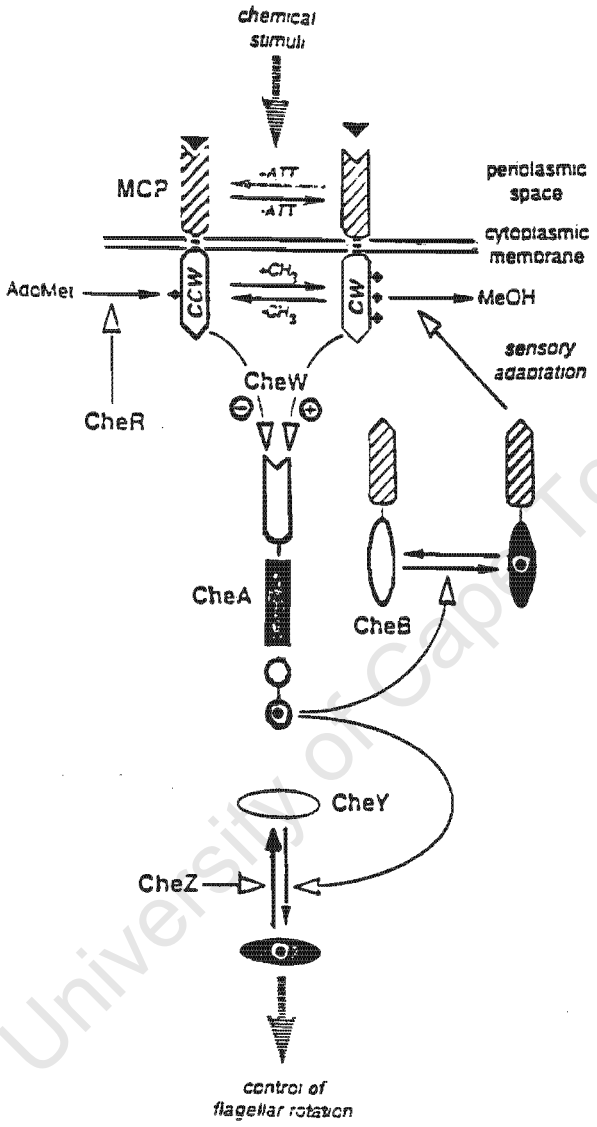



**Figure 1.6** Chemotaxis circuitry of *E. coli*. The signalling pathway handles responses to compounds detected by chemoreceptors of the MCP class. Receptor signals control the autophosphorylation rate of CheA to modulate the flux of phosphates to CheY and CheB. Phospho-CheY controls the flagella motors and phospho-CheB regulates sensory adaption through changes in MCP methylation state. The ligands are depicted as black triangles and the methyl groups are depicted as black diamonds. Open headed arrows denote control of the indicated signalling steps (Parkinson, 1993).

The function of each component in the chemosensory signalling pathway is well understood and will be described in the sections to follow.

### 1.2.1 Signal Transduction in Bacterial Chemotaxis

An overview of signal transduction in the *Escherichia. coli* and *Salmonella. typhimurium* chemotaxis systems is outlined in Figure 1.6. The membrane chemoreceptor-transducer protein, stimulate the flow of phosphoryl groups through CheA to CheY and CheB (Stock *et al.*, 1989). This process, which requires CheW, is inhibited by the binding of attractant ligands to the receptors and is stimulated by methylation of the receptors.

MCP molecules are proposed to control CheA through conformational changes that regulate the access of the phosphates to CheB and CheY (Hess *et al.*, 1988; Wylie *et al.*, 1988, Stock, 1988) both of which contain receiver modules. Phosphorylation enables CheY to interact with switch proteins to generate CW rotation (Barak and Eisenbach, 1992, Roman *et al.*, 1992). The motors rotate CCW by default, so the relative levels of P-CheY determine the cells swimming behaviour viz CCW or CW rotation (Parkinson, 1993). CheZ antagonises CheY activity by enabling the dephosphorylation of P-CheY (Hess *et al.*, 1988). CheB is part of a feedback circuit that terminates motor responses by altering the methylation states of MCP molecules, which in turn regulates their signaling properties. Thus, by controlling the flux of phosphate through CheA to CheY and CheB, the receptors not only trigger behavioural responses but also initiate the sensory adaptation process.



## 1.3 NITROGEN REGULATION

Bacteria assimilate nitrogen mainly through the synthesis of glutamine from glutamate and ammonia, an ATP-dependent reaction catalysed by glutamine synthetase (Stock *et al.*, 1989). Since no other pathway for the formation of glutamine other than the glutamine synthetase reaction exists, ammonia can be considered to be an essential metabolite. If an environmental source of ammonia is lacking, ammonia must be derived by the catabolism of nitrogenous compounds such as amino acids or urea, or from the reduction of atmospheric nitrogen.

Under conditions of limiting ammonia, there is a dramatic increase in the transcription of a number of genes (Reitzer and Magasanik, 1987). The Ntr regulon represents a subset of these

nitrogen-regulated genes that are regulated by common regulators. The individual Ntr operons are also subject to Ntr-independent controls such as induction by a specific nitrogen source and catalytic repression. The adaptive response to nitrogen starvation occurs in several stages. A central role is played by the *glnALG* operon that encodes glutamine synthetase, the histidine kinase of the Ntr regulon, $NR_{II}$, and the response regulator of the Ntr system, $NR_I$ (Backman *et al.*, 1981; Kustu *et al.*, 1979). Both, $NR_I$ and $NR_{II}$ regulate the transcription of *glnA* and the other Ntr and Nif genes.

In summary, the Ntr-dependent adaptive response to nitrogen starvation involves firstly an increase in the level of glutamine synthetase, which enhances the ability of the cell to use low concentrations of ammonia. Following this, the induction of Ntr operon products such as histidase and nitrogenase that provides ammonia from sources of organic nitrogen, occurs. This progression of events is tightly controlled by a cascade of trancriptional regulation that begins with the activation of $NR_I$ (Stock *et al.*, 1989).

### 1.3.1 Signal Transduction

The balance between nitrogen and carbon metabolism, as reflected by the intracellular ratio of glutamine and 2-ketoglutarate, appears to be the critical signal in the signal transduction cascade of the *glnALG* operon (Stadtman *et al.*, 1980). This ratio controls the activity of a bifunctional enzyme, the *glnD* gene product that catalyses the uridylylation and deuridylylation of tyrosine residues in another regulatory protein, $P_{II}$. Under conditions of high ammonia, the ratio of ketoglutarate/glutamine in relatively low and $P_{II}$ is predominantly in its unmodifed form. Ammonia-limiting conditions result in a relatively high ratio of ketoglutarate/glutamine and $P_{II}$ occurring predominantly in its uridylylated form (Stadtman *et al.*, 1980).

The ratio of $P_{II}/P_{II}$-uridine monophosphate controls the sensitivity of glutamine synthetase, to the allosteric effects of ligands, through its effect on the *glnE* gene product (Bueno *et al.*, 1985; Chock *et al.*, 1985; Standtman *et al.*, 1980). GlnE (AT) is a bifunctional enzyme that catalyses the adenylylation and deadenylylation of tyrosine residues in glutamine synthetase. Adenylation inhibits glutamine synthetase activity. Unmodified $P_{II}$ stimulates adenylylation and $P_{II}$-uridine monophosphate stimulates deadenylylation (Bueno *et al.*, 1985).

$P_{II}$ also serves to control transcription of the *glnALG* operon. Genetic evidence indicates that unmodified $P_{II}$ exerts its effects on *glnALG* trancription by controlling the activity of $NR_{II}$ (Bueno *et al.*, 1985; Chen *et al.*, 1982, Backman *et al.*, 1981). $NR_{II}$ is not required for activation of trancription from *glnAp2 in vivo* but has a dramatic effect on the timing of responses to changes in nitrogen availabilty. In wild-type cells, transcription from *glnAp2* rapidly adjusts to changes in nitrogen metabolism whereas in cells lacking $NR_{II}$, the regulation of this transcription is extremely slow. It is evident that the *glnD* (UT/UR)/$P_{II}$ signal transduction pathway acts to regulate the expression of Ntr genes entirely through $NR_{II}$ (Stock *et al.*, 1989).

$NR_{II}$ is a histidine kinase that regulates transcription from *glnAp2* by controlling the phosphorylation state of the transcription factor $NR_I$. The phosphorylation mechanism is similar to that of the CheA and CheY chemotaxis system. $NR_{II}$ is autophosphorylated on a histidine residue and can transfer this phosphate to $NR_I$ in the absence of nucleotides. $NR_I$ is phosphorylated within the N-terminal domain that is conserved in all response regulators. The addition of $P_{II}$ to reaction mixtures containing P-$NR_I$, $NR_{II}$, ATP and divalent cations, results in a rapid dephosphorylation of P-$NR_I$ (Keener and Kustu, 1988). $P_{II}$ thus appears to activate an ATP-dependent $NR_{II}$ phosphatase that hydrolyses P-$NR_I$ and thereby preventing the activation of transcription from *glnAP2* (Stock *et al.*, 1989).

## 1.4 PHOSPHATE REGULATION

### 1.4.1 Phosphate Uptake

In *E. coli*, phosphate transport is mediated by several proteins that exist within the cell envelope. Active transport across the cytoplasmic membrane takes place by low and high-affinity $P_I$ transport systems (PIT and PST) and other sugar phosphate transport systems (Gpt, Pgt and Uhp). An anion-specific porin (PhoE) mediates passage across the outer membrane into the periplasm. Within the periplasm, phosphatases such as alkaline phosphatase, PhoA and binding proteins, PstS and UgpB (Surin *et al.*, 1986) help to deliver usable phosphate to the transport systems in the cytoplasminc membrane (Surin *et al.*, 1985).

The expression of many of the phosphate uptake proteins is regulated by phosphates in the cell's environment. $P_I$ represses the *phoA* and *phoE* genes including the *upg* and *pst* operons. Sugar phosphates, such as glycerol phosphates and hexose phosphates, induce genes that encode their respective transport systems, *gptP* and *uhpT*. A number of histidine kinases and response regulators act to control these genes (Stock *et al.*, 1989). The PhoR kinase and PhoB regulator control the expression of *phoA*, *phoE*, the *pst* operon and the *phoBR* operon itself. These genes, all of whose promoters require PhoB, has collectively been termed the Pho regulon (Wanner, 1987).

### 1.4.2 Pho Regulon

The high-affinity PST system functions to detect high extracellular phosphate and this information is relayed to cause the repression of *phoA* as well as other genes of the Pho regulon, including PST itself (Stock *et al.*, 1989). The major histidine kinase of the Pho regulon is PhoR, with PhoB being its cognate response regulator. PhoB is essential for PhoA expression. The protein itself has been purified and shown to function as an acceptor for the phosphoryl group in P-PhoR. P-PhoB appears to have an inherent autophosphatase activity. PhoB in its phosphorylated form was shown to stimulate the *in vitro* transcription from the *phoA* promoter (Makino *et al.*, 1988). This stimulation is the result of enhanced binding of P-PhoB to the Pho box upstream of the *pstS* promoter (Stock *et al.*, 1989).



### 1.4.3 Signal Transduction Pathways That Regulate PhoB

In the Pho system, the kinase (PhoR) appears to be a typical membrane receptor with an extracellular sensory domain and an intracellular signalling domain (Figure 1.7). An analysis of the genetics of Pho regulation has indicated that all components of the *pst* operon are essential for Pho repression in high phosphate. Pst is a typical binding, protein-dependent bacterial transport system (Ames, 1986), composed of a periplasmic binding protein, PstS; a peripheral membrane protein, PstC; and two integral membrane proteins PstA and PstB (Stock *et al.*, 1989). In addition, the *pst* operon contains a fifth gene, *phoU*. PhoU is not essential for phosphate

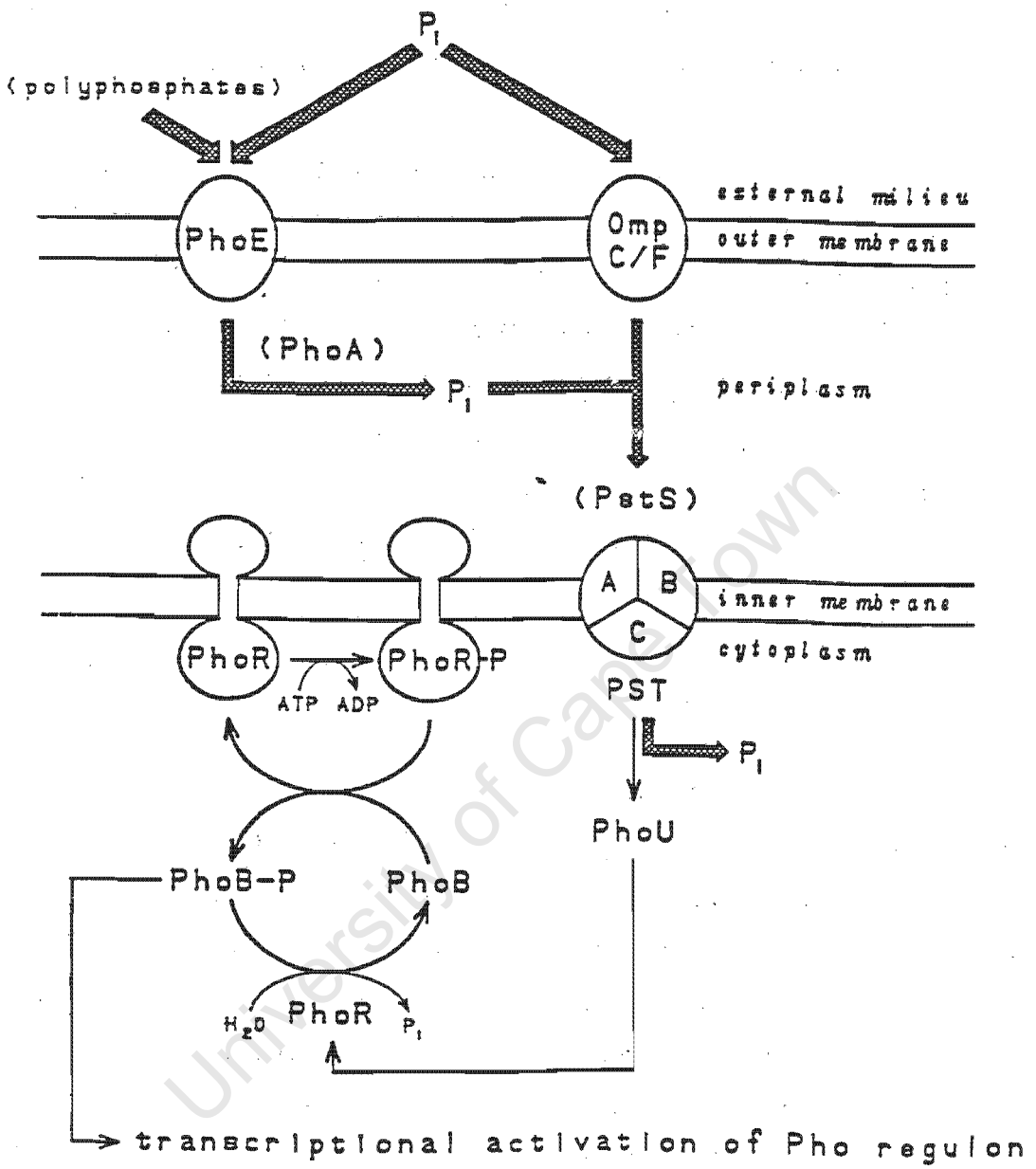





**Figure 1.7** Regulation of phosphate uptake in *E. coli*. Extracellular phosphate diffuses across the outer membrane through porins, PhoE, OmpF and OmpC. Alternatively, phosphoesters in the periplasm are hydrolysed to P through the action of alkaline phosphatase, PhoA. At low phosphate concentrations, phosphate is transported via a phosphate binding protein, PstS, through the PST sysetm into the cytoplasm. The PST system in conjunction with PhoU regulates the activity of the transcriptional activator, PhoB, that regulates the expression of all operons in the Pho regulon. The active form of PhoB is P-PhoB, produced through the action of the PhoR kinase. PhoU, together with PhoR acts to dephosphorylate P-PhoB (Stock *et al.*, 1989).

### 1.5.1 Mechanisms Underlying Osmoregulation

*Escherichia coli* cells are composed of two compartments, the periplasm and the cytoplasm delineated by the cell wall outer membrane and the inner cytoplasmic membrane. The cytoplasmic membrane is not rigid and cannot support an osmotic pressure gradient (Csonka, 1989). This function is supplied by the cell wall. The rigid outer structure is relatively impermeable to solutes of molecular weight > 600, thus trapping proteins and other polymers within the periplasm. These molecules, including polyanions such as membrane-derived oligosaccharides, support the osmotic strength of the periplasm through their attraction of high concentrations of cations such as sodium and potassium (Stock *et al.*, 1989). Since the cytoplasmic membrane cannot sustain a pressure gradient, the osmotic strength of the periplasm and cytoplasm must remain equal through movements of water between these compartments (Csonka, 1989). This process of maintaining equilibrium is facilitated by the roles played by cytoplasmic solutes known as compatible solutes, osmoprotectants and to a large extent, that played by the porin regulon.



### 1.5.2 Compatible Solutes

Exposure of cells to high external osmolarity results in an efflux of water from the interior. This efflux of water brings about a reduction in the turgor pressure and shrinkage of the cytoplasmic volume. As a consequence of cytoplasmic volume shrinkage, the concentrations of all the intracellular metabolites increase, thus decreasing intracellular water activity. An elevation in the concentrations of various intracellular molecules may in certain cases be inhibitory to cellular processes. Thus, passive alteration of the cell volume is not adequate for adaptation to changes in the osmolarity of the environment (Walderhaug *et al.*, 1987).

Instead of passive regulation, organisms generally respond to osmotic stress by increasing the concentrations of a limited number of solutes. Since these molecules that are accumulated during conditions of osmotic stress are not greatly inhibitory to cellular processes, they have been termed compatible solutes. Compatible solutes in general are solutes that are unable to cross the cell membranes rapidly without the aid of transport systems and for the most part do not carry a net electrical charge near pH7 (Brown and Simpson, 1972). The prominent compatible solutes

found in bacteria are potassium ions ($K^+$), the amino acids glutamate, glutamine, proline, γ-aminobutyrate and alanine, the quaternary amines glycinebetaine and the sugars sucrose, trehalose and glucosylglycerol (Csonka, 1989; Csonka and Hanson, 1991).

### 1.5.2.1 Potassium ions

Potassium ions ($K^+$) are the most prevalent cations in the cytoplasm of bacteria and serve as the major intracellular osmolytes that maintain turgor. The major contributions to the understanding of the role of $K^+$ in osmoregulation emerged from the work of Epstein and Kim (1971). Their findings revealed that intracellular concentrations of $K^+$ increased as the osmolarity of the medium increased. Furthermore, increased accumulation of $K^+$ was elicited only by high concentrations of solutes that could not diffuse across the cell membrane (e.g. glucose, sucrose and NaCl) and the intracellular concentrations of $K^+$ was dependent only on the osmolarity of the medium, regardless of the solute used. Accumulation of $K^+$ was not elicited by glycerol, which diffuses freely across the cell membrane. These findings suggested that the signal for enhanced $K^+$ accumulation is not the decrease in the intracellular water activity, but the loss of turgor or possibly the reduction in cytoplasmic volume.

Radioisotope studies (Epstein, 1986) monitoring $K^+$ concentration demonstrated that during steady-state growth of *E. coli*, there was a rapid exchange of intra- and extracellular $K^+$. This indicated that the intracellular concentration of $K^+$ is determined by both its rates of uptake and efflux. The stimulation of both the influx and efflux processes in response to media of hyper or hypoosmolarity, respectively, was found to occur rapidly and without the requirement of an energy source. Although the mechanism of regulation of these two processes is not known, it has been proposed to involve a direct effect of turgor on the proteins mediating $K^+$ entry and exit (Meury and Kepes, 1982).

*Escherichia. coli* has a number of $K^+$ transport systems of which two have been extensively characterised. The Trk system has a relatively low affinity for $K^+$ and is constitutively expressed (Walderhaug *et al.*, 1987; 1989). Its activity is enhanced in response to hyperosmotic shock. The *kdp* system has a much higher affinity for $K^+$ and is subject to transcriptional regulation. Its

activity is also stimulated by hyperosmotic shock in a manner similar to that observed with the Trk system (Rhoads and Epstein, 1978).

### 1.5.2.2 Glutamate and Glutamine

The cytoplasmic levels of glutamate increase in most prokaryotes after exposure to media of high osmolarity (Csonka, 1989). Richey *et al.* (1987) reported that the steady-state intracellular glutamate concentration in exponentially growing *E. coli* cells was directly proportional to the osmolarity of the growth medium. The levels of glutamine also increase in response to osmotic stress in Gram-negative bacteria, but because glutamine is present at much lower levels than glutamate it is probably not as important for the maintenance of cytoplasmic osmolarity (Csonka, 1989; Csonka and Hanson, 1991). The accumulation of glutamate and glutamine was observed in several species of bacteria grown in media devoid of exogenous amino acids, indicating that the increase in the levels of these amino acids is due to an enhancement of their rate of synthesis (Csonka, 1989). However, it is not known to what extent the increase in these amino acid levels is due to stimulation of their synthesis or inhibition of their turnover. Measures (1975), observed that $K^+$ at high concentration stimulated glutamate dehydrogenase activity in several bacterial species and therefore suggested that the accumulation of $K^+$ under conditions of osmotic stress, is the regulatory signal for the synthesis of glutamate. However there have been few reports on the possible transcriptional control of either glutamate synthase or glutamate dehydrogenase by osmotic stress.



### 1.5.2.3 γ-Aminobutyrate

Measures (1975) reported that γ-aminobutyrate levels increased in response to osmotic stress in a variety of bacteria. Accumulation of these amino acids was not seen in enteric bacteria grown in minimal medium, thus suggesting that its increase in levels is the result of uptake from the medium, rather than synthesis. Mutations that abolish the transport or catabolism of γ-aminobutyrate have been obtained in *E. coli*, but their effect on osmotic stress tolerance still has to be determined.

### 1.5.3 Osmoprotectants

Osmoprotectants are solutes that alleviate the osmotic stress imparted on the cell by the external environment (Csonka and Hanson, 1991). In the bacterial kingdom, only a few photoautotrophic species are able to carry out the complete synthesis of some of these osmoprotectants. Other species are dependent on the exogenous supply of these solutes or its precursor forms. Glycinebetaine, proline and choline are all model examples of osmoprotectants utilised by bacteria.

#### 1.5.3.1 Proline

Bacteria can accumulate proline to high intracellular concentrations by increased net synthesis or by enhanced uptake from the medium. It was found that osmotic stress resulted in large increases in the intracellular levels of proline in a large variety of bacteria. In general, Gram-negative bacteria achieve high intracellular concentrations of proline during osmotic stress only by enhanced transport (Csonka, 1989). *E. coli* and *S. typhimurium* have three independent proline transport systems: PutP, ProP and ProU (Wood, 1988; Haardt *et al.*, 1995).

The PutP system is required for the transport of proline when this metabolite is used as a carbon or nitrogen source (Maloy, 1987). It is not important for the transport of proline as an osmoprotectant because mutants lacking this system are stimulated normally by proline media of elevated osmolarity. However, the other two systems, ProP and ProU are responsible for the accumulation of proline to high levels under conditions of hyperosmotic stress (Csonka, 1989; Haardt *et al.*, 1995). The ProP system was first described as a minor proline permease in *E. coli* mutants lacking the PutP system. The activity of this system is also enhanced in response to amino acid starvation by a mechanism that is not understood. The ProP system has a rather low affinity for proline in cells grown in media of low osmolarity (Culham *et al.*, 1993).

The existence of the ProU system was inferred from the observation that proline was able to function as an osmoprotectant for *proPputP* double mutants of *S. typhimurium*. This third proline permease is proposed to function only in media of elevated osmolarity. The activity of this permease is enhanced by osmotic stress by at least hundred-fold as a result of an increase in the

level of transcription of the *proU* operon. The ProU system also has a relatively low affinity for proline. The existence of multiple transport systems for a substrate as is the case with proline, raises the question as to whether some control mechanism coordinate the operation of the independent porters. Whether some higher-order mechanism is coordinating the activities of the three, proline transport systems is still unknown and has to be addressed.

### 1.5.3.2. Glycinebetaine

A second important osmoprotectant compound accumulated by bacteria under conditions of hyperosmolarity is glycinebetaine (*N,N,N* trimethylglycine). Most bacteria are unable to synthesise glycinebetaine and are therefore dependent on the transport of this compound for its accumulation (Lucht and Bremer, 1994). The transport of glycinebetaine is stimulated by osmotic stress and mediated by the ProP and ProU systems. The ProU system is probably more important than the ProP system for the transport of glycinebetaine. Besides these systems it appears that an additional glycinebetaine permease exists in *S. typhimurium* (Ekena, 1990).

### 1.5.3.3 Choline

Although enteric bacteria are unable to synthesise glycinebetaine from glucose or other carbon sources, *E. coli* K12 can convert choline to glycinebetaine under conditions of osmotic stress (Strom *et al.*, 1986). Thus, choline is also an osmoprotectant for *E. coli*. The formation of glycinebetaine from choline involves two oxidative steps with glycinebetaine aldehyde as intermediate (Landfald and Strom, 1986). In *E. coli*, a single enzyme, which has both choline and glycinebetaine aldehyde dehydrogenase activities is encoded by the *betA* gene. This enzyme is membrane-bound and its activity is coupled to electron transport, therefore, requiring electron acceptors such as oxygen. This requirement for oxygen means that choline cannot be used as an osmoprotectant anaerobically. The uptake of choline is mediated by two transport systems, one of which is encoded by the *betT* gene.

### 1.5.4 Osmoregulation of the Periplasmic Space

In Gram-negative bacteria, the periplasmic space is the site where a number of hydrolases, metabolite binding proteins and receptors of chemotactic signals (Oliver, 1987) accumulate. Since solutes of less than 500Da are able to diffuse readily into the periplasmic space through porin proteins (Csonka, 1989; Csonka and Hanson, 1991), there are unique problems in the maintenance of the osmotic potential of the periplasm. To overcome these problems, Gram-negative bacteria accumulate large quantities of highly ionic polysaccharides, known as membrane-derived oligosaccharides. The presence of these anionic polymers in the periplasm gives rise to an electric potential across the outer membrane. This potential, known as the Donnan potential, results in the accumulation of cations at a higher concentration in the periplasm than in the medium, resulting in hydrostatic pressure in the periplasm (Kennedy, 1982). The synthesis of membrane-derived oligosaccharides is subjected to osmotic regulation so that they are synthesised maximally in media of low osmolarity. The increasing of the osmolarity results in the reduction of their synthesis.

In *E. coli*, the enzymes involved in the formation of membrane-derived oligosaccharides are expressed constitutively, suggesting that the osmotic control of their synthesis entails the regulation of the catalytic activity of at least one of these enzymes (Kennedy, 1987). Mutations in the *mdoA* or *mdoB* genes block the synthesis of membrane-derived oligosaccharides (Fiedler and Rottering, 1988). However, strains carrying these mutations do not show any growth defects in media of high or low osmolarity. This would indicate that an alternative mechanism besides the synthesis of membrane-derived oligosaccharides exists for maintaining the osmotic potential of the cytoplasm. These pathways are yet to be defined.



### 1.5.5 Osmotic Control of Transcription

#### 1.5.5.1 *Kdp* Operon

The *kdp* operon of *E.coli* is repressed by high concentrations of exogenous $K^+$ (Rhoads *et al.*, 1976). Recent findings have shown, however, that the operon could be induced even in the presence of excess $K^+$ by high concentrations of any ionic or nonpolar solutes that were excluded

by the membrane. There appeared to be interplay between hyperosmotic shock and the intracellular $K^+$ concentration in the regulation of expression of the *kdp* operon. To account for this observed transcriptional regulation it was proposed that the turgor pressure of the cell was the only signal that regulates the expression of this operon (Laimins *et al.*, 1981).

The transcription of the *kdpABC* genes is under positive control of the KdpD and KdpE proteins, which are involved in sensing the turgor and transferring the signal to the *kdpABC* promoter. The KdpD product has been proposed to span the periplasmic space, forming contacts with both the inner and outer membrane (Epstein, 1986). Here it serves as the sensor protein and is proposed to monitor cell turgor by measuring the distance between the inner and outer membrane (Laimins *et al.*, 1981). This signal is then transmitted to the KdpE protein, which then acts as a transcriptional activator of the *kdpABC* operon, in response to the loss of turgor. Although this turgor control model appears attractive, there are experimental evidence suggesting that turgor may not be the sole regulator. Evidence suggests that the operon, is at least in part, regulated by some factor that is either directly or indirectly related to the availability of $K^+$ (Gowrishanker, 1987).



### 1.5.5.2 *proU* operon

Studies have demonstrated that hyperosmotic stress elicits induction of transcription of the *proU* operon of *E. coli* and *S. typhimurium.* Similar to the *kdp* operon, induction of *proU* can be triggered by solutes that are excluded by the membrane, but not by substances traversing freely across this barrier. However, in contrast to the *kdp* operon, the *proU* operon is continuously induced in the presence of media of high osmolarity, whereas the *kdp* operon is only temporarily induced (Dunlap and Csonka, 1985).

*proU* regulation can be divided into 3 stages: After exposure of the cells to hyperosmotic shock, a 15-20 minute lag before increased transcription of *proU* is detectable (Gowrishanker, 1985). Following this is a rapid stimulation of transcription of the *proU* operon at a differential rate that greatly exceeds the steady state differential rate observed in cells growing in a medium of equal osmolarity. The final stage is characterised by the gradual decrease of the differential rate of transcription of *proU*, to a steady state value.

Since the *proU* operon is expressed at a high level under conditions where high osmolarity is maintained, the transient loss of turgor alone cannot be the regulatory signal for the expression of this operon. In principle the osmotic strength (or water activity) of the cytoplasm could be the signal for the transcription of *proU*. However, the fact that solutes that diffuse freely across the membrane do not induce *proU*, indicates that some other signal controls the transcriptional regulation of this operon. Based on their observations that the concentration of $K^+$ increases with increasing external osmolarity, Epstein (1986) have proposed that the intracellular concentration of $K^+$ may be the regulatory signal for *proU* induction. Since it is not completely established whether osmotic stress causes a transient or permanent increase in the $K^+$ levels of *E. coli*, no definitive role for this cation in osmotic adaptation has been proposed. Other studies have also demonstrated that supercoiling of the proU promoter in response to changes in the intracellular osmolarity could also be important in *proU* induction (Higgins *et al.*, 1988). However, the question as to whether the osmotic control of transcription of the *proU* promoter, is affected primarily by supercoiling, still needs to be explored further.

### 1.5.6 Regulation of porin expression

Gram-negative bacteria contain a number of porin proteins embedded in their outer membranes that act as channels to facilitate the diffusion of hydrophilic molecules across this hydrophobic barrier. In *E. coli* and *S. typhimurium*, two major porin proteins, OmpF and OmpC have been characterised and found to be differentially regulated (Parkinson, 1993; Nikaido, 1994). Regulation of these porin proteins is determined by a variety of environmental conditions such as osmolarity of the medium, carbon source and temperature. The total cellular level of OmpF and OmpC remain relatively constant but the proportion of the two varies. Thus, conditions favouring the synthesis of OmpF result in the repression of OmpC and vice versa (van Alphen and Lugtenberg, 1977).

The two proteins share extensive homology at the amino acid level but differ in the size of the pore that it forms (Nikaido and Vaara, 1987). At low osmolarity, poor carbon source and low temperature, the larger pore-sized OmpF protein (1.16nm) is preferentially expressed. However, at high osmolarity, good carbon sources and high temperature, OmpF expression is repressed while the smaller pore-sized OmpC protein (1.08nm) is increasingly expressed.

Early studies identified three genetic loci involved in this reciprocal regulation: *ompF*, *ompC* and *ompB* (Sarma and Reeves, 1977; Sato and Tura, 1979; Verhoef *et al.*, 1977). The ompF and *ompC* genes are structural, encoding for the OmpF and OmpC proteins respectively, while the *ompB* locus plays a regulatory role. The *ompB* operon is comprised of two genes, *envZ* and *ompR*, both being important for porin regulation (Hall and Silhavy, 1979; 1981).

### 1.5.6.1 The Role of EnvZ and OmpR

EnvZ and OmpR are members of a large family of homologous proteins that are collectively referred to as two-component regulatory systems. In the osmosystem, EnvZ and OmpR form a connected signal transduction pathway that enables cells to sense the external osmolarity and respond to this stimulus by regulating the transcription of the genes encoding the OmpF and OmpC proteins (Pratt and Silhavy, 1995).

EnvZ is a 450 amino acid protein that is localised to the inner membrane where it functions as an osmosensor (Comeau *et al.*, 1985; Hall and Silhavy, 1979). It possesses several enzymatic activities, including autophosphorylation, OmpR-kinase and OmpR-phosphate-phosphatase. Sequence analysis and β-lactamase fusion studies (Forst *et al.*, 1987), indicate that EnvZ possesses two membrane-spanning domain, placing its N-terminal domain in the periplasm and its C-terminal domain in the cytoplasm (Figure 1.8). The periplasmically-located N-terminal is ideally situated to monitor the surrounding osmolarity. This is corroborated by mutational studies where it was shown that a truncated form of EnvZ was found not be able to respond to changes in osmolarity (Igo and Silhavy, 1988). Furthermore a series of small deletions and insertions in the periplasmic domain that result in a constitutive $OmpF^{-}$-$OmpC^{+}$ phenotype are consistent with this region of the protein being essential in sensing osmolarity (Tokishita *et al.*, 1991; Russo, 1992). Additional studies, using chimeric proteins in which the N-terminal domain of EnvZ (consisting of the periplasmic domain, both transmembrane domains and a small cytoplasmic segment) is replaced with the corresponding region of the chemotaxis receptor Tar, also confirmed the importance of this region in osmolarity (Utsumi *et al.*, 1989). Here, the resulting chimeric protein, Taz1 was shown to no longer respond to varying osmolarity but rather to the presence or absence of aspartate. These results strongly support the idea that it is the N-termini of

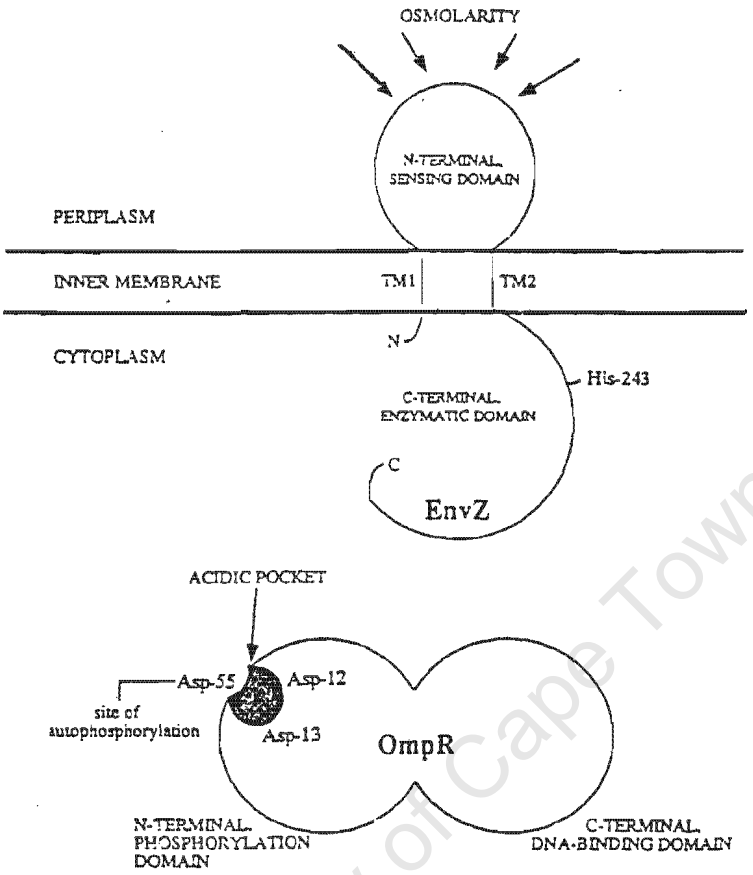





**Figure 1.8** Domain structure of OmpR and EnvZ. The sensor, EnvZ is located in the inner membrane with its N-terminal domain in the periplasm and its C-terminal domain in the cytoplasm. The N-terminal domain of EnvZ monitors the osmolarity and this information is transduced across the inner membrane to the C-terminal cytoplasmic domain. The C-terminal portion of EnvZ relays this information to the response regulator, OmpR, via kinase and phosphatase activities. OmpR is cytoplasmically-located directly responsible for regulating the transcription of the porins, OmpF and OmpC. The N-terminal half of OmpR is the phosphorylation domain, containing the acidic pocket and the presumed site of phosphorylation (Asp-55) and the C-terminal portion of OmpR is the DNA binding domain (Pratt and Silhavy, 1995).

the transmembrane sensors that possess the sensory function and that the N-terminal periplasmic domain functions directly to monitor medium osmolarity.

Once the N-terminal domain of EnvZ receives the stimulus, it transduces it across the membrane to the C-terminal cytoplasmic domain. The specific stimulus that EnvZ responds to has been a source of debate. To date, this question remains unanswered. No single molecule that functions as a direct and specific stimulus of the osmosystem has been found (Pratt and Silhavy, 1995). What is known is that the stimulus is not found in high osmolarity (Tokishita *et al.*, 1991; Russo, 1992) and it is not a membrane-derived oligosaccharide (Geiger *et al.*, 1992). If a molecular signal does exist, it is not known whether this signal is chemical (some small molecule) or mechanical (interaction with the cell wall).

Whatever the nature of the stimulus, its presence or absence is monitored by EnvZ. In response to changes in osmolarity, EnvZ is autophosphorylated at a conserved His-243 residue in the presence of ATP (Aiba *et al.*, 1989; Forst *et al.*, 1989; Igo and Silhavy, 1988). In addition to this conserved histidine residue, other regions that play important roles in the signal transduction process have also been identified. These include the transmembrane domains and the linker region of EnvZ. The importance of these regions will be addressed in Chapter 4. Following the phosphorylation of EnvZ, the phosphoryl group is transferred from the histidine site in EnvZ to its cognate regulator protein, OmpR (Pratt and Silhavy, 1995).



OmpR is directly responsible for eliciting the internal response, specifically the regulation of the transcription of the *ompF* and *ompC* genes (Fig. 1.9). OmpR is a 239 amino acid (Wurtzel *et al.*, 1982), cytoplasmic protein that is homologous to the family of transcriptional regulators that include PhoB, ArcA, PhoM-ORF2, VirG, PhoP and TctD (Stock *et al.*, 1989). As the response regulator, OmpR performs several important functions. Firstly, OmpR gathers information from EnvZ concerning osmolarity and then responds to this information. Depending on the signal it receives from EnvZ, OmpR favours expression of either *ompF* or *ompC*. It is, therefore, proposed that OmpR must be capable of assuming at least two states, the state found in low osmolarity and that found in high osmolarity (Figure 1.9, top). Currently, three genetically distinguishable states of OmpR have been described, namely the inactive unphosphorylated OmpR, the low-osmolarity form of OmpR and the high-osmolarity form of OmpR. OmpR in low osmolarity functions as a transcriptional activator of *ompF* whereas the OmpR in high osmolarity

functions as both a transcriptional repressor of *ompF* and a transcriptional activator of *ompC* (Slauch and Silhavy, 1989). EnvZ is required to act as both an activator of OmpR and to regulate the transition between the low and high osmolarity forms. Several models have been proposed to explain how the different forms of OmpR are involved in the regulation of the porin genes. The model that has generally been accepted is that a varying concentration of a single species of OmpR-P creates the necessary internal signal responsible for the reciprocal regulation of the porin genes (Russo and Silhavy, 1991).

In addition to assuming various forms, OmpR has DNA binding ability and plays a role in the activation and repression of transcription. Since OmpR can activate transcription of both the *ompF* and *ompC* genes, as well as repress transcription of *ompF*, it is implied that both positive DNA binding sites at both promoters and negative DNA binding site(s) at the *ompF* promoter, exist (Pratt and Silhavy, 1995). DNA protection studies reveal that OmpR binds to large regions of DNA at both the *ompF* and *ompC* promoters (Norioka *et al.*, 1986; Mizuno *et al.,* 1988; Maeda and Mizuno, 1988; Tsung *et al.*, 1989). However, no commonly accepted consensus sequence or DNA sequences that OmpR recognises have been identified. The large regions of DNA protected by OmpR (more that 60bp at each promoter) are suggestive of cooperative binding, but no evidence has been reported to support or refute these ideas. Furthermore, very little is known concerning how OmpR recognises its target DNA sites. No recognisable helix-turn-helix or other DNA binding motif (Nara *et al.*, 1986) have been identified, but it is proposed that the C-terminal contains the DNA binding domain. OmpR is suggested to bind to different sites at the porin promoters. Phosphorylation has been implicated in altering the affinity of OmpR for these various sites, in response to changing osmolarity. The mechanism through which this differential occupation of sites is achieved is not well understood.

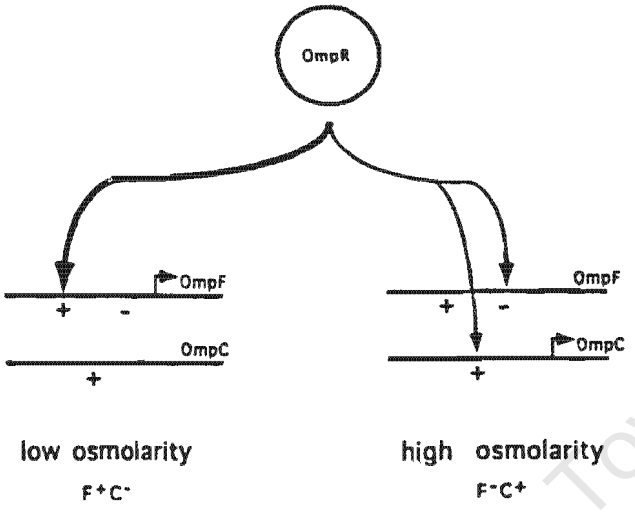



**Figure 1.9** Nature of the internal signal. (Top) OmpR assumes different roles under varying osmotic conditions. In low osmolarity (thick line), OmpR functions as a transcriptional activator of *ompF*, whereas in high osmolarity (thin line), OmpR functions to repress *ompF* transcription and activate ompC transcription. (Bottom) The distinction between the OmpR in low versus high osmolarity is though to be difference in the concentration of OmpR-phosphate. Low concentrations of OmpR favour transcriptional activation of *ompF,* whereas higher concentrations of OmpR-phosphate repress *ompF* transcription and activate *ompC* transcription (Pratt and Silhavy, 1995).

The binding of OmpR-P to the promoter regions of the porin proteins subsequently leads to either activation or repression thereof depending on the osmoregulatory stimulus received. OmpR-mediated transcriptional activation was shown to be dependent on the integrity of the C-terminal domain of the alpha subunit of RNAP (Russo and Silhavy, 1992; Slauch *et al.*, 1991; Sharif and Igo, 1993). Although the process has not been mechanistically elucidated, it is proposed that there is a direct protein-protein communication between OmpR and the C-terminal domain of alpha. This interaction appears to be a critical component of OmpR-mediated transcriptional activation. OmpR-mediated repression of the *ompF* promoter occurs under conditions of high osmolarity (Slauch and Silhavy, 1989). The formation of a repressive loop appears to be important in transcriptional repression. It is unclear how, in molecular terms,

OmpR mediates the formation of this loop and whether multerization or cooperative binding is involved. Furthermore, it is not known how this loop results in a decrease in transcription from the *ompF* promoter. The formation of this DNA loop implies that OmpR may oligomerise in the formation of this loop. Phosphorylation of OmpR is proposed to be a critical factor in the formation of the high-order OmpR oligomers. Another central issue concerning OmpR-mediated repression is whether it involves communication with the transcriptional machinery or simply the prevention of RNAP from binding to the promoter. Although evidence suggests that OmpR-mediated transcriptional activation and repression involves communication with the transcriptional machinery, it has not been proven conclusively (Slauch and Silhavy, 1991).

Porin regulation, as can be deduced by this review, is a complex sensory process (Pratt and Silhavy, 1995). Despite this complexity, the underlying mechanisms are slowly being unravelled. All the sensory mechanisms of microbes appear to be similar in design. In certain cases some low-level crosstalk between different signalling systems have been demonstrated (Igo *et al.*, 1989). This may connect the osmoregulatory circuitry to other physiologically relevant signalling pathways.



## 1.6 TWO-COMPONENT SIGNAL TRANSDUCTION AND BACTERIAL VIRULENCE

Bacterial pathogens must change their gene expression to survive the hostile environments faced during infection (Mekalanos, 1992). This adaptive response is often mediated by two-component regulatory systems. It has been estimated that enteric bacteria harbour some 40 different two-component systems that facilitate the response to a variety of chemical and physical signals (Stock *et al.*, 1995). However, the mechanism by which the various signals are combined into a co-ordinated cellular response has remained largely unknown. Studies have shown that an intricate network linking various two-component systems with the regulation of bacterial virulence factors, exist (Dziejman and Mekalanos, 1995). It follows, therefore, that a better understanding of these regulatory mechanisms used by pathogenic microbes would provide further insight into the regulation of bacterial virulence and, therefore, the control of the pathogen.

Common themes have emerged from the study of the control of bacterial virulence gene expression (Mekalanos, 1992). Among these are that certain environmental factors are frequently associated with the control of virulence gene transcription (Groisman *et al.*, 1990). Low iron concentration and increased temperature have been considered as parameters signalling entry of the microbe into host tissue. Other common signals regulating virulence gene expression include calcium, changes in pH or osmolarity and the levels of gases such as oxygen and carbon dioxide. The regulation of virulence by two-component systems is a strategy employed by many bacterial pathogens.

### 1.6.1 Regulation of *Shigella flexneri* Virulence

*Shigella flexneri* is an invasive pathogen that causes bacilliary dysentery. The pathogen has adapted the OmpR-EnvZ system for its own use to regulate both the expression of virulence factors and the OmpF and OmpC porins (Bernardini *et al.*, 1993). The *ompR-envZ* genes lie in the *ompB* operon as in *E. coli* but the regulation thereof differs from that of *E. coli*. OmpF is produced only under conditions of low osmolarity, but OmpC was found to be highly expressed under conditions of both high and low osmolarity. OmpF pores are slightly larger than that of OmpC and this has led to the hypothesis that a smaller pore is more advantageous in the host environment. The smaller pore is proposed to reduce the diffusion of toxic and potentially harmful molecules elicited by the host, thus protecting the pathogen within the host environment. *In vitro* studies showed that both *ompB* and *ompC* mutants were defective in two important steps in pathogenesis: spread and host cell death. Although these studies (Bernardini *et al.*, 1993) indicate that the *ompB* locus regulates additional genes important for pathogenesis, they also indicate that OmpC itself contributes significantly to the ability to establish infection.



### 1.6.2 Regulation of *Salmonella* virulence

*Salmonellae* are Gram-negative microorganisms that give rise to a variety of disease conditions, collectively known as human salmonellosis. These include diseases such as gastroenteritis, enteric fever and bacteremia (Groisman *et al.*, 1990). To establish a successful infection, the bacterium must resist death by the acid pH of the stomach and by the bile salts, antimicrobial

peptides and immunoglobin A in the small intestine. They must also resist clumping by the viscous intestinal mucin. These challenges are met, in part, by the use of two-component regulatory systems. The OmpR-EnvZ and the PhoP-PhoQ systems were found to be absolutely required for *Salmonella* virulence (Groisman *et al.*, 1990). In addition, other systems such as PmrA/PmrB and ArcA/ArcB have also been implicated in contributing to virulence (Roland *et al.*, 1993).

The PmrA/PmrB system controls modification of the positive charge of the lipopolysaccharide (LPS), affecting resistance of *Salmonella* to positively charged antibiotics such as polymyxin and to cationic microbicidal peptides such defensins (Vaara, 1981). *pmrA* encodes the response regulator of the system, while *pmrB* encodes the sensory element (Roland *et al.*, 1993). Missense mutations within the *pmrA* gene caused an increase the substitution of both 4-aminoarabinose and phosphorylethanolamine in the lipid A moeity of LPS (Helander *et al.*, 1994). This modification results in the outer membrane becoming less negatively charged and consequently not able to bind as much polymyxin, therefore, enabling the bacterium to persist.

As a pathogen, *Salmonella* is faced with hostile environments such as low oxygen conditions. Lee *et al.* (1992) determined that *S. typhimurium* requires these low oxygen conditions for the induction of their invasive determinants. The bacterial response to low oxygen conditions is proposed to be mediated by ArcA/ArcB, a two-component system that represses the expression of many genes under anaerobic conditions. However, the virulence role and the signal that is detected by this system, are still unknown (Lee *et al.*, 1992; Groismann and Ochman, 1993).

In response to changes in pH (osmolarity), *S. typhimurium*, as does *E. coli*, utilises the EnvZ/OmpR system. *S. typhimurium* strains defective in the EnvZ/OmpR system were found to be attenuated for mouse virulence (Groisman *et al.*, 1992). Although OmpR mutants were attenuated for mouse virulence, strains with single mutants in the OmpR-regulated genes, *ompF*, *ompC* or *tpp*, retained their virulent properties (Chatfield *et al.*, 1991). This indicates that there must be other OmpR-regulated genes that contribute to the pathogenic properties of virulent *S. typhimurium*.

The PhoP/PhoQ two-component system is one of the key systems involved in *Salmonella* virulence. The system includes the regulator, PhoP and a large array of about 40 PhoP-regulated

genes that are essential for various stages of infection (Garcia-Vescori *et al.*, 1994). PhoP is the best characterised of the eight proteins (RpoS, Crp, OmpR, SpvR, Fur, FlgM and FruR) identified as controlling virulence gene transcription within *Salmonella* (Groisman and Ochman, 1993).

Like several other two-component regulatory systems, the PhoPQ system is autophosphorylated. Chromosomal *lac* trancriptional fusions to *phoP* and *phoQ* have demonstrated that both PhoP and PhoQ are required for autophosphorylation. Once the environmental signal is received, PhoQ is predicted to autophosphorylate at a histidine residue that leads to the transfer of the phosphoryl group to an aspartic acid residue in PhoP. Several environmental cues control expression of various PhoP-regulated genes. Recently it has been identified that extracellular $Mg^{2+}$ is the key signal that governs the PhoP/PhoQ system. Garcia-Vescori *et al.*(1994) showed that the transcription of PhoP-regulated genes is induced in micromolar concentrations of $Mg^{2+}$, whereas the repression of PhoP-activated gene expression and the attenuation of the virulent properties of wild-type *Salmonella* species occurred under conditions of millimolar concentrations of $Mg^{2+}$. The PhoP/PhoQ system was also demonstrated to interact with other two-component systems to regulate virulence. Soncini and Groisman (1996) showed that the PhoP/PhoQ system controlled the expression of the PmrA/PmrB two-component system, thus illustrating that two-component systems can interact to process multiple environmental signals.



### 1.6.3 *Erwinia chrysanthemi* as a pathogen

The enterobacterium, *Erwinia chrysanthemi* is a ubiquitous plant pathogen of a wide variety of plant species. These bacteria are commonly found in soil or associated with plants. They are responsible for important economic losses since they cause softrotting, wilting and dwarfing in a wide range of plants, including maize, potatoes and carrots (Perombelon and Kelman, 1980). The main characteristic of soft rot *Erwiniae* is their ability to produce large quantities of plant cell-wall degrading enzymes. *E. chrysanthemi* synthesizes a battery of these enzymes including pectin methylesterases, plant pectate lyases, cellulases and proteases, that cause tissue maceration, resulting in the disease referred to as bacterial soft rot (Chatterjee and Starr, 1980).

The maceration process is dependent on complex regulatory pathways that fine-tune the gene expression of the pectinolytic machinery. The enzymes involved in this complex process have been well characterised for the pathogen, *E. chrysanthemi*, strain 3937 (Garibaldi and Bateman, 1971). However, very little is known about the factors that impact on the regulation of these genes. Physiological studies demonstrated that pectate lyase synthesis is subjected to factors such as catabolite repression or induction in late exponential growth phase (Hugouvieux-Cotte-Pattat *et al.*, 1986; Ji *et al.*, 1987). Other environmental factors that have also been implicated are temperature (Perombelon, 1990) oxygen tension, iron concentration, as well as osmolarity (Sauvage *et al.*, 1991). Research has been done relating pectinase gene expression to varying environmental conditions (Hugouvieux-Cotte-Pattat *et al.*, 1992). Mildenhall *et al.* (1988) have investigated specifically the effect that osmotic stress had on pectate lyase transcription (Mildenhall *et al.*, 1988). Their studies have prompted interest in the role that osmolarity plays in the pathogenesis of *Erwinia chrysanthemi*.

Physiologically, it has been shown that osmoregulation within *E. chrysanthemi* is similar to that of *E. coli* and *S. typhimurium* (Crampton, 1996). Furthermore, genetic evidence suggests that an *ompB* locus exists within *E. chrysanthemi* (Crampton, 1996). The question as to how osmoregulation is linked to pathogenesis at the genetic level still remains unanswered. However, by gaining an understanding of the underlying osmoregulatory mechanisms involved within *Erwinia chrysanthemi*, further insight into the role that osmolarity plays in pathogenesis could be obtained. Therefore, the genetic characterisation of the osmoregulation system would be beneficial in elucidating the complexities of pathogenesis of this pathogen.

# CHAPTER 2

# Confirmation of the presence of an *ompB* locus in *Erwinia chrysanthemi*

## 2.1 SUMMARY

Primers specific for conserved regions in *ompR* were designed and used to PCR amplify a 631 bp fragment from *Escherichia coli*. End-sequencing confirmed that the PCR product was derived from the *E. coli ompR* gene. PCR analysis, under the same conditions, using *E. chrysanthemi* DNA as template, yielded no product. Southern hybridisation analysis, using the PCR-generated *E. coli ompR* probe, subsequently confirmed the presence of an *ompR* homologue in *E. chrysanthemi*. These results combined with a prior identification of an *envZ* homologue established that an *ompB* locus exists in *E. chrysanthemi*.

## 2.2 INTRODUCTION

Osmoregulation in Gram-negative bacteria is characterised by the strict regulation of porin proteins, in response to changes in the environment (Hall and Silhavy, 1979;1981). In *Escherichia coli* the *ompB* locus, which encodes the *envZ* and the *ompR* genes, plays a pivotal role in these regulatory processes (Aiba *et al.*, 1989). EnvZ and OmpR are members of a conserved family of two-component proteins, and have been found in more than 50 different bacterial species (Parkinson and Kofoid, 1992; Stock *et al.*, 1989).

*Erwinia chrysanthemi* was shown to respond to changes in medium osmolarity in a similar manner to that observed in *E. coli* (Crampton, 1996). Furthermore, evidence that confirms the presence of an *envZ* homologue in *E. chrysanthemi*, suggested that an *ompB* locus may be present. The sequence conservation within regions of *ompR* among different bacterial species provided the tools for primer design. Since the entire *ompB* operon has been sequenced for both *E. coli* (Mizuno *et al.*, 1982) and *Salmonella typhimurium* (Liljestrom, 1988), an *ompR* probe, based on sequence identity could be designed and generated. This probe could be used in Southern hybridisation analyses of *Erwinia* genomic DNA, to identify whether an *ompR* homologue exists in *E. chrysanthemi*.

The aim was thus to generate a suitable probe to identify the existence of an *ompR* homologue and subsequently to confirm the presence of an *ompB* operon in *E. chrysanthemi*.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Bacterial strains, plasmids and culture conditions.

*Erwinia chrysanthemi* cells were grown in liquid culture (LB broth) or on solid agar (LB plates) and maintained for long-term storage in sterile distilled water in McCartney bottles at RT.

*Escherichia coli* strains used in this study are listed in Table 2.1. All *E. coli* strains were stored in 20% glycerol at -20 °C. Cultures were grown and maintained in LB and on LB plates. *E. coli* strain JM109, used for transformation studies, was made competent (see Appendix A) using the method outlined by Draper *et al.* (1988). The plasmid vector, pBluescriptSk was used for end-sequencing of the amplified *ompR* PCR product.

**Table 2.1.** *E. coli* strains used in this study.

| *E. coli* strains | Genotype | Source/reference |
|---|---|---|
| JM109 | *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, ($r_{K-}$, $m_{K+}$), Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac* $I^q$ ZΔ M15) | Yanisch-Perron *et al.* (1985). |
| K12 | Wild-type strain | laboratory stock |



### 2.3.2 Extraction of genomic and plasmid DNA

Genomic DNA from *E. coli* and *E. chrysanthemi* was extracted using the technique outlined by Ausubel *et al.* (1991). Plasmid DNA was prepared using the Nucleobond kit (Machery-Nagel) following the manufacturers' protocol. Both small-scale (miniprep) and large-scale (maxiprep) preparation of plasmid DNA are detailed in Appendix A .

### 2.3.3 Primer design and PCR analysis

The *ompR* genetic sequences for *E. coli* and *S. typhimurium* have both been determined (Mizuno and Wurtzel, 1982; Liljestrom, 1988). These sequences were aligned, using the GCG package version 8.0, to determine regions of homology (Figure 2.1). A forward and reverse primer with the respective sequences (5' GAATTCCAGGTTCGAAGCGTCGC 3') and (5' GATATCGGATCCGGTACAAAGA CGTAGCCCAG 3'), were designed and used to amplify a 631bp fragment from *E. coli.* Both primers contained restriction enzyme linker sequences to facilitate cloning of the PCR product into the plasmid vector.

PCR amplification was performed, using 0.5 µM of each primer, 200 µM of dNTP mixture, 3 mM $MgCl_2$ and 50 ng of *E. coli* and *E. chrysanthemi* DNA as template. 1 ng of DNA was used when the cloned, *ompR* PCR product was used as template. Applied Biotechnology *Taq* DNA polymerase (1U) and the supplied buffer were used in a total reaction volume of 100 µl. PCR amplification cycles were performed using a programmable thermocycler (JDI model 8012). Reaction mixtures were subjected to an initial denaturation step of 94 °C for 60s, followed by 30 cycles of amplification with a denaturation step of 94 °C for 60s, an annealing step of 58 °C for 30s and an elongation step of 72 °C for 60s. A final extension step of 72 °C for 300s was also included. PCR amplification was checked by electrophoresizing one-tenth of the reaction volume on a 0.8% agarose gel in TBE buffer, followed by ethidium bromide staining and visualisation over a 264 nm light source.



### 2.3.4 Cloning and end-sequencing of the *ompR* PCR product

To facilitate the cloning of the PCR product into the plasmid, pBluescriptSk (pSK), restriction endonuclease linker sequence was included in the design of the primers. The forward primer contained the *Eco*RI site and the reverse primer, the *Bam*HI and *Eco*RV sites. All three restriction endonuclease sites corresponded to sites found in the multiple cloning site of the plasmid, pBluescriptSk (see Appendix C for the plasmid map) and not in the *ompR* genes of *E.coli* and *S. typhimurium*

```
282 atgcaagagaattataagattctggtggttgatgacgatatgcgtctgcg 331 St
    |||||||||||  ||  ||||||||||||||  ||||||||  |||||  |||||
251 ATGCAAGAGAACTACAAGATTCTGGTGGTCGATGACGACATGCGCCTGCG 300 Ec
          .         .         .         .         .
332 ggcgctactggaacgttatctgaccgagcagggcttccaggttcgaagcg 381 St
     |||||  ||||||||||||||||  |||||  ||  ||||||||||||||||||||
301 TGCGCTGCTGGAACGTTATCTCACCGAACAAGGCTTCCAGGTTCGAAGCG 350 Ec
          .         .         .      forward primer
382 tcgctaacgctgagcagatggatcgtctgctgacccgtgaatctttccat 431 St
    |||||||  ||  ||  ||||||||||  ||||||||  ||||||||||||||||
351 TCGCTAATGCAGAACAGATGGATCGCCTGCTGACTCGTGAATCTTTCCAT 400 Ec
          .         .         .         .         .
432 ctcatggtactggatttaatgctgccaggtgaagatggtctgtcgatttg 481 St
    ||  ||||||||||||||||||  |  ||  ||||||||||  ||||||||||
401 CTTATGGTACTGGATTTAATGTTACCTGGTGAAGATGGCTTGTCGATTTG 450 Ec
          .         .         .         .         .
482 tcgtcgcctgcgtagtcaaagtaatccaatgccgatcattatggtcacgg 531 St
     ||  ||  ||  ||||||||  ||  ||  ||  ||||||||||||||||  ||||
451 CCGACGTCTTCGTAGTCAGAGCAACCCGATGCCGATCATTATGGTGACGG 500 Ec
          .         .         .         .         .
532 cgaagggtgaagaggttgaccgtatcgtcgggctggaaatcggcgccgat 581 St
    ||||  ||  |||||  ||  ||||||||||  ||  |||||  ||  |||||  ||
501 CGAAAGGGGAAGAAGTGGACCGTATCGTAGGCCTGGAGATTGGCGCTGAC 550 Ec
          .         .         .         .         .
582 gactacattcctaaaccgtttaacccgcgcgagctgttggcgcgtattcg 631 St
    ||||||||||||  ||||||||||||||||||  ||  |||  ||||  |||||  ||
551 GACTACATTCCAAAACCGTTTAACCCGCGTGAACTGCTGGCCCGTATCCG 600 Ec
          .         .         .         .         .
632 gcccgtgttacgtcgtcaggcaaacgaactgcccggcgcgccgtcgcagg 681 St
      |  |||  |  ||||||||||||  ||||||||||||  |||||  |||||  ||||
601 TGCGGTGCTGCGTCGTCAGGCGAACGAACTGCCAGGCGCACCGTCACAGG 650 Ec
          .         .         .         .         .
682 aagaggccgttatcgcgttcggtaagtttaaactgaacctcggtacgcgc 731 St
    |||||||  ||  ||  ||  ||||||||||||  |||||  ||||||||||||||||
651 AAGAGGCGGTAATTGCTTTCGGTAAGTTCAAACTTAACCTCGGTACGCGC 700 Ec
          .         .         .         .         .
732 gagatgttccgtgaagatgaaccgatgccgctgaccagcggcgagtttgc 781 St
    ||  ||||||||  |||||  ||  ||||||||||  ||||||||  ||||||||
701 GAAATGTTCCGCGAAGACGAGCCGATGCCGCTCACCAGCGGTGAGTTTGC 750 Ec
          .         .         .         .         .
782 ggtactgaaagcgttagtcagccatccgcgcgagccgctctctcgcgaca 831 St
    |||||||||  ||  |  ||||||||||||||  ||||||||||  |||||  |
751 GGTACTGAAGGCACTGGTCAGCCATCCGCGTGAGCCGCTCTCCCGCGATA 800 Ec
          .         .         .         .         .
832 agctgatgaatctggcccgtggccgcgagtattccgcgatggaacgctcc 881 St
    ||||||||||  ||  ||||||||  ||  ||  ||||||||  ||||||||||||
801 AGCTGATGAACCTTGCCCGTGGTCGTGAATATTCCGCAATGGAACGCTCC 850 Ec
          .         .         .         .         .
882 atcgacgtccagatctcccgcctgcgccgtatggtggaagaagatccggc 931 St
    ||||||||  |||||  ||  ||  ||||||||  ||||||||||||||||||  ||
851 ATCGACGTGCAGATTTCGCGTCTGCGCCGCATGGTGGAAGAAGATCCAGC 900 Ec
          .         .         .         .         .
932 acatccgcgttatattcagaccgtctggggcctgggctacgtctttgtac 981 St
     ||||||||||  ||||||||||||||||||  ||||||||||||||||||||
901 GCATCCGCGTTACATTCAGACCGTCTGGGGTCTGGGCTACGTCTTTGTAC 950 Ec
          .         .         .    reverse primer
982 cggacggttctaaagcatga                                  St
    |||||||  ||||||||||||
951 CGGACGGCTCTAAAGCATGA                                  Ec
```

**Figure 2.1.** DNA sequence alignment of the *E. coli* (Ec) and *S. typhimurium* (St) *ompR* genes to determine regions of homology for primer design. The underlined sequence represents the forward and reverse primer sequence selected.

Following PCR amplification, the band corresponding to the correct size (631 bp) was extracted from the 0.8% agarose gel in TBE, using the Geneclean$^{R}$ kit and following the manufacturers' (BIO 101, Inc) instructions. The PCR product and the vector, pBluescriptSk, was digested with the enzymes *Eco*RI and *Eco*RV. Following digestion, the PCR product was precipitated by adding 3 M NaOAc (one-tenth of the reaction volume) and two volumes of absolute ethanol, followed by microcentrifugation at 13000 rpm. The resulting PCR product was resuspended in TE and then ligated into the pBluescriptSk vector, following standard cloning and ligation procedures (Sambrook *et al.*, 1989), to generate recombinant DNA clones.

Recombinant DNA clones were transformed into competent *E. coli* JM109 cells. White colonies, indicative of insertional inactivation of the β-galactosidase gene of the vector (Vieira and Messing, 1982), were selected on LA$^{Amp}$ plates containing X-gal. Clones were screened for the presence of the 631 bp PCR product by digestion of the construct with *Eco*RI and *Eco*RV. Electrophoresis of the digested clones on a 0.8% agarose gel at 100V, was followed by the visualisation of the DNA with EtBr staining.

The selected recombinant clones were further analysed by the sequencing of the 5' and 3' ends of the insert DNA (end-sequencing). Nucleotide sequencing was carried out using the dideoxynucleotide, triphosphate, chain-termination method of Sanger *et al.* (1977). Reactions were performed with Sequenase$^{R}$ Version 2.0 sequencing kit and M13 forward and reverse primers. Analyses of DNA sequence was performed on a GCG package, version 8.0 (Devereux *et al.*, 1984), run on a DEC/VAX 6000-330 mainframe computer.



### 2.3.5 Southern hybridisation analysis of *E. chrysanthemi* genomic DNA

A DIG labelled *E. coli ompR* probe was generated (as described below) by PCR, using the recombinant *ompR* clone as template. The nucleotides in the PCR reaction were replaced with an equal concentration of digoxigenin-II-dUTP (DIG) DNA labelling mix (Boehringer Mannheim). The amplified product was electrophoresized on a 0.8% agarose gel and the DNA band corresponding to the correct size, was extracted using the Geneclean$^{R}$ kit as described previously.

Both *E. coli* and *E. chrysanthemi* genomic DNA (10 µg) was digested at 37 °C, O/N with the "frequent cutter", *Eco*RV. The *ompR* clone was digested with the enzymes, *Eco*RI (2U) and *Eco*RV (2U) to release the *ompR* insert. The digested genomic DNA as well the digested *ompR* clone was electrophoresised on a 0.8% agarose gel and was capillary transferred with 0.4 M NaOH onto a Hybond $N^+$ nylon membrane (Amersham). After transfer, the membrane was probed under conditions of high stringency (68 °C) with the DIG-labelled *E. coli ompR* probe (Boehringer Mannheim). Hybridisation signals were detected via chemiluminescence using the CSPD substrate (Boehringer Mannheim).

## 2.4 RESULTS AND DISCUSSION

### 2.4.1 PCR analysis and the generation of an *ompR* probe from *E. coli*

PCR amplification of *E. coli* genomic DNA yielded the expected 631 bp fragment (Figure 2.2). PCR analysis of *E. chrysanthemi* genomic DNA under the same conditions yielded no PCR product (Figure 2.2). This result suggests that if an *ompR* gene exists in *E. chrysanthemi*, the DNA sequences differ significantly within the region of primer binding.

Cloning of the 631 bp PCR product into pSK resulted in the creation of an *ompR* clone, designated, pA51 (see Appendix C for restriction map). Sequence analysis of both the 5' and 3' ends of the pA51 insert, further corroborated the fact that an *E. coli ompR* clone was generated (Figure 2.3). Restriction enzyme analysis of pA51 with *Eco*RI and *Eco*RV, yielded the expected 631 bp DNA insert (Figure 2.4), thereby confirming the successful cloning of the *E. coli ompR* PCR product.

To confirm the presence of an *ompR* homologue in *E. chrysanthemi*, the clone pA51 was used as template, in the PCR generation of an *ompR* probe for Southern hybridisation analysis.

### 2.4.2 Southern hybridisation analysis to confirm the presence of an *ompR* homologue in *E. chrysanthemi*

Southern hybridisation analysis, using the *E. coli ompR* probe, confirmed the presence of an *ompR* homologue in *E. chrysanthemi* (Figure 2.4). In both cases, the *ompR* probe hybridised to a single *Eco*RV-*Eco*RV DNA fragment. This indicates that no *Eco*RV sites exist in the *ompR* homologue in *E. chrysanthemi*, within the region spanned by the primers. The hybridisation of the *E. coli ompR* probe to *E. chrysanthemi* genomic DNA also confirms that sequence homology exists between these two microorganisms. However, the difference in the *Eco*RV banding pattern suggests that in addition to regions of homology, sequence variation exist within the gene for these two organisms. This supports the results obtained from PCR analysis.

In summary, the primers designed to regions of sequence homology between *E. coli* and *S. typhimurium ompR* genes were unsuccessful in amplifying an *ompR* homologue from *E. chrysanthemi* genomic DNA. However, Southern hybridisation analysis of *E. chrysanthemi* genomic DNA with an *E. coli ompR* probe confirmed that an *ompR* homologue exists for this microorganism. These results, in combination with the identification of an *envZ* homologue in *E. chrysanthemi* confirm that an *ompB* locus exists in *E. chrysanthemi*.

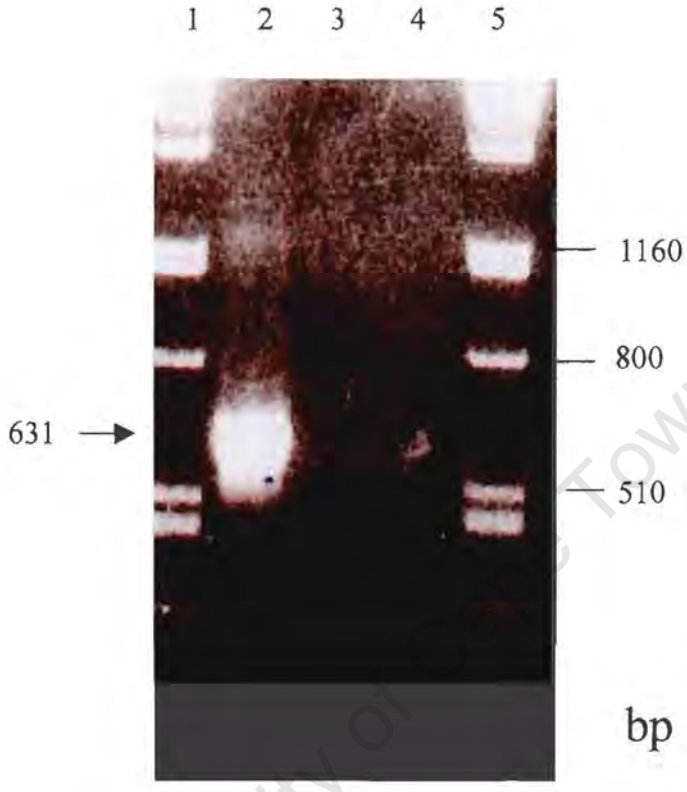





**Figure 2.2.** Polymerase chain reaction of a portion of the *ompR* gene of *E. coli* (lane 2) and *E.chrysanthemi* (lane 3). Lanes 1 &5, λ-*Pst*I molecular weight marker and lane 4, the negative control.

(A)

```
                                        139          129          119
PCRf.                                   GTAGTGAA-CTTGCC--TGG-CGT-AATATTCCG
                                        |||| |||||| ||| ||| |||||||||
E.coli CGCGTGAGCCGCTCTCCCGCGATAAGCTGATGAACCTTGCCCGTGGTCGTGAATATTCCG
        780       790       800       810       820       830

            109        99        89        79        69        59
PCRf.  -AATGGAGCGCTCCATCGACGTGCAGATTTC-CGTCTGCGCCGCATGGTGGAAGAACATC
       |||||| |||||||||||||||||||||||| |||||||||||||||||||||| |||
E.coli CAATGGAACGCTCCATCGACGTGCAGATTTCGCGTCTGCGCCGCATGGTGGAAGAAGATC
       840       850       860       870       880       890

             49        39        29        19         9
PCRf.  CAGCGCATCCGCGTTACATT-AGACCGTCTGGGGTCTGGGC-ACGTCTTTGTACCGGACC
       |||||||||||||||||||| |||||||||||||||||| ||||||||||||||||||
E.coli CAGCGCATCCGCGTTACATTCAGACCGTCTGGGGTCTGGGCTACGTCTTTGTACCGGACG
       900       910       920       930       940       950

E.coli GCTCTAAAGCATGAGGCGATTGCGCTTCTCGCCACGAAGTTCATTTGCCCGTACGTTATT
       960       970       980       990      1000      1010
```

**92.6% identity in 149 bp overlap**

(B)

```
                                        10        20        30
PCRr.                                   GAATTCCAGGTTCGAAGCGTCGCTAATGCAGAA
                                        ||||||||||||||||||||||||||||||||
E.coli CTGCTGGAACGTTATCTCACCGAACAAGGCTTCCAGGTTCGAAGCGTCGCTAATGCAGAA
          310       320       330       340       350       360

            40        50        60        70        80        90
PCRr.  CAGATGGATCG-CTGCTG-CTCGTGAATCTTTCCATCTTATGGTACTGG-TTTAATCTTA
       |||||||||||| |||||| ||||||||||||||||||||||||||| |||||| |||
E.coli CAGATGGATCGCCTGCTGACTCGTGAATCTTTCCATCTTATGGTACTGGATTTAATGTTA
          370       380       390       400       410       420

             100       110
PCRr.  CCTGGTGAACATGGCTTGTCGA
       ||||||||| ||||||||||||
E.coli CCTGGTGAAGATGGCTTGTCGATTTGCCGACGTCTTCGTAGTCAGAGCAACCCGATGCCG
          430       440       450       460       470       480
```

**95.5% identity in 112 bp overlap**



**Figure 2.3.** DNA sequence alignment of the *ompR* PCR fragment and the reported sequence of the *E. coli ompR* gene (A) shows the sequence alignment obtained using the universal M13 forward primer and (B) the alignment using the reverse primer. Note the high sequence identity indicating that the PCR product is the correct portion of the *E. coli ompR* gene.

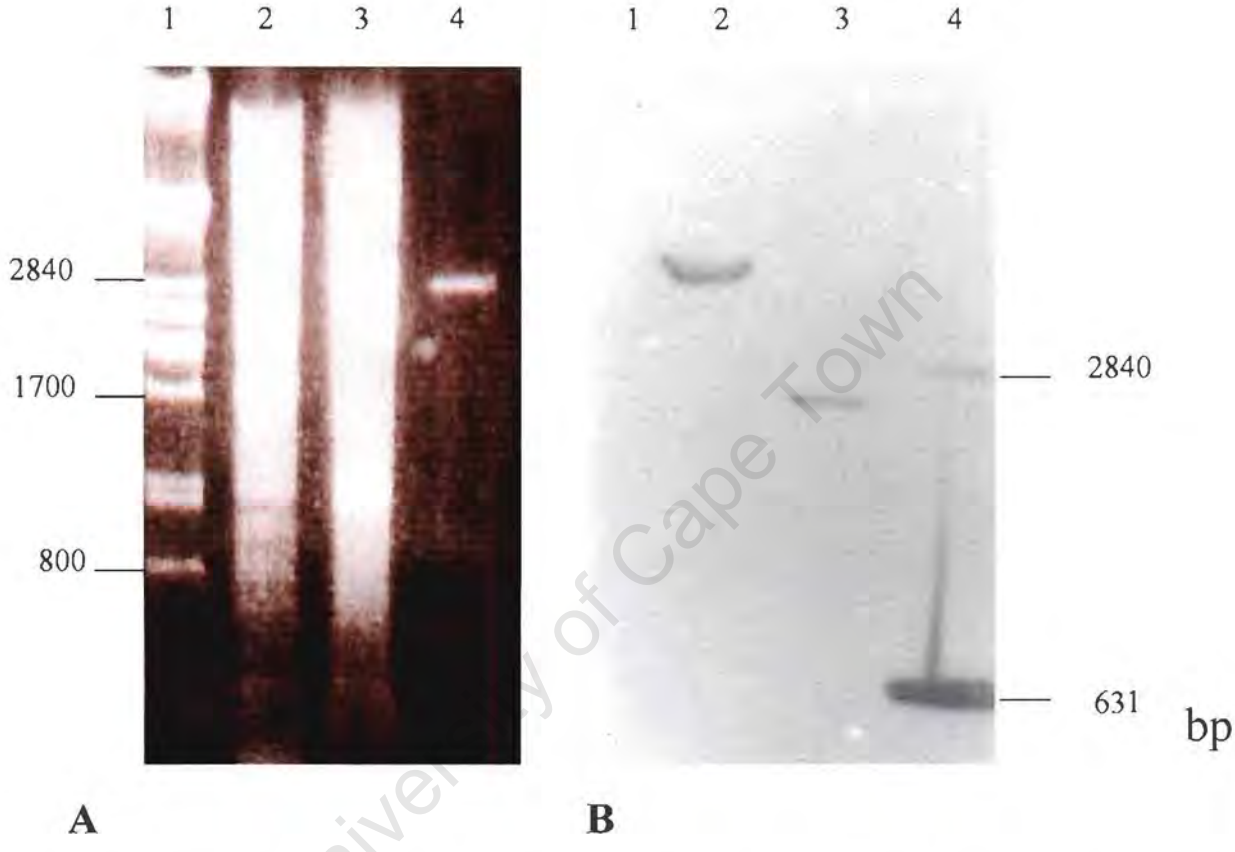





**Figure 2.4.** Southern hybridisation analysis of restricted *E. coli* and *E. chrysanthemi* genomic DNA. (A) Lanes 2 and 3 show the *E. coli* and *E. chrysanthemi* genomic DNA respectively, restricted *with Eco*RV. The λ-*Pst*I marker was loaded in lane 1 and the positive control, pA51 restricted with *Eco*RV and *Eco*RI, in lane 4. (B) Southern hybridisation analysis, using the 631 bp *E. coli ompR* DIG-labelled probe.

# Chapter 3

# Construction and screening of an *Erwinia chrysanthemi* genomic library in *Escherichia coli*, for clones encoding the *ompB* locus.

## 3.1 SUMMARY

A genomic library of *Erwinia chrysanthemi* was established in the positive-selection vector, pEcoR251, and maintained in the host, *Escherichia coli*. Screening of the *E. chrysanthemi* genomic library by DNA hybridisation analyses identified positive *ompR*-containing clone-pools, of which one was selected for further analyses. Recombinant clones representative of this positive *ompR* clone-pool was transformed in the *E. coli* host, JM109, for further screening by Southern hybridisation analyses. A number of putative *E. chrysanthemi ompR* clones were isolated of which four were chosen for further characterisation.

The putative *ompR* clones were analysed by Southern hybridisation analyses, using an *envZ*-specific probe, to identify those with both *ompR* and *envZ* genes. The clone, designated pRZ69, containing an insert size of 5000 bp was selected as the candidate *ompR-envZ* (*ompB*) encoding clone. Preliminary characterisation of the insert DNA, by Southern hybridisation analyses, was done to confirm that the clone was derived from *E. chrysanthemi*.

## 3.2 INTRODUCTION

Recombinant DNA technology has provided researchers with the tools to manipulate genetic material from different organisms in order that their individual genes are studied In principle, DNA fragments representing the entire genome of the organism are cloned into suitable vectors. Initially, researchers made use of negative selection of plasmid containing vectors such as pBR322 by the insertional inactivation of an antibiotic resistance marker. Improvements on these systems introduced the method of positive selection (Bochner *et al.*, 1980; Kiel *et al.*, 1987) which is a more efficient strategy for screening of genomic libraries (Kuhn *et al.*, 1986). These vectors either rely on the depression of an antibiotic resistance function (Roberts *et al.*, 1980; Nilsson *et al.*, 1983; Nikolnikov *et al.*, 1984), inactivation of a dominant function conferring cell sensitivity to an antibiotic or metabolite (Hennecke *et al.*, 1982; Ahmed, 1984; Burns and Beachan, 1984), removal of a lethal DNA sequence (Hagan and Warren, 1982) or inactivation of a lethal gene (Schuman, 1979; O' Conner and Humphreys, 1982; Kuhn *et al.*, 1986). The vector, pEcoR251 used in this study, is an example of a positive selection vector where a lethal gene is inactivated (Zabeau and Stanley, 1982) to facilitate genomic DNA library construction.

The cloned fragments are transferred to viable host cells where propagation of the recombinant plasmids take place. Screening for the clones containing the desired gene(s) is the next critical step. This can be done by employing either of two general strategies (i) assaying clones for the expression of the desired gene products (specific proteins), or (ii) by employing the use of gene-specific probes. The latter strategy was employed in this study.

*Erwinia chrysanthemi* is closely related to enteric bacteria such as *Escherichia coli* (Leary and Fulbright, 1982) and it has proteins analogous to the *E.coli* outer membrane porins (Crampton, 1996). Genetic evidence also suggests that *E. chrysanthemi* may contain a regulatory system analogous to the *E. coli ompB* locus (this study, Chapter 2). To determine whether this putative *ompB* locus is functionally analogous to that of *E. coli*, the osmoregulatory *envZ* and *ompR* homologues of *E. chrysanthemi* had to be isolated and

characterised. The aim of this study was thus to construct and screen an *E. chrysanthemi* genomic library for clones encoding these genes. Since both *E. coli ompR* and *envZ* probes were generated, screening of the *E. chrysanthemi* library by Southern hybridisation analyses could be achieved. This method of screening is described in this chapter.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Bacterial strains, plasmids and culture conditions

*Erwinia chrysanthemi* cells were grown in LB broth or on LB plates and maintained for long-term storage in sterile distilled water in McCartney bottles at RT.

*Escherichia coli* strains used in this study are listed in Table 3.1 and the restriction maps of vectors mentioned are shown in Appendix C. DNA fragments were cloned into the suicide vector, pEcoR251 (Zappe *et al.*, 1986) and maintained in the *rec*A$^-$, *E. coli* strain, JM109 (Table 3.1). A *rec*A mutant strain was selected to reduce the frequency of homologous recombination between recombinant plasmids and host chromosomal DNA.

**Table 3.1** *E. coli* strain used in this study.

| *E. coli* strain | Genotype | Reference |
|---|---|---|
| JM109 | *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 ($r_K$,$m_K^+$), *rel*A1, *sup*E44, Δ(*lac-pro*AB) [F', *tra*D36, *pro*AB, *lac* $I^qZ$ ΔM15] | Yanisch-Perron, *et al.* (1985) |

### 3.3.2 Construction and screening of the *E. chrysanthemi* genomic library

Genomic DNA from *E. chrysanthemi* was extracted using the technique described by Ausubel *et al.*, (1991). Plasmid DNA was prepared, using a Nucleobond Kit and following the manufacturer's recommendations (see Appendix A for a detailed description). Genomic DNA (100 ug) was partially digested with *Sau*IIIA restriction endonuclease and size fractionated on a 10% to 40% sucrose gradient. Fractions with fragment sizes in the range of 5 kb to 10 kb were pooled, ethanol precipitated at –20 °C, O/N, and resuspended in TE buffer, pH8. These fragments were cloned into the *Bgl*II site of the positive selection vector, pEcoR251, and transformed into competent JM109 cells (Draper *et al.*, 1988).

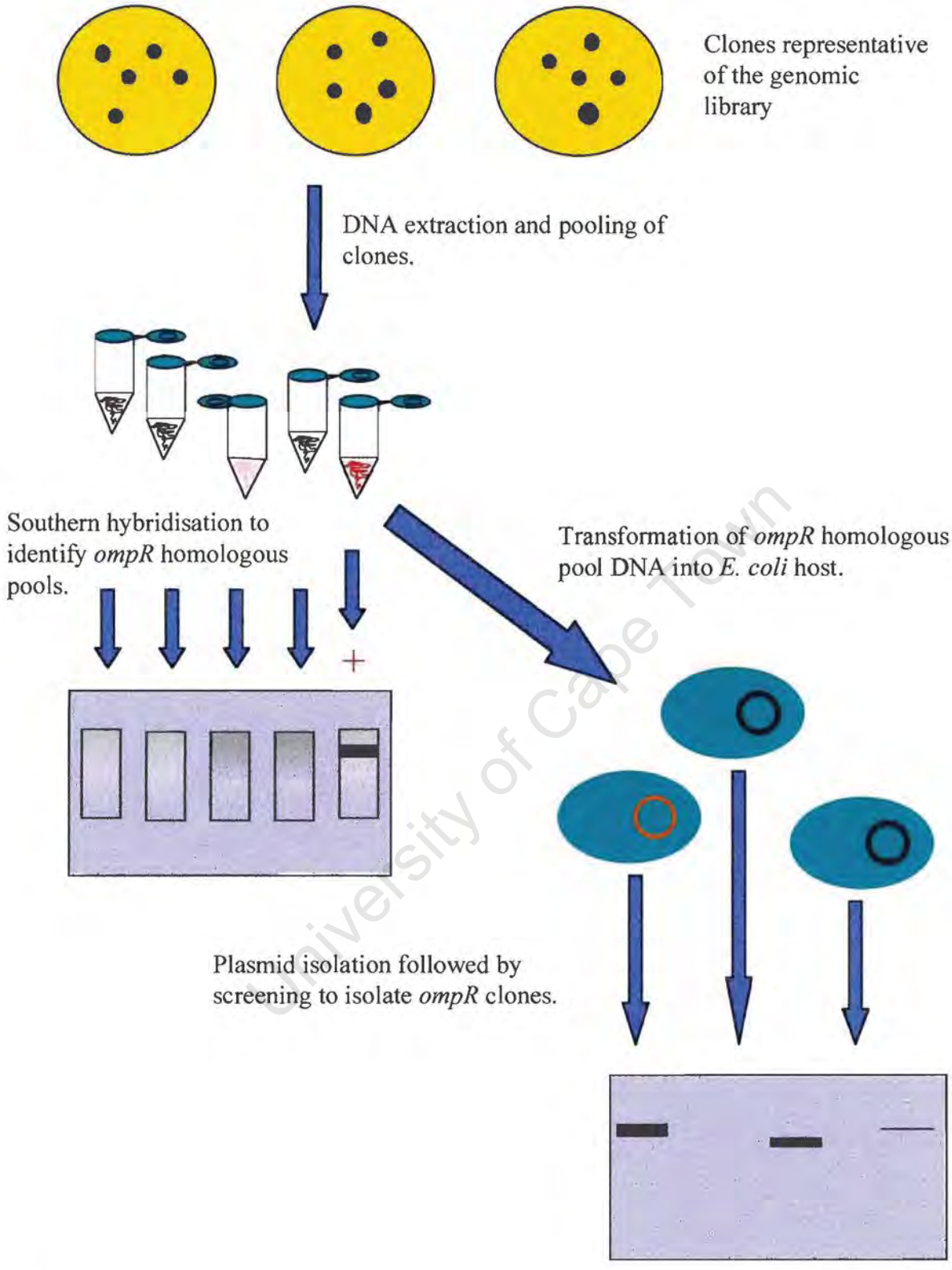





**Figure 3.1.** Schematic representation of the strategy followed for the screening of the *E. chrysanthemi* genomic library for *ompR* clones (see text for more details).

Transformations were optimised to obtain approximately 200-300 colonies per plate. These plates were flooded with 1 ml LB and the colonies were scraped and pooled for large-scale preparation of plasmid DNA. Fourteen pools, consisting of a total of 3704 colonies were obtained, which represented approximately 99% of the *E. chrysanthemi* genome (Clarke and Carbon, 1976). Five micrograms of uncut plasmid DNA of each pool was electrophoresed on a 0.8% agarose gel in TBE buffer and subjected to Southern hybridisation analyses under low stringency (Tm = 55 °C), using the PCR-generated *ompR* probe as described in section 2.3.5. Positive *ompR*-hybridising DNA pools, identified by Southern hybridisation analyses, were selected for further analyses to isolate *ompR*-encoding clones. Plasmid DNA from *ompR* homologous pools was transformed into JM109, to isolate single colonies with putative *ompR*-encoding recombinant plasmids. Small-scale preparation of plasmid DNA from individual colonies was done, prior to screening (see Figure 3.1 for a schematic outline of the screening method) for *ompR* clones by Southern hybridisation analysis. Putative *ompR*-encoding clones, identified by Southern hybridisation analyses, were further analysed by using the *envZ* probe to identify clones with both *ompR* and *envZ* homologues.

### 3.3.3 Southern hybridisation analyses of the *ompB* clone, pRZ69

Genomic and plasmid DNA was digested according to standard procedures (Sambrook *et al.*, 1989) using restriction endonucleases and their recommended buffers (Boehringer-Mannheim). Ten micrograms of *E. chrysanthemi* and *E. coli*, K12 chromosomal DNA was digested separately with the restriction endonuclease, *Eco*RV. The plasmid, pRZ69, was similarly subjected to restriction enzyme analysis with *Eco*RV. The resulting fragments were separated on a 0.8% agarose gel and transferred to Hybond $N^+$ membrane by capillary transfer, according to the Blotting and Hybridisation protocol for Hybond $N^+$ (Amersham).

The DIG-labelled probe used to confirm the origin of the insert DNA of the clone, pRZ69 was prepared by purifying a 2 kb *Eco*RV-*Eco*RV pRZ69-derived fragment, from an agarose gel, according to the instructions outlined in the Gene-clean$^R$ Kit (B101, USA) and labelling the resulting DNA fragments with digoxigenin. The non-radioactive labelling was done

according to the instructions outlined in the Gene-clean$^{R}$ Kit (B101, USA) and labelling the resulting DNA fragments with digoxigenin. The non-radioactive labelling was done according to the random-priming method outlined in the DNA labelling and Detection Kit (Boehringer-Mannheim,).

Prehybridisation, hybridisation and immunological detection procedures were performed according to the manufacturer's instruction that accompanied the Non-radioactive DNA labelling and Detection kits (Boehringer-Mannheim). All hybridisation steps were performed under conditions of either high stringency of 68 °C, or low stringency of 55 °C.

## 3.4 RESULTS AND DISCUSSION

### 3.4.1 Identification of homologous *ompR* DNA pools by Southern hybridisation

Approximately 3704 clones with an average insert size of 5 kb were obtained from the *E. chrysanthemi* genomic library. Fourteen plasmid pools, representing the total genomic library, were constructed and screened by Southern hybridisation analyses, to identify pools homologous to the *ompR*-probe (Figure 3.2). Screening under high stringency (Tm = 68 °C) proved too stringent (results not shown), therefore a low stringency (Tm = 55°C) step was undertaken. Screening under low stringency identified a number of *ompR*-homologous plasmid pools. Pool one (Figure 3.2, lane 2) which showed a distinct DNA band hybridising to the *ompR* probe was chosen for further charactersation.

### 3.4.2 Screening of the genomic library for *ompR*- and *envZ*-encoding clones

Preliminary screening of the genomic library, localised putative *ompR* clones to plasmid pool 1. The DNA of this pool was transformed into JM109 and 100 plasmid clones were prepared for Southern hybridisation analysis with the *ompR* probe. Of the 100 clones, four putative *ompR* clones were isolated (Figure 3.3A). These four clones were further analysed by Southern hybridisation analyses (Figure 3.3B) using the *envZ*-specific probe, to identify those clones encoding both *ompR* and *envZ* on the same plasmid.

Two of the four clones were found to hybridise to both the *ompR* (Figure 3.3A, lanes 2&3) and *envZ* probe (Figure 3.3B, lanes 2&3). The clone designated pRZ69 and containing the smaller of the two inserts (approximately 5 kb) was selected for further analyses.

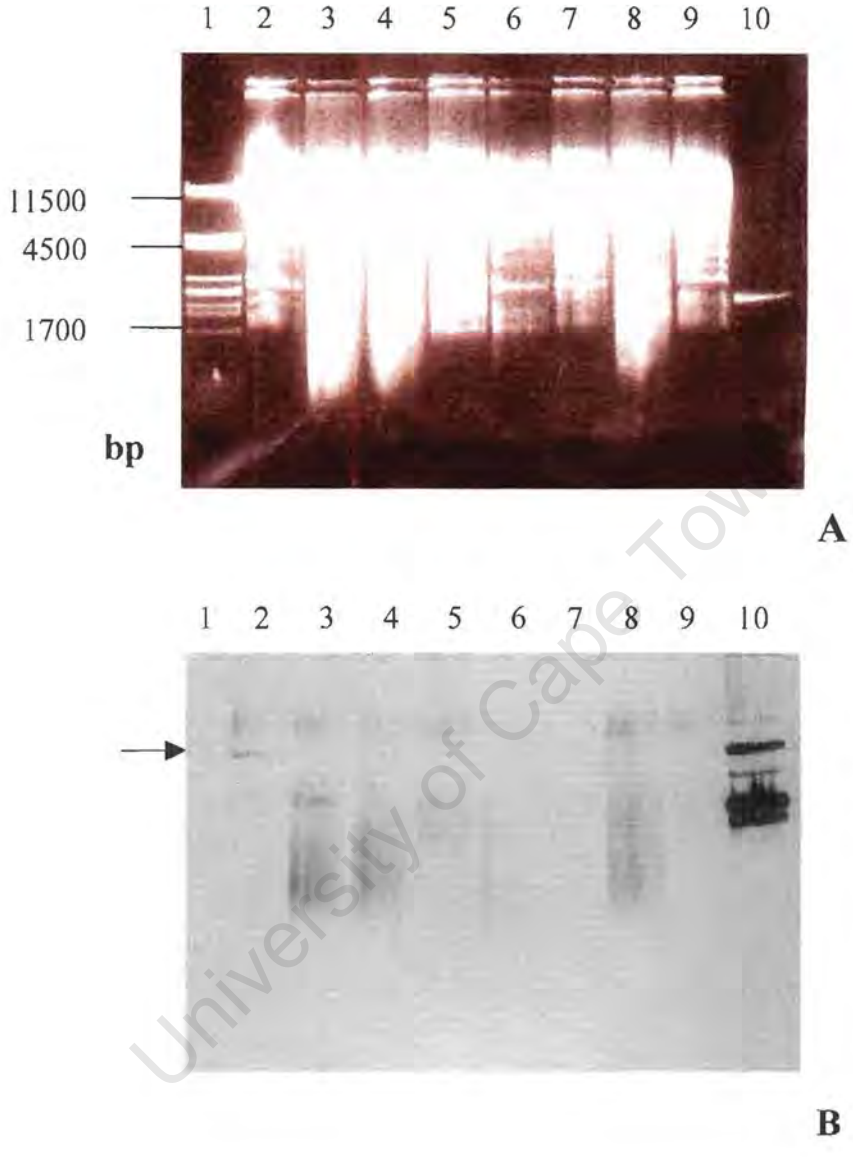





**Figure 3.2.** Southern hybridisation analysis of plasmid pools. (A) Uncut plasmid pools (lane 2-9) representing a portion of the *E. chrysanthemi* genomic library. Lane 1, the λ-*Pst*I marker and lane 10, the positive *ompR* control, pA51. (B) Southern hybridisation analysis using the *E. coli ompR* DIG-labelled probe, to identify *ompR*-hybridising plasmid pools. Note the distinct DNA band (lane 2) indicated by the arrow, showing the *ompR*-homologous plasmid pool chosen for further investigation.

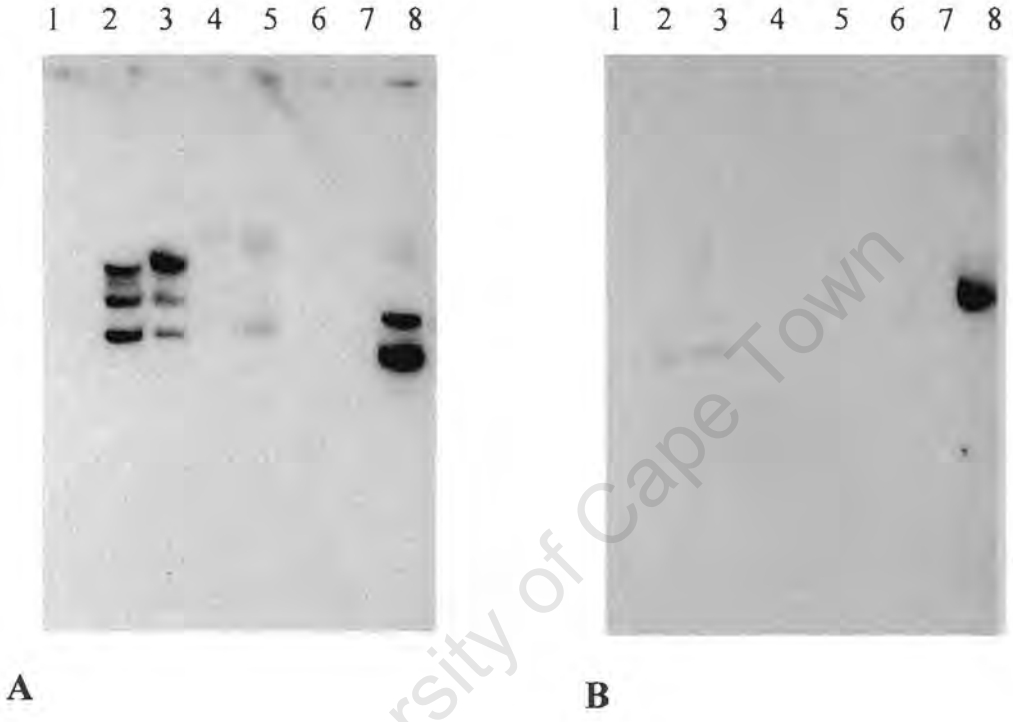





**Figure 3.3.** Screening of the genomic library for *ompB* homologues. (A) Southern hybridisation analysis of uncut DNA of putative clones, probed with the *ompR* probe. Lane 8, the *ompR* positive control and lanes 2 and 3 indicate two clones with homology to the probe, of which one (pRZ69, lane 2) was chosen for further characterisation. (B) Southern hybridisation analysis of the above clones digested with *Pst*I-*Eco*RI, probed with the DIG-labelled *envZ* probe. Lane 8, the *envZ* positive control and lanes 2 and 3 indicate two clones (one being pRZ69, lane 2) showing homology to the *envZ* probe.

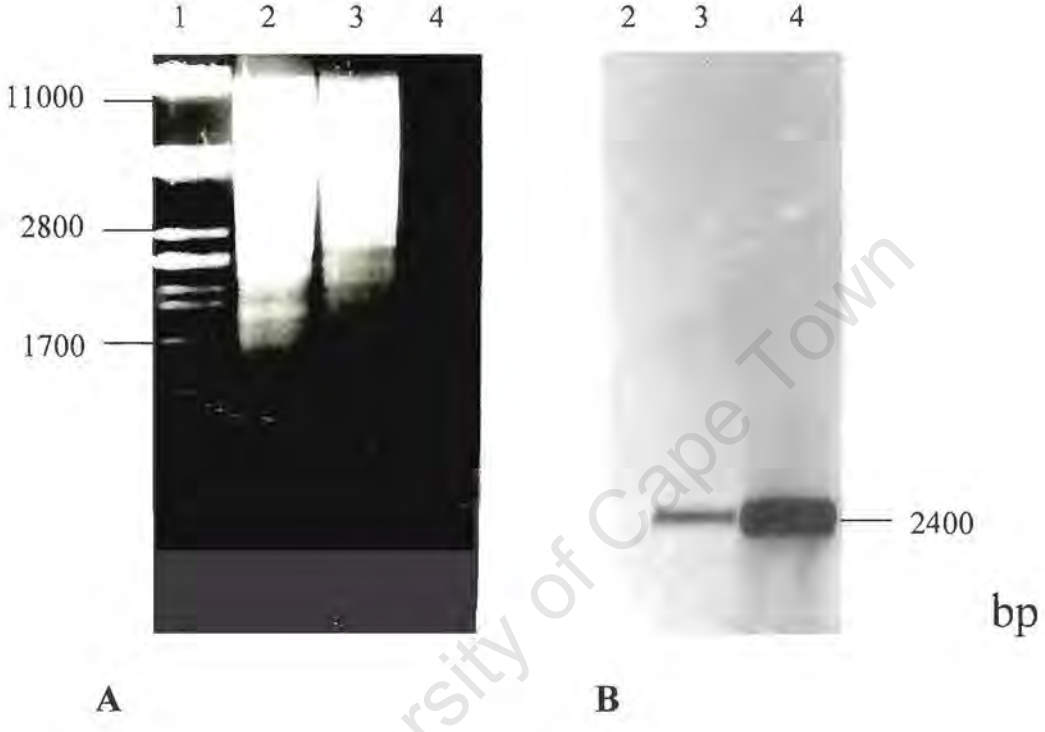





**Figure 3.4.** Confirmation of the origin of pRZ69. (A) *E. coli* and *E. chrysanthemi* genomic DNA digested with *Eco*RV (lane 2 and 3 respectively). Lane 1, the λ-*Pst*I marker and lane 4, pRZ69 digested with *Eco*RV. (B) Southern hybridisation analysis using a DIG-labelled pRZ69-derived *ompR* probe, showing that the clone, pRZ69, was derived from *E. chrysanthemi*.

### 3.4.3 Southern hybridisation analyses to confirm the origin of the insert DNA of pRZ69

Southern hybridisation analyses, using a 2 kb *Eco*RV-*Eco*RV pRZ69-derived fragment, was employed to confirm that the insert DNA of that clone was derived from *E. chrysanthemi* (Figure 3.3).

Chromosomal DNA of *E. coli* (Figure 3.4A, lane 2) and *E. chrysanthemi* (Figure 3.4A, lane 3), restricted with *Eco*RV, was compared with the banding pattern of the clone, pRZ69, restricted with the same enzyme (Figure 3.4A, lane 4). The Southern hybridisation analysis (Figure 3.4B) shows that homologous DNA sequences at a corresponding molecular weight exist for the clone, pRZ69 (lane 4) and *E. chrysanthemi* chromosomal DNA (lane 3) restricted with the same enzyme. No sequence identity with the *E. coli* chromosomal DNA under high stringency was noted (Figure 3.4B, lane 2). This apparent lack of sequence identity between the pRZ69-derived probe and the *E. coli* chromosomal DNA suggests that pRZ69 was not derived from *E. coli*. This is indicative of heterologous hybridisation under high stringency conditions, as was used in this study.

The high degree sequence identity (as determined by the strong chemiluminence signal) between the pRZ69-derived probe and the *E. chrysanthemi* chromosomal DNA (Figure 3.4B, lane 3) suggests that pRZ69 is derived from *E. chrysanthemi*. This was corroborated by the hybridisation of the probe to DNA bands at corresponding molecular weights for both pRZ69 and *E. chrysanthemi* chromosomal DNA, restricted with the same enzyme.

In summary, the construction and screening of an *E. chrysanthemi* genomic library yielded a clone, pRZ69, encoding both *ompR* and *envZ* homologues. Preliminary characterisation by Southern hybridisation analyses confirmed that the clone was derived from *E. chrysanthemi*. Further characterisation of the insert DNA was required to investigate the structural and functional similarity of this *ompB* homologue with that of those already characterised.

The genetic and physiological characterisation of pRZ69 is detailed in chapters four and five to follow.

# Chapter 4

# Genetic characterisation of the *ompRZ* locus of *Erwinia chrysanthemi*

## 4.1 SUMMARY

An *ompRZ* locus proposed to be involved in controlling outer membrane protein synthesis was cloned from *Erwinia chrysanthemi*, and the clone designated, pRZ69. The restriction map of this clone was determined and the *ompR* and *envZ* homologues were localised on the plasmid, by Southern hybridisation analyses. A 1200 bp, *Eco*RV-*PstI* fragment, containing the *envZ* homologue (pZS2) and a 2000 bp *Eco*RV-*Eco*RV fragment containing the *ompR* homologue (pRS1) were subcloned into the pBluescriptSk vector to facilitate DNA sequence analyses. Nested clones of both subclones were generated by EXOIII shortening and the DNA sequences of these overlapping clones were determined. DNA sequence analyses revealed that the *E. chrysanthemi ompRZ* locus was structurally similar, at the genetic level, to the *ompB* locus of *E. coli*. The *ompR* and *envZ* homologues were encoded as an operon and showed a high degree of sequence identity with that of the *ompB* locus of *E. coli,* at both the nucleotide and protein level.

## 4.2 INTRODUCTION

The reciprocal regulation of the porin proteins (as described in Chapter 1) in response to changes in the surrounding osmolarity, has been identified to involve three genetic loci: *ompF*, located at 21 minutes on the *E. coli* chromosome, *ompC*, at 48 minutes and *ompB* at 74 minutes (Sarma and Reeves, 1977; Sato and Tura, 1979; Verhoef *et al.,* 1977). The *ompF* and *ompC* proteins, are structural, encoding the OmpF and OmpC proteins respectively and the *ompB* locus plays a regulatory role. The *ompB* locus encodes two genes, *envZ* and *ompR* that function as an operon in porin regulation (Hall and Silhavy, 1979).

Both the *ompR* and *envZ* genes of *E. coli* have been cloned and characterised (Wurtzel *et al.,* 1982; Mizuno *et al.,* 1982), and their nucleotide sequences determined (Nara *et al.,* 1986). Based on both sequence and functional similarities, EnvZ and OmpR have been classified as members of a large family of homologous proteins referred to as two-component regulatory systems (Nixon *et al.,* 1986; Parkinson and Kofoid, 1992; Stock *et al.,* 1990). These regulatory systems generally include membrane-spanning sensor proteins and cytoplasmically-located response regulatory proteins (Figure 4.1). EnvZ is a transmembrane signal transducer (Figure 4.1) composed of a periplasmic sensor domain, two transmembrane domains, a linker domain and a cytoplasmic signalling domain. Sensors like EnvZ possess extensive homology in their C-terminal domains, while their N-terminal domains may vary (Nixon *et al.*, 1986). The histidine site at position 243 in EnvZ has been determined to be invariant among all known sensors in two-component systems. This site is functionally important and represents the site of EnvZ autophosphorylation. Similarly, the regulator *ompR* also possesses extensive homology with response regulators of other two-component systems, particularly in the N-terminal domain (Stock *et al.,* 1990). The N-terminal half of OmpR is the phosphorylation domain and contains the acidic pocket. The acidic pocket is characterised by a conserved aspartic acid residue at position 55, which is presumed to be the site of phosphorylation. In addition to the conserved aspartic acid residue, a lysine residue at position 109, has been reported to be conserved to the same

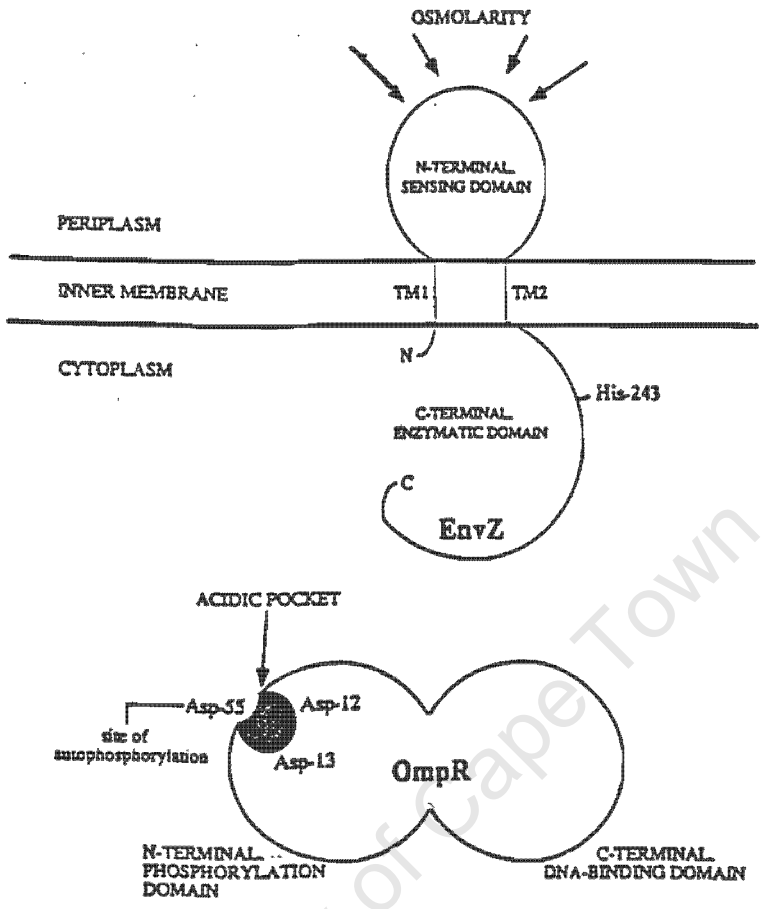



**Figure 4.1 Domain structure of OmpR and EnvZ.** The sensor, EnvZ, is located in the inner membrane with its N-terminal domain in the periplasm and its C-terminal domain in the cytoplasm. It possesses two transmembrane segments extending from 16 to 46 (TM1) and from 163 to 179 (TM2). OmpR is a cytoplasmic protein directly responsible for regulating transcription of the *ompF* and *ompC* genes. The N-terminal half of OmpR is the phosphorylation domain, containing the acidic pocket and the presumed site of phosphorylation (Asp-55), and the C-terminal portion of OmpR is the DNA binding domain.

(Modified from Forst *et al.*, 1987)

degree (Volz and Matsumura, 1991). Lysines are commonly involved in kinase function, however, no conserved lysine residues have been found associated with the histidine kinase domains of sensor proteins. This lack of a conserved catalytic lysine distinguishes the histidine protein kinase superfamily from other protein kinases that have been characterised.

Almost 100 different histidine protein kinases (sensor proteins) and their cognate response regulator proteins have now been sequenced (Stock *et al.,* 1995). Comparative sequence analyses have identified structural similarities among members of both sensor and response regulator proteins. Conserved amino acid residues that have functional significance have been identified, as described above, and its these conserved residues that will be investigated in the genetic characterisation of the *ompB* locus described in this chapter.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study have been listed previously (Tables 2.1 and 3.1). The *E. coli* strain, JM109 served as the host strain for all plasmid manipulations. The cloning vector, pBluescriptSk (see Appendix C for restriction map) was used in all subcloning experiments. All *E. coli* strains were grown and maintained at 37 °C in LB or on LB agar plates, with the appropriate antibiotics added (100 µg/ml for Amp) for the maintenance of the plasmid, pSK.

### 4.3.2 DNA manipulations and sequencing

Plasmid DNA isolation was performed using the Nucleobond kit (Machery-Nagel) and following the manufacturer's protocol. For the small scale preparation (miniprep) of plasmid DNA, the method outlined by Ish-Horowicz and Burke (1981) was followed. Restriction endonuclease analysis of the recombinant clones were performed at the appropriate temperature, using the restriction endonucleases and their recommended buffers, supplied by Boehringer Mannheim. Standard procedures were followed for plasmid propagation (see Appendix A for the method of transformation), restriction endonuclease mapping and subcloning procedures (Sambrook *et al.*, 1989). Subcloning was done by generating blunt-end insert fragments and cloning these into the *Eco*RV site of the vector, pBluesckriptSk. All digests were electrophoresized on 0.8 % agarose gels in TBE buffer at 100V. The subcloning and sequencing strategy is outlined in the legend of Figure 4.4. The complete sequence of the *ompRZ* locus was assembled from the sequences obtained from the subclones, pZS2 (1200 bp *envZ*-containing fragment) and pRS1 (2000 bp *ompR*-containing fragment). The DNA was sequenced in both directions using clones carrying overlapping deleted fragments obtained by EXOIII exonuclease degradation (Henikoff, 1984). The GCG software package was used to analyse the sequence data (Devereux *et al.*, 1984).

### 4.3.3 Characterisation of the insert in pRZ69

To localise the *ompR* and *envZ* homologues, the insert in pRZ69 was characterised by restriction endonuclease analyses, followed by the hybridisation of these restricted fragments to probes of the *ompR* and *envZ* genes. Southern hybridisation analyses were done as described in section 2.3.5, using the previously generated *E. coli ompR* and *envZ* probes.

## 4.4 RESULTS AND DISCUSSION

### 4.4.1 Characterisation of the *E. chrysanthemi ompRZ* clone, pRZ69

Preliminary characterisation of the insert in pRZ69 (Chapter 3) confirmed that an *ompB* locus was cloned from *E. chrysanthemi*. The insert was further characterised by restriction endonuclease analyses and a restriction map was constructed (Figure 4.2). Restriction endonucleases were selected, based on the restriction map of the *E. coli ompB* locus. Restriction endonuclease analyses revealed differences in the restriction maps of the two *ompB* loci compared. Restriction sites (for example, *Ssp*I and *Cla*I) that were observed in the *E. coli ompB* locus were absent in the putative *ompB* locus cloned from *E. chrysanthemi* (results not shown). This suggested that sequence variation existed, which agreed with the result showing failure to amplify an *ompR* homologue from *E. chrysanthemi* with the *E. coli* PCR primers. However, confirmation of sequence variation had to be obtained by DNA sequence analyses.

Southern hybridisation analyses, using the *E. coli ompR* and *envZ* probes respectively, were performed to localise the respective homologues on the plasmid, pRZ69. The *envZ* homologue was localised to the region between the 1200 bp *Eco*RV-*Pst*I fragment, while the *ompR* homologue was found to exist on a 2000 bp *Eco*RV-*Eco*RV fragment (Figure 4.3). These fragments, the 1200 bp *Eco*RV-*Pst*I and the 2000 bp *Eco*RV-*Eco*RV fragment were subcloned into the vector, pSK to facilitate the sequence analyses of the *ompB* homologue isolated (Figure 4.4). The plasmids, pZS2 containing the *envZ* homologue and pRS1, containing the *ompR* homologue, were thus constructed.

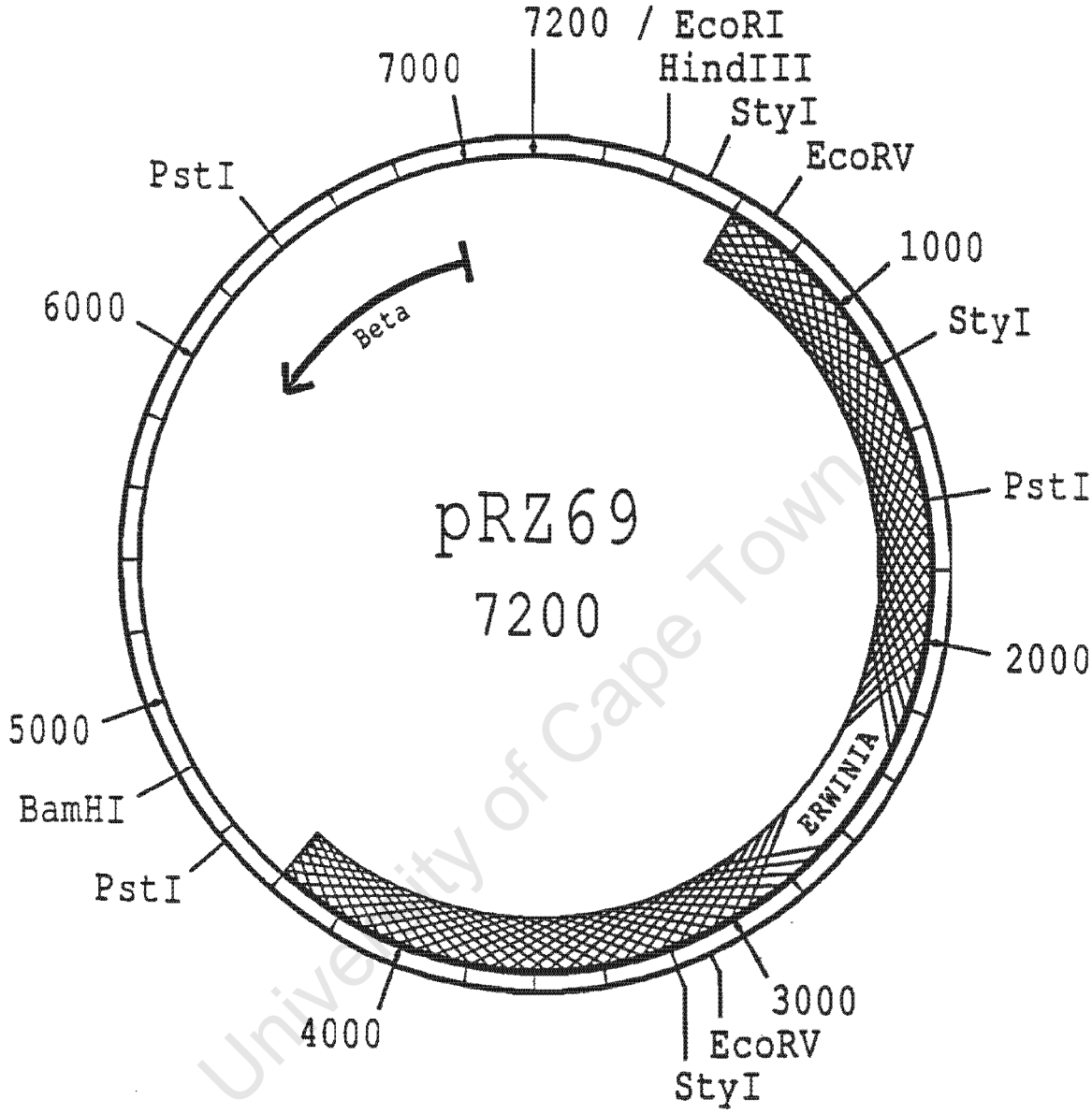





**Figure 4.2.** A restriction endonuclease map of pRZ69, a pEcoR251-derived clone, isolated from an *E. chrysanthemi* gene library. The gene conferring $Amp^r$ is indicated by "beta" with the arrowhead indicating the direction of transcription. The *Erwinia* insert DNA, encoding the putative *ompR* and *envZ* genes, is also indicated by the shaded box designated, "ERWINIA".

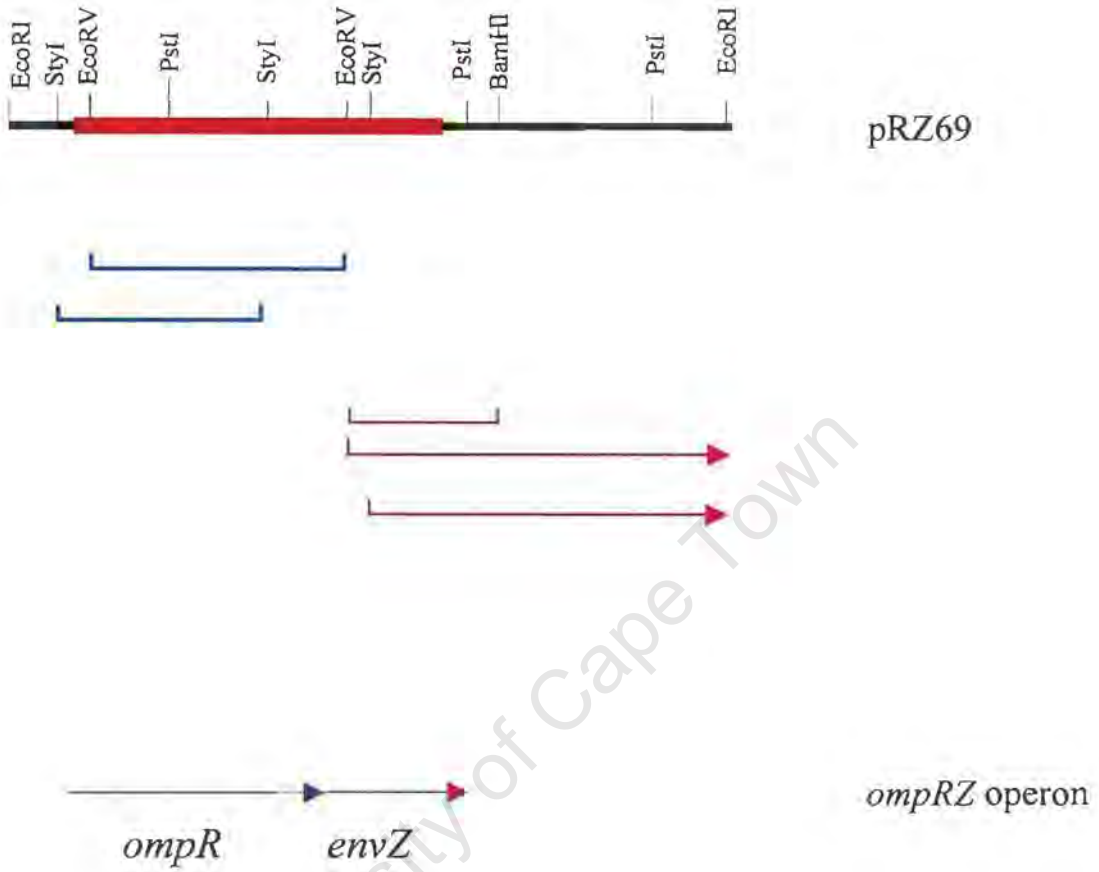





**Figure 4.3.** Localisation of the *ompR* and *envZ* homologues on the *E. chrysanthemi ompRZ*-carrying insert of pRZ69. The restriction map of pRZ69 is shown with the insert DNA represented as a red line and the vector sequence as a black line. The bar-lines below the restriction map indicate fragments of pRZ69 that hybridised to the *E. coli ompR* (blue) and *envZ* (pink) probes. The probes used were both portions of the *ompR* (631 bp) and *envZ* (918 bp) genes of *E. coli*, respectively. The suggested location and direction of transcription of the *E. chrysanthemi ompRZ* operon are indicated at the bottom of the figure.

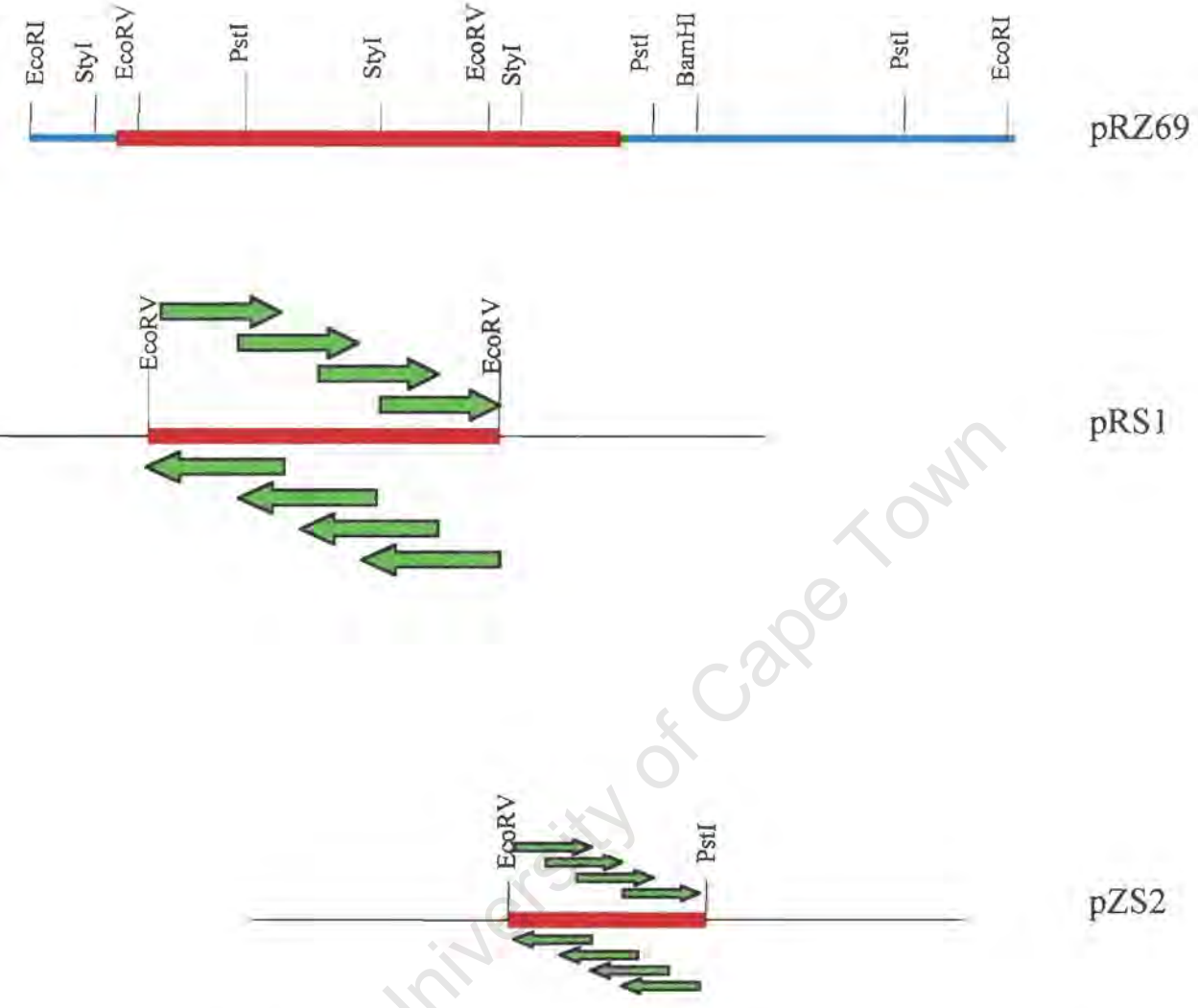





**Figure 4.4**. Subcloning and sequencing strategy followed to assemble the nucleotide sequences of the *ompR* and *envZ* genes of *E. chrysanthemi*. The full-length clone, pRZ69 was subcloned into pBluescriptSk (black line), generating the *ompR* and *envZ*-containing clones, pRS1 and pZS2, respectively. Insert DNA fragments are indicated by red lines, while the pEcoR251 vector sequence is indicated in blue. The clones were sequenced in both directions by assembling overlapping sequences of the EXO III deleted fragments (green arrows).

### 4.4.2. DNA sequence analyses of the *E. chrysanthemi ompRZ* clone

A number of overlapping fragments of the 1200 bp and 2000 bp inserts of the subclones pRS1 and pZS2 respectively, were sequenced (Figure 4.4). A contiguous sequence of approximately 3000 bp was obtained by assembling the sequences of these fragments (Figure 4.5). Translation of the nucleotide sequence into the six possible open reading frames (ORFs) by Frames analyses (GCG component) identified two prominent open reading frames (Figure 4.6). The first open reading frame, which starts at position 690 and ends at base pair position 1400, is proposed to encode the *E. chrysanthemi ompR* gene. The second open reading frame, which starts at position 1400 and ends at position 2800, is not encoded in the same frame as ORF1 and is proposed to encode the *E. chrysanthemi envZ* homologue. The *ompR* and *envZ* homologues were structurally similar in arrangement to the *ompB* operon of *E. coli*. The genes were encoded as an operon (Figure 4.5) with the *ompR* gene assumed as the promoter proximal gene. The entire nucleotide sequence was subjected to Blast search analyses and sequence identity with *ompB* loci of several bacterial species in the GenBank database was obtained (Table 4.1). The regions spanned by the *E. coli ompR* primers were also investigated to determine the degree of sequence variation (Figure 4.5). A high percentage (85% identity) of sequence conservation was observed within the region spanned by the *E. coli ompR* reverse primer (Figure 4.5). Sequence conservation within the region spanned by the forward primer was far lower, with only 8 of 18 nucleotides (44.4 %) sharing sequence identity with the *E. coli* sequence. This confirmed that sequence variation existed between *E. chrysanthemi* and *E. coli* and therefore accounted for the failure to PCR amplify an *ompR* homologue from *E. chrysanthemi* with the selected primer set.

```
1      CACTATAGGGCGATTGGGTCCACCGCTTTTTTCAGCAGGGCGCGGGCCATTGGCGCGTCA
61     ATCGAGATGTAATCTTTACGGCCATAGATTTCATCCGGCCCGACGATACGAAAGCGTTTA
121    ACCTCGCCCTCTTCGTTTTCCACCTCGACCCAGGCGCCAAAGAATACCTTGCCGTCCTGC
181    TGGGGGGAATAATCCACCACCCGCACTACCTGCAGGCGTTTGCGCAAGTAACGTACCCGG
241    CGGTCGATTTCGCGCAACAAGCGTTTGTTGTAGAGGTAATCGGCGTTTTCGCTGCGATCG
301    CCCAAACTGGCCGCCCAGGCTACCTTCTCTGTGATTTCCGGGCGACGCTCTTTCCACAGA
361    TAGTTCAGTTCCTGATGTAGGGCTTCATAGCCTTCGCGAGTGATCAGATCGGTTTTCATA
421    CGCTTTTCTGAAATGTGAAGATCATAAAATCGTTATAATCGACGACGGTGAGATAACATA
481    GCGGATAAATCGTCATTTGTAGCGAGGGTTGCGGCGTATTATGGCGGGAACGGCTTGATT
541    ACAACGTATTTTTATATCCTTTGTAACAATTTTGCCTAGAATGTATACCAGAAACGGCTG
601    TCAGTTCGTTGCGGGTTTCTGAACAATACTAGATTGTGTTTCGTGATGGAACCAAGCGCT
661    GGCAGCTATCATGTCTTTGGAGAAGAGAGATGCCAGAGAATTATAAAATTCTGGTTGTGG
                                       M  P  E  N  Y  K  I  L  V  V  D
                  (Fp)- ccaggttcgaagcgtcgc
721    ATGACGATATGCGCCTGCGAGCGTTATTGGAACGCTATCTGACCGAACAGGGTTTTCAGG
          D  D  M  R  L  R  A  L  L  E  R  Y  L  T  E  Q  G  F  Q  V
781    TACGCAGTGTTGCCAATGCCGAACAGATGGATCGCCTGCTGACGCGCGAATCGTTCCATC

          R  S  V  A  N  A  E  Q  M  D  R  L  L  T  R  E  S  F  H  L
841    TGATGGTGTTGGATTTGATGCTGCCGGGCGAGGATGGGTTGTCCATCTGCCGTCGTCTGC
          M  V  L  D  L  M  L  P  G  E  D  G  L  S  I  C  R  R  L  R
901    GTAGTCAAAGCAACCCGATGCCGATCATCATGGTGACGGCGAAAGGCGAAGAAGTGGACA
          S  Q  S  N  P  M  P  I  I  M  V  T  A  K  G  E  E  V  D  R
961    GGATCGTGGGGCTGGAAATCGGCGCGGACGATTATATTCCCAAACCGTTTAACCCACGCG
          I  V  G  L  E  I  G  A  D  D  Y  I  P  K  P  F  N  P  R  E
1021   AACTGCTGGCCCGTATTCGTGCGGTACTGCGCCGTCAGGCGAATGAATTGCCCGGTGCGC
          L  L  A  R  I  R  A  V  L  R  R  Q  A  N  E  L  P  G  A  P
1081   CGTCACAGGAAGAAGCGGTCATCGCATTCGGTAAATTTAAACTGAATCTGGGCACGCGCG
          S  Q  E  E  A  V  I  A  F  G  K  F  K  L  N  L  G  T  R  E
1141   AGATGTTCCGTGACGATGAACCCATGCCGCTGACCAGTGGCGAGTTCGCGGTACTCAAGG
          M  F  R  D  D  E  P  M  P  L  T  S  G  E  F  A  V  L  K  A
1201   CGCTGGTGAGCCATCCACGTGAGCCGCTGTCTCGCGACAAACTGATGAATCTGGCTCGTG
          L  V  S  H  P  R  E  P  L  S  R  D  K  L  M  N  L  A  R  G
1261   GCCGTGAATACAGCGCCATGGAACGTTCCATTGACGTGCAGATTTCGCGCTTGCGCCGCA
          R  E  Y  S  A  M  E  R  S  I  D  V  Q  I  S  R  L  R  R  M
                                                  (Rp)- ctgggctacg
1321   TGGTAGAGGAAGACCCGGCACATCCGCGTTATATCCAGACCGTGTGGGGACCTGGGTTACG
          V  E  E  D  P  A  H  P  R  Y  I  Q  T  V  W  G  L  G  Y  V
       tctttgtacc
1381   TTTTTGTGCCGGACGGCAGTAAAGCATGATTCGATGGCGCTTCTCACCACGCAGCGCCTT
                                       M  I  R  W  R  F  S  P  R  S  A  F
          F  V  P  D  G  S  K  A  *
1441   CGCCCGCACGCTGTTGCTGATTGTTACTCTGCTATTCGTTAGCCTTGTCACCACCTATCT
          A  R  T  L  L  L  I  V  T  L  L  F  V  S  L  V  T  T  Y  L
1501   GGTGGTGCTTAACTTCGCGATTCTGCCCAGTCTGCAACAGTTCAACAAAGTTCTGGCCTA
          V  V  L  N  F  A  I  L  P  S  L  Q  Q  F  N  K  V  L  A  Y
1561   TGAGGTCAGAATGTTGATGACGGACAAGCTGCAACTGGAAGATGGTTCCACGCTGGACGT
          E  V  R  M  L  M  T  D  K  L  Q  L  E  D  G  S  T  L  D  V
1621   CCCCCCGGCGTTCCGGCGCGAGATTTATCGCGAACTGGGTATTTCGCTCTACACCAACGC
          P  P  A  F  R  R  E  I  Y  R  E  L  G  I  S  L  Y  T  N  A
1681   CGCAGCGGAAGAGAGTGGGTTGCGCTGGGCGCAGCACTACAAGTTTCTGAGCCAGCAGAT
          A  A  E  E  S  G  L  R  W  A  Q  H  Y  K  F  L  S  Q  Q  M
1741   GGCGCAGCAAGTGGGTGGGCCGACCGAAGTGCGGGTTGAGGTCAGCAAGAATACGCCGGT
          A  Q  Q  V  G  G  P  T  E  V  R  V  E  V  S  K  N  T  P  V
1801   GGTATGGCTGAAAACCTGGCTATCGCCAGATATCTGGGTTCGGGTGCCGCTAACGGAAAT
          V  W  L  K  T  W  L  S  P  D  I  W  V  R  V  P  L  T  E  I
1861   CCATCAGGGCGACTTCTCGCCACTGTTTCGCTATACCCTGGCTATTATGCTGCTGGTGAT
          H  Q  G  D  F  S  P  L  F  R  Y  T  L  A  I  M  L  L  V  I
1921   CGGTGGCGCCTGGCTATTTATTCGGGTGCAGAATCGGCCCTTGGTGGAGCTAGAGCACGC
          G  G  A  W  L  F  I  R  V  Q  N  R  P  L  V  E  L  E  H  A
```

```
1981  TGCCATTCAGGTGGGGAAAGGCATTATTCCGCCGCCGTTACGTGAATATGGTGCTTCGGA
        A  I  Q  V  G  K  G  I  I  P  P  P  L  R  E  Y  G  A  S  E
2041  GGTCCGTTCGGTGACACGGGCCTTCAACCAGATGGCATCTGGGGTCAAGCTACTGGCGGA
        V  R  S  V  T  R  A  F  N  Q  M  A  S  G  V  K  L  L  A  D
2101  CGATCGAACGTTACTGATGGCTGGCGTCAGCCATGATTTACGCACGCCGTTGACCCGCAT
        D  R  T  L  L  M  A  G  V  S  H  D  L  R  T  P  L  T  R  I
2161  TCGTCTGGCAACCGAGATGATGGGCCAGGAAGATGAATACCTGGCGGAATCTATCAATAA
        R  L  A  T  E  M  M  G  Q  E  D  E  Y  L  A  E  S  I  N  K
2221  AGATATTGAAGAGTGTAACGCCATCATCGAGCAGTTCATCGACTACCTGCGCACGGGTCA
        D  I  E  E  C  N  A  I  I  E  Q  F  I  D  Y  L  R  T  G  Q
2281  GGAAATGCAGATGGAGGTTGCTGACCTCAATGCGATTCTGGGTGAAGTGGTCGCGTCTGA
        E  M  Q  M  E  V  A  D  L  N  A  I  L  G  E  V  V  A  S  E
2341  AAGCGGTTATGAGCGGGAAATCGACAGTGAATTGACTCTGGGCGAACTGCCGATGAAGGT
        S  G  Y  E  R  E  I  D  S  E  L  T  L  G  E  L  P  M  K  V
2401  CAGCCCCTTGTCGATTAAACGGGCGGTAGCGAATCTGGTCGTGAACGCCGCGCGTTATGG
        S  P  L  S  I  K  R  A  V  A  N  L  V  V  N  A  A  R  Y  G
2461  TAATGGCTGGATTCGTGTCAGTAGCGGCCGGGAATTACAGCGTGCCTGGTTCCAGGTGGA
        N  G  W  I  R  V  S  S  G  R  E  L  Q  R  A  W  F  Q  V  E
2521  AGATGATGGCCCCGGTATCGACCCATCGCAGTTGGCGCACCTGTTTCAGCCGTTTGTTCG
        D  D  G  P  G  I  D  P  S  Q  L  A  H  L  F  Q  P  F  V  R
2581  TGGTGATAGTGCCCGCAGTACCAGCGGCACTGGGTTAGGCCTGGCGATCGTGCAGCGTAT
        G  D  S  A  R  S  T  S  G  T  G  L  G  L  A  I  V  Q  R  I
2641  CATTGACGCACATAACGGATCGTTGGATGTGGGGAAAAGTGAGCGCGGTGGACTGCGCGT
        I  D  A  H  N  G  S  L  D  V  G  K  S  E  R  G  G  L  R  V
2701  GCGGGCTTATTTACCACTGGCGTTCCCGAACCCATCCGGCACGGCTTCAGTCCGTGTGCG
        R  A  Y  L  P  L  A  F  P  N  P  S  G  T  A  S  V  R  V  R
2761  TGAGGGCCATCGACGTAGCTCGACGCAGACAGGTCGCACCCGGCAAGACAAAGAGTAATA
        E  G  H  R  R  S  S  T  Q  T  G  R  T  R  Q  D  K  E  *
2821  TCGACCCACCGAGGTGAAATCGGTGAAAATAACAAAGAAAAGGCGCGTTATCCGTTTTTT
2881  GCTGTCGGAAAAACCGTCGTCGGGAAAACGCCGCCGGATGAGCGGCAGGCGATCGTCGAG
2941  GTTAGCGTTTCGGCCCGGCGGATACCAGTGCCGCACCGGCTGGGGTGTCGGTGTATTTAT
3001  CGAAGTTGGTGATAAAACGTGCGCCAGATCTCATAAGAATATATCAGAGATAGCGCAATT
3061  TTATGCTCTCTGAGAGCCACTTTCCTTACGTCCTTTTCTTAGAGGGGTCTAACTTTTTAA
3121  CAGAAAATATCTCA
```

**Figure 4.5.** Nucleotide sequence of the *E. chrysanthemi ompRZ* locus and adjacent regions. The derived amino acid sequences are indicated in single letter code below the coding sequence of the *ompR* and *envZ* genes. The start codon of the *ompR* gene is underlined and the *envZ* start codon sequence is underlined and in bold. Asterisks indicate the stop codons for both genes (*). Note that the genes are encoded as an operon (with the stop codon of *ompR* overlapping the start codon of *envZ*). The regions spanned by the *ompR* forward ($F_p$) and reverse complemented primer ($R_p$) of *E. coli* is also shown (boxed sequence) with nucleotides identical to the *E. coli* sequence at that position, highlighted in bold.

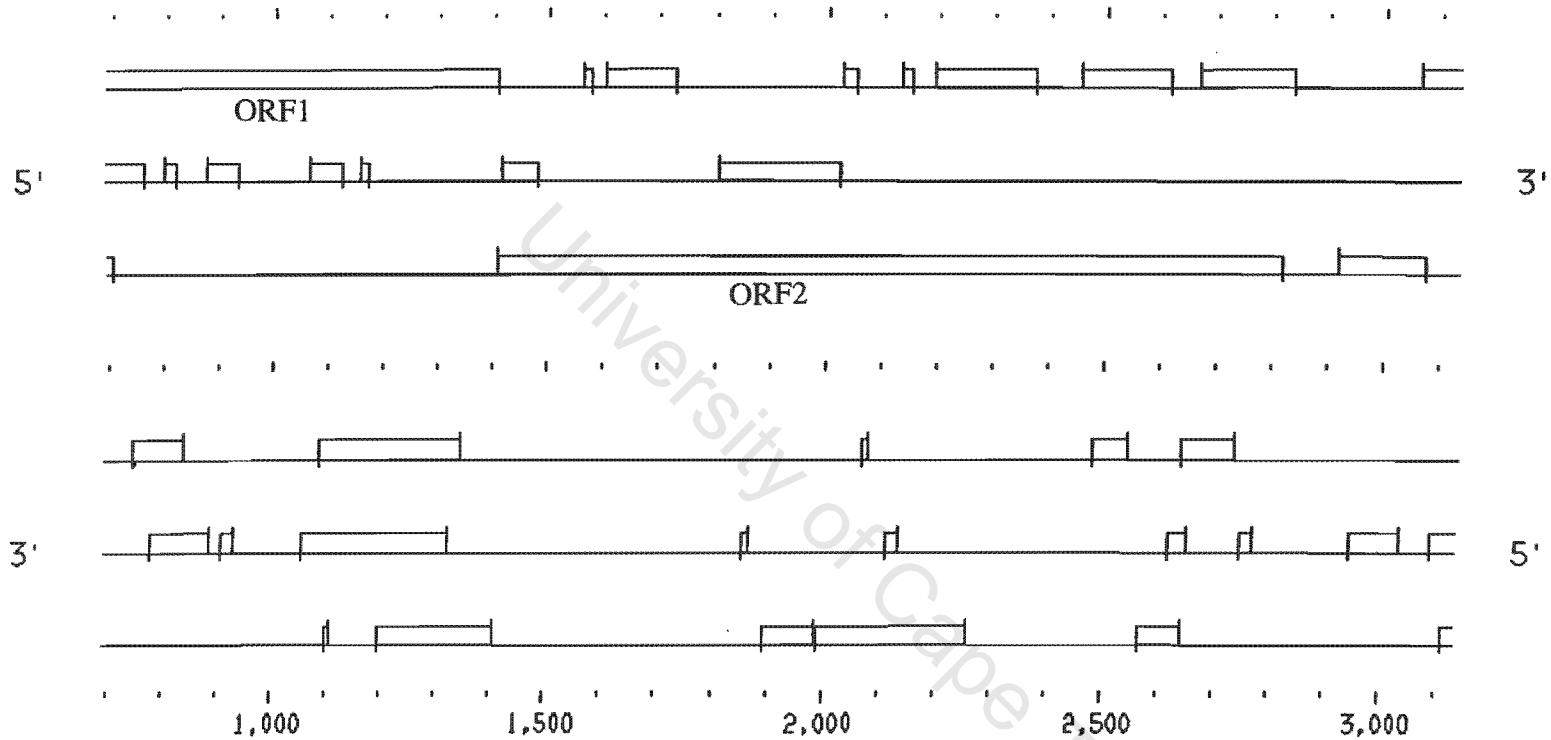





**Figure 4.6.** Frames analyses of the *E. chrysanthemi ompRZ* locus, showing the translation of the nucleotide sequence into the six possible open reading frames. Two prominent open reading frames are shown, ORF1 encodes OmpR and ORF2 encodes the EnvZ protein.

**Table 4.1.** Sequences producing significant alignments with the *E. chrysanthemi ompB* nucleotide sequence.

| Accession Number | GenBank nucleotide sequence | Regions of gene showing homology | | Percentage sequence identity |
|---|---|---|---|---|
| | | Query[a] | Subject[b] | |
| Y08950 | *Y.enterocolitica ompR* and *envZ* genes | 1-584 | 741-1324 | 83% |
| | | 771-1250 | 1463-1942 | 78% |
| | | 620-736 | 1344-1460 | 88% |
| X78270 | *S. typhi ompR* and *envZ* genes | 1-575 | 282-856 | 84% |
| | | 1316-1676 | 1517-1877 | 81% |
| | | 1005-1231 | 1238-1464 | 81% |
| J01656 | *E. coli ompB* operon. | 1-581 | 251-831 | 83% |
| | | 1322-1682 | 1492-1852 | 81% |
| | | 1089-1270 | 1291-1472 | 85% |
| AF030314 | Shigella ompR gene | 834-1409 | 4-579 | 83% |
| AF030416 | *Enterobacter cloacae ompR* gene | 912-1409 | 82-579 | 82% |
| AF030416 | *Enterobacter cloacae* histidine kinase sensor | 1417-2154 | 12-749 | 79% |
| AF030415 | *Shigella flexneri* histidine kinase sensor | 1730-2100 | 325-695 | 81% |
| | | 1496-1599 | 91-194 | 82% |
| J01675 | Plasmid RI13 from *E. coli, EcoRI* endonuclease | 3026-3134 | 774-881 | 99% |
| AF030417 | *Y. enterocolitica* histidine kinase sensor | 1412-2076 | 7-671 | 78% |
| AF030317 | *Proteus vulgaris ompR* gene | 1218-1390 | 388-560 | 81% |
| | | 852-1031 | 22-201 | 78% |
| U07746 | *Xenorhabdus nematophilus* AN6/1 *ompR* gene | 1213-1385 | 790-962 | 79% |
| | | 690-809 | 267-386 | 81% |
| Z97065 | *Bordetella bronchiseptica risA* and *risS* genes | 856-901 | 271-316 | 89% |

[a]Query is the *E. chrysanthemi* sequence, [b]Subject is the GenBank database sequences showing homology.

The *E. chrysanthemi* sequence was further analysed by aligning both the nucleotide and the translated sequence of the putative *ompR* (Figure 4.7) and *envZ* (Figure 4.8) genes respectively, with that of several other reported *ompB* sequences found in the GenBank database.

Multiple sequence alignment of both the putative *ompR* and *envZ* sequences of *E. chrysanthemi* with other species at the nucleotide level showed a high percentage of sequence identity. *E. chrysanthemi* shared 83% and 63% identity with the *ompR* and *envZ* sequences of *E. coli* respectively. It was shown that the *E. chrysanthemi* sequence shared a relatively high percentage of identity with the *ompR* and *envZ* sequences of *Yersinia enterocolitica* (84% and 59% respectively) but less so with *Xenorabdus nematophilus* (73% and 31%). This suggested that *E. chrysanthemi* was more closely related to *Y. enterocolitica* and *E. coli* than to *X. nematophilus*.

To relate sequence conservation with function, a closer investigation at the protein level was required. Multiple sequence alignment of the *ompR* and *envZ* peptide sequences (Figure 4.7 and 4.8) confirmed the high percentage of sequence identity that existed amongst different species. Despite the sequence variation at the nucleotide level, it was notable that a higher degree of sequence conservation at the protein level existed. *E. chrysanthemi* shared 83% and 63% sequence identity with the *ompR* and *envZ* sequences of *E. coli* respectively at the nucleotide level, compared to 99% and 71% respectively at the protein level. This high percentage of sequence conservation at the protein level, underlines the importance of the relationship between sequence structure and protein function. Multiple sequence alignment at the protein level (Figure 4.7) showed that the *ompR* sequences were generally well conserved throughout the entire protein sequence. *E. chrysanthemi* only differed from the consensus sequence at the second amino acid residue (where a P is substituted for a Q) and at position 155 (where a D is substituted for E). The generally conserved aspartic acid residue, at position 55, which is presumed to be the site of phosphorylation, was identified in the *E. chrysanthemi ompR* homologue.

The lysine residue at position 109, demonstrated to be conserved to the same degree (Volz and Matsumura, 1991), was also identified in the *ompR* homologue of *E. chrysanthemi*. This

conservation strongly supports the observations that Asp-55 and Lys-109 are functionally significant in osmoregulation.

EnvZ, based on sequence alignment (Figure 4.8), was found to be generally less conserved among species than *ompR*. The EnvZ protein sequence of *X. nematophilus* was found to be considerably shorter and more diverse than the peptide sequence of other *envZ* species. However, a high percentage of sequence conservation existed in the C-terminal region. This conservation of residues in the C-terminal region is common among sensor proteins (Nixon *et al.*, 1986; Gross *et al.*, 1989). Blocks of conserved residues were identified at amino acid positions 240-260 and at positions 280-290. Amongst the conserved residues, the invariant histidine site at position 243 (autophosphorylation site) was also identified in the *envZ* homologue of *E. chrysanthemi*. Further analyses of the *envZ* homologue were done to investigate whether the sequence variation altered the overall protein structure. EnvZ in *E. coli* is a model membrane-bound protein, characterised by its two transmembrane regions and cytoplasmically-located C-terminal region. To investigate whether this structure is maintained in the *E. chrysanthemi envZ* homologue, a hydrophobocity plot was constructed to identify whether transmembrane regions were present (Figure. 4.9). Hydropathy analyses (Figure. 4.9a) identified two transmembrane regions (similar to *E. coli*, see Figure 4.1), both consisting of a length of 23 amino acids, with one transmembrane region extending from amino acid position 17-39 and the other from position 160-182 (see Figure. 4.9b). These results therefore confirmed that the protein structure typical of a membrane-spanning protein was still maintained despite the variation in sequence.



In summary, the data presented in this chapter provides evidence that an *ompB* locus was cloned from *E. chrysanthemi*. DNA hybridisation and protein sequence analyses, showed that sequence identity between the clone isolated from *E. chrysanthemi* (pRZ69) and the *ompB* locus of *E. coli*, was present. The conserved residues that characterise the proteins of the two-component, sensor-regulator family were also successfully identified in the *E. chrysanthemi* clone. To support the genetic evidence that an *ompB* locus was cloned from *E. chrysanthemi*, physiological characterisation of the *ompRZ* locus was required.

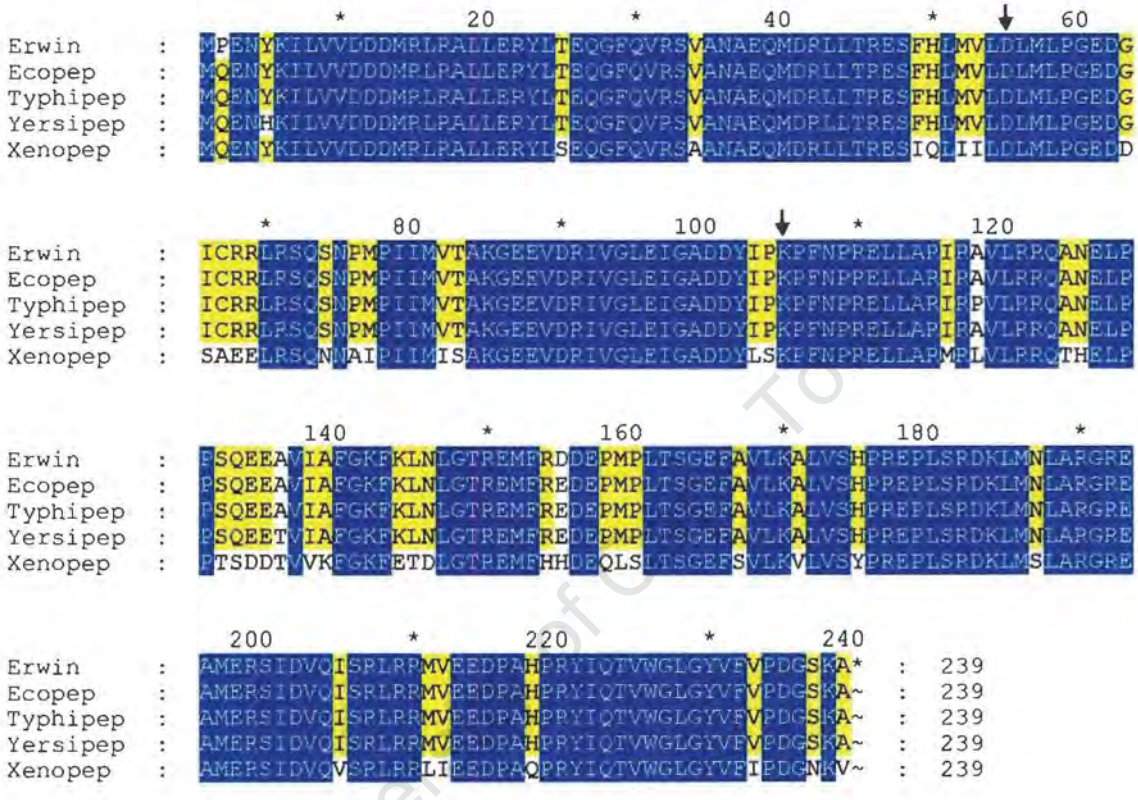



**Figure 4.7.** Multiple sequence alignment of OmpR peptide sequences. Identical amino acid residues are shaded in blue, while in less conserved regions, amino acid residues are shaded in yellow. The invariant aspartic acid and lysine residues at positions 55 and 109 respectively, are indicated by arrows. OmpR peptide sequences included are: *E. chrysanthemi* (Erwin), *E. coli* (Ecopep), *S. typhimurium* (Typhipep), *Y. enterocolitica* (Yersipep) and *X. nematophilus* (Xenopep).

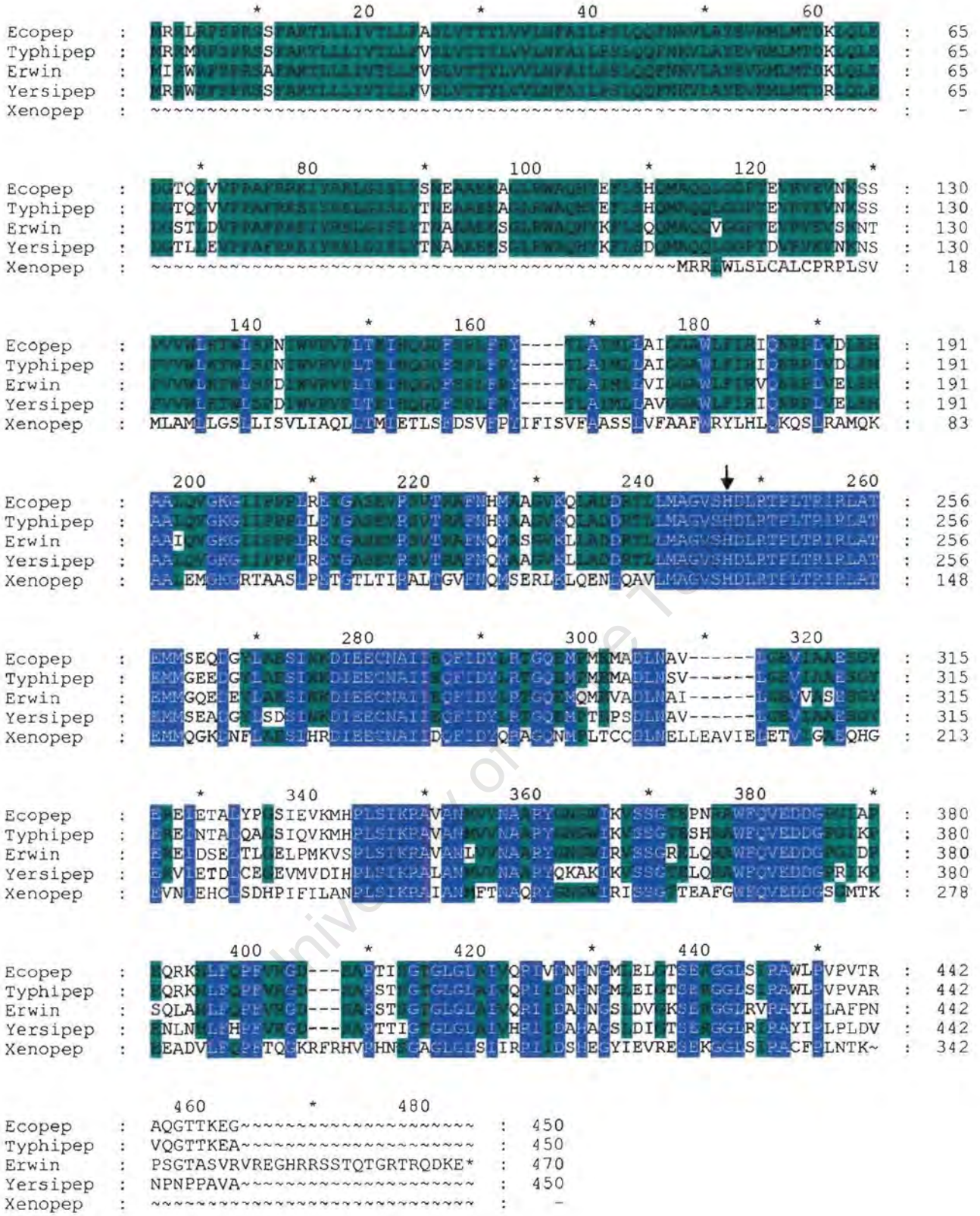
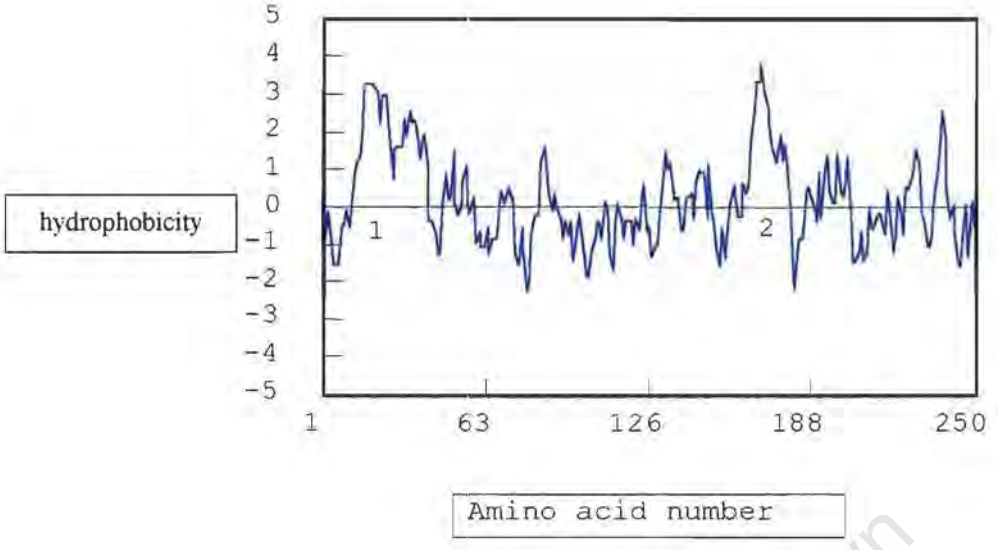



**Figure 4.8.** Multiple sequence aligment of EnvZ peptide sequences. Identical amino acid residues are shaded in blue, while in less conserved regions, amino acid residues are shaded in green. The invariant histidine at position 243 is indicated by an arrow. EnvZ peptide sequences included are: *E. chrysanthemi* (Erwin), *E. coli* (Ecopep), *S. typhimurium* (Typhipep), *Y. enterocolitica* (Yersipep) and *X. nematophilus* (Xenopep).




(a)

MIRWRFSPRSAFARTL$_{17}$**LLIVTLLFVSLVTTYLVVLNFAI**$_{33}$$^{1}$LPSLQQFNKVLAYEVRMLMTDKLQLEDGSTLDV
PPAFRREIYRELGISLYTNAAAEESGLRWAQHYKFLSQQMAQQVGGPTEVRVEVSKNTPVVWLKTWLSPDIWVRV
PLTEIHQGDFSP$_{160}$**LFRYTLAIMLLVIGGAWLFIRVQ**$_{182}$$^{2}$NRPLVELEHAAIQVGKGIIPPPLREYGASEVRSVTR
AFNQMASGVKLLADDRTLLMAGVSHDLRTPLTRIRLATEMMGQEDEYLAESINKDIEECNAIIEQFIDYLRTGQE
MQMEVADLNAILGEVVASESGYEREIDSELTLGELPMKVSPLSIKRAVANLVVNAARYGNGWIRVSSGRELQRAW
FQVEDDGPGIDPSQLAHLFQPFVRGDSARSTSGTGLGLAIVQRIIDAHNGSLDVGKSERGGLRVRAYLPLAFPNP
SGTASVRVREGHRRSSTQTGRTRQDKE *

(b)

**Figure 4.9. Hydrophobicity plot** (a) of the *envZ* homologue of *E. chrysanthemi*, showing the transmembrane regions numbered 1 and 2 respectively. The peptide sequence of the *envZ* homologue is given below (b) with the transmembrane regions illustrated by the boxed peptide sequences, numbered 1 and 2.

# CHAPTER 5

# Physiological characterisation and preliminary mutagenesis of the *ompRZ* locus of *Erwinia chrysanthemi*

## 5.1 SUMMARY

The *ompRZ* locus of *Erwinia chrysanthemi* was determined to be structurally similar to the *ompB* locus of *Escherichia coli*. To determine whether the *ompRZ* locus is functionally analogous to the *ompB* locus, a mutagenesis strategy to inactivate the locus in *E. chrysanthemi* was employed. Primers specific for the *envZ* homologue was designed from sequence data and the resulting PCR product constituting the C-terminal region was cloned into the suicide vector, pGP704. The resulting suicide construct, pMUT1, was utilised in attempts to inactivate the *envZ* gene in *E. chrysanthemi* by homologous recombination. Attempts to introduce the suicide construct into *E. chrysanthemi* by electroporation and conjugation were unsuccessful and, therefore, mutagenesis proved impossible for this study. As an alternative, complementation studies were employed to confirm functional analogy of the *ompRZ* locus to the *ompB* locus of *E. coli*. The *ompRZ* clone, pRZ69, was shown to complement an *E. coli ompR-envZ* mutant as determined by the expression of the *ompF-lacZ* transcriptional fusion. Furthermore, the introduction of pRZ69 into the mutant *E. coli* (Δ *ompR-envZ*) strain restored the osmoregulation of the major outer membrane protein genes, *ompC* and *ompF*.

## 5.2 INTRODUCTION

The outer membrane of Gram-negative bacteria serves as a permeability barrier to a large array of molecules. Molecules larger than 600-dalton are excluded by the outer membrane (Decad and Nikaido, 1976) and, therefore, bacteria have had to devise ways of exchanging nutrients and waste products with their environment. This flux between cell and environment takes place through different groups of protein channels, termed porins.

Porins consist of open water-filled channels that allow the influx of small hydrophilic molecules (Nikaido and Nakae, 1979). Porins are generally stable trimeric structures and are highly permeable to small hydrophilic molecules (Hancock, 1987). The outer membrane of *E. coli* contains two major porin proteins, OmpC and OmpF (Nikaido and Vaara, 1985). These porin proteins are regulated in a reciprocal manner in response to various environmental signals such as medium osmolarity (van Alphen and Lugtenberg, 1977; Hall and Silhavy, 1979). The *ompB* locus, which is comprised, of the *ompR* and *envZ* genes, plays a critical role in the regulation of porin expression. In this two-component system, the cytoplasmically-located EnvZ protein, monitors changes in medium osmolarity and transmit the signal by protein phosphorylation to OmpR (Igo *et al.*, 1989), the transcriptional activator of the porin genes. The total amount of OmpF and OmpC protein remains constant in response to surrounding osmolarity, whereas the relative levels of the two proteins fluctuate in a reciprocal manner. Under conditions of low osmolarity, expression of OmpF is favoured, whereas in high osmolarity, OmpC predominates (van Alphen and Lugtenberg, 1977). In addition to osmolarity, environmental stimuli like temperature and pH also affect porin expression. High osmotic pressure, high temperature and acidification of the growth medium have been shown to result in a switch from the expression of the OmpF to the OmpC porin (Mizuno and Mizushima, 1990; Heyde and Portalier, 1988). This switch has been proposed to be the result of changes in the cellular level of the phosphorylated form of OmpR (Mizuno and Mizushima, 1990).

Studies using gene and operon fusions to both OmpF and OmpC revealed that the regulation of porin expression occurs at the transcriptional level (Hall and Silhavy, 1979). In low

osmolarity, OmpR activates transcription of OmpF, resulting in an $OmpF^{+}OmpC^{-}$ ($F^{+}C^{-}$) phenotype. In high osmolarity, OmpR represses transcription at OmpF and activates OmpC transcription, resulting in an $F^{-}C^{+}$ phenotype (Slauch and Silhavy, 1985). Furthermore, genetic approaches involving mutagenesis have also provided clues as to which regions of the sensor and regulator proteins are functionally important in osmoregulation (Tokishita *et al.*, 1991; Nara *et al.*, 1986; Pratt and Silhavy, 1994). These combined approaches provide an effective way of characterising genes or regions of genes that are physiologically significant in osmoregulation. These strategies were employed to aid in the physiological characterisation of the putative *ompB* clone isolated from *E. chrysanthemi*.

Osmoregulation in *E. chrysanthemi* is proposed to involve a two-component system similar to that observed in *E. coli* (Crampton, 1996). Furthermore, porins analogous to the OmpF and OmpC porins of *E. coli* have been isolated from *E. chrysanthemi*. Preliminary studies (this study) identified a regulatory locus, *ompRZ*, in *E. chrysanthemi* that is structurally similar to the *ompB* locus of *E. coli*. To determine functionally similarity between these two loci, either of two approaches could be employed. The first approach involved targeting regions of the *E. chrysanthemi ompRZ* clone for mutagenesis, followed by the phenotypic screening for mutants in osmoregulation. Since the *ompR* and *envZ* genes play critical roles in regulating the outer membrane porins, any mutation(s) in the putative *ompB* clone affecting porin expression could be physiologically linked to the osmoregulatory genes. To mutagenise the chromosomal *ompR* and *envZ* genes, regions of the genes could be cloned into the suicide vector, pGP704 that only replicates in $pir^{+}$ strains (Miller and Mekalanos, 1988). Under these circumstances, the maintenance of pGP704 recombinants in *E. chrysanthemi* ($pir^{-}$ strain) is dependent on the integration of the plasmid into the chromosome. Integration into the chromosome, which involves the homologous recombining of the inserted fragment and the chromosomal gene copy by Campbell-like cross-over, leads to the inactivation of that gene (Niaudet *et al.*, 1982). Preliminary mutagenesis in *E. chrysanthemi* was attempted but was unsuccessful and, therefore, the alternative approach, complementation studies, was employed.

The second approach to determine functional analogy to the *E. coli ompB* locus involved the complementation of mutants in the *ompR* and *envZ* genes of *E. coli*. In this study an *E. coli* mutant harbouring an *ompR-envZ* deletion and an *ompF-lacZ* transcriptional fusion (Karlsson *et al.*, 1991) was used. The β-galactosidase fusion provided an easy assayable means (i.e. by the $Lac^+$ phenotype) of detecting complementation of the *ompR-envZ* deletion by the *ompRZ* clone of *E. chrysanthemi*. This approach proved efficient in the characterisation of the genes involved in osmoregulation.

The aims of this chapter were to show that the *ompRZ* clone of *E. chrysanthemi* was functionally similar to the *ompB* locus of *E. coli* and thereby to confirm that osmoregulation in *E. chrysanthemi* is regulated by a similar two-component system.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used are listed in Table 5.1. The cloning vector used for mutagenesis was the suicide vector, pGP704 (Appendix C). The *E. coli* strain, SY327λpir served as the recipient strain for initial cloning experiments because of its high transformation efficiency. The strain lacked the mobilisable elements needed for conjugation, therefore, the *E. coli* strain, SM10λpir that possessed the necessary trans acting factors for mobilisation, was used for subsequent conjugation and homologous recombination studies.

Pirhonen (personal communication) supplied the *E. coli* mutant strain, TK2040, which was used in complementation studies. To study the osmoregulation of porin proteins, the bacteria were grown in YS (Mildenhall *et al.*, 1981) or YS supplemented with sucrose (Bassford *et al.*, 1977). Ampicillin (Amp) was added to media at 100 µg/ml, kanamycin (Kan) at 50 µg/ml and rifampicin (Rif) at 25 µg/ml. All *E. coli* strains were grown in LB or on LB plates at 37 °C. *E. chrysanthemi* cells were similarly grown in LB or on LB plates but at 30 °C. Long term storage of *E. coli* cells were done in 20% glycerol at –20 °C, while *E. chrysanthemi* cells were maintained in distilled water at room temperature. Recombinant strains were maintained in media with the appropriate antibiotics added.

**Table 5.1** *E. coli* strains and plasmids used in this study

| *E .coli* strain | Genotype | Source/reference |
|---|---|---|
| SY327 λpir | F⁻ *ara*D, Δ(*lac*, *pro*), *arg*E, *rec*A56, rif$^r$, *nal*A, λpir | Miller and Mekalanos, 1988; Kolter *et al.*, 1978 |
| SM10λpir | (*thi*1, *thr*1, *leu*B6, *sup*E44, *ton*A21, *lac*Y1, *rec*A⁻:RP4⁻ 2-Tc::Mu Kan$^r$) | Simon *et al.*, 1983 |
| TK2040 | MH513 (ompR-envZ)7 zhe::Tn10 | Karlsson *et al.*, 1991. |
| Plasmids | Chromosomal genes carried on the plasmid | Source/reference |
| pSK | | Stratagene |
| pGP704 | | Miller and Mekalanos, 1988 |
| pMUT1 | *EnvZ* | This study |
| pRZ69 | *ompR envZ* | This study |
| pDA3 | β-agarase | Schroeder, personal communication |



### 5.3.2 Genetic techniques

Preparation of competent cells by the rubidium chloride treatment (Armitage *et al.*, 1988) and transformation with ligated DNA were done as described by Sambrook *et al.* (1989). All standard cloning techniques were according to Sambrook *et al.* (1989). Restriction endonuclease analyses were performed by using the restriction endonucleases and buffers supplied by Boehringer Mannheim.

Mutagenesis by homologous recombination was done as follows: Primers specific for the N- and C-terminal regions of the *envZ* gene were designed from the derived *E. chrysanthemi* nucleotide sequence, using the Prime component of the GCG package. PCR conditions, using the primer pairs, PP1F (5′ TTGCTGATTGTTACTCTG 3′) and PP1R (5′ TATTCTAGCTGACCTCAACC 3′) and CP1F (5′ CTACAAGTTTCTG AGCCAGC 3′) and CP1R (5′ AATCGCATTGAGGTCAGC 3′) respectively, were optimised for the amplication of the *envZ* fragments from the full-length *ompRZ* clone, pRZ69. The subsequent PCR products, the 700 bp N-terminal fragment and the 600 bp C-terminal fragment, were used in cloning experiments using the suicide vector, pGP704. This was followed by transformation into the *E. coli* strain, SY327λpir, for initial characterisation by restriction endonuclease analyses. The cloned PCR products were subsequently transformed into either electroporation-competent *E. chrysanthemi* cells (see Appendix A for electroporation procedure) or into the *E. coli* strain, SM10λpir, for homologous recombination studies. In recombination studies where conjugation was employed, the transformed *E. coli* strain represented the donor strain and contained the $Kan^r$ antibiotic marker for selection in conjugation studies. The recipient strain was $Rif^r$ *E. chrysanthemi* cells obtained by selecting for $Rif^r$spontaneous mutants (see Appendix A for detailed procedure) as described by (Luria and Delbrück, 1943). Donor and recipient strains were combined in varied ratios and incubated overnight on LB plates at 30 °C, prior to antibiotic selection. Selection of transconjugants was done on media containing both Amp (marker on pGP704) and Rif (marker on *E. chrysanthemi* chromosome).



### 5.3.3 Outer membrane protein extraction and SDS-PAGE analysis

Outer membrane proteins from both *E. coli* and *E. chrysanthemi* were extracted by using the technique of Lohia *et al.* (1984) with modifications (Mizuno, personal communication). The extraction procedure included a sonication step to lyse the cell, after which cells were treated with sodium lauroylsarcosine (see Appendix A for detailed protocol).

Protein concentrations of extracted OMPs were determined by Bradfords assay (Bradford, 1976) with Bovine serum albumin serving as the standard. Protein samples were prepared by adding an equal volume of loading buffer (Sambrook *et al.*, 1989). Denaturation of the protein samples were done in a boiling water bath for 10 minutes before electrophoresis on a 10% denaturing polyacrylamide gel as described by Laemmli (1970). To facilitate the separation of *E. coli* OmpC and OmpF proteins, 4 M urea was added to the separation gel. The gel was stained with Coomasie Brilliant Blue (Sigma) as described by Sambrook *et al.* (1989). Pharmacia low molecular weight standard markers were used to determine protein sizes and consisted of the following: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD) and α-lactalbumin (14.4 kD).

### 5.3.4 Assay of β-galactosidase activity

The enzyme activity was assayed essentially as described by Miller (1972). Cells were grown overnight in appropriate medium at 37 °C, subcultered (1:100) into fresh medium (100 ml) and then grown to mid-log phase at 37 °C. The β-agarase clone (pDA3) that was used as the negative control in the assay was supplied by Schroeder (personal communication). Assays were performed on 1 ml culture with three independent assays being done for each strain. The remaining culture was used for outer membrane protein extractions. The results obtained for the 3 independent β-galactosidase assays were averaged for display as bar graphs.

## 5.4 RESULTS AND DISCUSSION

### 5.4.1 Preliminary mutagenesis of the putative *ompB* clone of *E. chrysanthemi*

To determine functional similarity between the *ompB* loci of *E. coli* and *E. chrysanthemi*, a mutagenesis strategy to confirm the role of the *ompRZ* locus in osmoregulation was devised. The *envZ* gene of the *ompRZ* locus of *E. chrysanthemi* was targeted for mutagenesis and primers specific for the N- and C-terminal regions respectively were designed. PCR amplication yielded the expected 700 bp (N-terminal fragment) and the 600 bp (C-terminal fragment) PCR products. Blunt-end cloning of the PCR products into the *EcoR*V site of pGP704 was only successful for the C-terminal fragment. The resulting suicide construct, pMUT1, was used further in mutagenic studies in attempts to inactivate the *envZ* gene in *E. chrysanthemi*. Two methods, electroporation and conjugation, were used in attempts to introduce the suicide construct into *E. chrysanthemi*.

**Table 5.2** Electroporation of *E. chrysanthemi* with the suicide vector, pGP704 and its derivative, pMUT1.

| | Allele on plasmid | Time constant | Number of transformants |
|---|---|---|---|
| pSK[a] | - | 2.8 | 0 |
| pGP704 | - | 2.5 | 0 |
| pMUT1 | *envZ*$^+$ | 2.5 | 0 |

[a]pSK served as the positive control in this experiment

The electroporation procedure was not successful for introducing the suicide construct into *E. chrysanthemi* (Table 5.2). No transformants were obtained for both pSK and the pGP704 construct, as observed by the lack of growth on LA$^{Amp}$ plates. The pGP704 control (vector only) served as the negative control and, therefore, no growth was expected, as the plasmid is unable to be maintained in the pir$^-$ *E. chrysanthemi* strain. Cell viability for *E. chrysanthemi* was therefore tested to rule out the possibility of the cells being non-viable prior to

electroporation. Growth of competent *E. chrysanthemi* on LA plates was noted to assess cell viability. The presence of growth on LA plates confirmed that the cells were viable and that the lack of growth on $LA^{Amp}$ plates was the result of failure to introduce the recombinant plasmids into *E. chrysanthemi*. Electroporation conditions were altered by the use of different field strengths, however no transformants were obtained. As a consequence an alternative method of conjugation was employed.

The conjugation strategy necessitated the selection of a $Rif^r$ resistant strain of *E. chrysanthemi* to serve as the recipient strain. The selection for $Rif^r$ strains was performed on Rif gradient plate with a concentration range of 0-25 µg/ml. Further selection on Rif plates resulted in the isolation of a strain that was resistant at 50 µg/ml. Outer membrane protein analyses of the $Rif^r$ strain was done and comparisons with the wild-type $Rif^s$ strain were made (Figure 5.1). This was done to confirm that the rifampicin selection did not affect the outer membrane profile. This had to be established prior to mutagenesis of the *ompB* locus, to ensure that any changes identified in the outer membrane protein profile post-mutagenesis, could be related directly to mutations introduced into the osmoregulatory genes. No difference in the outer membrane profiles was detected for the $Rif^r$ strain compared to the $Rif^s$ strain (Figure 5.1). This confirmation allowed the next step, mutagenesis, to be undertaken. The first step in mutagenesis was to introduce the suicide construct, via conjugation, into *E. chrysanthemi*. Rifampicin resistant *E. chrysanthemi* (recipient) and kanamycin resistant *E. coli*, SM10λpir (donor) cells were allowed to conjugate before selection for transconjugants. Selection on rif/amp plates yielded no transconjugants, even after varying the donor/recipient ratio. The absence of transconjugants could be the result of the following:

(i) No transfer of the suicide construct to *E. chrysanthemi* during conjugation,

(ii) No integration of the suicide construct into the chromosome post-conjugaton, or

(iii) The mutation in the gene is lethal to the bacterium resulting in non-viability.

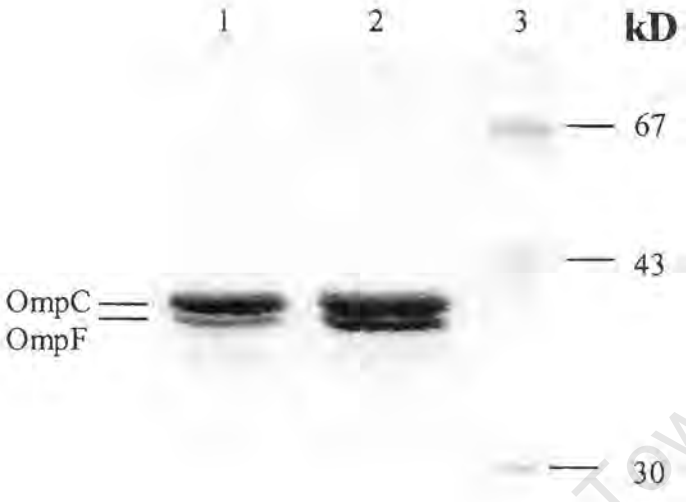



**Figure 5.1.** Osmoregulatory profile analysis. Outer membrane proteins of the wild-type *E. chrysanthemi* strain (lane 1) was separated by SDS-PAGE and compared to the outer membrane protein pattern of the Rif$^r$ mutant strain (lane 2) to confirm that no differences in the osmoregulatory profiles existed. Lane 3, low molecular weight protein marker with sizes given in kilodaltons (kD). OmpC and OmpF indicate the positions of the OmpC and OmpF proteins, respectively.



No evidence exists to support the hypothesis that the mutation in the gene is a lethal mutation. Studies have demonstrated that mutations in *envZ* could be introduced without resulting in non-viability (Hsing *et al.*, 1998; Park and Inouye, 1997). The C-terminal region has been well characterised by mutational analyses, and has been proven to be important in regulating the kinase and phophatase activities of *envZ* (Hsing *et al.*, 1998). No studies have reported on lethal mutations in *envZ*, particularly in the C-terminal region. Thus, it seems improbable that the absence of transconjugants is the result of a lethal mutation in *envZ*. Since integration of the construct into the chromosome is dependent on sequence identity between the construct and the chromosomal gene (Niaudet et al., 1982), it seems unlikely that there would be a lack

of integration, considering the high degree of sequence identity over the 600 bp region. Thus, it seems that the absence of transconjugants is the result of inefficient conjugation. The introduction of the construct into *E. chrysanthemi* thus proved to be the limiting step in the successful creation of a mutant in the osmoregulatory genes. The efficiency of introducing the suicide construct into *E. chrysanthemi* has to be improved, before mutagenesis can be successfull. This optimisation proved to be too time-consuming for this study and thus an alternative means of confirming functional similarity between the *ompRZ* locus of *E. chrysanthemi* and the *ompB* locus of *E. coli*, was investigated.

### 5.4.2 Complementation of the *E. coli ompR-envZ* mutant by pRZ69

In *E. coli*, the relative amounts of OmpC and OmpF, in response to medium osmolarity is mediated by the *ompB* locus. OmpC expression is favoured under conditions of high osmolarity (e.g. medium supplemented with 20% sucrose), while OmpF expression predominates at low osmolarity (Bassford *et al.*, 1977; Hall and Silhavy 1979, 1981). Therefore, it was of interest to determine whether the *E. chrysanthemi ompRZ* clone (pRZ69) would also restore the osmoregulatory properties to the *E. coli ompR-envZ* deletion mutant.

The results of these analyses are summarised in Table 5.3 and indicate that the putative *ompB* clone from *E. chrysanthemi* is able to complement the *E. coli* deletion mutant. Under conditions of low (0% sucrose) and high (20% sucrose) osmolarity, the mutant strain, TK2040, exhibited basal levels of β-galactosidase activity, as a result of the lack of expression of the *ompF-lacZ* transcriptional fusion. This lack of expression is the direct result of the deletion in the osmoregulatory genes, *ompR* and *envZ*, that regulate the OmpF and OmpC porins. A similar basal level of β-galactosidase activity was observed for the β-agarase clone (pDA3) used as a negative control in this study. This result demonstrates that the neither the β-agarase nor any of the pEcoR251-encoding sequence is able to regulate the expression of the transcriptional fusion (*ompF-lacZ*$^+$).

The introduction of the putative *ompB* clone (pRZ69) into the mutant strain resulted in a 200-300 fold increase in β-galactosidase activity (see Table 5.3 and Figure 5.2). This significant increase in β-galactosidase activity (OmpF expression) demonstrates that the clone pRZ69 is able to restore the osmoregulation of the porin proteins, OmpF and OmpC. No significant difference in β-galactosidase activity was noted (Figure 5.2) for cells grown in high osmolarity compared to cells grown under conditions of low osmolarity. Since a significant decrease in OmpF expression is expected under conditions of high osmolarity it appears that some unknown factor is interfering with the regulation of the porin genes. This has to be confirmed experimentally and requires further investigation.

**Table 5.3** Complementation of an *E. coli ompR-envZ* mutant by the *ompRZ* clone of *E. chrysanthemi*.

| Strain | Chromosomal *ompR-envZ* allele | Porin gene fusion | Gene(s) carried on plasmid | β-galactosidase activity[a] | |
|---|---|---|---|---|---|
| | | | | -sucrose | +sucrose |
| TK2040 | Δ(*ompR-envZ*)7 | (*ompF-lacZ*$^+$) | - | 2.5 | 1.4 |
| TK2040/pDA3 | Δ(*ompR-envZ*)7 | (*ompF-lacZ*$^+$) | β-agarase | 2.8 | 2.3 |
| TK2040/pRZ69 | Δ(*ompR-envZ*)7 | (*ompF-lacZ*$^+$) | *ompRZ*[b] | 469.9 | 436.6 |

[a] The cells were grown in YS or YS supplemented with 20% sucrose and the appropriate antibiotics at 37 °C to mid-exponential phase prior to ß-galactosidase assay. The assay was done according to Miller (1972) and the activity is expressed in Miller units.

[b] *ompRZ* are the *ompR* and *envZ* homologues isolated from *E. chrysanthemi*.

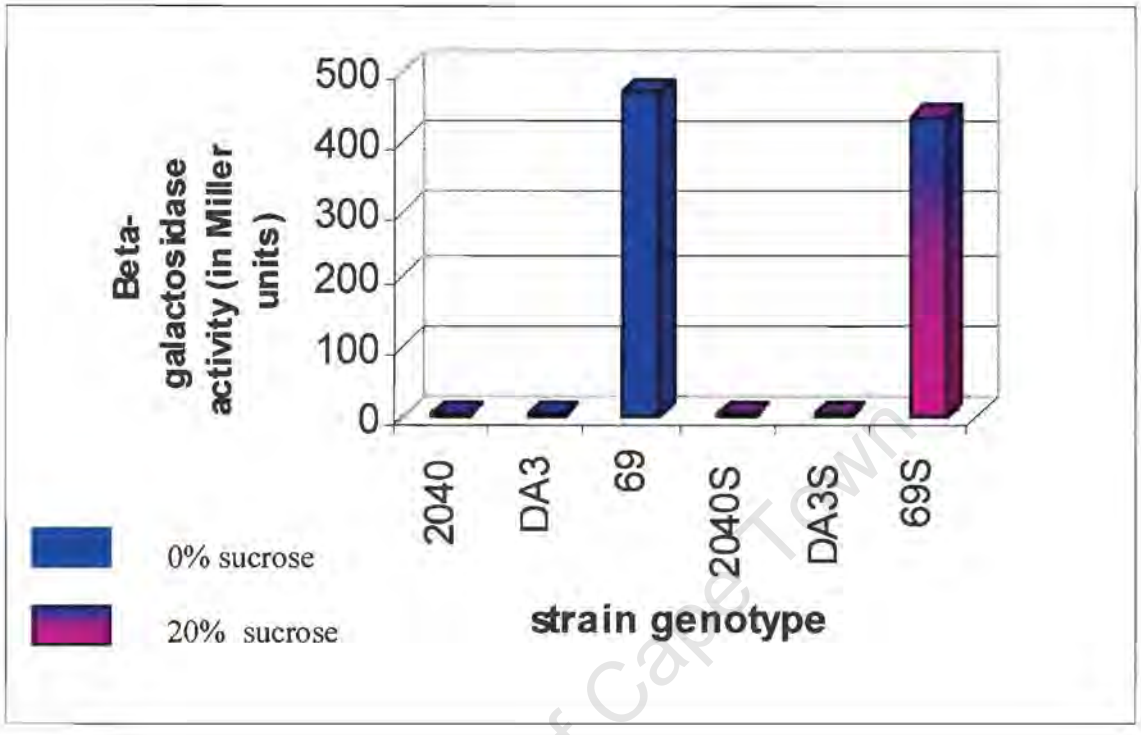



**Figure 5.2.** Complementation of an *E. coli ompR-envZ* mutant by the *ompRZ* clone of *E. chrysanthemi*. Complementation was determined by the transcription of *ompF*, as monitored through an *ompF-lacZ*$^{+}$ fusion. ß-galactosidase assays were performed with cells grown under conditions of either high (20% sucrose) or low (0% sucrose) osmolarity at log phase. 2040, represents the mutant strain grown in low osmolarity, whereas 2040S, represents the mutant strain grown under high osmolarity. 69, represents the mutant strain complemented with the clone, pRZ69 (under low osmolarity) and 69S, the complemented mutant strain under high osmolarity. DA3 represents the mutant strain with the β-agarase clone introduced, grown under low osmolarity, while DA3S is the same strain grown under high osmolarity.

To further investigate the restoration of the osmoregulatory properties to an *E. coli ompR-envZ* mutant, outer membrane protein analysis was done (Figure 5.3). Outer membrane proteins were extracted from *E. coli* strain K12 grown in low (0% sucrose) and high osmolarity (20% sucrose) and the observed patterns on SDS-PAGE gels, served as the wild-type osmoregulatory profiles (Figure 5.3, lanes 1 and 2). The mutant strain, TK2040 (lane 3 and 4), as well as the complemented mutant (lane 5 and 6) strain (TK2040 with the *ompRZ* clone, pRZ69) was similarly subjected to outer membrane profile analyses. Under conditions of low osmolarity (lane 1), OmpF expression in the K12 strain was favoured over OmpC expression, which is the expected wild-type profile. However, the expression of OmpF and OmpC under conditions of high osmolarity (lane 2) was equivalent and did not exhibit the expected reciprocal regulation, where OmpC is favoured. This lack of reciprocal regulation cannot be satisfactorily explained, however the OMP profile does show that some form of regulation is taking place, since the pattern for low osmolarity (lane 1) differs from that of high osmolarity (lane 2).

The mutant strain, TK2040 (lane 3) exhibited an OmpF-deficient phenotype. In addition, when comparing lanes 3 (low osmolarity) and 4 (high osmolarity), an OmpC constitutive phenotype is noted. This OmpF-deficient/OmpC constitutive phenotype is a result of the mutation in the *ompR-envZ* regulatory locus (Pirhonen *et al.*, personal communication). When the putative *ompB* clone (pRZ69) is introduced into the mutant strain the OmpF-deficient phenotype is restored (lanes 5 and 6). This corroborates the results obtained for the β-galactosidase assays, showing an increase in OmpF expression for the complemented mutant compared to the mutant strain. No significant difference in OmpF expression (as determined by OmpF protein levels) was noted under conditions of high osmolarity compared to low osmolarity. This confirms the result obtained for the β-galactosidase assays. The osmoregulatory profile of the complemented mutant under high osmolarity, was identical to the profile for the selected wild-type strain under the same conditions (compare lanes 2 and 6). This confirms that the introduction of the clone, pRZ69 into the mutant strain, restored the osmoregulatory properties.

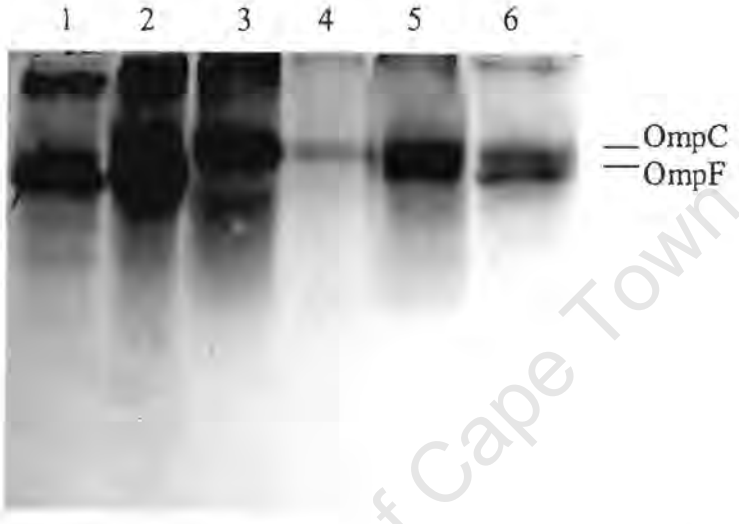





**Figure 5.3.** Restoration of porin expression by the *ompRZ* clone of *E. chrysanthemi*. The osmoregulatory profiles of an *E. coli* K12 strain (wild-type profile), mutant strain, TK2040 and the pRZ69-complemented mutant strain were determined under conditions of low osmolarity (0% sucrose) and high osmolarity (20% sucrose). Lanes 1 and 2, OMPs of *E. coli* strain grown in 0% and 20% sucrose, respectively (0 and 20 in the figure). Lane 3 and 4, mutant *E. coli* strain TK2040, in 0% and 20% sucrose, respectively and lane 5 and 6, mutant strain TK2040 complemented with the clone, pRZ69, in 0% and 20% sucrose, respectivley. C and F denotes the positions of the OmpC and OmpF proteins, respectively.

In summary, these results strongly suggest a physiological role for the regulatory locus isolated from *E. chrysanthemi*, in osmoregulation. Furthermore, based on both structural and functional studies, it can be concluded that the regulatory locus isolated is an *ompB* osmoregulatory locus. Thus, the results of this chapter confirm that the *ompRZ* locus of *E. chrysanthemi* is functionally similar to the *ompB* locus of *E. coli* and that osmoregulation in *E. chrysanthemi* is regulated by a two-component system.

# CHAPTER 6

# GENERAL DISCUSSION

## 6.1 Discussion and Conclusions

Many of the interactions of bacteria with their environment are controlled by two-component regulatory systems. One of the most extensively studied sensory systems, is the osmoregulatory system of *Escherichia coli* that controls the expression of the porins, OmpF and OmpC (Stock *et al.*, 1989; Csonka, 1989; Csonka and Hanson, 1991; Parkinson and Kofoid, 1992; Parkinson, 1993). In response to fluctuations in certain environmental parameters, *E. coli* initiates a complex process that leads to the appropriate adaptive response. The *ompB* locus of *E. coli*, that is physiologically important in the adaptation of the bacterium to osmolarity, is characterised by two proteins, *ompR* and *envZ* (Hall and Silhavy, 1979, 1981a). EnvZ is a cytoplasmically-located sensor protein that monitors external medium osmolarity and relays the information to its cognate response regulator, OmpR, which in turn, regulates the porins, OmpC and OmpF (Hall and Silhavy, 1981b). Regulation of the porins occurs in a reciprocal manner, where OmpF predominates over OmpC under low osmolarity and vice versa under high osmolarity (van Alphen and Lugtenberg, 1977).

A similar locus has been identified in many Gram-negative bacteria, including *Salmonella typhimurium*, *Yersinia enterocolitica* and many others. Studies have also identified a regulatory locus, *ompRS*, controlling porin expression in *Erwinia carotovora subsp carotovora* (Karlsson *et al.*, 1991). Thus, a similar system was sought in a related soft-rot *Erwinia* species, *Erwinia chrysanthemi*. *E. chrysanthemi* is a pathogen of a large variety of economically important crops (Perombelon and Kelman, 1980). The underlying mechanisms of pathogenicity are complex and probably involve several regulatory systems. The production of a variety of extracellular plant cell wall-degrading enzymes appears to play the most important role in determining pathogenicity of these bacteria (Collmer and Keen, 1986; Kotoujansky, 1987). However, the role of other cellular components in pathogenicity of *Erwinia* species is less well characterised. A lack of certain outer membrane proteins involved in iron acquisition has been shown to lead to reduced virulence in *Erwinia chrysanthemi* (Expert and Toussaint, 1985). Outer membrane porins and *ompR* have also been shown to be required for virulence in the animal pathogen, *S. typhimurium* (Dorman *et al.*, 1989) but their role in determining virulence in plant pathogens has not been that extensively studied. *E. chrysanthemi* is closely related to enteric bacteria such as *E. coli* and *S. typhimurium* (Leary and Fullbright, 1982) and has

proteins analogous to the *E. coli* outer membrane porins (Crampton, 1996). Crampton (1996) also demonstrated that the outer membrane porins of *E. chrysanthemi* was regulated similarly to the porins of *E. coli* under conditions of fluctuating medium osmolarity. Thus, compelling evidence exists that *Erwinia chrysanthemi* would benefit from an osmoregulatory system such as the *ompB* locus of *E. coli*.

To confirm the presence of an *ompB* locus in *E. chrysanthemi*, primers were designed to conserved regions within the *ompR* gene and used in PCR analyses of both *E. coli* and *E. chrysanthemi* genomic DNA. PCR amplication of *E. chrysanthemi* genomic DNA yielded no PCR product, therefore, the existence of an ompB locus in *E. chrysanthemi* had to be determined by an alternative strategy. PCR amplication of *E. coli* genomic DNA yielded the expected 631 bp product. This fragment was cloned into the vector, pBluescriptSk, end-sequenced to confirm its autheniticity and used to generate a DIG-labelled *ompR* probe for Southern hybridisation analyses. Southern hybridisation analyses of *E. chrysanthemi* genomic DNA confirmed the presence of an *ompR* homologue in *E. chrysanthemi*. A similar strategy was followed to generate an *E. coli envZ* probe to confirm the presence of an *envZ* homologue in *E. chrysanthemi* (Crampton, 1996). These results provided the genetic evidence that an *ompB* locus was present and, therefore, an *E. chrysanthemi* genomic library was constructed in order that the osmoregulatory genes, *envZ* and *ompR*, could be isolated.



Approximately 3704 clones were obtained for the *E. chrysanthemi* genomic library and these were subdivided into fourteen clone pools. Screening of the library by Southern hybridisation analyses, localised putative *ompR* clones to clone pool one. Further characterisation by Southern hybridisation, resulted in the isolation of the candidate *ompR* clone, pRZ69. Genetic characterisation of pRZ69 confirmed its origins from *E. chrysanthemi* and established, by restriction endonuclease analyses, that differences between the *ompB* locus of *E. coli* and the putative *ompB* locus of *E. chrysanthemi* (*ompRZ*), existed. Southern hybridisation analyses were employed to localise the *ompR* and *envZ* homologues on the plasmid. Fragments containing these homologues were subcloned into the vector, pBluescriptSk to facilitate sequence analyses. Nucleotide sequence analyses revealed two open reading frames encoded as an operon, proposed to be the *ompR* and *envZ* genes, respectively. The regulatory locus, *ompRZ*, was structurally similar to the *ompB* locus of *E. coli*, with *ompR* being the promoter proximal gene. Multiple sequence

alignment of the *ompR* and *envZ* homologues of *E. chrysanthemi* with other GenBank *ompB* sequences identified a high degree of sequence identity among the sequences. Despite sequence variation at the nucleotide level, a higher degree of sequence conservation at the protein level was observed. This can be attributed to codon degeneracy as a result of "wobble" at the third base pair (Crick, 1966) allowing for the same amino acid to be specified by more than one codon. Furthermore, the higher degree of sequence conservation at the protein level underlines the importance of structure-function relationship (i.e. the structure of the protein, which is determined by peptide sequence, is important for the functioning of the protein in certain instances). In general, the OmpR protein was observed to be generally more conserved among species than the EnvZ protein. A high degree of sequence conservation was noted in the C-terminal region of the EnvZ peptide sequences, which is the region found to be commonly conserved among sensor proteins (Kofoid and Parkinson, 1988; Stock *et al.*, 1990). This region represents the enzymatic domain and contains the invariant histidine residue at position 243, the site of autophosphorylation (Forst *et al.*, 1989; Igo and Silhavy, 1988). The invariant histidine site was identified in the putative EnvZ peptide sequence of *E. chrysanthemi*, confirming that a sensor protein had been cloned. Furthermore, the transmembrane regions identified by hydropathy analysis also provided strong evidence that a membrane-spanning protein that could act as a sensor was cloned from *E. chrysanthemi*. The conserved aspartic acid residue at position 55 (phosphorylation site) and the lysine residue at position 109 were also identified in the putative OmpR peptide sequence of *E. chrysanthemi*. These conserved residues are characteristic of response regulator proteins and therefore it can be concluded that a regulator protein was cloned from *E. chrysanthemi*. The data presented here provides genetic evidence that both a sensor and regulator protein were cloned from *E. chrysanthemi*. DNA hybridisation and nucleotide sequence analyses support the evidence that a two-component osmoregulatory system (*ompR-envZ*) was cloned. The next step was to prove functional similarity between the *ompRZ* locus of *E. chrysanthemi* and the *ompB* locus of *E. coli*.

Several lines of evidence exist to support the functional similarity between the two loci. Complementation studies, using an *E. coli ompR-envZ* mutant, demonstrated that the introduction of the *ompRZ* clone, pRZ69 (putative *ompB* clone of *E. chrysanthemi*) complemented the mutation in the osmoregulatory genes. Furthermore, the OmpF-deficient phenotype was restored with the introduction of the clone, pRZ69. Although the

complemented mutant did not show the expected reciprocal regulation under high osmolarity as described for *E. coli*, it was consistent with the osmoregulatory profile of the selected wild-type strain, under the same conditions. Studies showed that some form of regulation occurred, since the outer membrane profile under low osmolarity differed from the profile under high osmolarity. These results, therefore, suggest that some unknown common factor played a role in affecting the regulation pattern of both the selected wild-type and the complemented strain, under conditions of high osmolarity. Despite these experimental shortcomings the results are still conclusive in showing functional similarity between the *ompRZ* locus of *E. chrysanthemi* and the *ompB* locus of *E. coli*. Preliminary mutagenesis was attempted to inactivate the *ompRZ* locus in *E. chrysanthemi*. The initial aim was to identify whether mutations in the *ompRZ* locus affects the bacterium's ability to respond to fluctuating medium osmolarity, thus investigating the role of the *ompRZ* locus in osmoregulation. Mutagenesis was unsuccessful because of the lack of efficient transfer of the suicide construct into *E. chrysanthemi*. Therefore, future research should entail the refining of the mutagenesis strategy for *E. chrysanthemi*.

In conclusion, both structural and physiological evidence supports the findings that an *ompB* locus controlling osmoregulation in *E. chrysanthemi* is present. These findings therefore suggest that osmoregulation in *E. chrysanthemi* is mediated by a similar two-component system as in *E. coli*.

## 6.2 FUTURE RESEARCH PERSPECTIVES

The use of two-component systems to regulate virulence has proved to be an important aspect of pathogenesis. In this study, the pathogenesis of *E. chrysanthemi* with respect to osmolarity was of interest. The pathogenicity genes of *E. chrysanthemi* was observed to be affected by changes in medium osmolarity (Mildenhall and Prior, 1983; Hugouvieux-Cotte-Pattat *et al.*, 1992), suggesting a link between osmoregulation and pathogenicity. The isolation of the *ompRZ* locus of *E. chrysanthemi* provides the basis for further investigation into the role that osmoregulation plays in pathogenicity. The genetic characterisation of the *ompRZ* locus allows for specific regions to be targeted for homologous recombination studies or other mutagenic strategies. The information gained from the preliminary mutagenic studies will prove invaluable in further attempts at mutagenesis. If mutagenesis proves successful, pathogenicity tests can be performed to investigate if virulence is

attentuated. In this way the putative link between pathogenicity and osmoregulation can be elucidated.

Understanding the regulatory mechanisms used by pathogenic microbes may provide useful information for the development of effective control measures. This study may assist in unravelling the complexity of the regulatory mechanisms used by *E. chrysanthemi* as a pathogen.

# APPENDIX A

## Methods

# APPENDIX A

## A1. Preparation of bacterial genomic DNA

Genomic DNA was prepared according to the method of Ausubel *et al.* (1989).

1. A 100 ml culture was grown to saturation at 30 °C with shaking.
2. Bacterial cells were collected by centrifuging samples for 5 min at 5000 rpm.
3. Cells were resuspended in 9.5 ml TE buffer. Cell lysis was performed by adding 500 µl of 10% SDS and 50 µl of 20 mg/ml proteinase K and incubating the sample for 1 hr at 37 °C.
4. 1.8 ml of NaCl was added and the sample was mixed thoroughly before the addition of 1.5 ml CTAB/NaCl solution.
5. After an incubation of 20 min at 65 °C, an equal volume of chloroform/isoamyl alcohol was added, and the sample was centrifuged at 7000 rpm for 5 min to separate the phases.
6. The aqueous supernatant was transferred to a fresh tube using a wide-bored pipette, and the DNA was precipitated out of solution with 0.6 volumes of isopropanol.
7. The DNA was pelleted at 10 000 rpm for 10 min, and washed with 70% ethanol.
8. The pellet was resuspended in 1 ml TE, pH8 and the concentration of DNA was determined spectrophotometrically by measuring the $A_{260}$ between 210 nm and 310 nm of a 1 in a 100 dilution. The relationship of $A_{260}$ = 1 for 50 µg/ml DNA was used.

## A.2 Large scale isolation of plasmid DNA

DNA isolations were performed according to Ish-Horowicz and Burke (1981).

1. A 200 ml culture was grown overnight with shaking at 37 °C in the presence of 100 µl/mg ampicillin.
2. The cells were harvested in a GSA centrifuge tube at 5000 rpm for 5 min.
3. The pellet was resuspended in 4 ml of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0), transferred to a SS34 centrifuge tube and left for 5 min at room temperature.

4. 8 ml of Solution III (0.2 M NaOH, 1% (w/v) SDS) was added. The tube was rolled gently to mix the solutions and was incubated on ice for 5 min.
5. 6 ml of Solution III (5M potassium acetate, pH 4.8) was added, the tube was mixed well and incubated for 5 min on ice.
6. The debris (lysed cells and proteins) was spun down at 15 000 rpm for 15 min and the supernatant decanted into a new SS34 tube.
7. An equal volume of isopropanol was added and the tube was centrifuged at 15 000 rpm for 15 min.
8. The pellet was washed with 70% ethanol and the tube was inverted to drain off the excess ethanol, before resuspending the pellet in 4.2 ml Tris-EDTA (TE) buffer, pH 8.0.

10 4.4 g of cesium chloride (CsCl) was added and dissolved and 400 µl of ethidium bromide (EtBr) (10 mg/ml) was added.

11. The tube was centrifuged at 15 000 rpm for 15 min to precipitate any remaining protein debris.
12. The refractive index of the solution was adjusted to 1.394.
13. The sample was sealed in a Beckman Quickseal ultracentrifuge tube (5 ml) and centrifuged for a minimum of 6 h at 55 000 rpm in a Beckman Vti 65 rotor at 15 °C.
14. The plasmid band was extracted in the smallest possible volume from the side of the tube using a 1 ml syringe and a 1.2 mm gauge needle under long wavelength UV light (350 nm).
15. The EtBr was extracted at least three times using equal volumes of salt saturated isopropanol.
16. Two volumes of water were added to the DNA solution. One volume of isopropanol was added to this, and DNA was pelleted at 13 000 rpm in a microfuge for 10 minutes at room temperature.
17. The pellet was washed with 1 ml of 70% ethanol at 12 000 rpm for 10 min.
18. The DNA was resuspended in 200 µl TE, pH 8 and the concentration of DNA was determined spectrophotometrically as described in A1.

**OR**

**Large scale isolation of plasmid DNA (Nucleobond kit)**

The procedure as outlined by Machery - Nagel was followed.

1. Cells were grown in 100 ml LB (with selection) at 37 °C, O/N.
2. The cells were harvested in a GSA centrifuge tube at 5000 rpm for 5 min.
3. The pellet was resuspended in 4 ml of buffer S1.
4. Buffer S2 (4ml) was added and the contents of the tube were mixed gently at room temperature for 5 min, to disrupt the cells.
5. Buffer S3 (4 ml) was added, the tube was mixed thoroughly and incubated on ice for 5 - 10 min.
6. Centrifugation at 15000 rpm for 40 min was done to pellet the cell and protein debris.
7. The nucleobond cartridge was equilibrated with 2 ml of buffer, N2 and the supernatant from step 6, was loaded onto the AX100 column.
8. After allowing the supernatant to flow through the column, 2X 4 ml wash steps were done, using buffer, N3.
9. The plasmid DNA was eluted from the column with 2 ml of buffer, N5.
10. The plasmid DNA was precipitated with 0.7 volumes of isopropanol and centrifugation at high speed (13 000 rpm, 5 min).
11. The resulting pellet was washed with 70% ethanol, briefly dried and resuspended in 50 - 100 µl of TE buffer.

**A3. Small scale isolation of plasmid DNA (miniprep)**
The method is a scaled down version of the above maxiprep method.

1. LB broth (700 µl) containing 100 µg/ml ampicillin was pipetted into Eppendorf microcentrifuge tubes which were then inoculated and grown at 37 °C with vigorous shaking overnight.
2. Cells were harvested by centrifugation at 13 00 rpm for 1 min.
3. Solution I (150 µl) was added and the tube was vortexed until the pellet was resuspended.
4. Solution II (300 µl) was added, the tube inverted and incubated for 5 min on ice.
5. Solution III (225 µl) was added and the tube was inverted gently a few times.
6. After 5 min on ice, the cellular debris was collected by centrifugation at 10 000g for 5 min.
7. The supernatant was removed to a new Eppendorf tube. One volume of isopropanol was added, mixed and spun at 13 000 rpm for 15 min.

8. The DNA pellet was washed with 70% ethanol, and resuspended in TE, pH 8 containing 10 µg/ml ribonuclease A (RNAse).

## A4. Restriction endonuclease digestion of DNA

Restriction digests were performed as outlined by Sambrook *et al.* (1989). Restriction digests of miniprepped DNA typically contained 10 µl of DNA, 1 unit of endonuclease and 2 µl of the appropriate restriction endonuclease buffer in a total volume of 20 µl. Restriction digests of maxipreped DNA were performed in 20 µl and contained 1 unit of endonuclease for every microgram of plasmid and 2 µl of the appropriate restriction endonuclease buffer. All digests were left at 37 °C for 2 hr unless stated otherwise by the manufacturer.



## A5. Construction of a gene-library

### . A5.1 *Sau*IIIA digestion

1. Set up a pilot *Sau*IIIA digestion study to estimate the units of enzyme required to obtain DNA fragment sizes in the range of 10 kb (see Figure A1).
2. For the pilot study, prepare a stock tube containing the following:

   10 µl of genomic DNA (5 µg)

   10 µl of Buffer H (Boehringer Mannheim, high salt buffer)

   80 µl of distilled water
3. Before *Sau*IIIA digestion, divide contents of the stock tube into 5 tubes as follows:

   Tube 1 - 30 µl of stock tube

   Tube 2 - 20 µl

   Tube 3 - 20 µl

   Tube 4 - 20 µl

   Tube 5 – 10 µl
4. Place on ice.
5. Add 5 U of *Sau*IIIA to tube 1and dilute serially (10 µl of tube1 to tube 2, etc).
6. Incubate tubes at 37 °C for 15 min.
7. Estimate the amount of enzyme units required to obtain a DNA range of 5 - 10 kb, by electrophoresising an aliquot of the digested samples on an agarose gel (see Figure A1).

8. Scale up the digestion, using 100 µg of genomic DNA and the optimal units of enzyme (as determined above) for 5 – 10 kb DNA range.

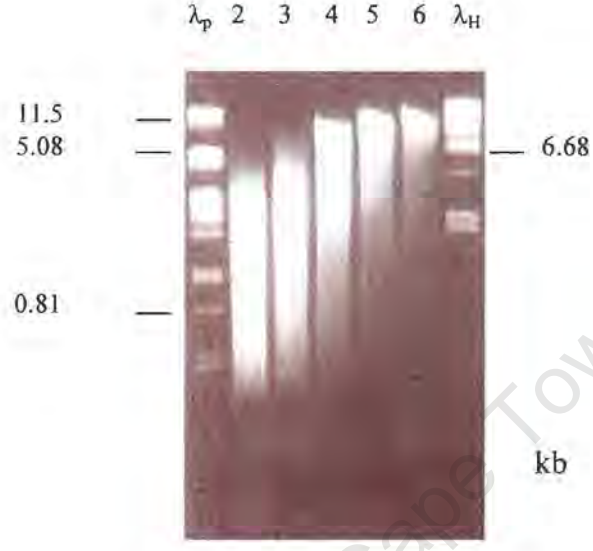



**Figure A1**. Pilot *Sau*IIIA digestion of genomic DNA to estimate the optimal units of enzyme to use to obtain a DNA range of 5 – 10 kb. Lanes 2 – 4, represents over-digestion and lane 6, under-digestion of genomic DNA. Lane 5, optimal units of enzyme required to obtain a 5 – 10 kb DNA range. $\lambda_p$- lambda *Pst*I and $\lambda_H$- lambda *Hind*III markers



### A5.2 Sucrose gradient fractionation

1. Load 1 ml of the *Sau*IIIA-digested DNA sample onto a sucrose gradient (10% -40%) and centrifuge in a *Swi* 27, swinging bucket rotor at 23 000 rpm for 16 – 21 h.
2. Fractionate 500 µl aliquots of the sucrose gradient sample into sterile 1.5 ml eppendorfs, by using the fractionation pump.
3. Take 20 µl aliquots of every seventh sucrose fraction tube, dilute in distilled water, and electrophorese on a 0.8% agarose gel to determine the fraction(s) containing DNA in the10 kb range.
4. Precipitate the DNA of these fractions by adding one tenth of the volume of 3 M sodium acetate, two volumes of ethanol and placing the tubes at – 20 °C, overnight.

5. Resuspend the precipitated DNA in distilled water and determine DNA concentration spectrophotometrically
6. Digest 10 µg of the suicide vector, pEcoR251, with the enzyme, *Bgl*II and set up ligations using the digested vector and the *Sau*IIIA-digested DNA fragments.

### A6. DNA ligation reactions

Ligation reactions were allowed to proceed overnight at 16 °C. Ligation reaction volumes were usually 20 µl containing 2 µl of ligation buffer with ATP (Boehringer Mannheim) and 1 unit of T4 ligase.

### A7. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using horizontal submerged gels (Sambrook *et al.* 1989). Agarose was dissolved in either Tris-acetate EDTA (TAE) or Tris-borate EDTA (TBE) buffer to a final concentration of 0.7, 0.8 or 1%. Electrophoresis loading buffer, containing the dye bromophenol blue, was added to the DNA samples (one sixth the final volume) before they were loaded into the wells of agarose gels. Long gels were run at 100 V constant voltage for about 5 h or overnight at 30 V. Minigels were run at 100 V constant voltage for 2 h. Gels contained 5µl of EtBr (10 mg/ml stock) in 100 ml of agarose so that DNA bands could be visualised using a 264 nm transilluminator. If DNA fragments were to be isolated from the agarose gel for subsequent ligation reactions, the 310 nm transilluminator was used for only a few seconds. Gel photography was carried out using a Polaroid CU-5 Land camera fitted with a red filter. Polaroid type 667 film (ASA-3000) was used with an exposure time of 1-2 sec at f4.7.

### A8. Purification of DNA fragments

The Geneclean$^{R}$ Kit (Bio 101) was used according to the manufacturer's instructions to isolate DNA fragments from agarose gel slices.

1. The appropriate agarose gel slice (0.8% agarose) was cut out of the gel under long wave UV light (310 nm).
2. 2.5 volumes of saturated sodium iodide solution were added to the gel slice. The

tube was left at 50 °C for 5 min.

3. Glassmilk (5 µl) was added and the solution was mixed well and left on ice for 5 min.
4. The sample was spun for 5 s to pellet the glassmilk and the supernatant was discarded.
5. The pellet was washed three times with 500 µl ice cold NEW buffer.
6. The solution was spun as before. The DNA was eluted from the glassmilk by dissolving the pellet in 10 µl TE, pH8.

**A9. Alkali DNA blotting**

1. The DNA fragments to be blotted were separated by 0.8% agarose gel electrophoresis in TBE buffer.
2. The DNA was depurinated by soaking the gel in 0.25M hydrochloric acid (HCl) for 15 min with gentle shaking. The gel was briefly rinsed in distilled water.
3. A capillary transfer system was set up. A Whatmann 3MM filter wick was wetted with 0.4M NaOH and placed over a glass bridge so that the wick ends were touching the bottom of a reservoir tank containing 0.4M NaOH. The gel was placed on the wick, on top of which was placed a wetted nylon Hybond $N^+$ membrane and three sheets of Whatmann 3MM filter paper. All air bubbles were removed before placing a stack of absorbent pads ten centimetres in height on top of the membrane. A one kilogram weight was used to compress the pads. DNA transfer from the gel to the nylon membrane took place via capillary action for 20 h with periodic replacement of the absorbent pads.
4. The membrane was dried at 37 °C for 1 h. The membrane was then ready for prehybridisation.



**A10. Southern hybridisation of DNA to a DIG-labelled probe**

Prehybridisation, hybridisation and detection procedures were carried out as according to the instructions of the manufacturer of the Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim)

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**A10.1 Hybridisation**

1. The membrane was allowed to prehybridise for a minimum of 4 h in prehybridisation buffer in a plastic container at 65 °C with gentle shaking.
2. The DIG-labelled DNA probe (25 ng DNA/ml buffer) was heated in a boiling water bath for 10 min to denature the DNA, and then immediately chilled on ice.

3. The membrane was placed in a hybridisation bag, which had been sealed on three sides. Hybridisation buffer (20 ml) was added and the bag was sealed making sure that all the air bubbles had been removed. Hybridisation was allowed to proceed overnight at 65 °C.
4. The probe was poured into a capped plastic tube and was frozen at –20 °C for further use.
5. The membrane was given two 5 min washes in 2X SSC/0.1% SDS at room temperature, and was then washed twice for 15 min at 65 °C in 0.1X SSC/0.1% SDS.

### A10.2 Chemiluminescent detection

All volumes are for a membrane size of 100 $cm^2$

1. After hybridisation and post-hybridisation washes, the membrane was equilibrated in wash buffer for approximately 5 min.
2. The membrane was incubated in buffer 2 for 30 min with gentle agitation.
3. Anti-DIG-alkaline phosphatase fragments were diluted 1:100000 in buffer 2. The membrane was incubated with 20 ml of this solution for 30 min.
4. The antibody solution was discarded, and unbound conjugate was removed by 2X 15 min washes with 100 ml wash buffer.
5. The membrane was equilibrated for 5 min in buffer 3.
6. The CSPD$^R$ stock solution (10 mg/ml) was diluted 1:200 in buffer 3. The membrane was incubated with the lumigen for 5 min with gentle agitation.
7. The damp membrane was sealed in a hybridisation bag and was exposed to XAR-5 autoradiographic film for 10-30 min.



## A11. Exonuclease III shortening of insert DNA (Henikoff, 1984)

1. Plasmid DNA (12 µg) was double digested to completion with the appropriate restriction endonucleases generating a 3' overhang which is susceptible to the exonuclease III enzyme, and a 5' overhang adjacent to vector sequence which is resistant the enzyme.
2. The DNA was precipitated by adding one tenth volume of 5 M sodium perchlorate and an equal volume of isopropanol. The pelleted DNA was resuspended in 100 µl of exonuclease III buffer.
3. Eleven Eppendorf tubes each containing 12 µl of freshly prepared S1 mix were placed on ice.

4. The tube containing the DNA mixture was equilibrated at 37 °C, and at T=0, a 4.5 µl sample was removed and placed in the first of the 11 tubes as an undigested control sample.
5. The shortening reaction was started by the addition of 150 U of exonuclease III to the DNA tube.
6. While at 37 °C, samples (4.5 µl) were removed from the DNA tube at 30 s intervals and were added to the S1 tubes. Solutions were mixed well by vigorous pipetting.
7. The Eppendorf tubes containing S1 mixes were raised to room temperature and incubated for 30 min.
8. The action of S1 nuclease was inhibited by the addition of 1.8 µl of S1 stop. Tubes were placed at 70 °C for 10 min.
9. To confirm that shortening reactions were successful, 4 µl from every second tube was electrophoresed on a 0.8% agarose gel.
10. Klenow mix (1.8 µl) and 1 µl of klenow enzyme were added to each tube and left for 5 mins at room temperature to blunt the ends of the DNA fragments.
11. dNTPs (1µl of 0.125 mM solution) were added to each tube and left for 15 min at room temperature.
12. Half of the sample from each tube was added to 60 µl of ligation mix (ligation buffer, T4 ligase and sterile water). Ligations were carried out overnight at 16 °C. Competent *E. coli* cells were transformed. Small-scale preparation and digestion of plasmid DNA was performed to find the appropriate shortened plasmids.



## A12. Nucleotide sequencing

Nucleotide sequencing was according to the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977). The Sequenase$^{R}$ Version 2.0 DNA Sequencing Kit was used according to the manufacturer's instructions. This kit uses the T7 DNA polymerase (Sequenase$^{R}$) enzyme.

### A12.1 Primer annealing reaction

1. 10 µg of freshly maxiprepped DNA resuspended in TE, pH 8, was diluted to 18 µl with sterile distilled water.

2. 2 N NaOH (2 µl) was added and the tube was incubated at 37 °C for 30 min to denature the double stranded DNA.
3. The tube was placed on ice immediately and 4 µl 3 M sodium acetate and 150 µl absolute ethanol was added.
4. The tube was placed at –70 °C for 15 min and then microcentrifuged for 15 min at 4 °C.
5. 70% ethanol (500 µl) was added and the tube was centrifuged again so that the pellet was spun through the ethanol wash. The supernatant was carefully discarded.
6. The DNA pellet was resuspended in 7 µl sterile distilled water to which 2 µl of reaction buffer and 1 µl primer (2 pmol) was added.
7. The tubes were incubated at 65 °C for 10 min and then cooled to 37 °C.

### A12.2 Sequencing reactions

1. Termination tubes were prepared as follows: 1.5 µl extension mix and 1 µl dideoxy-termination mix was added to G and C termination tubes. A and T termination tubes contained 1 µl extension mix and 1.5 µl dideoxy-termination mix. If more than one template was sequenced, these volumes were scaled up and 2.5 µl aliquots were added to each termination tube.
2. The labelling mix was prepared on ice and the amount per template was as follows:
   1.0 µl DTT
   2.0 µl Labelling mix minus dATP
   1.7 µl DMSO
   0.5 µl SSB
   2.0 µl T7 polymerase
   0.5 µl $^{35}$S-dATP
3. The template tubes were placed at room temperature and 8 µl of the prepared labelling mix was added to each tube. The labelling reaction was left for 20 min.
4. The termination tubes (A, C, G, T) were prewarmed in a heating block to 48 °C for 30 s.
5. An aliquot of 3.6 µl from each labelled template tube was added to each of the 4 prewarmed termination tubes. Termination reactions were left to proceed for 10 min at 48 °C.
6. Klenow enzyme (1 µl of 0.25 U solution) was placed in each termination and tube and the incubation continued for 4 min.

7. Stop solution (5 µl) and 1 µl of proteinase K (0.1 µg/ml) mixed into each tube. After 5 min, termination reactions were placed at 65 °C for 20 min to inactivate the proteinase K.
8. Prior to electrophoresis, the samples were denatured at 95 °C for 3 min and then placed on ice.
9. Aliquots (3-4 µl) were run on 6% polyacrylamide gels with Tris-taurine-EDTA running buffer for 2 to 6 h at 80 Watts.
10. The gel was dried at 80 °C onto Whatmann 3MM blotting paper using a Dual Temperature Slab Gel Dryer (Hoefer Scientific Instruments) for 30 min and then exposed to XAR-5 autoradiographic film for a minimum of 16 h.

### A12.3 Sequitherm cycle sequencing

**ALF-EXPRESS Cy5 end labelled primer method.**

Use only DNA transformed into an end$^-$ *E. coli* strain.

All labelling reactions must be done with flourescent lights off.

1. Label 200 µl coloured PCR tubes.
2. Add 3 µl termination mixes (A, C, G, T) to labelled tubes.
3. On ice make DNA up to 12.5 µl total volume with sterile milliQ water.
   Add:1 µl primer
   2.5 µl 10X sequencing buffer
   1 µl Sequitherm DNA polymerase
   Mix well and spin down.
4. Aliquot 3.8 µl from the eppendorf to each termination tube.
5. Place reactions in a thermal cycler (Hybaid Thermal Cycler) and run under the following conditions:
   93 °C for 5 minutes
   93 °C for 30 seconds
   55 °C for 30 seconds
   70 °C for 60 seconds
   Run for thirty cycles.
6. Remove tubes and add 3 µl Stop/loading solution.
7. Incubate at 95 °C for 5 minutes to denature before running.

8. Spin and Load 4 µl onto 6% polyacrylamide gel with TBE (Tris-Borate Buffer). All gels were run in 0.5% TBE tank buffer.

**ALF-EXPRESS Running Conditions**

All running conditions were kept constant.

Voltage: max. 1500 Volts

Current: 50 Milliamps

Power: 25 Watts

Laser Value: 650-800

**A13. Preparation and transformation of competent *E. coli* cells**

The method of Draper *et al.* (1988) was used for the transformation of *E. coli* cells.

1. A 1/100 dilution of an overnight culture was made into 100 ml of LB broth, and was grown with shaking at 37 °C until the $OD_{600}$ reached 0.35.
2. The culture was transferred into a GSA bottle and chilled on ice for 15 min.
3. Cells were pelleted at 2500 rpm for 5 min at 4 °C.
4. Cells were gently resuspended in 21 ml ice-cold TFB1, and incubated on ice for 90 min.
5. Samples were centrifuged at 2500 rpm for 5 min at 4 °C. Cells were resuspended in 35 ml chilled TFB2. These cells were aliquoted (100 µl) into 1.5 ml Eppendorf tubes. These cells were competent and were either used immediately for transformation or frozen in liquid nitrogen and stored at –70 °C.
6. Plasmid DNA (10 ng) was added to 100 µl of competent cells and incubated on ice for 20 min.
7. Cells were heat shocked by placing the tubes in a 37 °C water bath for 60 s. Tubes were returned immediately to ice for a further 2 min.
8. LB broth (800 µl) was added to each Eppendorf tube, which was left to shake at 37 °C for half an hour.
9. Cell aliquots (100 µl) were plated onto LB plates containing 100 ug/ml ampicillin or the relevant antibiotic.

## A14. Outer Membrane Protein Extraction Techniques

### A14.1 OMP extraction technique (Ditandy and Imhoff, 1993)

1. Grow cells overnight in 100 ml broth.
2. Spin in GSA tubes at 6000 rpm for 5 minutes.
3. Wash pellets in 50mM Tris/HCl pH9 containing 5% (W/V) NaCl.
4. Spin at 8000 rpm for 5 minutes.
5. Resuspend vigorously in same buffer plus 30% (W/V) sucrose to 1/20 culture volume.
6. Spin at 8000 rpm for 10 minutes to remove murienoplasts.
7. Dialyse the supernatant in water overnight.
8. Centrifuge for 1 hour at 40 000 rpm in an Ultracentrifuge using a 50Ti rotor.
9. The pellet was resuspended in 100 µl Phosphate Buffer pH7.
10. Electrophorese samples (10 µg) on a 12% SDS-polyacrylamide gel

### A14.2 OMP Extraction Technique (Lohia *et al.*, 1984; Mizuno, pers. comm.)

1. Grow cells in 100 ml broth.
2. Centrifuge at 6000 rpm for 5 minutes.
3. Rapidly resuspend cells in 7ml cold 0.75M sucrose-10mM Tris/HCl (pH 7.8).
4. Add lysozyme to a final concentration of 100 µg/ml (50 µl of a 2mg/ml stock to 1 ml of cells suspension).
5. Incubate on ice for 2 minutes.
6. Dilute the suspension slowly with 2 volumes 1.5mM EDTA, pH 7.5.
7. Sonicate at maximum power for 4 minutes to burst the spheroplasts.
8. Intact cells were removed using low speed centrifugation (4000 rpm for 20 minutes).
9. The spernatant was the spun at 40 000 rpm in a 50 Ti rotor.
10. The pellet was resuspended in 0.75 ml phosphate buffer (pH 7.5).
11. Add an equal volume of 2% sarcosyl and incubate at 37 °C for 30 minutes.
12. Make up to a total volume of 8 ml with phosphate buffer and spin at 40 000 rpm.
13. Resuspend pellet in 100 µl phosphate buffer (pH 7.5).
14. Run samples on a 12% SDS-polyacrylamide Gel

## A15. Protein Concentration Determination

The method of Bradford (1976) was used.

1. Add 1, 5, 10, 15, 20, and 25 µl of 0.5 mg/ml BSA to 6 microfuge tubes.
2. Make up to 100 µl with 0.15M NaCl.
3. Add 1ml Coomasie Brilliant Blue Solution and allow to stand for 2 mins.
4. Determine $OD_{595}$. generate standard curve and determine concentration of your unknown.

## A16. β-galactosidase Assays (Miller and Stadtman, 1972)

1. Grow cells overnight at 37 °C in the presence of selection.
   Dilute cells 1:100 and grow for a further 4 hours.
2. Centrifuge 2 ml cells and resuspend in 2 ml 0.1M Phosphate buffer and measure $OD_{600}$.
3. Incubate on ice for 20 mins.
4. To 200 µl of cells add: 200 ; Z-buffer,
   20 µl chloroform
   10 µl 0.1% SDS
   Vortex for 10 seconds.
5. Add 100 µl of fresh ONPG (4 mg/ml) made up in 0.1M Phosphate buffer (ONPG requires about 5 mins vortexing to dissolve).
6. Keep on ice till this point.
7. Incubate at 28 °C for up to 1 hour (until solution goes pale yellow).
8. Stop the reaction by adding 500 µl of 0.5 M $Na_2CO_3$.
9. Microfuge for 1 min to clarify solution.
10. Remove 800 µl of supernatant and measure its $OD_{420}$ (ONPG) and $OD_{550}$ (cell debris)
11. Determine activity by the following equation:

$$\text{Activity in Miller Units} = \frac{1000 \times [OD_{420} - (1.75 \times OD_{550})]}{\text{Reaction time (mins)} \times OD_{600} \times \text{volume of cells (ml)}}$$

## A17. Isolation of antibiotic resistant mutants (Spontaneous Mutants)

Spontaneously arising mutants were isolated by following the principles as outlined by Luria and Delbrück, 1943.

### A17.1 Preparation of gradient plates

1 Measure 10 ml of autoclaved nutrient agar into sterile petri dishes and allow to cool with one edge elevated (see Figure A2).

2 After the agar has hardened, level the plates and add an additional 10 ml of antibiotic agar.

3 Allow the plates to cool on a level table, thus producing a gradient of antibiotic from 0 µg/ml at one edge to the maximum concentration on the other edge.

4 Mark the edges to show which way the gradient is oriented.

5 Allow the plates to stand overnight so that the antibiotic gradient can be established.



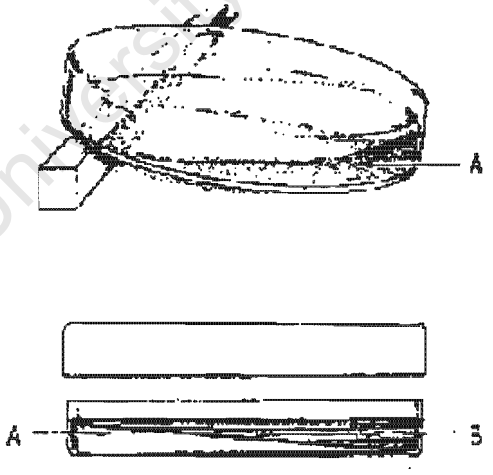



**Figure A2.** Preparation of gradient plate for isolation of antibiotic-resistant mutants. Top, step 1, bottom, step 2. (A) Nutrient agar, (B) antibiotic agar. The resulting antibiotic gradient extends from a low concentration on the left to high on the right of the plate.

### A17.2 Mutant isolation procedure

1 Inoculate a single colony in LB and grow to mid-logarithmic phase.
2 Spread 0.1 ml of sample on the antibiotic gradient plates.
3 Incubate the plates for 24 to 36 h.
4 Pick resistant colonies with a sterile loop and streak out on a second gradient plate in the direction of increasing antibiotic concentration.
5 After a second incubation, pick single colonies and test on nongradient antibiotic plates (various antibiotic concentrations), to confirm levels of resistance.

## A18 Electroporation

### A18.1 Preparation of electrocompetent cells (Metzger *et al.*, 1992)

1 Grow cells in LB at 37 °C (*E. coli*) or 28 °C (*E. chrysanthemi*) to a density of 0.5- 0.8 $OD_{590}$.
2 Harvest the cells by centrifugation, 6000 rpm for 10 min.
3 Wash three times with 10 mM Hepes (pH 7).
4 Wash once with 10% (w/v) glycerol and resuspend in 10% glycerol such that the original culture is concentrated 500 fold (3 to $5X10^{10}$ cfu/ml).
5 Store cells at -70 °C.



### A18.2 Electroporation procedure

1 Thaw electrocompetent cells on ice and to 40 µl of cells, add cold DNA solution (approximately 700 ng of plasmid DNA in $H_2O$).
2 Incubate for 3 min on ice before transfer to electroporation cuvette.
3 For *E. chrysanthemi*, adjust the settings to 25 kV/cm (field strength), 200 ohms (controller setting) and 25 µF (capacitance).
4 After pulsing, dilute cells immediately in 960 µl of SOC medium and incubate at 28 °C for 1 h in a rotatory shaker.
5 Dilute cells further and plate on selective media.

# APPENDIX B

## Buffers and solutions

Standard buffers and solutions were prepared as outlined in Sambrook *et al.* (1989). Solutions were autoclaved at 121 °C for 15 min or were filter sterilised through a 0.22 µm Millipore filter.

**B1. Tris-EDTA (TE) buffer (pH 8.0)**

| | |
|---|---|
| Tris base | 1.21 g |
| EDTA (0.5 M, pH 8.0) | 2.0 ml |
| Distilled water to | 1.0 L |

The pH was adjusted to 8.0 with 0.1 M HCl

**B2. 50 X Tris Acetate (TAE) buffer (pH8.0)**

| | |
|---|---|
| Tris base | 242.0 g |
| Glacial acetic acid | 57.1 ml |
| EDTA (0.5 M, pH8.0) | 100.0 ml |
| Distilled water to | 1 L |

**B3. 10 X Tris-Borate EDTA (TBE) buffer (pH 8.0)**

| | |
|---|---|
| Tris base | 108.0 g |
| Boric acid | 55.0 g |
| EDTA | 20.0 ml |
| Distilled water to | 1 L |

**B4. 10 X Tris-Taurine EDTA (TTE) buffer**

| | |
|---|---|
| Tris base | 108.0 g |
| Taurine | 36.0 g |
| EDTA | 3.72 g |
| Distilled water to | 1 L |

**B5. Gel electrophoresis loading buffer**

| | |
|---|---|
| Bromophenol blue | 62.5 g |
| Sucrose | 10.0 g |
| EDTA (0.5 M, pH 8) | 1.0 ml |
| Distilled water to | 25.0 ml |

**B6. Solutions for bacterial genomic DNA prepartion**

**CTAB/NaCl solution**

| | |
|---|---|
| NaCl | 4.1 g |
| CTAB | 10.0 g |
| (hexadecyltrimethyl ammonium bromide) | |
| Distilled water to | 1 L |

**B7. Alkaline lysis buffers for preparation of plasmid DNA**

**Solution I**

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

**Solution II**

0.2 N NaOH (freshly diluted form a 10 N stock)

1 % SDS

**Solution III**

| | |
|---|---|
| 5 M potassium acetate | 60.0 ml |
| Glacial acetic acid | 11.5 ml |
| Distilled water | 28.5 ml |

## B7.1 Buffer solutions for nucleobond kit (Machery-Nagel)

**S1**

| | |
|---|---|
| Tris-HCl | 50 mM |
| EDTA | 10 mM |
| RNAse A | 100 µg/ml, pH 8 |
| (store at 4 °C) | |

**S2**

| | |
|---|---|
| NaOH | 200 mM |
| SDS | 1% |

**S3**

| | |
|---|---|
| KAc | 2.80 mM |
| (store at 4 °C) | |

**N2**

| | |
|---|---|
| Tris | 100 mM |
| Ethanol | 15% |
| KCl | 900 mM |
| (Adjusted with $H_3PO_4$ to pH 6.3) | |

**N3**

| | |
|---|---|
| Tris | 100 mM |
| Ethanol | 15% |
| KCl | 1150 mM |
| (Adjusted with $H_3PO_4$ to pH 6.3) | |

N5

| | |
|---|---|
| Tris | 100 mM |
| Ethanol | 15% |
| KCl | 1000 mM |

(Adjusted with $H_3PO_4$ to pH 8.5)

**B8. Restriction enzyme buffers**

Boehringer Mannheim buffers A, B, L, M and H were used with the appropriate restriction endonuclease. Final concentration in mmol/l.

| Stock solution | A | B | L | M | H |
|---|---|---|---|---|---|
| Tris acetate | 33 | - | - | - | - |
| Tris-HCl | - | 10 | 10 | 10 | 50 |
| Mg-acetate | 10 | - | - | - | - |
| $MgCl_2$ | - | 5 | 10 | 10 | 10 |
| K-acetate | 66 | - | - | - | - |
| NaCl | - | 100 | - | 50 | 100 |
| Dithioerythritol | - | - | 1 | 1 | 1 |
| Dithiothreitol | 0.5 | - | - | - | - |
| 2-mercaptoethanol | 1 | 1 | - | - | - |
| pH at 37 °C | 7.9 | 8.0 | 7.5 | 7.5 | 7.5 |

**B9. DNA hybridisation solutions**

**20 X SSC**

| | |
|---|---|
| NaCl | 175.3 g |
| Trisodium citrate dihydrate | 88.2 g |
| Distilled water | 1 L |

pH to 7.0

**Prehybridisation and hybridisation buffer**

| | |
|---|---|
| 20 X SSC | 50 ml |
| Skim milk powder | 3 g |
| N-lauroylsarcosine, Na | 0.2 g |
| SDS (0.02%) | 400 µl |
| Distilled water to | 200 ml |

**B10. Chemiluminescent detection solutions**

**Buffer 1**

| | |
|---|---|
| Maleic acid | 23.2 g |
| NaCl | 17.6 g |

Adjust pH to 7.5 with NaOH pellets, make volume up to a litre and autoclave.

**Wash buffer**

| | |
|---|---|
| Buffer 1 | 997 ml |
| Tween 20 | 3 ml |

**Block buffer**

1% skim milk powder in buffer 1.

**Buffer 3**

| | |
|---|---|
| 1 M Tris-Cl (pH 8) | 50 ml |
| 5 M NaCl | 10 ml |
| 1 M $MgCl_2$ | 25 ml |
| Distilled water to | 500 ml |

**B11. Exonuclease III shortening solutions**

**10 X S1 buffer**

| | |
|---|---|
| 3 M potassium acetate | 1.1 ml |

5 M sodium chloride 5.0 ml

Glycerol 5.0 ml

$ZnSO_4$ 30 mg

Autoclave

**Exo III buffer**

1 M Tris-Cl, pH 8.0 660 µl

100 mM $MgCl_2$ 66.4 µl

Distilled water 9.27 ml

Autoclave

**S1 mix**

10 X S1 buffer 41 µl

Sterile water 258 µl

S1 nuclease 60 U

**S1 stop**

0.3 M Tris base

0.05 M EDTA

Autoclave but do not pH since high pH is needed to inactivate the S1 nuclease.

**Klenow mix**

20 mM Tris-Cl, pH 8.0

7 mM $MgCl_2$

Autoclave.

**Ligase mix**

10 X ligation buffer 90 µl

T4 ligase 12 µl

Sterile water 618 µl

Enough for 12 tubes. Use 60 µl per tube.

**B12. DNA sequencing gel mix (Manuel Sequencing)**

A 6% polyacrylamide gel mix was used.

| | |
|---|---|
| Urea | 12.0 g |
| Acrylamide | 4.8 g |
| Bis acrylamide | 0.21 g |
| 10 X TTE | 8.0 ml |
| Distilled water to | 80.0 ml |

55 ml of the mix was filtered through a 0.8 µm Millipore filter. TEMED (50 µl) and 50 µl of 50% ammonium persulphate was added and mixed before the gel was poured.

**B13. DNA Sequencing Gel Mix (Automated Sequencing)**

| | |
|---|---|
| Urea | 21 g |
| Long Ranger Acrylamide | 5.5 ml |
| MilliQ water to a total volume of | 70 ml |

Filter 40 ml through a 0.22µm CV millipore filter

Add 10 ml filtered 10 X TBE

Degas for 5 minutes

Add: 250 µl 10% Ammonium persulfate

40 µl TEMED

Pour gel rapidly.

**B14. Solutions for the preparation of competent *E. coli* cells**

**TFB1**

100 mM RbCl

50 mM $MnCl_2.4H_2O$

30 mM potassium acetate

10 mM $CaCl_2$

15% glycerol

**TFB2**

100 mM MOPS, pH 7.0

100 mM RbCl

75 mM $CaCl_2$

15% glycerol

**B15. Media**

Solid media contained 1.5% (w/v) agar. Media were autoclaved at 121 °C for 20 min.

**Luria-Bertani medium (LB)**

| | |
|---|---|
| Bacto tryptone | 10 g |
| Yeast extract | 5 g |
| NaCl | 10 g |
| Distilled water to | 1 L |

**Modified Scott's medium (YS)**

| | |
|---|---|
| $Na_2HPO_4$ | 1.42 g |
| $KH_2PO_4$ | 0.27 g |
| $MgSO_4.7H_2O$ | 0.24 g |
| $NH_4NO_3$ | 0.4 g |
| Yeast extract | 0.9 g |
| $H_2O$ | 1000 g |

**SOC medium**

| | |
|---|---|
| Bacto-tryptone | 2% |
| Yeast extract | 0.5% |
| NaCl | 10 mM |
| KCl | 2.5 mM |
| $MgCl_2$ | 10 mM |
| $MgSO_4$ | 10 mM |

| | |
|---|---|
| Glucose | 20 mM |

**B16. Antibiotics and media additives**

| Concentration | Stock |
|---|---|
| Ampicillin | 100 mg/ml in water (used at 100 µg/ml) |
| Rifampicin | 50 mg/ml in methanol (used at 25 - 50 µg/ml) |
| Kanamycin | 100 mg/ml in water (used at 50 µg/ml) |

Stock solutions of antibiotics dissolved in water were sterilised by filtration throµgh a 0.22-micron filter. All antibiotics were divided into 1 ml aliquots and stored at –20 °C.

**IPTG (isopropyl-b-D-thio-galactopyranoside)**

| | |
|---|---|
| IPTG | 23.4 mg |
| Distilled water | 1.0 ml |

The soluton was aliquoted and stored –70 °C.

**X-Gal (5-bromo-4-chloro-3-indolyl-b-galactoside)**

| | |
|---|---|
| X-gal | 0.2 g |
| Dimethylformamide | 10 ml |

The solution was stored at –70 °C.

**40% sucrose**

| | |
|---|---|
| Sucrose | 40 g |
| Distilled water | 100 ml (Filter sterilise) |

**B17. SDS-polyacrylamide Gel Electrophoresis Solutions**

**30% Acrylamide/0.8% bisacrylamide**

30 g acrylamide and 0.8 g of *N,N'*-methylene-bisacrylamide in a total volume of 100 ml water. Must discard after 30 days.

**4X Tris-Cl/SDS, pH 6.8**

Dissolve 6.05 g Tris base and 0.4 g SDS in 40 ml water. Adjust pH to 6.8 with 1N HCl.

Add water to 100 ml total volume.

**4X Tris-Cl/SDS, pH 8.8**

Dissolve 91 g tris base and 2 g SDS in 300 ml water. Adjust pH to 8.8 with 1 N of HCl. Add water to 500 ml total volume.

**5X SDS/electrophoresis buffer**

| | |
|---|---|
| Tris base | 15.1 g |
| glycine | 72 g |
| SDS | 5 g |

Add water to 1000 ml.

**2X SDS/sample buffer**

To 40 ml water add:

| | |
|---|---|
| Tris base | 1.52 g |
| glycerol | 20 ml |
| SDS | 2 g |
| Bromophenol blue | 1 mg |
| 2-mercaptoethanol | 2 ml |

Adjust pH to 6.8 with 1N HCl

Add water to 100 ml.

**10% Ammonia persulfate**

Dissolve 10 g of ammonia persulfate in 10 ml water. Aliquot into 1 ml into 1.5 ml eppendorf tubes. Store at –20 °C.

**12% Separating gel**

12 ml 30% acrylamide/0.8% bisacrylamide

7.5 ml 4X Tris-Cl/SDS pH 8.8

10.5 ml Water

100 µl 10% ammonium persulfate

20 µl TEMED

**Stacking Gel**

1.3 ml 30% acrylamide/0.8% bisacrylamide

2.5 ml 4X Tris-Cl/SDS pH 6.8

6.1 ml Water

50 µl 10% ammonium persulfate

10 µl TEMED

**B18 Bradfords Reagents**

**Coomasie Brilliant Blue Solution**

| | |
|---|---|
| Coomasie Brilliant Blue | 100 mg |
| 95% ethanol | 50 ml |
| 85% phosphoric acid | 100 ml |

Bring volume to 1 L with water. Filter, and store at 4 °C

**B19. OMP protein extraction buffers**

**Phosphate Buffer Saline**

| | |
|---|---|
| NaCL | 137 mM |
| KCl | 2.7 mM |
| $Na_2HPO_4.7H_2O$ | 4.3 mM |
| $KH_2PO_4$ | 1.4 mM |

Bring volume to 1L with water.

**B20. β-galactosidase assay buffers**

Phoshate Buffer

17 ml 1 M $Na_2HPO_4$

33 ml 1 M $Na_2HPO_4$

150 ml water to make volume to 200 ml

pH 7.0

or

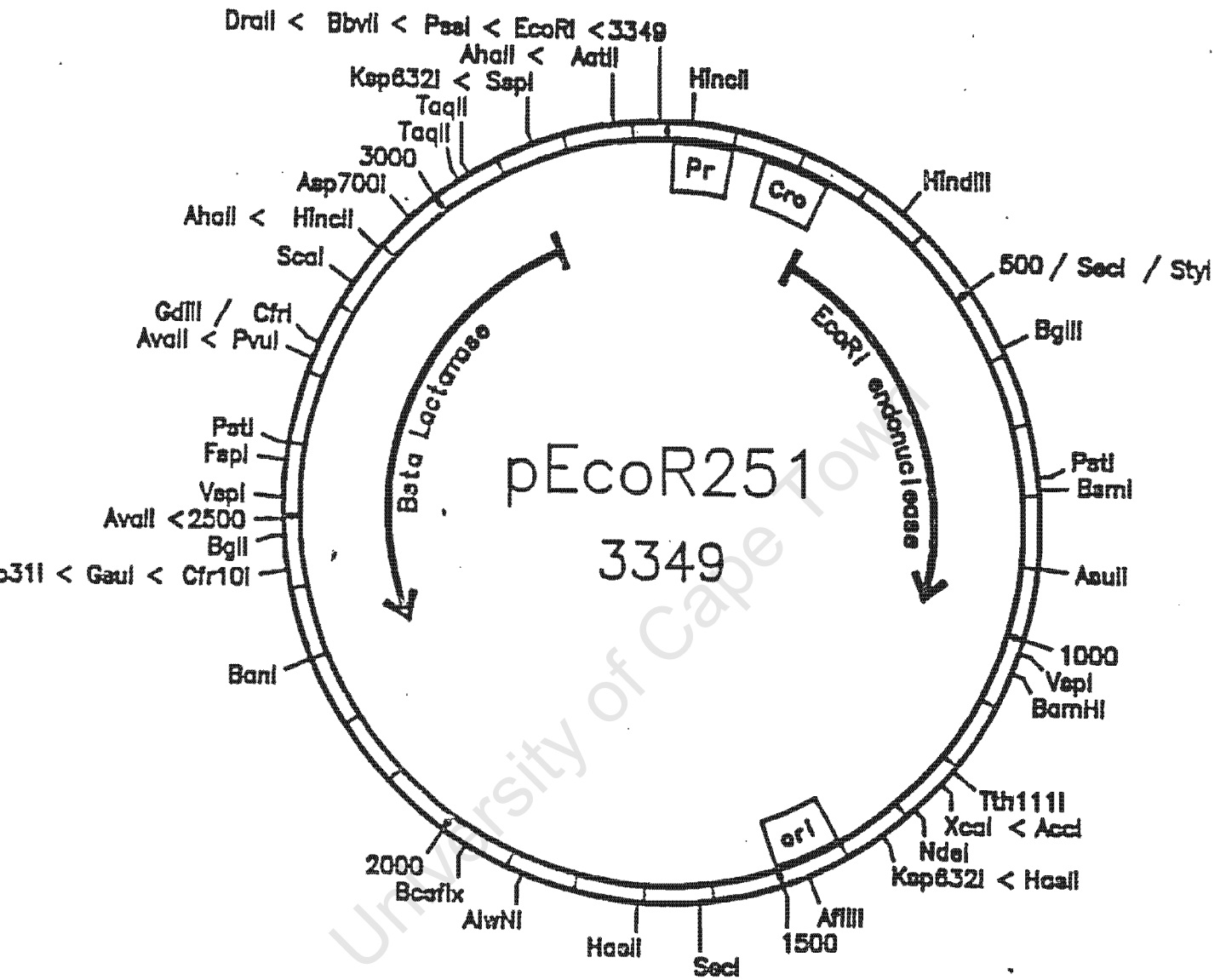
| | |
|---|---|
| $Na_2HPO_4$ | 16.1 g |
| $NaH_2PO_4$ | 5.5 g |
| Add water to | 1 L (adjust to pH 7) |

**Z- Buffer**

| | | |
|---|---|---|
| KCl | | 0.75 g |
| $MgSO_4$ (1 M) | | 1 ml |
| β-mercaptoethanol | 2.7 ml | |
| $Na_2HPO_4.7H_2O$ | 16.1 g | |
| $NaH_2PO_4.H_2O$ | | 5.5 g |
| Add water to | | 1 L (do not autoclave) |

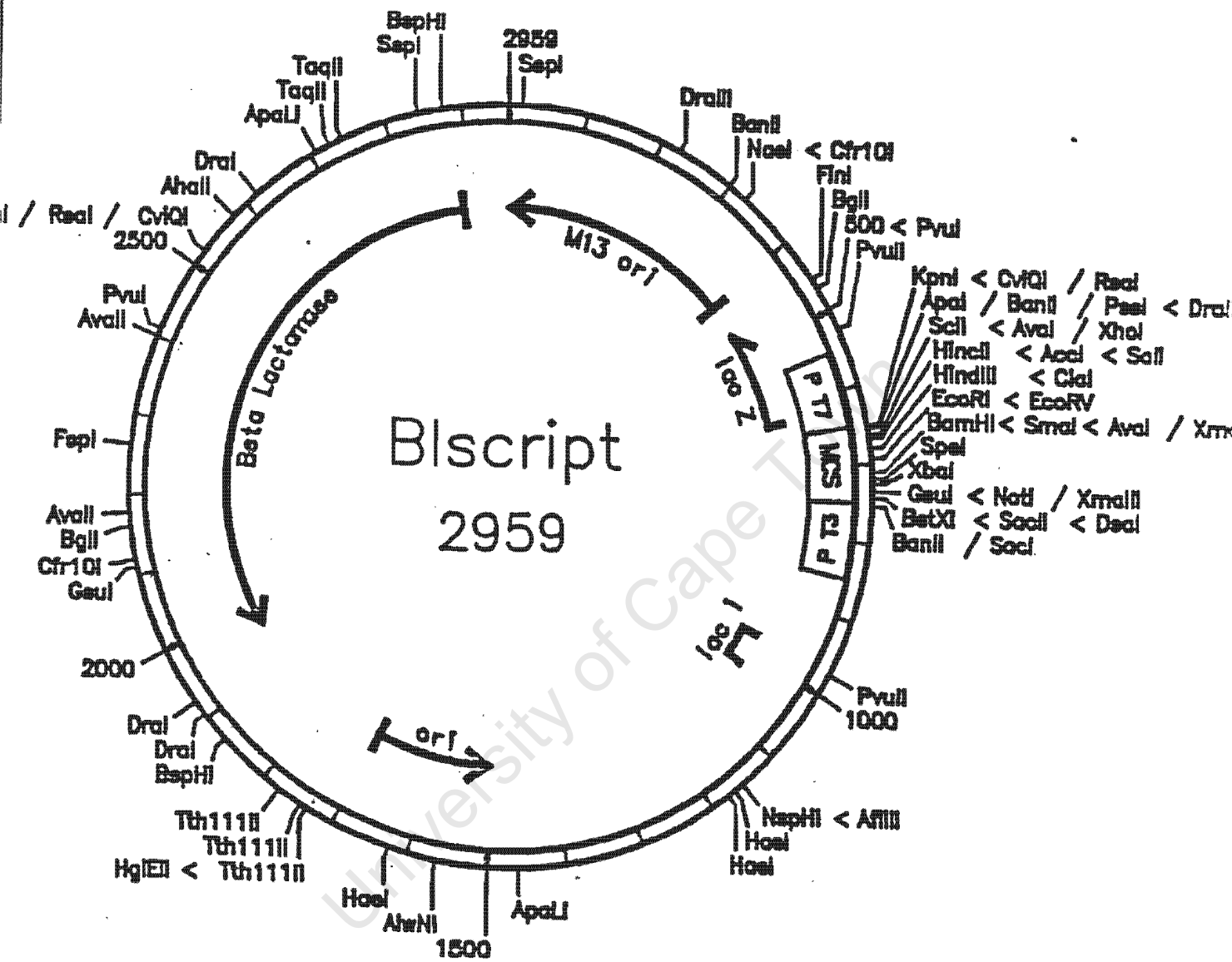
# APPENDIX C

## Plasmid maps

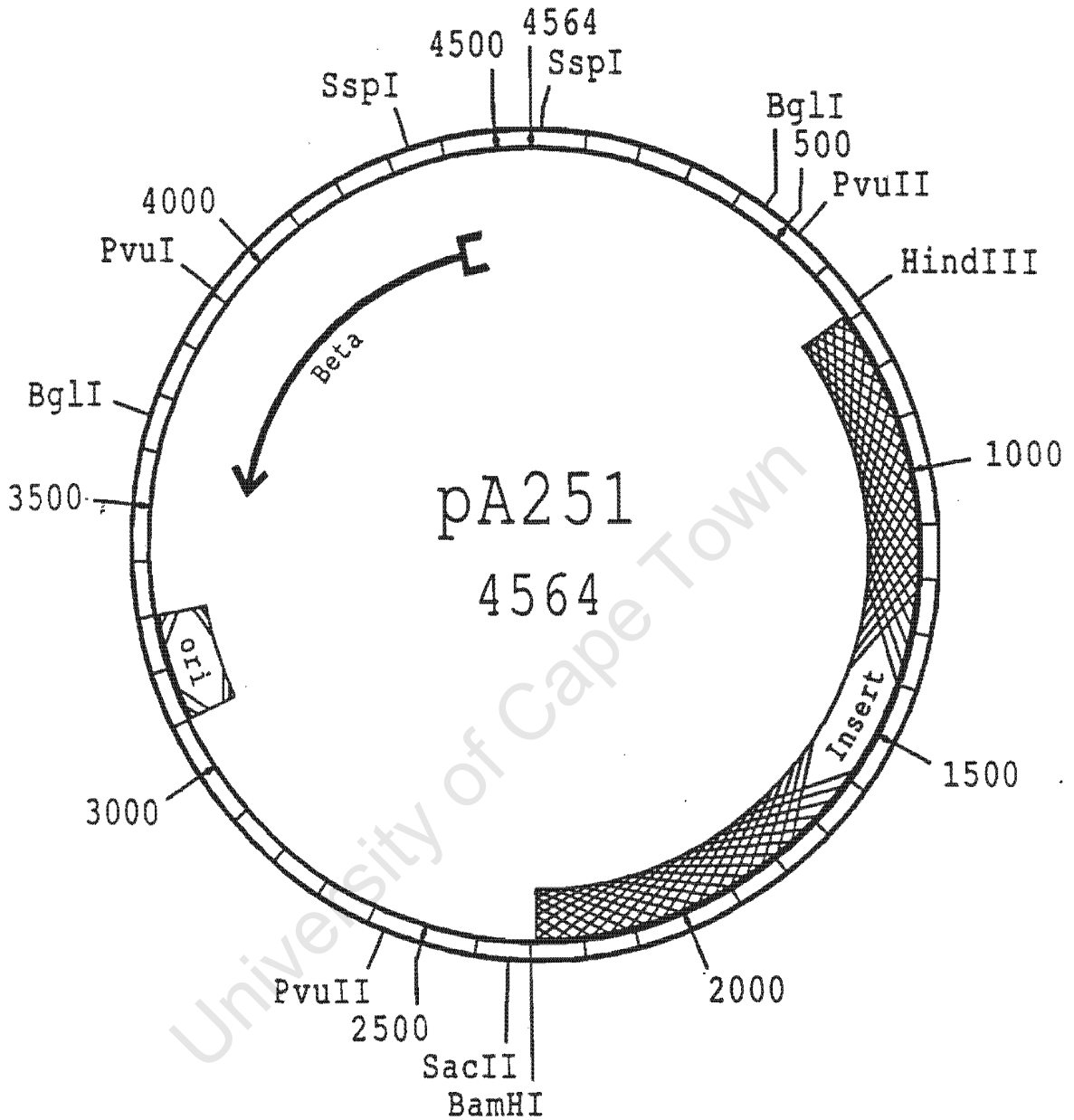





C1. Restriction endonuclease map of pEcoR251. DNA is cloned into the gene coding the restriction endonuclease, *Eco*RI, resulting in the inactivation of the gene. This prevents *Eco*RI endonuclease-initiated cell death and is an example of a positive-selection vector. The beta-lactamase gene (Beta), the origin of replication (ori) and the *Eco*RI endonuclease gene are shown.

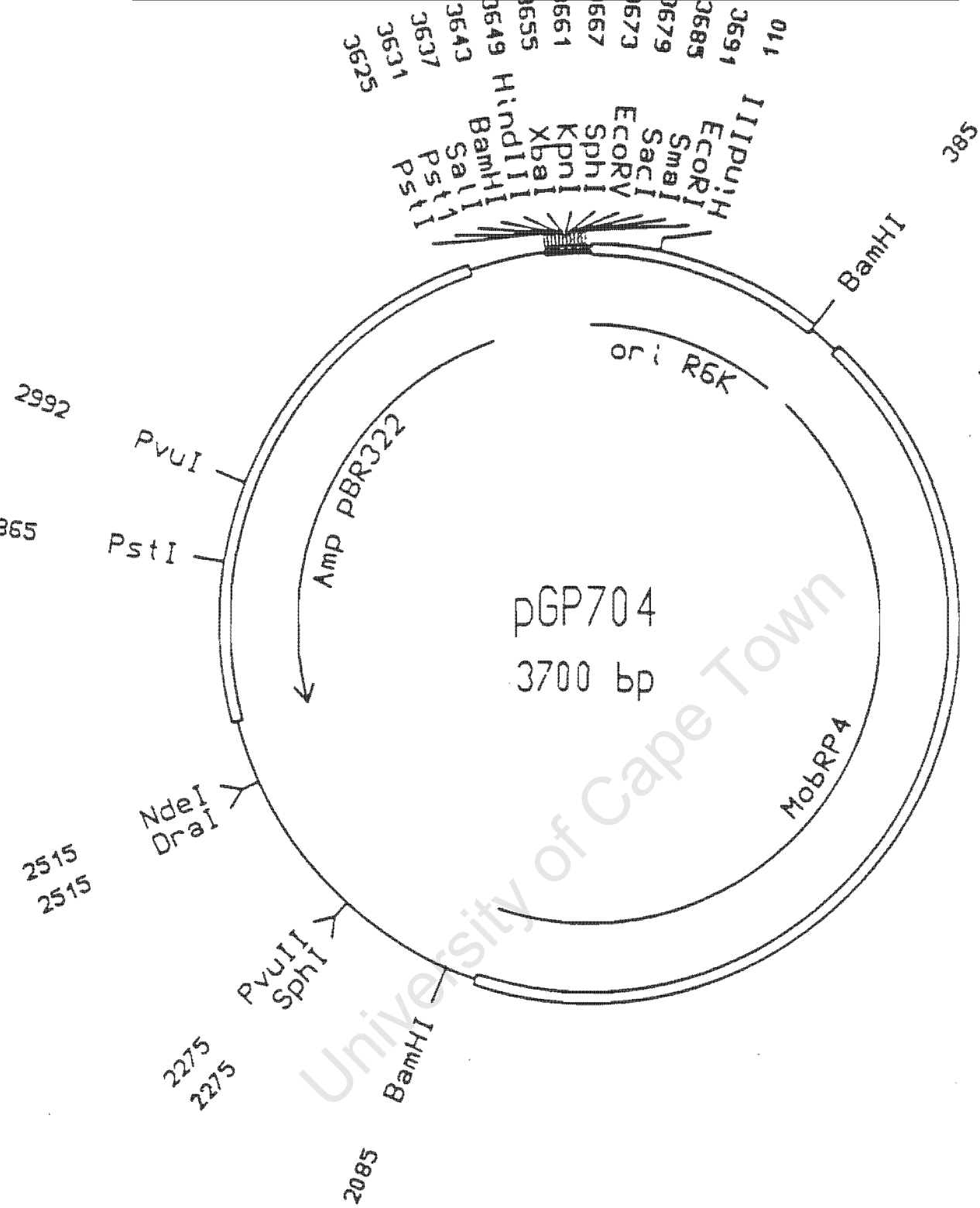



C2. Restriction endonuclease map of pBluescriptSK (Stratagene) showing the relative positions of the M13 origin of replication, the plasmid's origin of replication (ori), the beta-lactamase gene, the *lacI* promoter (laci) and the *lac Z'* gene (lacZ), the lambda phage T7 (p T7) and T3 (p T3) promoters and the multiple cloning site (mcs).






**C3.** Restriction endonuclease map of pA251, the pBluescriptSK-derived clone containing 631 bp of the *ompR* gene of *E. coli* K12. The relative positions of the Beta lactamase gene (Beta), the origin of replication (ori) and the recognition sites of various restriction sites are shown.




C4. Restriction endonuclease map of pGP704, a novel suicide vector used in the construction of insertion mutations (Miller and Mekalanos, 1988).

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