Physiological and genetic evidence for an OmpB signal transduction system in *Erwinia chrysanthemi*

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CONTENTS

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ABSTRACT

In order for bacteria to survive in their environment they must continuely sense signals such as, presence of host organisms, chemical concentrations, or variations in other physiological parameters. Many bacteria sense their environment through the use of a two component regulatory systems. These systems usually employ the use of two different proteins, a sensor protein and its cognate response regulator.

Some bacteria can survive fluctuations in medium osmolarity through the use of a two component signal transduction system. In *Escherichia coli* and *Salmonella typhimurium* this two component system includes the EnvZ sensor protein and its cognate response regulator, OmpR. The two genes that code for these proteins are *envZ* and *ompR* genes respectively. The two genes together form the *ompB* operon respectively. This operon regulates the expression of two outer membrane proteins, OmpF and OmpC in response to medium osmolarity in *E. coli.*

Erwinia chrysanthemi has been found to be sensitive to desication. Proliferation of soft rot, caused by this organism, has also been associated with irrigation. *E. chrysanthemi* has also been observed to respond to changes in medium osmolarity. Evidence of an *ompB* operon was thus sought. Outer membrane proteins were isolated using sodium lauroylsarcosine. Three major outer membrane proteins were isolated, namely Omp1 (37.5 kd), Omp2 (35.5 kd) and Omp3 (34.5 kd). Increase in medium osmolarity resulted in an increase in expression of Omp3, while Omp1 was suppressed. This lends support to the presence of an *ompB* like signal transduction system in *E. chrysanthemi.* Growth temperature was shown to have no effect on the expression of the major OMP. Similarly, culture growth phase had no effect on major OMP expression. However, two induced OMP were present from mid log phase onwards.

Since *E. chrysanthemi* is able to use pectin as a carbon source, the possible induction of an outer membrane in response to polygalacturonic acid was examined. An induced OMP was identified, and designated OmpG (27 kd). This

outer membrane protein may enhace the uptake of mono- and di-galacturonic acid, the breakdown products of pectin and polygalacturonic acid.

Primers were designed to amplify a portion of the *envZ* gene from *E. coli.* Attempts were also made to amplify a portion of *E. chrysanthemi envZ* gene homologue. However, PCR amplification of an *envZ* homologue from *E. chrysanthemi* under high or low stringency PCR was not successful. PCR amplification of the *envZ* gene from *E. coli* was successfull, yielding a 913-bp fragment. This 913-bp fragment was used for Southern blot analysis of *E. chrysanthemi* and *E. coli* genomic DNA cut with a number of restriction endonucleases. Southern blot analysis showed different banding patterns for *E. coli* and *E. chrysanthemi.* These positive results confirmed the presence of an *envZ* gene homologue in *E. chrysanthemi.*

Since sequence alignment of both the *envZ* genes from *E. coli* and *S. typhimurium* showed high sequence similarity, the *E. coli ompB* operon was isolated for homologous recombination mutagenesis studies in *E. chrysanthemi.* This was done by generating an *E. coli* K12 genomic DNA library in the suicide vector pEcoR251. Libraries were screened using the PCR generated probe and a positive clone was isolated. Plasmid pE18, containing the *envZ* gene was used for further studies. Plasmid pA251 was kindly supplied by Mr C. Adams, from the *E. coli* library, containing the *ompR* gene.

Sequencing a 1.7 kb *BglII* and *SacI* generated subclone of pE18 indicated that the *E. coli* K12 *envZ* gene showed 99.7% identity to the reported sequence. Similar sequence analysis of pA251 showed that the *E. coli* K12 *ompR* gene had 98.6% identity to the reported sequence. These sequence variation and resulting amino acid variations could be strain related. The sequenced *ompB* operon can now be subcloned into a suitable suicide vector and homologous recombination mutagenesis studies in *E. chrysanthemi* can be carried out. These studies will allow for the determination of the function of the *ompB* homologue in *E. chrysanthemi.*

CHAPTER 1

General Introduction: Environmental Sensing in Bacteria

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GENERAL INTRODUCTION

Environmental Sensing of Bacteria

To survive in the environment, bacterial cells must continually sense signals, such as chemical concentrations, presence of host organisms or variation in physiological parameters. Physiological parameters may include temperature, osmolarity, viscosity, pH or light. Bacteria must adjust their structure, physiology, and behaviour accordingly. An elaborate array of environmental sensors and response regulators, usually proteins, thus exist for these adaptive behaviours. These proteins are typically known as signalling proteins and communicate by means of two distinctive domains termed transmitters (T) and receivers (R) (Kofoid and Parkinson, 1988). These communication systems are able to effect a wide variety of signalling tasks including physiological responses to changes in medium osmolarity (Tokishita and Mizuno, 1994); chemotaxis (Parkinson, 1993; Bourret *et al.*, 1991) and many more (Lossik, *et al*, 1986; Iuchi and Lin, 1988; Saier *et al.*, 1975; Stock *et al.*, 1989).

1.1 Two Component Regulatory System

The mechanism that is commonly used by many bacteria to sense the environment usually consists of a two component regulatory system. This system involves at least two different proteins, a sensor protein and its cognate response regulator (Figure 1) (Bayles, 1994).

It would seem that both these proteins are highly conserved in all bacterium, and contain both transmitter and receiver domains. The sensor protein is usually associated with the bacterial membrane and detects the environmental stimuli. The transmitters have an autokinase activity that attaches a phosphoryl group from ATP to its histidine residue. The phosphate is then transferred to an aspartate residue in the target receiver protein (Parkinson and Kofoid, 1992). Receiver

phosphorylation stimulates the output domain and in so doing, activates the function of the response regulator. These regulators are often transcriptional regulators but may possess other functions. For example, the regulation of flagellar motion and methylesterase activity (Bayles, 1994).

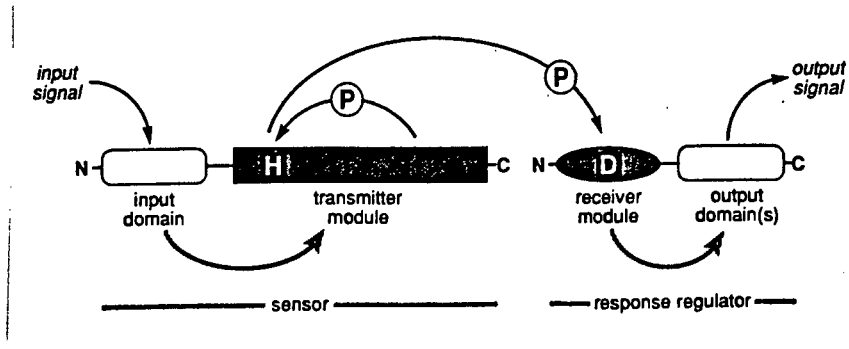

Figure 1. A two component paradigm for sensory signalling via communication modules. Information flows through noncovalent controls exerted by one domain upon another (larger arrows) and by phosphorylation reactions (arrows labelled P) involving histidine (H) and aspartate (D) residues (Parkinson and Kofoid, 1992).

Most sensor proteins are located in the cytoplasmic membrane with their transmitters facing inwards towards the cell cytoplasm. Nearly all of them have two membrane spanning segments (Parkinson and Kofoid, 1992), but at least one, UhpB, has more (Island *et al.* 1992). The periplasmic domains are structurally unrelated and have diverse receptor functions. Examples of these functions are, EnvZ senses osmolarity (Tokishita and Mizuno, 1994), KdpD is thought to respond to turgor pressure (Epstein *et al.*, 1990), and UhpD detects periplamsic hexose phosphates (Western and Kadner, 1987).

1.1.1 Receivers

Receivers recognise and interact with their cognate transmitters by accepting signals from them, and then modulate the output activity. Most of these interactions involve changes in the phosphorylation state of the receiver even though it has not been demonstrated in all signal transducing systems (Parkinson and Kofoid, 1992).

Once a transmitter has autophosphorylated, the transfer of the phosphate from the histidine residue to the receiver is catalyzed by the receiver itself rather than by the transmitter (Parkinson and Kofoid, 1992). Thus receivers are enzymes in their own right and are capable of using various phosphate sources, including available phosphohistidines. The *in vitro* half lives of phosphorylated receivers varies greatly, from a few seconds for CheB and CheY (Hess *et al.*,1988) to several hours for OmpR (Igo *et al.*, 1989). Receiver dephosphorylation also appears to be autocatalytic as it can take place in the presence of other proteins. As with the autophosphorylation reaction, it is dependent on the presence of Mg^{2+} or other divalent cations, and is effectively halted on denaturation (Weiss and Magasanik, 1988).

Structure-Function Relationships

All structure-function relationships that have been elucidated to date have been derived from the CheY model (Parkinson and Kofoid, 1992). Asp-57 has been shown by direct chemical analyses to be the phosphorylation site in CheY (Saunders *et al.*, 1989), EnvZ (Tokishita and Mizuno, 1994) and VirG (Jin *et al.*, 1990). Mutants with amino acid replacements at this position in various receiver proteins are incapable of being phosphorylated and have no signalling activity (Parkinson and Kofoid, 1992). Thus, Asp-57 seems to be the main phosphorylation site in receiver modules.

Another characteristic of receivers is the concentration of acidic amino acids which is known as the acid pocket. The acid pocket is a characteristic feature of receivers. Mutations that affect the acid pocket residues generally reduce phosphorylation ability. Depending on the location and nature of the mutant amino acid, some alterations reduce function and others induce constitutive activity (Parkinson and Kofoid, 1992). Asp-13 is almost universally present, but about half of the receivers have a glutamate at position 12. In CheY, Asp-13 and Asp-57 contribute to the binding of divalent cations, especially Mg^{2+}, which plays a crucial role in CheY

phosphorylation activities (Lukat *et al.*, 1990). The function of the acid pocket may well have similar function in other known receivers.

Lys-109 is found in all orthodox receivers and probably plays a fundamental role in their behaviour. An arginine replacement at this position results in the complete loss of function, even though the mutant protein can still be phosphorylated (Lukat *et al.* 1991). Studies done using nuclear magnetic resolution have suggested that Lys-109 is repositioned in the Che-Y mutants. It would thus seem that phosphorylation might displace the side chain of Lys-109 from the acid pocket, which would then trigger conformational changes in other parts of the molecule which regulate its activity (Parkinson and Kofoid, 1992). In summary, it would seem that the defects described above alter the chemistry of the acid pocket directly, or indirectly via inhibition of various conformational changes.

Regulation of Output Activity in Response Regulators

Phosphorylation has been implicated in controlling output activity of a number of receiver subfamilies (Parkinson and Kofoid 1992). Phosphorylation leads to an activation of the output function, but in some response regulators the unphosphorylated form may also have a functional role. The two forms of DegU, for example, have different regulatory activities (Dahl *et al.*, 1992). An example of a receiver with a single output function is OmpR. Phosphorylation of OmpR enhances the protein's ability to bind specific regulatory sequences involved in transcriptional activation (Forst *et al.*, 1989). Since the activating effects of response regulator phosphorylation can be duplicated by mutational changes, phosphorylation may simply be a device for inducing conformational changes in the response regulator. Although little is known about how this control is accomplished, it does appear that receivers have developed both positive and negative strategies for controlling associated output domains (Parkinson and Kofoid, 1992). The output domain in most response regulators may be under positive control, but evidence so far is circumstantial. The removal of the receiver module in OmpR does not enhance

the DNA binding ability of its output domain, implying that the receiver is needed to activate the output function (Tsung *et al.*, 1989).

Two general classes of mechanisms would enable receivers to exert positive or negative control over output functions (Figure 2). Control could involve direct interaction of the receiver with the output domain, promoted through specific contacts modulated by the phosphorylation state (Figure 2a). Alternatively, output control might involve no direct contact between the receiver and the rest of the protein, in which case any particular receiver could in principle be functionally coupled to a variety of output domains (Figure 2b). A simple mechanism for indirect control could be through phosphorylation-induced changes in the receiver aggregation state (Kofoid and Parkinson, 1988). For example, phosphorylation might cause receiver subunits to dimerize, thereby altering their output domains by bringing them closer together, and in so doing alter their function (Figure 2b). The presence of flexible linkers joining receiver and output domains in many response regulators implies that some relative movement of the two domains may be necessary for proper control (Parkinson and Kofoid, 1992).

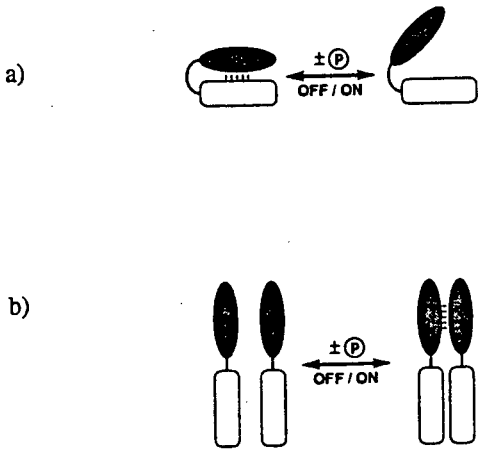

Figure 2. Two general mechanisms for controlling output domain activity (OFF or ON) through changes in receiver phosphorylation state. Receivers could modulate activity by direct interaction with the output domain (2a) or by mediating subunit association (2b) (Parkinson and Kofoid, 1992).

1.1.2 Transmitters

Transmitter modules recognise and interact with their cognate receivers. These reactions may involve changes in transmitter phosphorylation activities, but this has yet to be proven.

The only proven catalytic activity for transmitters is the formation of intramolecular high energy phosphohistidine (Forst *et al.*, 1989). The transmitter autophosphorylation reaction represents a possible control point for regulating the flow of phosphate into receivers. A number of proteins also exhibit an apparent phosphatase activity that provides a second method of regulating the phosphorylation state of their cognate receiver proteins.

Structure of Transmitters

Transmitters contain five short blocks of common sequence, similarly arranged but variably spaced (Figure 3). Block H is the most variable of these five regions and is located in the N-terminal half and includes the histidine residue that serves as the site of autophosphorylation. The other blocks are in the C-terminal half of the transmitter. Blocks G1 and G2 resemble glycine rich portions of nucleotide-binding domains (Rossman *et al.*, 1974). G1 and G2 are separated by a spacer of variable length, with block F situated roughly in the middle.

Replacement of the block H histidine eliminates both autophosphorylation and phosphatase activity (Yang and Inouye, 1991; Kanamaru *et al.*, 1990). Mutational changes at other block H residues can affect either activity, suggesting that local structure around the histidine influences the ratio of the two activities. Mutations in block N, G1 and G2 eliminate autokinase activity (Kanamaru *et al.*, 1989; Yang and Inouye, 1991). At present the function of block F is unknown (Parkinson and Kofoid, 1992). Parkinson and Kofoid (1992) have subdivided transmitters into two domains namely T_L and T_R. These two domains are connected by a hinge-like linker. It has been proposed that the hinge connecting T_L and T_R permit contact between

them that regulate the enzymatic activities of transmitters in much the same way as in eukaryotes (Parkinson and Kofoid, 1992).

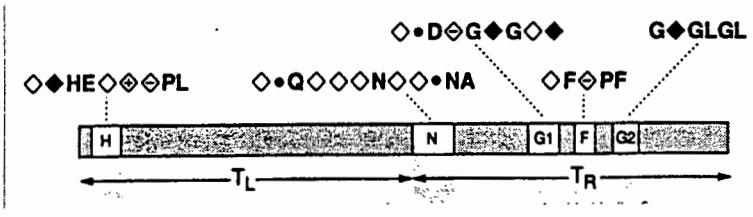

Figure 3. Structural features or orthodox transmitters. Five sequence motifs characteristic of transmitters are shown at the top. Letters indicate amino acids present in at least 70% of aligned transmitter sequences at that position. Diamond symbols indicate positions at which at least 50% of the amino acids belong to the same chemical family: white non-polar (I, L, M, V); black polar (A, G, S, P); plus sign, basic (H, K, R); minus, acidic or amidic (D, E, N, Q). Black dots indicate wild card positions with less than 50% family conservation (Parkinson and Kofoid, 1992).

Input Control of Transmitter Activity in Sensors

Input domains in sensor proteins appear to modulate the autokinase and phosphatase activities of their adjoining transmitters to control the phosphorylation state of response regulators. It has been postulated that these transmitters could have two autokinase states resulting from "ON" and "OFF" conformations (Parkinson and Kofoid, 1992). The overall level of autokinase activity in a sensor population would reflect the proportion of transmitters in each signalling state. Stimuli would modulate autokinase activity by shifting the OFF/ON equilibrium.

Sensor proteins have the same membrane topology and overall domain organisation as methyl accepting chemotaxis proteins (MCP) (Figure 4a). This has been demonstrated for EnvZ (Forst *et al.*, 1987) and VirG (Melchers *et al.*, 1989). It appears that nearly all transmitters have two membrane spanning segments that could arrange the molecule into an N-terminal periplasmic receptor domain and a C-terminal cytoplasmic signalling domain. These input and output domains are joined by a linker segment emanating from one of the transmembrane segments. This linker may effect conformational changes between the two domains during transmembrane

signalling as proposed by Stock *et al.* (1991) (Figure 4b). The proposal suggests that the input and output domains of sensors may individually undergo transitions between relaxed and tense conformations. When the domains are rigidly coupled, a conformational change in one domain is accompanied by an opposing change in the other domain.

Mutations in the linker region could also affect output activity in two ways. One method is to effectively uncouple the two domains leading to constitutive signalling activity. The other is to lock the transmitter in the inactive state by mimicking the conformational effects of input stimuli. Linker mutations in transmitter-containing proteins also bias signal output in one of two directions. A linker mutant of EnvZ behaves as though it is locked in the OFF state (Tokishita *et al.*, 1992), whereas four BvgS linker mutants are locked in the ON state (Miller *et al.*, 1992).

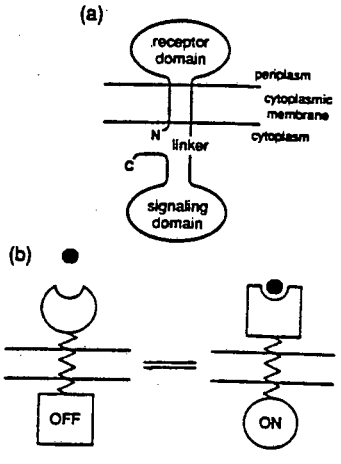

Figure 4. Models of membrane topology and transmembrane signalling in sensor proteins. (a) Membrane organisation of MCP chemoreceptors and EnvZ. (b) Possible mechanical coupling between periplasmic and cytoplasmic domains during transmembrane signalling (Parkinson and Kofoid, 1992).

Mutations in the trans-membrane portions of EnvZ protein produce analogous signalling defects, with some mutants locked in the autokinase OFF state and others in the ON state (Tokishita *et al.*, 1992). Tokishita and Mizuno (1994), concluded that there was an intermolecular interaction between the membrane-spanning

segments of EnvZ and this interaction is crucial for transmembrane signalling in response to an external osmotic stimulus.

1.1.3 Transmitter and Receiver Interactions

Characteristics of communication module circuits should reflect the interplay of several parameters that govern-transmitter-receiver interactions. The cycle of simple circuits where output signals are related directly to the receiver phosphorylation state is shown in Figure 5. Changes in receiver phosphorylation begin with the activation and autophosphorylation of the transmitter in response to sensory input. This is followed by association of unphosphorylated receivers with activated transmitters through specific recognition interactions. After engaging the transmitter, the receiver catalyses the transfer of the phosphate from the histidine residue to its own aspartate acceptor site. Receiver phosphorylation could result in reduced binding affinity for the dephosphorylated transmitter or lead to production of an output signal (Figure 2b). The change in response regulator activity would persist until the receiver loses its phosphate, either spontaneously, through its own autophosphatase activity, or through transmitter-stimulated dephosphorylation (Parkinson and Kofoid, 1992).

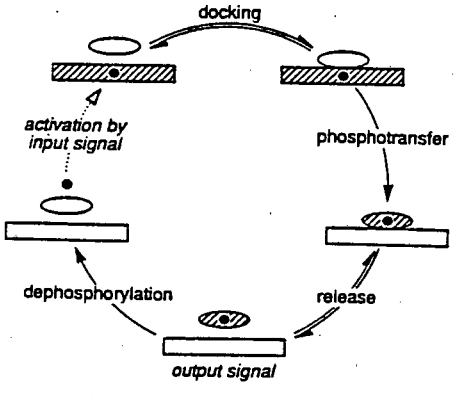

Figure 5. Critical events in the transmitter-receiver cycle. Docking and release are controlled by reversible equilibria, whereas phosphotransfer from transmitter to receiver and subsequent dephosphorylation of the receiver may be effectively unidirectional. Hatched shading denotes modules in an activated conformation; black circles represent phosphoryl groups (Parkinson and Kofoid, 1992).

A diverse range of examples exist for these two way regulatory systems. To obtain an understanding of the complexity of these systems, the sensing system in *E. coli* and other Gram-negative bacteria will be summarised below. Examples will include chemotaxis, nitrogen, phosphate and osmotic stress regulation. Physiological and genetic responses of bacteria to osmotic stress will also be discussed.

1.2 Chemotaxis

Motile bacteria swim by rotating their long filamentous flagella. Rotation in the counterclockwise (CCW) direction propels the cell forward while rotation in the clockwise (CW) direction causes a turn or tumble (Parkinson, 1993). These reversals and pauses in flagellar rotation result in changes in direction of motion. Cells heading towards an attractant or from a repellent, suppress tumbles and pauses thus maintaining a constant course (Macnab and Ornston, 1977). Attractants and repellents are sensed directly by means of specific chemoreceptors. These receptors have been found to occur in patches, often at the cell pole (Shapiro, 1993). Mutants have been selected that are either unable to change their direction or otherwise are defective in chemotaxis (Armstrong *et al*, 1967; Aswad and Koshland, 1975). Using this approach for *E. coli* and *S. typhimurium* six different types of *che* (chemotaxis) genes have been identified; *cheA, cheB, cheR, chew, cheY,* and *cheZ* (Stock *et al.*, 1989). Deletion of any one of these *che* genes produces a cell that is fully motile, but completely unable to migrate in a chemical gradient (Parkinson and Houts, 1982). All the above genes code for soluble cytoplasmic proteins which are able to participate in, or, modulate signal transduction between chemoreceptors and the flagellar motor (Stock *et al.*, 1989).

Three flagellar proteins, FliG, FliM and FliN seem to function as the flagellar switching apparatus. Interactions between the Che proteins and the flagellar switch cause a clockwise rotation, thereby inducing tumbly behaviour (Stock *et al.* 1989). Mutant strains that completely lack these proteins exhibit a Fla$^-$ phenotype, viz., they have lost their flagella motor function. The genes that encode these components are *fliG, fliM* and *fliN* (Parkinson *et al.*, 1983).

Most bacteria detect chemotactic stimuli with chemoreceptors known as methyl-accepting chemotaxis proteins (MCPs) (Hazelbauer, 1992). A family of these MCPs have been defined for *E. coli* and include, Tsr, Tar, Tap and Trg which are able to mediate responses to amino acids, peptides, and some sugars (Stewart and Dahlquist, 1987). These proteins have the same membrane topology as other receivers, and limited sequence similarities suggest that they are related to kinase receptors. However, the MCP signalling domain does not resemble an orthodox transmitter and, in fact, has no known catalytic function. It does however, modulate the activity of the transmitter-containing CheA protein to elicit the chemotactic responses (Borkovich *et al.,* 1989).

1.2.1 Phosphorylation and Signal Transduction

An overview of the signal transduction system for *E. coli* and *S. typhimurium* chemotaxis system is depicted in Figure 6 (Parkinson, 1993). The MCPs stimulate the flow of phosphoryl groups through CheA to CheY and CheB (Stock *et al.* 1989). This is thought to be brought about by conformational changes of the MCP's and as a result of ligand binding. These movements could occur between the two subunits or within the individual subunits of the MCP dimer (Parkinson, 1993). Conformational changes are thus thought to control phosphorylation of CheA by regulating the access of the phospho-acceptor site to the catalytic domain of the MCP's (Parkinson, 1993). The flow of phosphoryl groups requires CheW and is inhibited by the binding of attractant ligands to the receptors (Borkovich *et al.*, 1989). This process is also stimulated by the methylation of these receptors (Stock *et al.*, 1989). CheA transfers its phosphates to CheB and CheY, the latter is solely a receiver (Hess *et al.*, 1988). P-CheY interacts with the flagella switching apparatus to cause clockwise rotation and thereby inducing tumbling behaviour (Roman *et al.*, 1992). The motors rotate CCW by default, so the relative levels of P-CheY determines the cells swimming behaviour viz., CCW or CW rotation (Parkinson, 1993). CheZ antagonizes CheY activity by enabling the dephosphorylation of P-CheY (Hess *et al.*, 1988). CheB is part of a feedback circuit that terminates motor

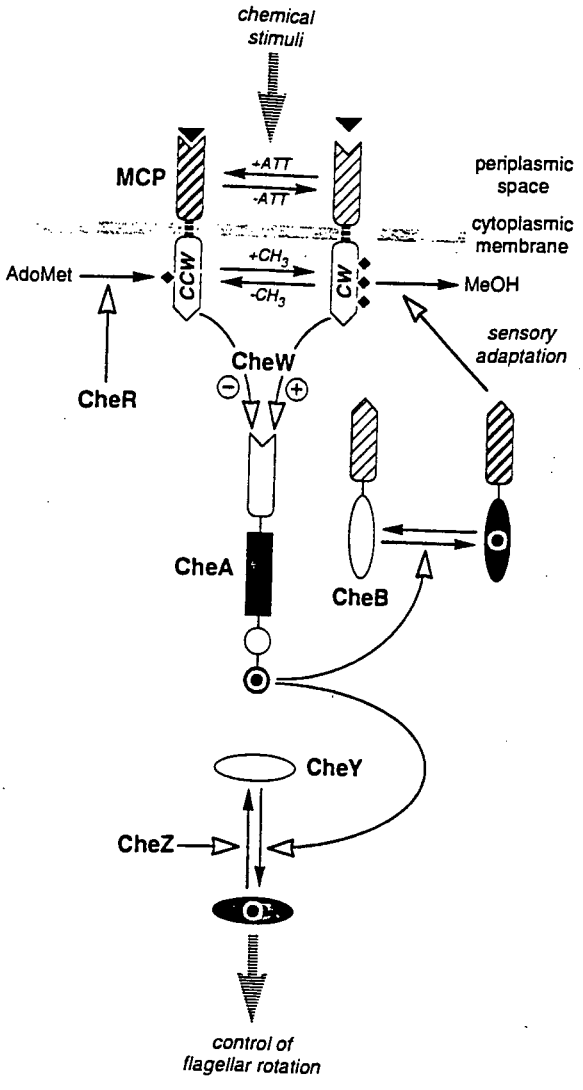

Figure 6. A model system for the mediation of chemotaxis in *E. coli* and *S. typhimurium*. This signalling pathway responds to compounds detected by chemoreceptors of the MCP class. Receptor signals control the autophosphorylation rate of CheA to modulate the flux of phosphates to CheY and CheB. Phospho-CheY controls the flagellar motors, and phospho-CheB regulates sensory adaption through changes in MCP methylation state. The ligand 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).

responses by adjusting the methylation states of MCP molecules, which in turn regulates their signalling properties. The levels of methylation act to control receptor sensitivity to the effects of the stimulatory ligands (Stock and Stock, 1987).

CheA is the central regulator of the chemotaxis system and controls flagellar switching through CheY. Simultaneously it is able to feed back through CheB to control receptor signalling. The N-terminal end of CheA contains the His-48 phosphorylation site and is able to interact with CheY and CheB. The C-terminal domain is thought to function as a receiver of information from the membrane chemoreceptor proteins and CheW. The central region of CheA is the kinase domain (Stock *et al.*, 1988). CheY has been found to be the response regulator of the chemotaxis system.

A requirement for ATP in the CheY-dependent generation of tumbling behaviour in living cells has been determined. Flagellar rotation has been shown to require an electrochemical proton gradient in *E. coli, S. typhimurium* and *Bacteroides subtilus* (Macnab, 1987). Depletion of the ATP within the cells using arsenate or depletion using adenine nucleotides, results solely in counter clockwise rotation of the flagella. Mutations in the *fliG* or *fliM* have demonstrated that ATP acts with the Che proteins to control the flagellar rotation switch rather than an energy source for rotation or switching. ATP is required for CheY activity independent of CheA (Smith *et al.*, 1988).

P-CheA transfers its phosphoryl group to CheY. CheY is subsequently hydrolysed to CheY and P. CheZ has been found to enhance the rate of CheY dephosphorylation (Hess *et al.*, 1988). P-CheA can also transfer its phosphoryl group to the CheB protein (Hess *et al.*, 1988). The rates of phosphotransfer from CheA to CheB measured with purified components are slightly faster than corresponding rates to CheY (Stock *et al.*, 1989).

At present there seems to be two levels of control for receptor methylation. Firstly, stimulatory ligands induce conformational changes in the receptors that appear to

change the accessibility of potential substrate residues to the methylating and demethylating enzymes (Springer *et al.*, 1982). The changes to methylation produced by this affect are specific to the receptor that is stimulated, and they persist for as long as the stimulus is present (Goy *et al.,* 1977). The second level of regulation occurs through a methylesterase enzyme and requires CheA and CheW (Stewart and Dahlquist, 1987). This effect is not receptor specific (Sanders and Koshland, 1988), rather its transient (Kehry *et al.*, 1985), is further dependent on intracellular ATP (Smith *et al.*, 1988) and requires the N-terminal regulatory domain of the CheB protein as well as the C-terminal catalytic domain (Stock *et al.*, 1989). CheW dramatically stimulates the rate of CheY phosphorylation in a reconstituted system containing CheA and a membrane preparation with high levels of the tar receptor-transducer protein (Borkovich *et al.,* 1989). In the absence of receptors, the purified CheW protein has no apparent effect on CheA autophosphorylation, phosphotransfer from P-CheA to CheY or CheB, CheZ phosphatase activity, or inherent stability of P-CheA, P-CheY, or P-CheB (Stock, 1989). CheW is able induce coupling of CheA to the chemoreceptor control by promoting the formation of ternary complexes containing an MCP dimer, two CheW monomers, and a CheA dimer (Gegner *et al.,* 1992).

1.3 Nitrogen Regulation

1.3.1 Bacterial Responses to Nitrogen Starvation

The preferred nitrogen source for most bacteria is ammonia. The assimilation of ammonia present in low concentrations is accomplished by the conversion of glutamate and ammonia to glutamine. This is an ATP-dependent reaction catalyzed by glutamine synthetase (Stock *et al.,* 1989). The glutamine amino group is the source for the biosynthesis of nucleotides, amino sugars, histidine, tryptophan and asparagine. The rest of the nitrogen source within the cell comes from glutamate, formed by glutamate synthase from glutamine and 2-ketoglutarate. If ammonia is lacking in the environment, ammonia must be derived by the catabolism of

nitrogenous compounds such as amino acids and urea or from the reduction of atmospheric N_2.

When cells are grown under conditions of ammonia starvation, there is a dramatic increase in the transcription of a number of genes. The Ntr regulon constitutes a subset of these genes that are controlled by common regulators (Reitzer and Magasanik, 1987). The individual Ntr operons are also subjected to Ntr-dependent controls such as induction by a specific nitrogen source and catabolite repression. The adaptive response to nitrogen starvation occurs in several stages. A central role is played by the *glnALG* operon, which encodes glutamine synthetase (*glnA* gene product), the histidine kinase of the Ntr regulon, NR_{II} (product of *glnL/ntrB*), and the response regulator of the Ntr system, NR_I (product of *glnG/ntrC*) (Backman *et al.*, 1981; Kustu *et al.*, 1979; MacFarland *et al.*, 1981). NR_I and NR_{II} regulate the transcription of *glnA* and the other Ntr and Nif (nitrogen fixation) genes.

The *glnALG* operon contains 3 distinct promoters. Two are upstream from the *glnA* gene, *glnAp1* and *glnAp2*; and the third is located in the *glnA-glnL* intergenic region (Ueno-Nishio *et al.*, 1984). Under conditions of nitrogen excess, a low rate of transcription from *glnAp1* produces a small amount of glutamine synthetase, and low transcription from the *glnL* promoter maintains a low intracellular concentration of NR_I and NR_{II}. Transcription from *glnAp1* and *glnLp* is repressed by NR_I, which binds to the two high affinity binding sites overlapping the -35 region and transcriptional start site of *glnAp1* and to a single high affinity site overlapping the -10 region *glnLp* (Reitzer and Magasanik, 1983; Ueno-Nishio *et al.*, 1984).

Starvation of nitrogen results in an increase in transcription of the *glnALG* operon from the *glnAp2* promoter (Reitzer and Magasanik, 1985). This increased transcription results in increased intracellular concentrations of the products of that operon. Transcription requires both NR_I and RNA polymerase containing a minor sigma factor, σ^{54}, the product of the *rpoN* gene, also termed *glnF* or *ntrA* (Hunt and Magasanik, 1985). Activation of transcription from *glnAp2* causes a 10-fold increase

in NR_I. Increased transcriptional activation is therefore moderated by the elevated intracellular concentrations of NR_I that accompanies it.

If ammonia is added to a nitrogen depleted bacterial culture, transcription from the *glnAp2* is quickly reduced and active glutamate synthetase is inactivated by the adenylylation of tyrosyl residues (Standtman *et al.*, 1980). If starvation for nitrogen continues, the increase in transcription of the *glnALG* operon is followed by an increase in the transcription of a number of other Ntr operons whose products facilitate the catabolism of various alternative nitrogen sources such as amino acids or urea (Magasanik, 1982). It would seem that some Ntr operons are activated directly by high concentrations of NR_I. In other cases, the expression of nitrogen regulated operons are activated by proteins whose expression is controlled by NR_I. An example is the Nif regulon of *Klebsiella pneumoniae* (Ausubal, 1984). Another example is the effect of NR_I on the expression of Ntr operons. A typical member of this class are the *hut* operons of *Klebsiella arogenes*, whose products facilitate the utilization of histidine as a sole nitrogen source (Bender *et al.*, 1983).

In summary, the Ntr-dependent adaptive response to nitrogen starvation involves an increase in the level of glutamine synthetase to enhance the ability of the cells to use low concentrations of ammonia. This is followed by the induction of Ntr operon products, such as histidase and nitrogenase, to provide ammonia from sources of organic nitrogen or from atmospheric N_2. This progression of events is controlled by a cascade of transcriptionally regulated reactions which begins with the activation of NR_I (Bourett *et al.*, 1991; Stock *et al.*, 1989).

1.3.2 Signal Transduction

The balance between the nitrogen and carbon metabolism as reflected by the ratio of glutamine and 2-ketoglutarate appears to be the critical signal for the induction of this signal transduction cascade (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} (Stadtman *et al.*, 1980). Thus, when ammonia is plentiful and the ratio of 2-ketoglutarate/glutamine is relatively low, P_{II} is in its unmodified form. However, when ammonia is limiting and the ratio of 2-ketoglutarate/glutamine is relatively high, P_{II} is 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 effects of ligands through its effects on the product of the *glnE* gene (Bueno *et al.*, 1985; Chock *et al.*, 1985; Standtman *et al.*, 1980). GlnE adenyltransferase, is a bifunctional enzyme that catalyses the adenylation and deadenylation of tyrosine residues in glutamine synthetase. Unmodified P_{II} stimulates the adenyl reaction, and P_{II}-urodine monophosphate stimulates the deadenylation reaction (Bueno *et al*, 1985). GlnE adenylyltransferase acts slowly in the absence of P_{II} and has a natural affinity for the adenylation reaction. The mechanism by which GlnE adenyltransferase responds to nitrogen limitation in the absence of P_{II} is not known. P_{II} has also been found to control transcription of the *glnALG* operon (Bueno *et al.*, 1985).

Genetic evidence indicates that unmodified P_{II} exerts its effect on *glnALG* transcription by controlling the activity of NR_{II} (Bueno *et al.*, 1985; Chen *et al.*, 1982). The latter has an effect on the timing of responses to changes in nitrogen availability.

In wild-type cells, transcription from *glnAp2* rapidly adjusts to changes in nitrogen metabolism. In cells which lack NR_{II}, the regulation of this transcription is extremely slow (Chen *et al.*, 1982). It has also become apparent through missense mutation in NR_{II}, that GlnD/P_{II} signal transduction pathway acts to regulate the expression of Ntr genes entirely through NR_{II} (Stock *et al.*, 1989). Studies on NR_{II} deficient strains indicate an NR_{II}-independent mechanism for Ntr regulation. It has also been found that NR_{II} is able to act as both a positive and a negative transcription regulator (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. In a cell-free transcription system consisting of purified components, phosphorylation of NR_I by NR_{II} confers upon NR_I the ability to activate transcription from *glnAp2 (*Ninfa and Magasanik, 1986). The phosphorylation system is similar to that of CheA and CheY. 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 (Keena and Kustu, 1988). P-NR_I is rapidly hydrolysed under physiological conditions to NR_I and P_i (Keena and Kustu, 1988). P_{II} appears to activate an ATP-dependent NR_{II} phosphatase that hydrolyses P-NR_I and thereby prevents the activation of transcription from *glnAp2* (Stock *et al.*, 1989).

1.4 Phosphate Regulation

1.4.1 Phosphate Uptake

In *E. coli* there are several proteins within the cell envelope that transport phosphate into the cytoplasm. Active transport across the cytoplasmic membrane is mediated by low and high-affinity phosphate transport systems (PIT and PST respectively) and several sugar phosphate systems (Gpt, Pgt, and Uhp) (Rossenber *et al.*, 1977). An anion specific porin (PhoE) mediates passage of phosphates across the outer membrane into the periplasm (Nikaido and Vaara, 1985). Within the periplasm, alkaline phosphatase, PhoA (Toriani, 1960), and binding proteins, PstS and UgpB (Surin *et al.*, 1986), help deliver usable phosphate to the transport systems in the cytoplasmic membrane (Surin *et al.*, 1985).

Expression of many proteins involved in phosphate uptake are regulated by the presents of phosphates in the cells environment. Phosphate represses *phoA* and *phoE* gene expression as well as the *ugp* and *pst* operons (Amemura *et al.*, 1985). Glycerol phosphates and hexose phosphates induce genes that encode their respective transport systems, *gptP* and *uhpT*. A number of different histidine kinases and response regulators act to control the above mentioned genes (Stock *et al.*, 1989).

The PhoR kinase and PhoB regulator modulate the expression of *phoA*, *phoE*, the *pst* operon, and the *phoBR* operon. This collection of genes, all of whose promoters require PhoB, have been termed the Pho regulon (Wanner, 1987).

1.4.2 Pho Regulon

The high-affinity PST system functions to detect high levels of extracellular phosphate and uses this information to cause the repression of *phoA* as well as other genes of the Pho regulon, including *pst* gene (Stock *et al.*, 1989).

PhoR can be autophosphorylated in the presence of ATP. The stability of PhoR-P is comparable to that found for CheA-P and NR_{II}-P (Stock *et al.*, 1989). PhoB is the response regulator of the Pho regulon. The latter is defined as the set of genes whose expression is dependent on PhoB. PhoB protein is homologous over its entire length to the subfamily of response regulators that includes OmpR. PhoB has also been purified and shown to function as an acceptor for the phosphoryl group in P-PhoR. The phosphorylation of PhoB results in the stimulation of the *phoA* promoter. Pho promoters have a -10 sequences typical for the major σ^{70} form of RNA polymerase holoenzyme, and P-PhoB dependent *pstS* occurs with σ^{70} (Amemura *et al.*, 1985). The -35 regions of Pho promoters contain consensus sequences, Pho boxes, that function as PhoB-binding sites (Makino *et al.*, 1988). Phosphorylation of PhoB has also been shown to enhance binding of PhoB to the Pho Box upstream from the *pstS* promoter (Stock *et al.*, 1989).

1.4.3 Signal Transduction Pathways That Regulate PhoB

Figure 7 shows the typical phosphate uptake system as found in *E. coli*. In the Pho system, the PhoR appears to be a typical membrane receptor with an extracellular sensory domain and an intracellular signalling domain. PhoR activity was first thought to be regulated either by phosphate in the periplasm or indirectly by an interaction of the receptor with the periplasmic phosphate binding protein, PstS.

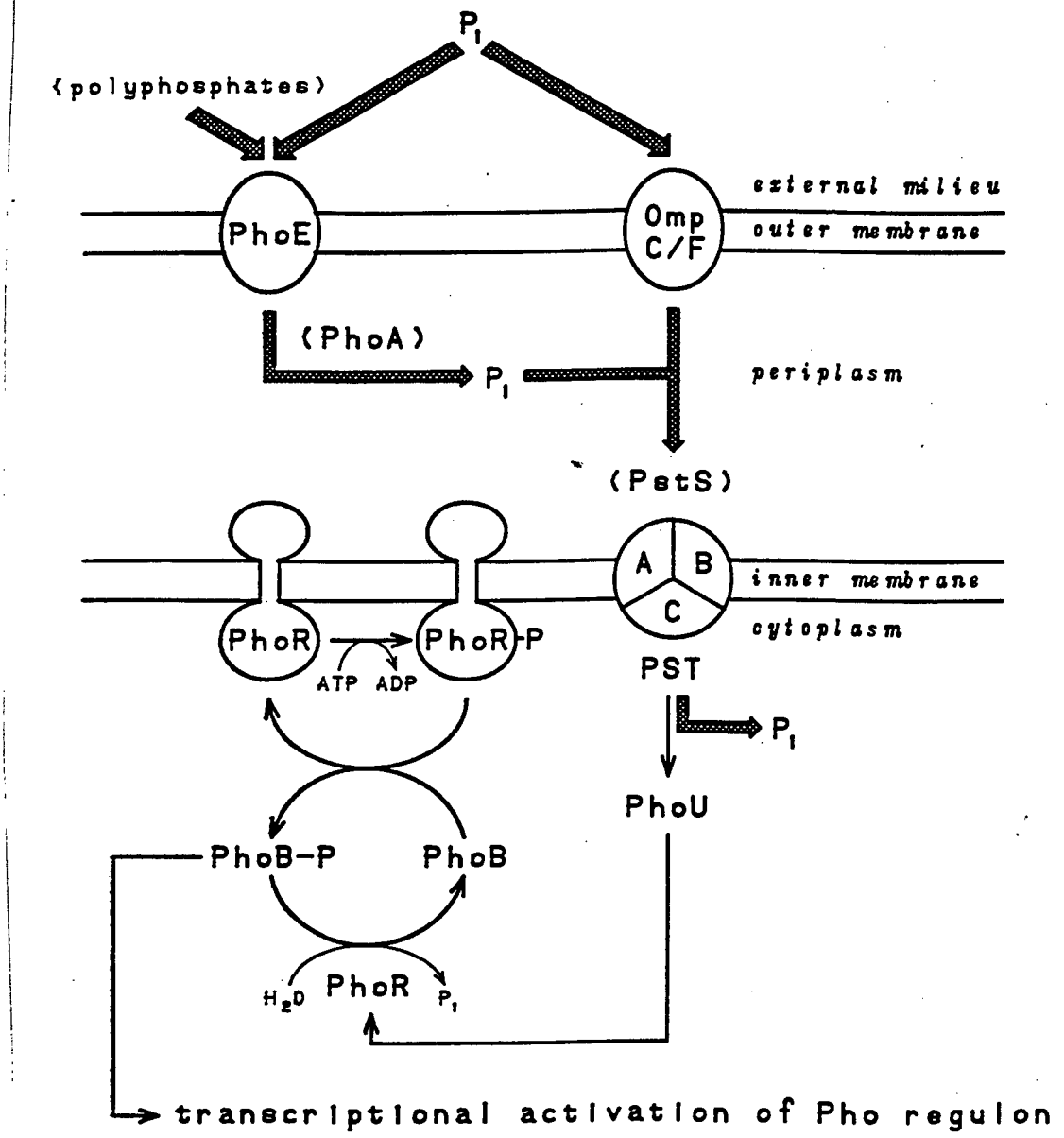

Figure 7. System that regulates phosphate uptake in *E. coli*. Extracellular P diffuses across the outer membrane through porins, PhoE, OmpF, and OmpC. Alternatively, phosphoesters in the periplasm are hydrolysed to P through the action of alkaline phosphatase, PhoA. At low phosphate concentrations, phosphate is transported via a phosphate binding protein, PstS, through the PST system 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, including *phoE, phoA, pstSCABphoU*,and *phoBR*. 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).

However, it was found that all the components of the *pst* operon are essential for Pho repression in a high phosphate environment (Amemura, 1985).

PST is a typical binding-protein-dependent bacterial transport system (Ames, 1986). It is composed of a periplasmic binding protein, PstS; a peripheral membrane protein, PstC; and 2 integral membrane proteins, PstA and PstB (Stock *et al.*, 1989). In addition, it contains a fifth gene *phoU*, that encodes a 21 kd cytoplasmic protein with no apparent homology to any other known protein (Amemura, 1985). PhoU is not essential for phosphate uptake, but is required for phosphate repression of the Pho regulon (Makino *et al.*, 1985). It thus seems that PhoU promotes the dephosphorylation of P-PhoB in response to a signal that reflects the activity of the PST system. In *phoR* mutants, *phoA* is constitutively expressed at one-third the maximal level observed in phosphate starved wild type cells (Makino *et al.*, 1985). This expression depends on the gene *phoM*. This is thought to be a typical receptor-histidine kinase. It is thought that PhoM can act in place of PhoR to provide phosphoryl groups for PhoB activation. *phoM* is not part of the Pho regulon (Wanner and McSharry, 1982) and does not seem to be controlled by PST. PhoR and PhoM probably both function to activate Pho expression by donating phosphoryl groups to PhoB. At high phosphate concentration, PhoR together with PST activates a PhoR-dependent phosphatase that dephosphorylates P-PhoB.

1.5 Osmoregulation

The osmotic strength of the environment is one of the many physical parameters that determines the ability of organisms to proliferate in a given habitat. Osmotic regulation can be defined as the active process carried out by organisms to cope with osmotic stress (Csonka, 1989).

Gram-negative bacteria are composed of two compartments, periplasm and cytoplasm. The cytoplasmic membrane is not rigid and cannot support an osmotic pressure gradient. To maintain an osmotic pressure gradient, cells must rely solely on the outer membrane complex of the cell wall. The outer wall is impermeable to

with a molecular weight greater than 600 daltons, thus polymers and other proteins are trapped within the periplasm. Among these molecules, polyanions such as membrane derived oligosacharides, support the osmotic strength of the periplasm through their attraction of high concentrations of cations such as Na^+ and K^+ (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 the two compartments (Csonka, 1989). In order to cope with osmotic stress bacteria have the ability to synthesise or accumulate compatible solutes and osmoprotectants.

1.5.1 Compatible Solutes

Exposure of cells to high external osmolarity results in the efflux of water from the interior. The decrease in the internal water content brings about a reduction in the turgor pressure and a shrinkage of the cytoplasmic volume. There is thus a concentration of intracellular metabolites which leads to a reduction in intracellular water activity. This increase in concentration of intracellular metabolites can lead to inhibitory effects on cellular processes (Waldehaug *et al.*, 1987).

Organisms generally respond to osmotic stress by increasing the concentrations of a limited number of solutes. Thus, the water activity of the cell interior can be reduced, and cell volume and turgor pressure can be restored. Compatible solutes accumulate by *de novo* synthesis or by transport from the culture medium. Molecules that are accumulated during conditions of stress are termed compatible solutes and are not greatly inhibitory to cellular processes (Brown and Simpson, 1972). Some examples of compatible solutes include K^+ ions, the amino acids glutamine, glutamate, proline, and alanine, the quaternary amines glycinebetaine, and the sugars sucrose and trehalose (Csonka, 1989; Csonka and Hanson, 1991, Lucht and Kelman, 1994). Compounds such as glycine betaine, proline and many more, which are accumulated in response to osmotic stress have specifically been called osmoprotectants. These will be discussed in section 1.5.2. Compatible solutes are unable to cross the cell membranes rapidly without the aid of transport systems.

Further, they for most part, do not carry a net electrical charge near pH7. This property can be beneficial as uncharged molecules can accumulate to a high intracellular concentrations without disturbing the structures of cellular macromolecules (Csonka, 1989; Csonka and Hanson, 1991).

Potassium Ions

Potassium ions are the most common cations in the cytoplasm of bacteria and are the major intracellular osmolytes that maintain turgor. There is a positive correlation between the intracellular content of this cation and the ability of bacteria to tolerate conditions of high osmolarity (Christian and Waltho, 1961). The signal for enhanced potassium uptake was found to be triggered as a result of loss of turgor or cytoplasmic volume (Csonka, 1989). It would seem that the intracellular concentration of potassium ions is regulated by relative rates of influx and efflux of this ion. The stimulation of both the efflux and influx processes in response to hyper- or hypo-osmolarity, occurs very rapidly and does not require an energy source (Csonka, 1989). High levels of glutathionine are required for the retention of potassium (Meury and Kepes, 1982), and the concentrations of this metabolite increase with osmotic stress (Munro *et al.*, 1972).

E. coli has a number of potassium transport systems of which two have been extensively characterized. The Trk system, which has a low affinity for potassium ions, and Kdp system, which has a higher affinity for the same ion (Walderhaug *et al.*, 1987; Walderhaug *et al.*, 1989). The former is produced constitutively, but its activity is enhanced in response to hyperosmotic shock. The latter is subjected to transcriptional regulation and its activity is also stimulated by hyperosmotic shock in a manner similar to the Trk system (Rhoads and Epstein, 1978).

Mutagenesis studies revealed that the Trk system consists of 7 loci namely *trkA, trkB, trkC, trkD, trkE, trkG* and *trkH*, which are required for potassium uptake and retention (Epstein and Kim,.1971; Walderhaug *et al.*, 1987). Bakker *et al.* (1987) has shown that *trkB* and *trkC* gene products are involved in potassium ion efflux but not

in potassium uptake. The function of the other components of the Trk system are as yet to be resolved.

The components of the Kdp system are encoded by the *kdpABC* operon, which is under positive transcriptional control by the *kdpD* and *kdpE* gene products (Epstein, 1986; Polarek *et al.*, 1987; Csonka and Hanson, 1991). The predicted amino acid sequence of the *kdpA* protein was determined and shown to have significant similarity to a mammalian sarcoplasmic Ca^{2+} ATPase. This result together with mutations in *kdpA*, which were found to cause decreased affinity of the Kdp system for potassium ions, suggests that the *kdpA* gene product is responsible for the binding of potassium ions during its transport (Siebers *et al.*, 1987).

Nucleotide sequence analysis of the *kdpD* gene indicated that the protein product resembles the transcriptional activator proteins of two component regulatory systems (Albright *et al.*, 1989). The KdpE protein could thus be its cognate signal sensor (Epstein *et al.*, 1990).

Glutamate and Glutamine

The cytoplasmic levels of glutamine and glutamate generally increase in all prokaryotes on exposure to high osmolarity (Csonka, 1989). However, glutamine is found at much lower levels than glutamate and is probably not important in maintaining cytoplasmic osmolarity (Csonka, 1989, Csonka and Hanson, 1991). Because glutamine is a precursor to glutamate biosynthesis, its accumulation in response to osmotic shock may be a consequence of increased need for the synthesis of glutamate. The accumulation of glutamate is not required for osmotic stress tolerance since *S. typhimurium* mutants have been found to overproduce proline did not have increased glutamate levels after being exposed to media of high osmolarity. They were nevertheless more tolerant of osmotic stress than the wild-type strains (Csonka, 1988).

1.5.2 Osmoprotectants

Osmoprotectants are solutes that alleviate osmotic stress when in the medium. In the bacterial kingdom only a few photoautotrophic species can carry out the complete synthesis of some of these osmoprotectants such as glycine betaine. Other species depend on exogenous glycine betaine, or its precursor (choline). Glycine betaine, proline betaine and 3-dimethylsulphoniopropionate are synthesized by cyanobacteria or algae and are found in fresh or salt water environments as a result of excretion or leakage from the producing organism. Glycine betaine, and proline betaine are present in the diet of many animals and thus available to intestinal bacteria. Glycine betaine is also excreted by the kidneys and can be taken up by uropathogenic bacteria (Csonka and Hanson, 1991).

Proline

Bacteria can accumulate proline to high intracellular concentrations by increased synthesis or by enhanced uptake from the environment. 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). 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 is a proline permease and consists of a single protein embedded in the cytoplasmic membrane (Culham *et al.*, 1993). Exposure of cells to osmotic stress results in stimulation of the activity of this permease, mainly as a result of some postranslational modification (Milner *et al.*, 1988). There is a two to three fold elevation in the steady state level of transcription of the structural gene of the ProP system upon osmotic stress, which also contributes to its increased activity. The activity of this system is also increased in response to amino acid starvation

(Anderson, *et al.*, 1980). The *proU* operon, on the other hand, encodes a multicomponent, binding-protein-dependent transport system (Gowrishanker, 1989). The ProU system is also enhanced under osmotic stress conditions. Its activity is increased 100-fold as a result of an increase in the steady state level of transcription of the *proU* operon (Dunlap and Csonka, 1985).

Glycinebetaine

Most bacteria are unable to synthesise glycinebetaine and therefore are dependent on the transport of this compound for its accumulation. The transport of glycinebetaine was observed to be stimulated by osmotic stress in a wide range of bacteria including *Enterobacteriaceae,* cyanobacteria, and *Lactobacillus acidophilus* (Csonka, 1989). This observation is unusual as glycinebetaine and proline do not have similar molecular structures. The ProU system is probably more important for the uptake of glycinebetaine as it has a higher affinity for this compound than the ProP system (Cairney *et al.*, 1985).

The ProU system of *E. coli* and *S. typhimurium* contains a periplasmic glycinebetaine binding (GBB) protein of 31 kilodaltons (kd) (Jebbar *et al.*, 1992). The purified GBB protein had no detectable affinity for proline. This suggests that a higher *in vivo* activity of the ProU system with a glycinebetaine substrate than with proline could be a consequence of the relative affinities of the binding protein for these two compounds. *proP* and *proU* double mutants did not however, prevent the recovery of cells under high osmotic shock conditions as glycinebetaine was still taken up by the cells (Csonka, 1989). These results indicate that there is an additional permease besides the ProP and ProU systems in *S. typhimurium.*

Choline

Although enteric bacteria are unable to synthesize glycinebetaine from glucose or other carbon sources, *E. coli* can convert choline to glycinebetaine under conditions of osmotic stress (Strom *et al.*, 1986). The formation of glycinebetaine involves two

oxidation steps, with glycinebetaine aldehyde as the 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, so that it requires some terminal electron acceptor, such as oxygen (Landfald and Strom, 1986). The uptake of choline is mediated by two transport systems. The first is coded for by the *betT*$^+$ gene, and the structural gene for the other has not been found.

1.5.3 Osmoregulation of the Periplasmic Space

In Gram-negative bacteria, the periplasmic space houses a number of hydrolases for macromolecular nutrients, binding proteins for metabolites, and receptors for chemotactic signals (Oliver, 1987). Because solutes of less than 600 daltons can readily diffuse into the periplasm through porin proteins located in the outer membrane, there are unique problems in the maintenance of the osmotic potential of the periplasm (Csonka, 1989; Csonka and Hansen, 1991).

The periplasmic space of enteric bacteria contains large quantities of highly ionic polysaccharides, known as membrane-derived oligosaccharides (MDO's) which are too large to diffuse through the porin proteins. In *E. coli* these molecules consist of 6 to 12 glucose units which are held together by β1,2 or β1,6 linkages. The presence of these anionic polymers in the periplasm give 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 periplasmic space (Kennedy, 1982). The synthesis of the membrane derived oligosaccharides is subject to osmotic regulation so that they are synthesized maximally in media of low osmolarity. Increasing osmolarity results in a decrease in their synthesis. In *E. coli*, the enzymes involved in the formation of membrane derived oligosaccharides are constitutive, suggesting that the osmotic control, of synthesis of these polysaccharides entails regulation of the catalytic activity of at least one of these enzymes (Kennedy, 1987). Mutations in the *mdoA* or *mdoB* gene block the synthesis of membrane derived

oligosaccharides (Fiedler and Rottering, 1988). Strains carrying these defects did not show altered growth rates to low osmolarity. This would indicate that there are alternate synthetic pathways for membrane-derived oligosaccharides for maintaining the osmotic potential of the cytoplasm. These pathways have as yet not been discovered.

1.5.4 Osmotic Control of Transcription

kdp Operon

The *kdp* operon of *E. coli* is repressed by high concentrations of potassium ions (Rhoads, 1976). It is however, induced by high concentrations of any ionic or non-polar solutes that are excluded by the membrane. Exposure of cells to hyperosmolarity in media containing concentrations of potassium ions sufficient for cells to maintain turgor resulted in only transient burst of elevated transcription of the *kdp* operon. Expression of the operon returned to its basal value 30 minutes after the hyperosmotic shock. It is thus proposed that turgor pressure is the sole signal that regulates the expression of this operon (Laimins *et al.*, 1981).

The transcription of *kdpABC* genes is under positive control of the *kdpD* and *kdpE* proteins, which are both involved in sensing the turgor and transferring the signal to the *kdpABC* promoter. The KdpD product has been proposed to span the periplasmic space and to form contacts between both inner and outer membranes (Epstein, 1986). This would seem logical as KdpD can then detect turgor pressure by measuring the distance between the two membranes and in so doing transmit a signal to KdpE, which in turn transcriptionally activates *kdpABC* operon (Csonka, 1989).

Choline is efficiently oxidized to glycinebetaine in media of high osmolarity, and the latter is a more effective compatible solute than potassium ions. If the expression of *kdp* were dependent solely on turgor, then the threshold for *kdp* repression would be expected to occur at a lower potassium ion concentration in the presence of choline chloride than in the presence of sucrose or in media of low osmolarity. This

is not the case and raises questions as to the validity of a single regulator for *kdp* expression and suggests that this operon is at least in part regulated by some factor that is related directly or indirectly to the availability of potassium ions (Gowrishanker, 1987).

proU Operon

The induction of *proU* can be triggered by solutes that are excluded by the membrane but not by substances that can freely traverse this permeability barrier. The *proU* operon is continuously induced as long as the cells are exposed to media of high osmolarity (Dunlap and Csonka, 1985). This is in contrast to the *kdp* operon.

The induction of *proU* is a three stage process. After exposure of the cells to hyperosmotic shock, there is a 15-20 minute lag before increased transcription of *proU* is detectable. There is a rapid stimulation of transcription of *proU* operon at a differential rate that greatly exceeds the steady state differential rate observed in cells growing in a medium of equal osmolarity. Finally, the differential rate of transcription of *proU* gradually decreases to a steady state value. It has also been suggested that both *kdp* and *proU* operons are regulated by a common signal (Gowrishanker, 1985).

Because *proU* is expressed as long as high osmotic conditions are maintained, transient loss of turgor alone cannot be the regulatory signal for the expression of this operon, as has been proposed for *kdp*. The water activity of the cytoplasm could then be the signal for the transcription of *proU*, but the fact that solutes that can freely diffuse across the cytoplasmic membrane do not induce *proU* indicates that some other signal governs the transcriptional regulation of this operon. Because the steady state concentration of potassium ions increases with increasing external osmolarity, Csonka (1989) proposed that the intracellular concentration of this cation might be the regulatory signal for the induction of *proU*. Since it is not fully settled as to whether osmotic stress causes merely a transient or permanent increase in the potassium ion levels of *E. coli*, no compelling proposals can yet be made for possible

regulatory roles of this cation in osmotic adaption. Higgins *et al.* (1988), have also demonstrated that supercoiling influences the level of transcription of *proU*. However, their data did not show that the osmotic control of *proU* transcription is necessarily exerted by supercoiling.

1.5.5 Regulation of Porin Expression

The inner membrane contains active transport systems and receptors that move material in and out of the cytoplasm and detect environmental signals. The permeability of the outer membrane is largely determined by channels formed by porins. There are 2 major porins in *E. coli*, OmpF and OmpC (Parkinson, 1993; Nikaido, 1994). OmpF and OmpC provide relatively nonspecific pores that facilitate movement of small hydrophilic molecules across the outer membrane. A system for the regulation of OmpC and OmpF is outlined in Figure 8.

E. coli, *S. typhimurium*, and many other Gram-negative bacteria are able to alter their porin composition of their outer membranes in response to change environmental conditions, and in so doing, change their permeability to solutes in the surrounding medium (Forst and Inouye, 1988). In *E. coli* the relative amounts of these porins are regulated in response to a number of environmental stimuli, which include medium osmolarity, temperature and carbon source. OmpF and OmpC are regulated in a reciprocal manner so that the total level of porin protein remains constant (Schnaitman, 1974).

The regulation of porin expression in relation to osmolarity and temperature has been ascribed to the differing environmental niches of a bacteria. For example, *E. coli* infects the intestinal tract of animals and is thus exposed to pH variation, relatively high temperatures and high osmolarity. Under these conditions OmpC expression is favoured in place of OmpF. This would seem logical as the smaller pore size of OmpC would significantly reduce the diffusion of larger hydrophobic and negatively

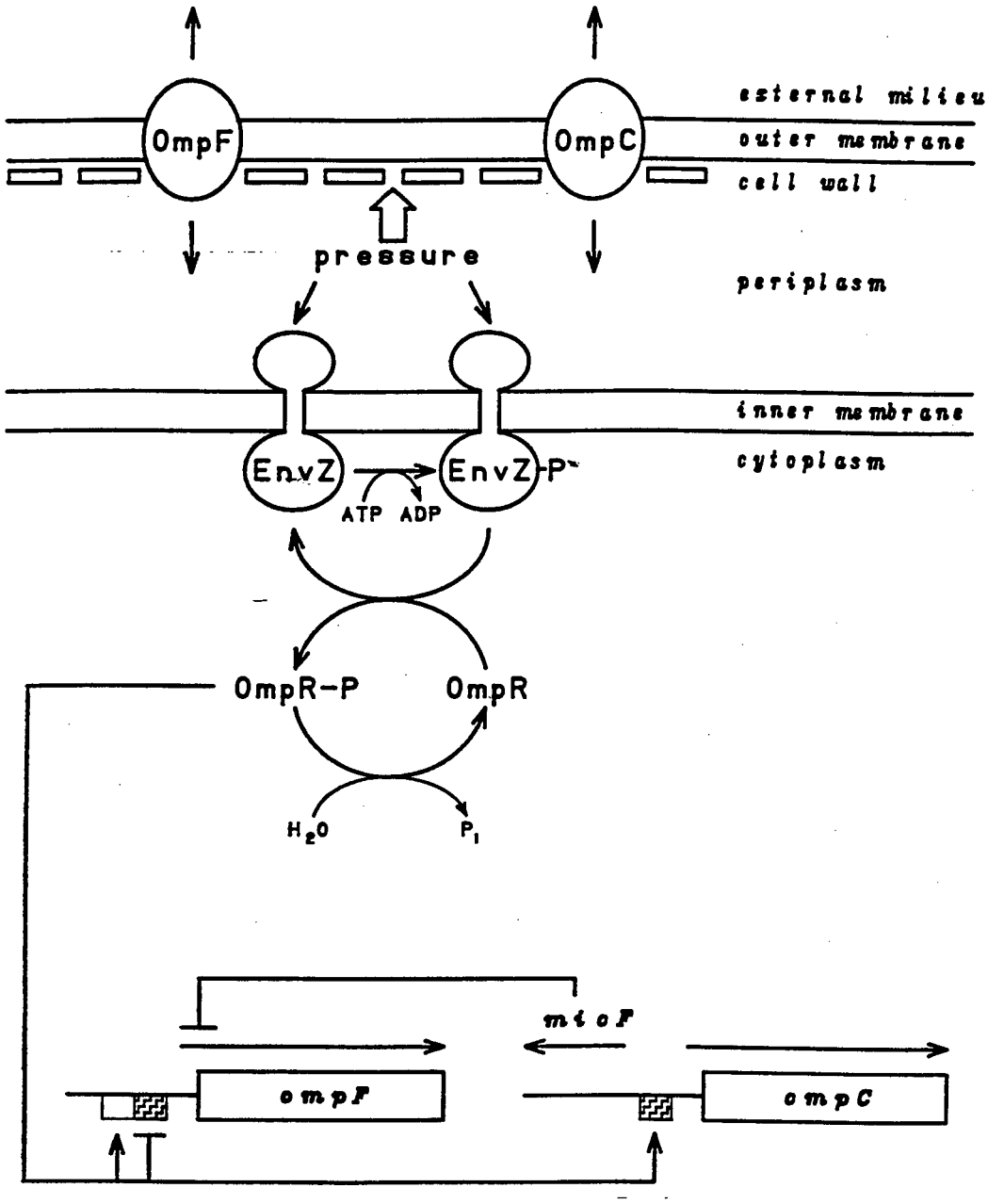

Figure 8. System that regulates expression of the porins OmpF and OmpC in *E. coli* and *S. typhimurium* outer membranes. The EnvZ receptor-kinase catalyses the phosphorylation of the transcriptional regulator OmpR in response to osmotic stress. Phosphorylated OmpR in turn controls porin expression. Open headed arrows denote control of the indicated signalling steps (Parkinson, 1993). K and P represent the kinase and phosphatase activities of EnvZ.

charged molecules through the membrane (Nikaido and Vaara, 1985). In the external environments with lower temperatures, pH, osmotic strength and concentrations of nutrients, the larger channel of OmpF may have a distinct advantage.

Biochemical and genetic studies have established that *ompF* and *ompC* encode the major *E. coli* porins and that the *ompB* locus encodes a positive activator required for *ompF* and *ompC* expression (Sato and Yura, 1979; Verhoef *et al*, 1977). It has also been established that *ompB* mediated porin regulation occurs at the transcriptional level (Hall and Silhavy, 1979). DNA sequence analysis has shown that the *ompB* locus is comprised of two genes, *ompR* (outer membrane protein regulation) and *envZ* (environmental sensing protein)(Hall and Silhavy, 1981).

1.5.6 Roles of EnvZ and OmpR

EnvZ is a 450-amino acid inner membrane protein which functions as an osmosensor. EnvZ is composed of a 115 amino acid periplasmic domain and a 270 amino acid cytoplasmic histidine kinase domain. The periplasmic domain of EnvZ is essential for the detection of changes in medium osmolarity (Tokishita *et al.*, 1991) and is generally assumed to be the input domain. However, how it senses osmolarity is still very much a mystery (Parkinson, 1993). Procaine has been found to mimic the effect of high osmolarity (Rampersaud and Inouye, 1991), suggesting that EnvZ may somehow sense membrane curvature or fluidity. EnvZ controls the activity of OmpR, the response regulator of the porin expression system.

OmpR is a 239 amino acid protein that is homologous over its entire length to the family of transcriptional regulators that includes PhoB, ArcA, PhoM-ORF2, VirG, PhoP, and TctD (Stock *et al.*, 1989). EnvZ is autophosphorylated in the presence of ATP and has been shown to involve a phosphohistidine linkage (Aiba *et al.*, 1989).

The phosphoryl group in EnvZ is readily transferred to OmpR (Aiba *et al.*, 1989). This phosphorylation event, which occurs within the N-terminal end of OmpR,

enhances the ability of the C-terminal region to bind to sequences upstream of the *ompF* and *ompC* promoters (Mizuno *et al.*, 1988).

Studies with purified components indicate that the cytoplasmic domain of EnvZ, by itself, catalyses the dephosphorylation P-OmpR (Aiba *et al.*, 1988). This activity resembles the P-NR_I dephosphorylating activity of NR_{II} in that the reaction requires ATP. In the case of EnvZ, it has been shown that the phosphatase reaction does not require ATP hydrolysis since ADP as well as several non-hydrolysable analogs can act in place of ATP in the EnvZ-dependent phosphatase reaction (Aiba *et al.*,1988).

More recently the importance of the two trans-membrane spanning regions of EnvZ, designated TM1 and TM2, have been elucidated. Mutations in TM1 yielded a phosphatase defective activity but did not alter kinase activity. In addition other mutations resulting in amino acid substitutions in TM1 and TM2 yielded both kinase and phosphatase defective activities (Tokishita and Mizuno, 1994). These results show the intimate intermolecular interaction between the membrane spanning segments of EnvZ to be crucial for transmembrane signalling in response to an external osmotic stimulus (Tokishita and Mizuno, 1994).

The *ompF* promoter, located approximately 110 bases upstream from the *ompF* ATG initiation codon, has a conventional Pribnow box at -12 to -7 and a -35 region that has weak homology to the consensus sequence (Inokuchi *et al.*, 1984; Taylor *et al.*, 1985). Deletion analyses have defined an essential upstream region extending to -90 that is required for expression from *ompF* promoter (Inokuchi *et al.*, 1984). DNA footprinting studies with purified OmpR protein have established multiple sites of OmpR binding between -40 and -95 (Maeda and Mizuno, 1988). These binding sites must be close to the -35 consensus regions of the *omp* promoters for proper regulation (Maeda and Mizuno, 1990), suggesting that OmpR controls transcription by influencing binding of RNA polymerase to the promoter (Parkinson, 1993). Some evidence supporting this theory are the findings of Slauch *et al.* (1991), who showed that mutations in *rpoA*, which encodes the α subunit of RNA polymerase, influence the ompR dependent transcription of *ompF* and *ompC*.

A similar genetic analysis as well as footprinting studies with OmpR indicate that the *ompC* promoter has a structure that is similar, but not identical, to that of *ompF* promoter. Sites for OmpR binding and activation are localized within a region extending from -35 to -95 base pairs 3' of the site of transcriptional initiation (Maeda and Mizuno, 1988).

At present it seems likely that the osmotically regulated switch from OmpF to OmpC is due to change in the level of P-OmpR. Both unphosphorylated and phosphorylated OmpR bind to the target sites, but P-OmpR has a higher affinity (Aiba *et al.*, 1989). Phosphorylation may promote oligolimerization of OmpR, which is normally monomeric, thereby enhancing its ability to bind at tandemly repeated target sites (Nakashima *et al.*, 1991). Genetic studies indicate that *in vivo*, the *ompF* promoter is more efficiently activated at relatively low OmpR phosphorylation states, whereas at high P-OmpR levels, the *ompC* promoter is activated and the *ompF* is repressed (Russo and Silhavy, 1991). The OmpF and OmpC expression patterns of different EnvZ and OmpR mutants are largely consistent with this control model (Russo and Silhavy, 1991). However, it has been shown through these mutants that OmpR can ordinarily acquire some of its phosphates from other sources (Forst *et al.*, 1990). At elevated levels of OmpR, osmoregulation of porin expression in the absence EnvZ was similar to that seen in wild-type cells. One explanation for this is that alternative histidine kinases can act in place of EnvZ to phosphorylate OmpR. The possibility of cross-talk has been demonstrated by showing that OmpR phosphorylation and transcriptional activation of the *ompF* promoter can be accomplished *in vivo* by using either the CheA or NR_{II} kinases in place of EnvZ (Igo *et al.*, 1989). This low level cross-talk may connect the osmoregulatory circuitry to other physiologically relevant signalling pathways.

All the sensory mechanisms of microbes are similar in design and yet capable of high affinity signal transduction. It has also been found that higher cells exhibit similar signalling tasks and may handle them with similar molecular signalling mechanisms. Bacterial signalling systems may well be universal in all signal transduction schemes and play a vital role in understanding signal transduction systems in higher cells.

1.6 *Erwinia chrysanthemi* and its Environment

Erwinia chrysanthemi is a ubiquitous plant pathogen and is known to infect a wide range plant species including potatoes, maize and other agriculturally important crops. It is thus part of a wide range of plant associated bacteria which include the agrobacteria, clavibacter, pseudomonads, rhizobia, streptomycetes, xanthomonads. All these organisms also possess the ability to survive in association with the host plant or as free living forms apart from the host.

E. chrysanthemi has the ability to produce a wide variety of enzymes, including pectinases, pectate lyase and cellulases, which are used to break down plant cell wall components resulting in the common disease known as bacterial soft rot (Chatterjee and Starr, 1980). Although much is known about the enzymes produced on infection of host plants and export of these enzymes, little is known about the organism's ability to adapt and sense its environment. Research has been done relating pectinase gene expression to differing environmental conditions (Hugouvieux-Cotte-Pattat *et al.*, 1992), as well as the effect of osmotic stress on pectate lyase secretion and transcription (Mildenhall *et al.*, 1988).

The underlying questions are thus, how does *E. chrysanthemi* respond to differing nutrient and osmotic stresses that it may incounter within or outside the host plant? Further, what are the underlying mechanisms that allow for the sensing of these environmental changes? Although much work has been done on *Staphylococcus aureus* (Bayles, 1994), *Escherichia coli* and *Salmonella typhimurium* (Csonka, 1989; Csonka, 1991; Parkinson and Kofoid, 1992; Parkinson, 1993; Stock *et al.*, 1989; Tokishita and Mizuno, 1994) relatively little has been done to answer these questions for the plant pathogen *E. chrysanthemi.* Research done to date on *E. chrysanthemi* has focused on environmental stimuli which affect enzyme secretion and transcription activation (Hugouvieux-Cotte-Pattat *et al.*, 1992) or inhibition of secretion (Mildenhall *et al*, 1988) of these plant degradative enzymes. It would thus be beneficial to obtain a better understanding of the various sensory mechanisms in *E. chrysanthemi*, that allow for their response to osmotic and nutrient stresses.

CHAPTER 2

Isolation of the Major Outer Membrane Proteins of *Erwinia chrysanthemi* Under Different Environmental Conditions

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2.1 SUMMARY

Outer membrane proteins (OMPs) of *E. chrysanthemi* were extracted using sodium lauroylsarcosine (SLS). Three major OMPs were isolated, namely Omp1 (37.5 kd), Omp2 (35.5 kd) and Omp3 (34.5 kd).

OMP profiles varied with the culture growth phase. Two additional outer membrane proteins were observed once mid log phase was reached. These two additionally expressed proteins may be involved in scavenging for limiting compounds, such as iron. Growth temperatures had no affect on the expression of the major OMPs. However, increased medium osmolarity resulted in the enhanced expression of Omp3 and the suppression of Omp1. Omp2 was unaffected by changes in growth conditions. An additional OMP was found to be expressed when *E. chrysanthemi* was grown on polygalacturonic acid (PGA) as the sole carbon source. This OMP, designated OmpG (27 kd), is thought to enhance the uptake of mono- and di-galacturonic acid, the breakdown products of PGA or pectin.

2.2 INTRODUCTION

The main function of the outer membrane of Gram negative bacteria is to act as a permeability barrier. Because the outer membrane excludes molecules larger than 600 daltons (Decad and Nikaido, 1976), the bacteria must somehow exchange nutrients and waste products with their environment. Much of the flux takes place through different groups of protein channels. These include porins, some of which allow nonspecific and spontaneous diffusion of small solutes. Another group of specific channels contain specific ligand binding sites within the channel and thus facilitate the uptake of certain compounds. A third type of channel forming protein are the high affinity receptor proteins. These proteins, together with the TonB coupling protein in the periplasmic space, facilitate energy-coupled translocation of large nutrient molecules that exist in very low concentrations in the environment (Nikaido, 1994; Kadner, 1990, Postle, 1990).

Porins consist of open, water filled channels that allow the influx of small, hydrophilic molecules (Nikaido and Nakae, 1979). Porins are usually stable trimeric structures and are highly permeable to small hydrophilic molecules (Hancock, 1987). Electron microscopy studies and atomic force microscopy (AFM) have shown that the trimer contains three separate openings at the surface, which unite into a single channel near the centre of the membrane (Engel *et al.*, 1985, Schabert *et al.*, 1995). Two examples of porins are OmpF and OmpC of *E. coli*. These are composed of protein subunits of molecular weights between 36,000-38,000 daltons. Similar trimeric porins are widespread among various evolutionary branches of Gram-negative bacteria (Nikaido, 1992). The estimated average pore size for *E. coli* OmpC and OmpF porins are 10 and 12Å respectively. Porin channels have also been found to have charge preference. OmpF and OmpC porins prefer neutral molecules and cations over anions. In contrast, the PhoE porin of *E. coli*, as well as the major porins of *Neisseria*, show a preference for anions (Nikaido, 1994). All porins from *E. coli* exclude lipophilic solutes (Parkinson, 1993).

2.2.1 Regulation of Porin Expression

The synthesis of outer membrane proteins is complex. In *E. coli,* changes in osmotic potential of the external environment is sensed by the cytoplasmic membrane protein EnvZ. The latter phosphorylates a cytoplasmic regulator protein, OmpR, which represses the synthesis of OmpF and increases that of OmpC (Stock *et al.*, 1989). OmpC produces a channel slightly smaller than that of OmpF. This is thought to benefit *E. coli* by decreasing the effects of the high osmotic potential simply by slowing down the efflux of water from the cell into the environment. As a result, *E. coli* can survive within the intestinal tract in the presence of bile salts.

E. coli is also able to sense temperature and pH fluctuations. Under conditions of relatively high temperatures and low pH, there is an increased expression of OmpC (Nikaido and Vaara, 1985, Heyde *et al.*, 1988, Thomas and Booth, 1992). Similarly, the *E. coli* PhoE porin, which permits the rapid influx of phosphate and phosphorylated compounds, is expressed when the organism is starved of phosphate (Nikaido, 1994).

Although the outer membrane proteins of *E. coli* and *S. typhimurium* have been well characterised, very little is known about the major outer membrane proteins of *E. chrysanthemi* and their respective expression under different environmental conditions. Outer membrane proteins have been implicated in iron acquisition for *E. chrysanthemi* and are thought to be important in the virulence of the organism (Expert and Toussaint, 1985). The possibility thus exists that certain porins may be differentially expressed under varying environmental conditions such as osmotic and temperature differences. These adaptions may be vital for *E. chrysanthemi* to establish upon invasion of the host plant. This study was therefore undertaken to isolate and partially characterise the outer membrane proteins of *E. chrysanthemi* and determine the expression of these porins under different environmental conditions.

2.2.2 Induced Outer Membrane Proteins

Some nutrient molecules are too large to enter through non-specific porins. For example, the uptake of maltose and maltodextrins is too slow in *E. coli* for the organism to use this compound as a carbon source due to their size. Specific channels are able to enhance the uptake of these compounds across the outer membrane. Some examples of these specific channels in *E. coli* include LamB, which enhances the uptake of maltose and maltodextrins, (Lucky and Nikaido, 1980) and ScrY, a sucrose channel (Schulien *et al.*, 1991). *Pseudomonas aeruginosa* lacks classical porins entirely and the need for specific channels is even greater. A number of specific porins thus exist for this organism and include OprB, a glucose channel, OprD, an amino acid channel, and OprP, a phosphate channel (Trias *et al.,* 1988; Trias and Nikaido, 1990; Hancock, 1990). The above porins have specific ligand binding sites within their channels. These channels also allow for non-specific diffusion of small hydrophilic compounds across the outer membrane.

2.2.3 *Erwinia chrysanthemi* and its Environment

E. chrysanthemi must survive within a host organism as well as independently from the host in the environment. The outer membrane of *E. chrysanthemi* might allow the bacterium to adapt to and survive under these different environmental conditions. The role of outer membrane proteins (OMPs) in virulence has also been reported for *E. chrysanthemi* (Expert and Toussaint, 1985). Water activity, as determine by solute concentration, is also thought to play a vital role in pathogenesis (Colhoun, 1973). The association of maize stalk rot and the availability of water suggested that *E. chrysanthemi* is sensitive to desiccation (Hoppe and Kelman, 1969). A number of studies were subsequently conducted to note the response of *E. chrysanthemi* to changing water activity (Mildenhall *et al.*, 1981; Mildenhall and Prior, 1983; Mildenhall *et al.*, 1988; Hugouvieux-Cotte-Pattat *et al.,* 1992; Prior *et al.*, 1994; Gouesbert *et al.*, 1995). These studies have shown contrasting results. Mildenhall *et al.* (1988) demonstrated that high osmolarity has a negative effect on pectate lyase secretion. However, increased medium osmolarity retarded the secretion of pectate

lyase but did not inhibit its transcription. Hugouvieux-Cotte-Pattat *et al.* (1992) found that increased osmolarity enhanced extracellular pectate lyase. This increase in pectate lyase production was associated with the induction of a major pectate lyase gene, *pelE*. In all cases, the addition of the osmoprotectants, proline, glycine betaine and pipecolic acid overcame the effects of high osmolarity. These osmoprotectants also alleviated the effect of high salt concentrations on the growth of *E. chrysanthemi* (Gouesbet *et al.*, 1995).

Since water potential plays a vital role in virulence, and OMPs are affected by changes in water potential in *E. coli*, a fuller understanding of the role of OMP in the initial interaction, virulence and subsequent survival of *E. chrysanthemi* in its host would be beneficial.

Like most plant pathogenic bacteria, *E. chrysanthemi* has the ability to use pectin and pectate as a carbon source of which the break down products are mono- and di-galacturonic acid. Pectin is degraded by the pectinase and pectate lyase enzymes whose secretion is affected by medium osmolarity (Gouesbet *et al.*, 1995; Mildenhall *et al.*, 1988). Mono- and di-galacturonic acid could possibly be taken up *via* induced OMPs with distinct ligand binding sites. These OMPs would thus enhance the uptake these compounds making them readily available for use as a carbon source. The induction of specific OMP for the above mentioned products was thus also investigated.

2.3 MATERIALS AND METHODS

All referenced techniques are outlined in Appendix A.

2.3.1 Bacterial Strains and Media

Erwinia chrysanthemi JM1 was supplied by Professor J. P. Mildenhall of Fort Hare University, South Africa. Cultures were maintained on LB plates at 4°C and a single colony was resuspended in sterile distilled water for long term storage at room temperature. *E. chrysanthemi* was grown on minimal media supplemented with 0.36% sodium polypectate (NaPP), to determine the presence of an induced outer membrane protein. *E. chrysanthemi* was also grown in minimal media supplemented with 0.5% glycerol for all other experiments. Glycerol was chosen as the carbon source as it does not induce any outer membrane proteins in *E. coli* (Lugtenberg *et al.*, 1976). *E. coli* K12 was used as a control for OMP extractions.

2.3.2 Growth Conditions

E. chrysanthemi was grown in 5 ml broths of minimal media supplemented with 0.5% glycerol at 30°C overnight (O/N). This temperature was found to be the optimum growth temperature for *E. chrysanthemi*. 1 ml of this culture was inoculated into 100 ml minimal media broths for further analysis.

Liesman *et al.* (1995) found certain OMPs were expressed at different culture growth phases of *Xenorhabdus nematophilus*. To determine whether this was the case in *E. chrysanthemi*, OMPs were isolated from bacteria at various stages of culture growth and samples were compared. Cells were harvested at early and mid log phase (OD_{600}=0.4 and 0.7) and stationary phase (OD_{600}=1.38). The growth curve of *E. chrysanthemi* was determined at 30°C. Samples were taken every 2 hours and the OD was determined using a Beckman DU-64 UV spectrophotometer at 600 nm.

To determine the effect medium osmolarity on the expression of the major outer membrane proteins of *E. chrysanthemi*, cells were grown under normal growth conditions (minimal media plus 0.5% glycerol) and conditions of high osmotic potential, by the addition of 0.3M NaCl.

E. chrysanthemi was also grown in minimal media at 30 and 37°C to determine the affect of temperature on the OMP profile.

To determine the presence of an induced OMP under different environmental conditions, *E. chrysanthemi* was also grown at 30°C in minimal media with NaPP as the sole carbon source.

2.3.3 OMP Extraction

Outer membrane proteins from *E. chrysanthemi* were extracted using the techniques of Ditandy and Imhoff (1993) and Lohia *et al.* (1984) with modifications (Mizuno, pers. comm.). The extraction of *E. coli* outer membrane proteins was also carried out using the latter technique as a control. The former technique separates the outer membrane from the cell using NaCl and sucrose resulting in murienoplasts. The latter technique requires the cells to be lysed by sonication and then treated with sodium lauroylsarcosine. Both techniques then allow for the selective isolation of OMP's.

Protein concentrations of extracted OMPs were determined using Bradfords reagent (Bradford, 1976), with bovine serum albumin as a standard. Proteins were denatured by adding an equal volume of loading buffer (Sambrook *et al.*, 1988). OMPs were further denatured by heating in a boiling water bath for 10 minutes before electrophoresis. Protein samples (10 μg) were electrophoresed in a 12% denaturing polyacrylamide gel as described by Laemmli (1970). The gel was stained with Coomasie Brilliant Blue (Sigma) as described by Sambrook *et al.* (1988). Pharmacia low molecular weight standard markers were used to determine protein sizes. The molecular weight markers consisted of phosphorylase b (94 kd), bovine serum

albumin (67 kd), ovalbumin (43 kd), carbonic anhydrase (30 kd), soybean trypsin inhibitor (20.1 kd) and ∝-lactalbumin (14.4 kd).

2.4 RESULTS AND DISCUSSION

2.4.1 Outer Membrane Protein Profile of *E. chrysanthemi*

The use of sodium lauroylsarcosine (SLS) for the selective isolation of outer membrane proteins has been extensively used (Filip *et al.*, 1973, Lohia *et al.*, 1984, Thurn and Chatterjee, 1982). The outer membrane protein profile of *E. coli* obtained using this technique corresponded favourably to reported literature thus confirming the reliability of the above SLS OMP extraction technique. As the isolation of *Erwinia amylovora* outer membrane proteins has already been achieved (Thurn and Chatterjee, 1982) using SLS, it was assumed that this technique would also result in successful isolation of OMP of *E. chrysanthemi.* As depicted in Figure 1, this isolation method yielded 3 distinct major OMPs. An alternate OMP isolation technique was also carried out to substantiate the results obtained when using SLS. The technique of Ditandy and Imhoff (1993) yielded similar results (Figure 1). Yields (μg/ml cell culture) of OMP were much lower for the same technique (data not shown). The sodium lauroylsarcosine technique was therefore used in all further experiments.

Table 1 lists the molecular weights of the major outer membrane proteins of *E. coli, Salmonella typhimurium, Erwinia amylovora,* and *E. chrysanthemi.* The sizes of the major outer membrane proteins of *E. chrysanthemi* were determined from calculating the R_F values for each protein, and that of the molecular weight markers. This information was subsequently processed on DNAFRAG version 2.1. The relative sizes of the three major outer membranes were determined to be 37.5kd (Omp1), 35.5 kd (Omp2) and 34.5 kd (Omp2). It is interesting to note that the molecular weights of these major OMP of *E. chrysanthemi* are similar to those recorded for the bacterial species mentioned in Table 1. The significance of this is as yet unknown.

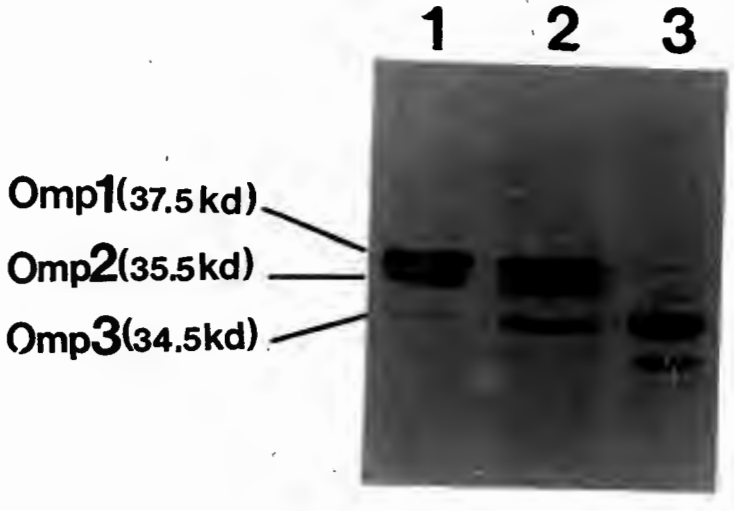


Figure 1. Outer membrane protein profile of *E. chrysanthemi* as extracted using the methods of Ditandy and Imhoff (lane 1) and Lohia and Mizuno (lane 2). *E. coli* OMP profile (lane 3).

Table 1. Major outer membrane proteins of *E. coli* (van Alphen and Lugtenberg, 1977), *S. typhimurium* (Nurminen et al., 1976), *Erwinia amylovora* (Thurn and Chatterjee, 1982) and *E. chrysanthemi.*

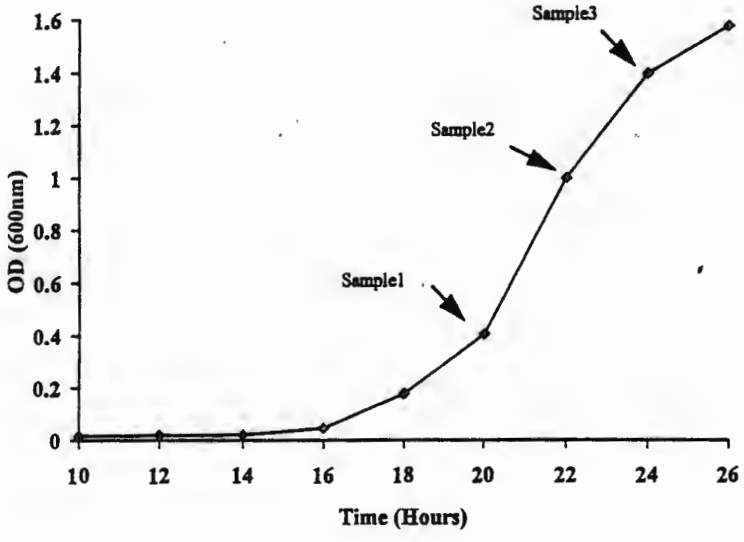
E. coli	*S. typhimurium*	*E. amylovora*	*E. chrysanthemi*
OmpF (37kd)	OmpC (36kd)	OmpX (40kd)	Omp1 (37.5kd)
OmpC (36kd)	OmpF (35kd)	OmpY (38kd)	Omp2 (35.5kd)
OmpA (35kd)	OmpD (34kd)	OmpZ (14.8kd)	Omp3 (34.5kd)

Differences in OMP profiles of *E. chrysanthemi* was also observed depending on the culture growth phase. Once the OD_{600} reaches 0.7 (mid log phase) two additional high molecular weight OMP of 74 and 79 kd are expressed (Figure 2a and b). This result is consistent with observations made by Liesman *et al.* (1995) on *Xenorhabdis nematophilus*. Because the *E. chrysanthemi* cells were grown in minimal media, it is hypothesized that these OMP may assist the bacteria in scavenging for nutrients such as iron which may be limiting. Expert and Toussaint (1985) found that iron limiting media enhances the production of a number of high molecular weight outer membrane proteins. The sizes of these proteins were determined to be between 78 and 90 k. A similar effect has been noted in *E. coli* (Lugtenberg et al., 1976), but the function of these additionally expressed OMP is also as yet unknown .

2.4.2 OMP Profile Under Different Environmental Conditions

Osmolarity, temperature and pH, have a well documented effect on OMP expression in *E. coli* (van Alphen and Lugtenberg, 1977; Lundrigan and Earheart, 1984; Heyde *et al.*, 1988). These environmental conditions alter the expression of OmpC and OmpF of *E coli.* It is thought that these adaptions assist *E. coli* in surviving in the intestine where high osmotic conditions and higher temperatures prevail. In *E. coli,* an increase in environmental temperature and osmolarity results in an increase in expression of OmpC. Conversely, a decrease in temperature and osmolarity causes an increase in the expression of OmpF. However, the absolute amount of the two porins in the outer membrane remain constant while the relative ratios vary.

a)



b)

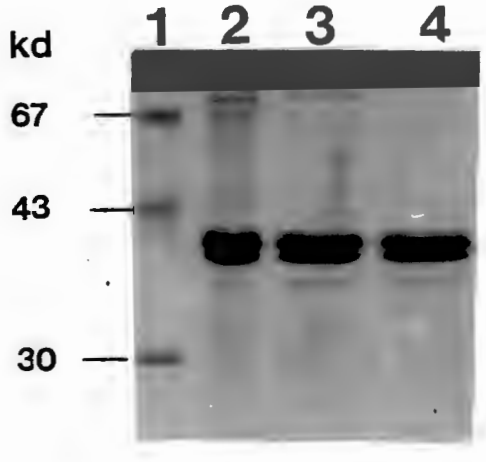


Figure 2. Growth curve and outer membrane profile of *E. chrysanthemi* at different growth phases. a) Growth curve of *E. chrysanthemi* and the respective sampling points (arrows); b) Molecular weight markers (lane 1). OMP profiles of the sampled points. Samples were taken at OD_{600} 1.38 (lane 2), 0.7 (lane 3) and 0.4 (lane 4).

The OMP profile of *E. chrysanthemi* did not change when cells were grown at 30 and 37°C respectively (Figure 3). This is in contrast to reported literature for *E. coli* (Stock *et al.*, 1989; Parkinson, 1993; Lundrigan and Earheart, 1984, Lugtenberg *et al.*, 1976). However, higher temperatures may affect the outer membrane profile as *E. chrysanthemi* has been observed to grow well at 37°C (Holt *et al.*, 1984). Alternatively, *E. chrysanthemi* may not have the need to sense temperature as both environmental and host temperatures are approximately the same. Conversely, *E. coli* must adapt to the high temperatures of the gut relative to the external environment and thus has developed a sensing system for this purpose.

Under relatively high osmotic conditions, a different OMP profile was observed for *E. chrysanthemi* when compared to the profile obtained from normal growth conditions (Figure 4a). Under these conditions, Omp3 is induced while Omp1 is suppressed. These porins could be the counterparts of OmpC and OmpF in *E. coli* respectively. The relative amount of Omp2 does not change and probably has the same structural function as OmpA of *E. coli.*

Scanning the polyacrylamide gel with a GS 300 reflectance\absorbance (585 nm) scanning densitometer (Hoefer Scientific Instruments), allowed the calculation of the percentage area variation in the expression of Omp1 and Omp3 (Figure 4b). The values of the percentage areas calculated from Figure 4b can be seen in Table 2. These results demonstrate the magnitude of the induction of Omp3 (≈ 5X greater) and the repression of Omp1 (≈ 1.6X lower).

From the above results, it appears that *E. chrysanthemi* has the ability to sense changing osmotic conditions in the environment. A signal transduction system similar to that of OmpB in *E. coli* might thus be present in *E. chrysanthemi*. It is also clear that Omp1 and Omp3 of *E. chrysanthemi* are expressed in a similar fashion to OmpF and OmpC of *E. coli* respectively. It would also seem that Omp2 may be similar to OmpA of *E. coli* as its expression was not affected by alterations in environmental conditions.

Table 2. Percentage area differences of the expression of the major outer membrane proteins Omp1, Omp2, and Omp3 of *E. chrysanthemi* as determined by GS 300 reflectance/absorbance scanner of the 12% denaturing polyacrylamide gel. *E. chrysanthemi* was grown in minimal media and minimal media supplemented with 0.3M NaCl to determine the effects of osmolarity on the expression of the major outer membrane proteins.

Major OMP's	Normal Growth Conditions (% Area)	Growth at High Osmolarity (% Area)
Omp1	55.2	35.6
Omp2	40.5	41.0
Omp3	4.3	22.4

2.4.3 Substrate Induced OMP

Unlike OmpC and OmpF of *E. coli* which have a low specificity for solutes (Nikaido and Vaara, 1985), other induced porins are specific for certain solutes. Of these, the best studied are the LamB and PhoE porin proteins of *E. coli*. These are specific for maltose and phosphate uptake in *E. coli* respectively (Szmelcman and Hofnung, 1975, Stock *et al.*, 1989). Since *E. chrysanthemi* has the ability to produce pectate lyase in response to macerated plant extract which contains pectin (Bourson *et al.*, 1992), as well as the addition of polygalacturonic acid (PGA) (Mildenhall *et al.*, 1981), it is probable that an induced OMP may also be present to facilitate the uptake of mono- and di-galaturonic acid. The latter two compounds are the breakdown products of polygalacturonic acid and pectin, which are important structural constituents of plant cell walls. In the presence of PGA, an additional OMP (OmpG) was observed relative to normal growth conditions (Figure 5). The molecular weight of this protein was determined to be 27 kd. As with LamB, which enhances the uptake of maltodextrins and maltose, it is suspected that the induced OMP, OmpG, would enhance the uptake of both mono- and di-galacturonic acid.

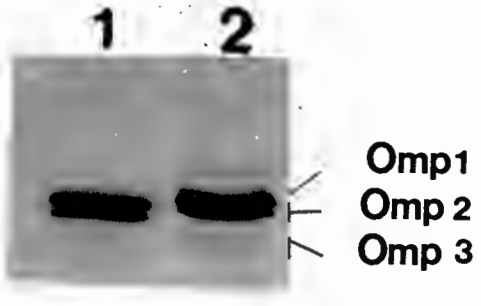
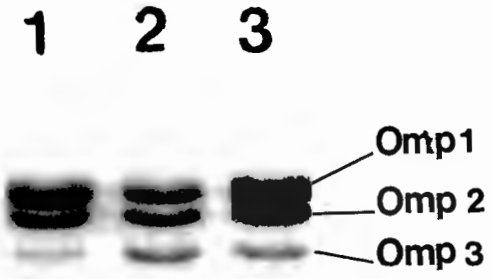


Figure 3. OMP expression of *E. chrysanthemi* at different growth temperatures. Cells were grown at 37°C (lane 1) and 30°C (lane 2) in minimal media.

a)



b)

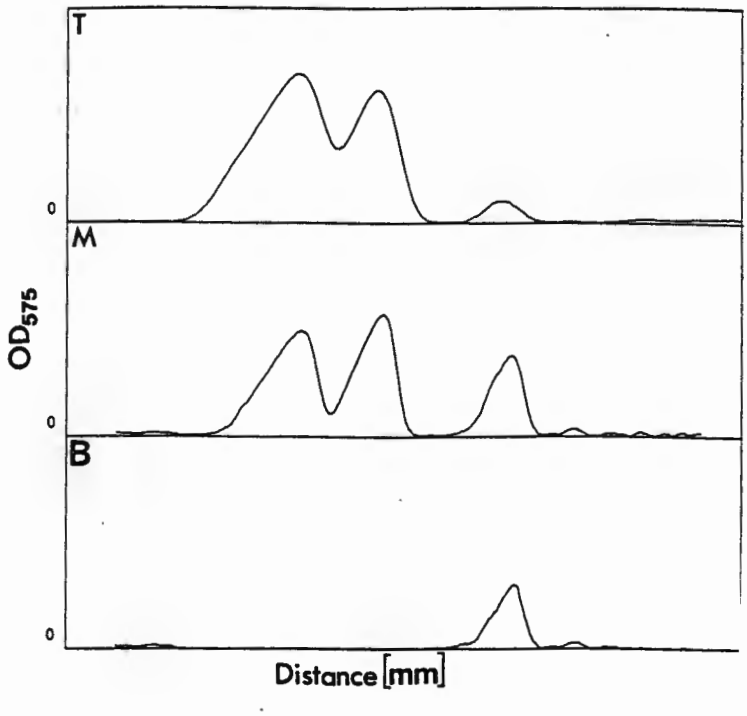


Figure 4. The differential expression of major OMP of *E. chrysanthemi* under high osmotic conditions. a) Cells were grown in minimal media with no NaCl at 30°C (lane 1). Cells were also grown at 37°C (lane 3) and 30°C (lane 2) in minimal media with the addition of 0.3M NaCl. b) GS 300 densitometer scan of the polyacrylamide gel of lane 1 (T=top) and lane 2 (M=middle). The bottom (B) graph shows the difference between the top and middle scan emphasising the induction of Omp3.

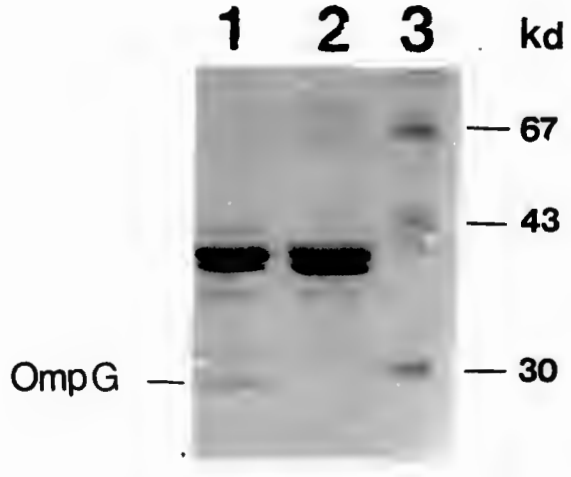


Figure 5. Expression of an induced OMP in *E. chrysanthemi* when cells were grown in the presence of 1.8% PGA in minimal media (lane 1) and the absence of PGA (lane 2). Molecular weight marker (lane 3).

Environmental conditions have been found to effect the expression of pectinase genes in *E. chrysanthemi* 3937 (Hugouvieux-Cotte-Pattat *et al.*, 1992), but variation in environmental conditions did not induce OmpG (Figures 3 and 4). It would appear that OmpG is induced by the presence polygalacturonic acid, as no other growth conditions induced OmpG. The ability of *E. chrysanthemi* to enhance the uptake of galacturonic acid *via* OmpG would have a direct effect on its pathogenicity. Pathogenicity is associated with nutrient availability and the ability to take up those nutrients (Expert and Toussaint, 1985). The absence of the OmpG porin may thus affect the uptake of mono and di-galacturonic acid and in so doing limit the rate of growth of *E. chrysanthemi.* This would ultimately reduce the virulence of *E. chrysanthemi.* The role of OmpG in pathogenicity or virulence and galacturonic acid uptake thus warrants further investigation.

In summary, the isolation of the outer membrane proteins of *E. chrysanthemi* produced 3 major outer membrane proteins. The protein sizes are 34.5 kd, 36.5 kd and 37.5 kd, and are similar in size to OmpC, OmpF and OmpA, respectively, of *E. coli.* Further, there is an alteration in OMP expression when cells are subjected to high osmotic conditions. Growth at 30 and 37°C did not alter the expression of any of the three major OMP as it does for *E. coli.* Samples at different stages of culture growth also showed variation in OMP profile with the additional expression of two OMP's of approximately 70 and 72 kd once mid log phase was reached. The presence of a substrate induced OMP, OmpG (27 kd), was also observed when cells were grown in the presence of polygalacturonic acid.

3.2 INTRODUCTION

The outer membrane of *E. chrysanthemi* contains 3 major outer membrane proteins, Omp1, Omp2, and Omp3 (Chapter 2). Two of these OMP, Omp1 and Omp3, are expressed in a reciprocal manner due to the osmolarity of the growth media. As osmolarity increases the expression of Omp3 is increased while that of Omp1 is decreased. This is also the case for *E. coli,* were OmpC expression is increased while OmpF is suppressed as there is an increase in osmolarity (van Alphen and Lugtenberg, 1977). In *E. coli,* this regulation is mediated at the transcriptional level by the *ompR* and *envZ* genes (Hall and Silhavy, 1979; Hall and Silhavy, 1981). These two genes make up the *ompB* operon (Nara *et al.*, 1986).

Since *E. chrysanthemi* exhibits a similar regulation of porin expression, a similar *ompB* operon could be present. The entire OmpB operon of *E. coli* (Mizuno, *et al.*, 1982; Wurtzel *et al.*, 1982), and *S. typhimurium* (Lijestroem *et al.*, 1988), have been sequenced. With this sequence information, a probe could be designed and used in screening *E. chrysanthemi* genomic DNA for the presence of a possible *envZ* gene homologue. This could be accomplished by using primers designed to amplify a portion of the *envZ* gene from *E. coli* K12. The amplified DNA could then be used to probe *E. chrysanthemi* genomic DNA.

The aim was thus to obtain a suitable probe to screen for an *envZ* gene homologue in *E. chrysanthemi.* This probe could then be used to identify the presence of an *envZ* gene homologue in *E. chrysanthemi.*

3.3 MATERIALS AND METHODS

All materials and methods are recorded in Appendix A and B. Plasmid maps are shown in Appendix C.

3.3.1 Bacterial Strains, Plasmids and Culture Conditions

Erwinia chrysanthemi JM1 cells were grown and maintained on LB broth and LB plates. This culture was stored in sterile distilled water in McCartney bottles at room temperature.

Escherichia coli strains used are listed in Table 3.1. All strains of *E. coli* were stored in 20% glucose at -20°C. Cultures were grown or maintained on LB and LB plates respectively. The plasmid vector pSK bluescript was used for nucleotide sequencing of the amplified *envZ* PCR product.

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

***E. coli* strains**	**Genotype**	**Reference**
DH5∝	*F-, recA1, endA1, gyrA96, thi, hsdR17, (r_{K-},m_{K+}), supE44, relA1, deoR, Δ(lacZYA-argF)U169, 080dlacZΔM15*	Hanahan (1983)
K12	*Wild Type Strain*	Mildenhall (pers. comm.)

3.3.2 Genomic and Plasmid DNA Extraction

Genomic DNA from *E. coli* and *E. chrysanthemi* was extracted using the technique of Ausubel *et al.* (1991). Plasmid DNA was prepared by the alkali hydrolysis method of Ish-Horowicz and Burke (1981). Both small scale (mini-prep) and large scale (maxi-prep) isolation of plasmid DNA are detailed in Appendix A.

3.3.3 Primer Design and PCR Amplification

Both *S. typhimurium* and *E. coli* K12 *envZ* genes have been sequenced (Liljestroem *et al.*, 1988; Mizuno *et al.*, 1982). These were aligned using GCG package version 8.0, and areas of homology were determined (Figure 3.1). A forward (5'-GGCGGCCCGACGGAAGTGCG-3') and reverse (5'-TCCAGCATCCCGTTATGG-3') primer were designed from this aligned sequence, and used to amplify a 913 bp fragment from the *E. coli* K12 *envZ* gene (Figure 3.1).

PCR amplification reaction contained 0.125μM of each primer, 200μM of each nucleotide, 10mM $MgCl_2$ and 15ng of *E. coli* and *E. chrysanthemi* template genomic DNA. 1ng of DNA was used when the cloned *envZ* PCR product was used as the template. Promega *Taq* DNA polymerase (1 unit) and propriety buffer were used in all reactions. The total reaction volume was 100μl.

Reaction mixtures were subjected to an initial denaturation step of 93°C for 180 seconds (sec). This was followed by 30 cycles of amplification using a denaturation step of 93°C (60 sec), an annealing step of 65, 60 and 55°C (30 sec), and a extension step of 72°C (60 sec). A final extention step of 180 sec at 72° was also included. DNA amplification cycles were run on a programmable thermocycler (JDI model 8012). 10μl of the reaction mix was then run on a 0.8% agarose gel, stained with ethidium bromide and viewed over a 264nm light source.

concentration of 10X digoxigenin-II-dUTP (DIG) DNA labelling mix (Boehringer Mannheim). After amplification, the sample was run on a 0.8% agarose gel in TAE buffer. The band corresponding to the correct size was eluted using the Geneclean^R kit as previously described.

Both *E. coli* and *E. chrysanthemi* genomic DNA (10μg) was digested overnight at 37°C with 10 units of *AccI*, *HinfI*, and *RsaI*, and run on a 0.8% agarose gel in TBE. The three enzymes were chosen because they would yield a known banding pattern in *E. coli* that could subsequently be differentiated from *E. chrysanthemi*. Genomic DNA was capillary blotted with 0.4 M NaOH onto Hybond N^+ nylon membrane (Amersham). The blot was probed under conditions of high stringency (68°C) by Southern blot hybridization with the DIG-labelled pENVZ generated probe (Boehringer Mannheim). Hybridisation signals were detected via chemiluminescence using the CSPD substrate (Boehringer Mannheim).

3.4 RESULTS AND DISCUSSION

3.4.1 PCR Generation and Sequencing of an *envZ* Probe from *E. coli*

PCR amplification of *E. coli* genomic DNA yielded a 913-bp fragment as expected (Figure 3.2). Amplification of *E. chrysanthemi* genomic DNA under the same conditions did not result in any PCR product (Figure 3.2). It can therefore be concluded that the *envZ* gene, in the region of primer binding, is significantly different to that of *E. coli*. Sequence analysis of both the 5' and 3' ends of the pENVZ insert indicated that the DNA fragment was indeed the correct portion of the *E. coli envZ* gene (Figure 3.3).

For this reason the amplified 913-bp fragment from *E. coli* K12 was thus used as a probe to ascertain whether an *envZ* gene homologue existed in *E. chrysanthemi*. Further PCR reactions performed to obtain a suitable probe involved the re-isolation of the 913-bp fragment from pENVZ. This 913-bp *envZ* fragment was used as a template in all further PCR reactions. This excluded the possibility of eronous PCR amplification products, which occurred when total genomic DNA was used as a template.

3.4.2 Southern Blot Analysis of *E. chrysanthemi* Genomic DNA

Southern blot analysis using the probe described above showed the presence of an *envZ* gene homologue in *E. chrysanthemi* (Figure 3.4). Different banding patterns were obtained when the 2 genomic DNA's were cut with the same enzyme, indicating significant variation between *E. chrysanthemi* and *E. coli envZ* genes. The fact that the K12 *envZ* probe binds to *E. chrysanthemi* genomic DNA shows that homology exists between the gene of these two organisms. From these results it can be concluded that *E. chrysanthemi* contains an *envZ* gene homologue. This would support the results from Chapter 2.

```
                    EC      ATGAGGCGATTGCGCTTCTCGCC
                            ||||||||| |||||||||| ||
                    ST      ATGAGGCGAATGCGCTTCTCACC

     990       1000      1010      1020      1030      1040      1049
EC     ACGAAGTTCATTTGCCCGTACGTTATTGCTCATCGTCACCTTGCTGTTCGCCAGCCTGGT
       |||||||||||||||| ||| | ||||||||||||||||||||||| |||||||||
ST     GCGAAGTTCATTTGCCCGCACGCTGTTGCTCATCGTCACCTTGCTGTTCGTCAGCCTGGT
            1030      1040      1050      1060      1070      1080

    1050      1060      1070      1080      1090      1100      1109
EC     GACGACTTATCTGGTGGTGCTGAACTTCGCGATTTTGCCGAGCCTCCAGCAGTTTAATAA
       |||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||
ST     GACGACCTACCTGGTGGTGCTGAACTTCGCGATTTTGCCGAGCCTCCAGCAGTTTAATAA
            1090      1100      1110      1120      1130      1140

    1110      1120      1130      1140      1150      1160      1169
EC     AGTCCTCGCGTACGAAGTGCGTATGTTGATGACCGACAAACTGCAACTGGAGGACGGCAC
        || || || |||||||| |||||| |||||||||| || ||||||||||||||||||||
ST     GGTTCTGGCTTACGAAGTCCGTATGCTGATGACCGATAAGCTGCAACTGGAGGACGGCAC
            1150      1160      1170      1180      1190      1200

    1170      1180      1190      1200      1210      1220      1229
EC     GCAGTTGGTTGTGCCTCCCGCTTTCCGTCGGGAGATCTACCGTGAGCTGGGGATCTCTCT
       |||||| || ||||| || || || || ||||| ||||| || ||||||||||||||||
ST     GCAGTTAGTCGTGCCGCCTGCGTTTCGCCGGGAAATCTATCGCGAGCTGGGGATCTCTCT
            1210      1220      1230      1240      1250      1260

    1230      1240      1250      1260      1270      1280      1289
EC     CTACTCCAACGAGGCTGCCGAAGAGGCAGGTCTGCGTTGGGCGCAACACTATGAATTCTT
       |||| ||||||| || |||||||||||| ||  ||||||||||||||||||| ||||||||
ST     CTACACCAACGAAGCCGCCGAAGAGGCCGGGTTGCGTTGGGCGCAACACTACGAATTCTT
            1270      1280      1290      1300      1310      1320

    1290      1300      1310      1320      1330      1340      1349
EC     AAGCCATCAGATGGCGCAGCAACTGGGCGGCCCGACGGAAGTGCGCGTTGAGGTCAACAA
       |||||| ||||||||||||||| | ||||||||||||||||||||||||||||||||||
ST     AAGCCACCAGATGGCGCAGCAATTAGGCGGCCCCGACGGAAGTGCGCGTTGAGGTCAACAA
            1330      1340      1350      1360      1370      1380

    1350      1360      1370      1380      1390      1400      1409
EC     AAGTTCGCCTGTCGTCTGGCTGAAAACCTGGCTGTCGCCCAATATCTGGGTACGCGTGCC
       ||| ||||| ||||| ||||| ||||||||||||||||||||||||||||| ||||||||
ST     AAGCTCGCCCGTCGTGTGGCTCAAAACCTGGCTGTCGCCCAATATCTGGGTGCGCGTGCC
            1390      1400      1410      1420      1430      1440

    1410      1420      1430      1440      1450      1460      1469
EC     GCTGACCGAAATTCATCAGGGCGATTTCTCTCCGCTGTTCCGCTATACGCTGGCGATTAT
       |||||||| |||||||||||||||| |||||||| ||||| || |||||||||| ||
ST     GCTGACCGAGATTCATCAGGGCGATTTTTCTCCGCTTTTCCGTTACACGCTGGCGATCAT
            1450      1460      1470      1480      1490      1500

    1470      1480      1490      1500      1510      1520      1529
EC     GCTATTGGCGATAGGCGGGGCGTGGCTGTTTATTCGTATCCAGAACCGACCGTTGGTCGA
       |||  |||||||||||||| |||||||||||||||||||| ||||| |||||||| || ||
ST     GCTCCTGGCGATAGGCGGCGCGTGGCTGTTTATTCGTATACAGAATCGACCGTTAGTGGA
            1510      1520      1530      1540      1550      1560

    1530      1540      1550      1560      1570      1580      1589
EC     TCTCGAACACGCAGCCTTGCAGGTTGGTAAAGGGATTATTCCGCCGCCGCTGCGTGAGTA
       ||| ||||| || |||||||||||| || || || ||||||||||||||||||  || ||
ST     TCTTGAACATGCCGCCTTGCAGGTAGGGAAGGGAATTATTCCGCCGCCGCTGCTGGAATA
            1570      1580      1590      1600      1610      1620

    1590      1600      1610      1620      1630      1640      1649
EC     TGGCGCTTCGGAGGTGCGTTCCGTTACCCGTGCCTTTAACCATATGGCGGCTGGTGTTAA
       |||||| || || ||||| || || |||||| || |||||||||||||| || || || ||
ST     TGGCGCCTCTGAAGTGCGCTCTGTGACCCGGGCGTTTAACCATATGGCAGCCGGCGTGAA
            1630      1640      1650      1660      1670      1680

    1650      1660      1670      1680      1690      1700      1709
EC     GCAACTGGCGGATGACCGCACGCTGCTGATGGCGGGGGTAAGTCACGACTTGCGCACGCC
       |||| |||| |||||||| |||||  |||||||||| || || ||||||||||||||||||
ST     GCAATTGGCCGATGACCGTACGCTATTGATGGCGGGCGTCAGCCACGACTTGCGCACGCC
            1690      1700      1710      1720      1730      1740
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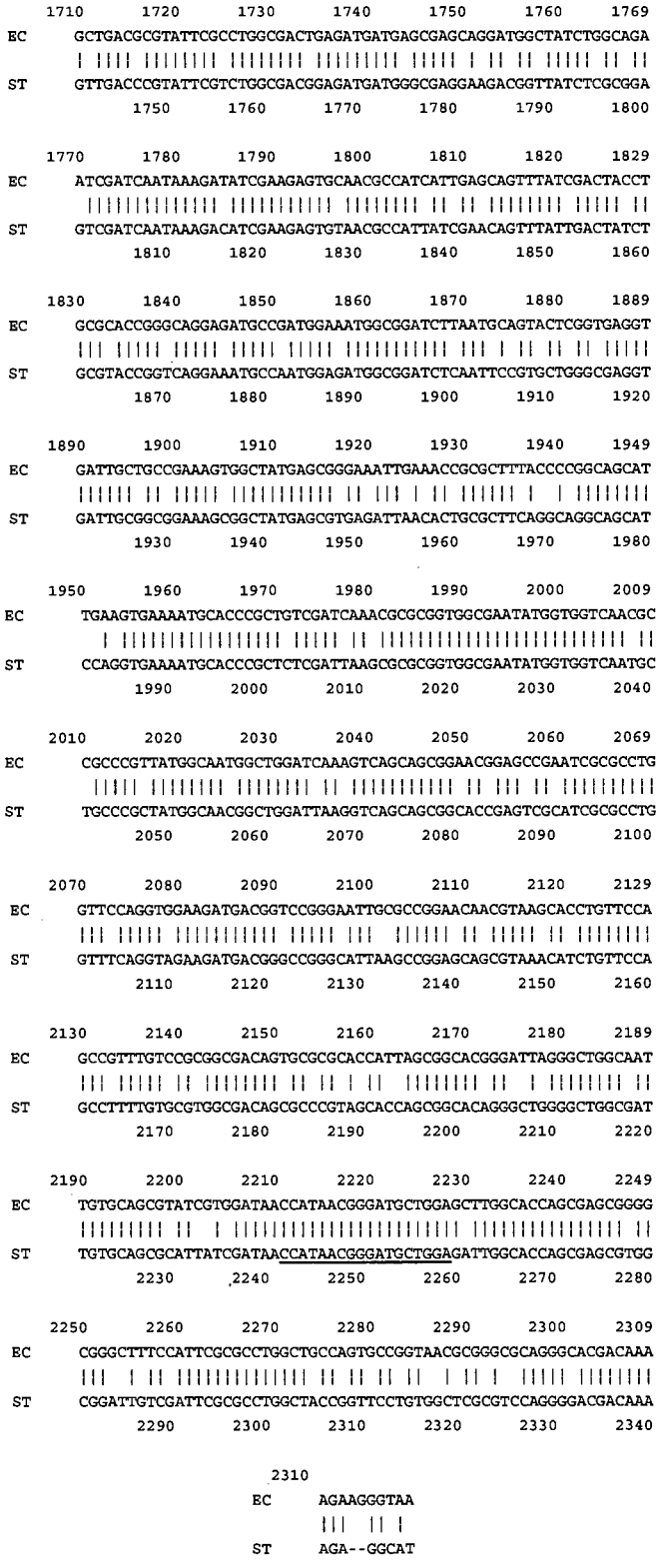


Figure 3.1. Sequence alignment of the *envZ* genes of *E. coli* (EC) and *S. typhimurium* (ST) (Mizuno *et al.* 1982, Lijestroem *et al.* 1988). Underlined areas indicate both the forward and reverse primers.

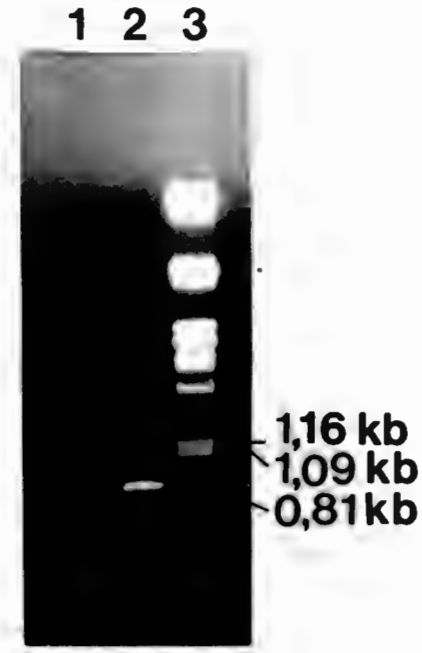
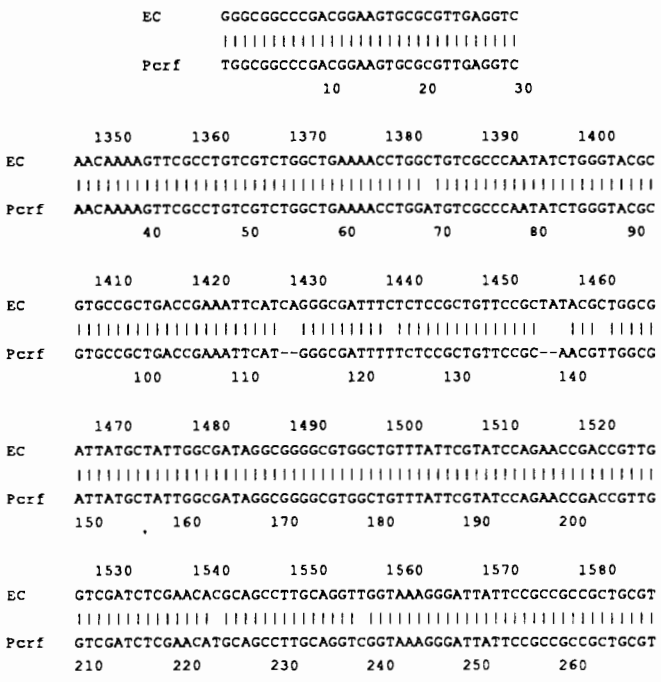


Figure 3.2. PCR amplification of a 913-bp fragment of the *envZ* gene from *E. chrysanthemi* (lane 1) and *E. coli* (lane 2). λ *PstI* marker in lane 3.

a)



b)

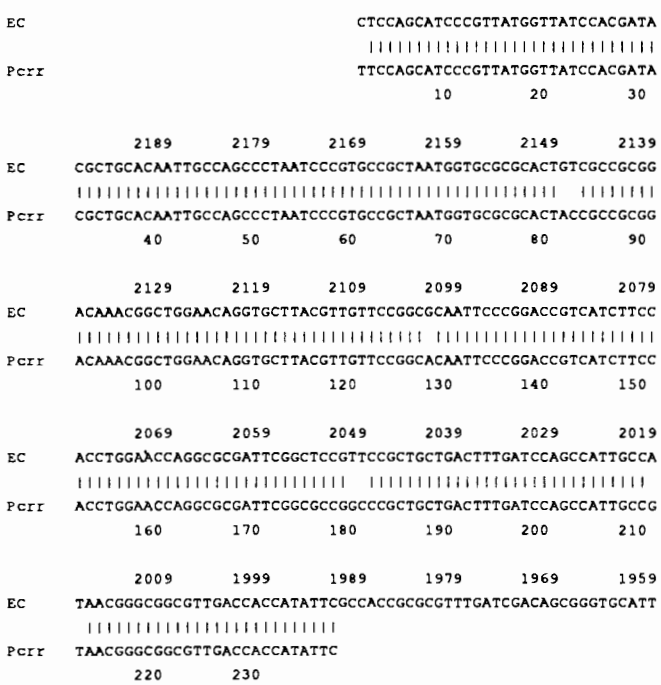
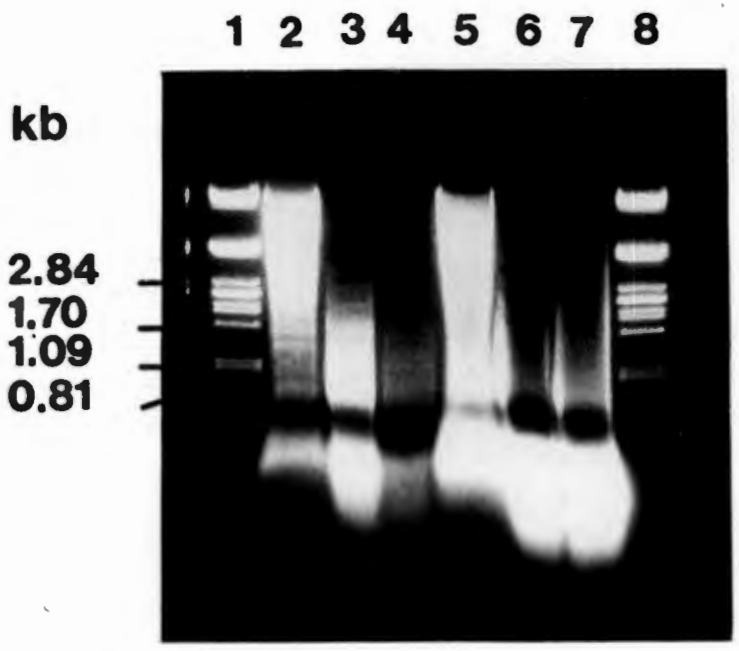


Figure 3.3. Sequence alignment of the *envZ* PCR fragment and the reported sequence of *E. coli envZ* gene. A) Sequence obtained using the universal M13 forward primer (Pcrf) and B) using the reverse primer (Pcrr). Note the high sequence similarity indicating that the PCR product is the correct portion of the *E. coli envZ* gene.

a)



b)

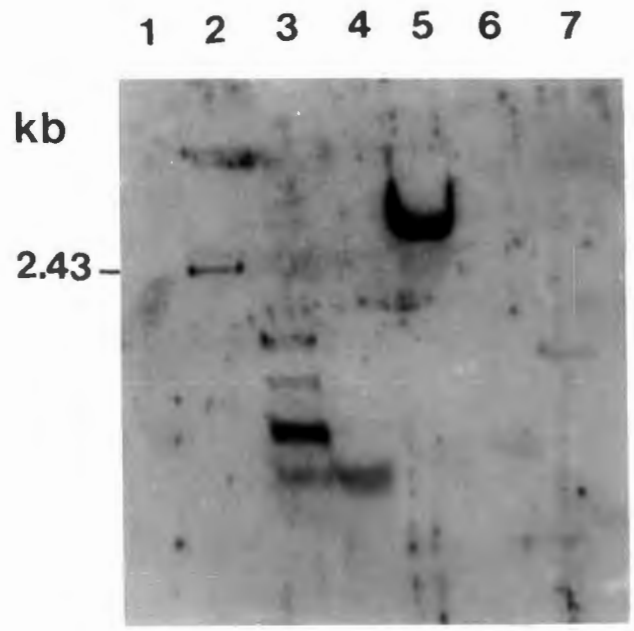


Figure 3.4. Agarose Gel (a) and Southern blot analysis (b) of *E. coli* and *E. chrysanthemi* genomic DNA restriction digests. a) *E. coli* genomic DNA digested with *AccI* (lane 2), *HinfI* (lane 3) and *RsaI* (lane 4). Similarly, *E. chrysanthemi* genomic DNA digested with *AccI* (lane 5), *HinfI* (lane 6) and *RsaI* (lane 7). λ *PstI* marker is in lane 1 and 8. b) Southern blot analysis of the above gel using the 913-bp PCR fragment from the *envZ* gene of *E. coli*. Note, Lane 8 was ommitted for the Southern blot as it is duplicated in lane 1.

In summary, the primers designed to regions of high homology between *E. coli* and *S. typhimurium envZ* genes were unsuccessful in amplifying an *envZ* gene fragment in *E. chrysanthemi* genomic DNA. However, Southern blot analysis of *E. chrysanthemi* with an *E. coli envZ* probe indicated that an *envZ* gene homologue is present in *E. chrysanthemi.*

CHAPTER 4

Isolation and Sequence Analysis of the *OmpB* Operon of *Escherichia coli* K12 for Homologous Recombination Studies in *Erwinia chrysanthemi*

TABLE OF CONTENTS

4.1 SUMMARY

Screening of an *E. coli* K12 gene library for the *envZ* gene yielded a single clone designated plasmid pE18. The size of the DNA insert was 3.7 kb and the gene was situated at the 3' end of the insert. Subcloning of a 1.7 kb fragment generated from a *ClaI/BglII* restriction enzyme digest yielded a plasmid pE18.1. Plasmid pA251 was kindly provided by Adams (pers. comm.) which contained a 1.6 kb DNA insert. This insert carries the *E. coli* K12 *ompR* gene. These two subclones were shortened in both the forward and reverse directions using exonuclease III. This resulted in a number of unidirectionally shortened nested deletions. Sequencing of pA251 resulted in a contiguous sequence of 958-bp. Similarly pE18.1 gave a 1792-bp contiguous fragment. Sequence alignment of the *E. coli ompB* operon from Genbank and that derived in this study showed 99.4% identity.

Sequence analysis of pA251 showed the presence of the RNA polymerase recognition site, the Pribnow box, and the ribosome recognition site for *ompR*. The *envZ* gene ribosome binding site, ATG start codon were also present on pA251. The stem loop structure and stop codon for *envZ* was however found on pE18.1. The use of this sequence has also been discussed with regards to homologous recombination mutagenesis of the *E. chrysanthemi ompB* homologue. These mutations will hopefully allow for a better understanding of the way *E. chrysanthemi* responds to its environment and what role these sensing systems play in virulence of this organism.

4.2 INTRODUCTION

The *envZ* and the *ompR* genes are located in the *ompB* operon in both *Escherichia coli* (Mizuno *et al.*, 1982; Wurtzel *et al.*, 1982) and *Salmonella typhimurium* (Lijestroem *et al.*, 1988). The *ompR* gene from *E. coli* codes for a protein of 32,489 daltons, consisting of 248 amino acid residues. The EnvZ protein consists of 395 amino acid residues and has a molecular weight of 43,963 daltons. Transcription studies on the DNA from this operon indicates that *ompR* and *envZ* are co-transcribed as a polycistronic mRNA in *E. coli*. Translation of the *ompR* gene terminates at two tandem TAA codons and translation of the *envZ* gene initiates 29 nucleotides downstream (Wurtzel *et al.*, 1982).

Sequence alignment of the *E. coli* and *S. typhimurium ompB* operons showed high identity (85%) between the two operons. For this reason, recombination mutagenesis studies of the *ompB* operon in *E. chrysanthemi* were proposed using the *E. coli ompB* operon. These studies will allow for a better understanding of the role of *envZ* and *ompR* gene homologues in the expression of the major OMPs during conditions of increased medium osmolarity in *E. chrysanthemi*.

In order to perform the recombination mutagenesis studies the full length *ompB* operon clone was required. The isolation of the *ompB* operon from our strain of *E. coli* K12 was thus carried out. Our aim was thus to clone and sequence the *E. coli* K12 *ompB* operon. The operon could then be used in future mutagenic studies on the *E. chrysanthemi envZ* and *ompR* homologues.

4.3 MATERIALS AND METHODS

Standard methods and materials are recorded in Appendix A and B. Plasmid maps of cloning vectors are shown in Appendix C.

4.3.1 Bacterial strains, plasmids and culture conditions

E. coli K12 was grown and maintained in LB broth or LB plates. Cultures were stored in 20% glycerol at -20°C.

E. coli strains used for all DNA manipulations are presented in Table 4.1. DNA fragments were cloned into pEcoR251 (Zappe *et al.*, 1986), and subcloned into the Bluescript vector pSK (M13-) (Stratagene). The plasmid pSK was used for exonulclease shortening and nucleotide sequencing.

Table 4.1 *E. coli* strains used in this study

***E. coli* strain**	**Genotype**	**Reference**
JM105	*thi, ppsL, endA, sbc15, hspR4, Δ(lac-proAB)[F', traD36, proAB, LacIZΔM15]*	Yanish-Perron *et al.*, (1985)
DH5∝	*F-, recA1, endA1, gyrA96, thi-1, hsdR17* (r_{K-},m_{K+}), *supE44, relA1, deoR, Δ(lacZYA-argF)U169, 80dlacZΔM15*	Hanahan (1983)
E. coli K12	*Wild Type Strain*	Mildenhall (pers. comm.)

4.3.2 Construction and screening of genomic DNA libraries

DNA from *E. coli* K12 was prepared according to the method of Ausubel *et al.* (1989). DNA (1mg) was partially digested with the restriction endonuclease *Sau3A* and size fractionated on a 10% to 40% sucrose gradient. Fractions containing fragments between 5 kb and 10 kb were pooled, ethanol precipitated and resuspended in TE buffer, pH 8. DNA fragments were cloned into the *BglII* site of the positive selection vector pEcoR251 and transformed into competent *E. coli* JM105 cells according to the method of Draper *et al.* (1989).

E. coli, JM105 transformants were patched in duplicate onto LB^{Amp} plates. 3400 clones were patched and plated on LA^{Amp} plates as this would represent 99% of the *E. coli* K12 genome (Clarke and Carbon, 1976). 200 clones were pooled for maxi-prep plasmid DNA isolation. Plasmid DNA was isolated using the alkali hydrolysis method of Ish-Horowicz and Burke (1981). The pools of plasmid DNA were run on a 0.8% agarose gel in TBE buffer, after which Southern blot analysis with the PCR generated *envZ* probe from *E. coli* was carried out as described in section 3.3.5. Transformants from the plasmid DNA batches that gave positive results were individually inoculated for mini-prep plasmid DNA extraction by the method of Ish-Horowicz and Burke (1981). These individual plasmids were run on a 0.8% agarose gel in TBE buffer and Southern blot analysis was performed to confirm the positive result. Both small scale (mini-prep) and large scale (maxi-prep) isolation of plasmid DNA are detailed in Appendix A.

4.3.3 Plasmid DNA manipulations and sequencing

Positive recombinant pEcoR251 derivatives were digested with restriction endonucleases and mapped according to Sambrook *et al.* (1988). All digests were run on a 0.8% agarose gel in TBE buffer and were subjected to Southern blot analysis.

Selected restriction enzymes were used for subcloning the insert DNA. Digests were run on a 0.8% agarose gel in TAE buffer and selected DNA fragments were gel purified. All DNA fragments were made blunt by using polynucleotide kinase as described in Appendix A. These blunt DNA fragments were ligated into the *EcoRV* site of the pSK polylinker. Plasmid DNA was transformed into *E. coli* DH5∝ cells. White colonies, indicative of insertional inactivation of the B-galactosidase gene of the vector (Viera and Messing, 1982), were selected on LB^{Amp} plates containing X-gal. These colonies were screened for the presence of the *envZ* gene using the Southern blot technique with the *E. coli* PCR generated *envZ* gene probe.

A single positive sub-clone was selected for shortening using exonuclease III according to the method of Henikoff (1984). This subclone along with clone pA251 from Adams (pers. comm) was digested with the endonuclease *SacI* that generates a 3' overhang which is not susceptible to exonuclease III, and *BamHI* that generates a 5' overhang which is susceptible to digestion by the same enzyme. Exonuclease III digestion produced a range of unidirectionally shortened nested deletions for nucleotide sequencing by an adaption of the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977). Reactions were performed with the Thermo-cycler Sequitherm sequencing kit (Epicentre) and CY-5 labelled M13 forward and reverse primers. A detailed description of the Cycle sequencing technique is given in Appendix A. Labelled DNA was run on an ALF-Express automated sequencer. Running parameters are also given in Appendix A. Compilation and analysis of genomic sequences was done on a GCG package version 8.0 (Devereux *et al.*, 1984). This package was run on a DEC/VAX 6000-330 mainframe computer.

4.4 RESULTS AND DISCUSSION

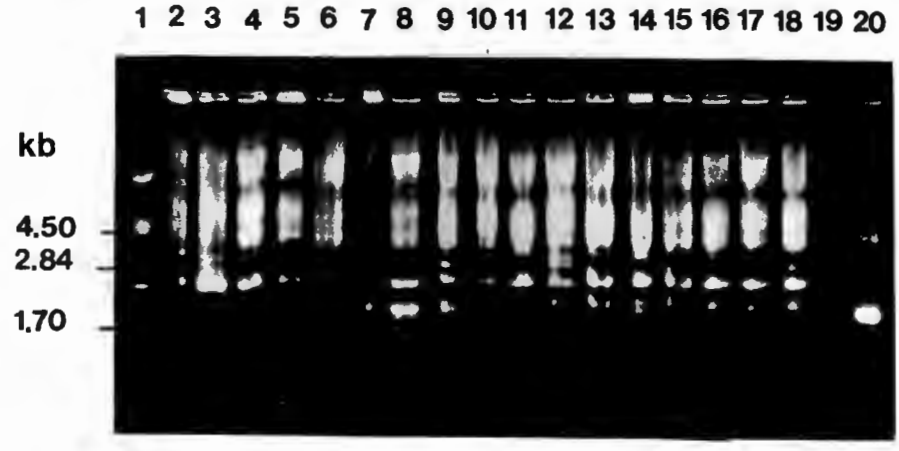
4.4.1 Screening of the genomic DNA libraries

The generation and isolation of the *envZ* gene from the library was done to obtain an accurate *envZ* gene sequence reading for our strain of *E. coli* K12. The reason being that sequence alignment of the PCR generated, *E. coli envZ* gene fragment (Figure 3.3) and that reported by Mizuno *et al.* (1982) from Genbank showed a number of sequence mismatches. Further, this would allow for the authentication of a clone carrying the *envZ* gene for use in homologous recombination studies. Therefore, a genomic library was generated and screened for the *envZ* gene from *E. coli* K12. JM105 derivatives harbouring plasmids with *E. coli* K12 inserts were pooled and screened for the *envZ* gene using the PCR generated probe described in section 3.2.5 (Figure 4.1). This procedure identified 5 positive pooled samples from the genomic library, one of these (pool 13) was selected for further characterization. The 200 clones from this pool were mini-preped and rescreened as above (Figure 4.2). The clone contained the plasmid pE18, which contains a 3.6 Kb insert DNA (Figure 4.3). Clone pE18 was used for all further studies on the *envZ* gene.

4.4.2 Subcloning pE18

Based on the restriction map (Figure 4.3) and Southern blot analysis of pE18 (Figure 4.4) a subcloning strategy could be devised to isolate the smallest fragment of DNA containing the *envZ* gene (Figure 4.5). Restriction digests using *BglII* and *ClaI* confirmed that a small 1700-bp DNA fragment at the 3' end of the pE18 insert contained the *envZ* gene (Figure 4.4). This fragments was subcloned into pSK and designated pE18.1. Southern blot analysis confirmed the presence of the *envZ* gene in pE18.1 (Figure 4.6). pE18.2 and pE18.2 yielded negative results, therefore did not contain the *envZ* gene. Adams (pers. comm.) kindly supplied a subclone of the *ompR* gene in the pSK vector (Figure 4.7). This subclone contained the plasmid pA251 with a 1.6 kb insert of the *ompR* gene also situated at the 3' end of the insert.

a)



b)

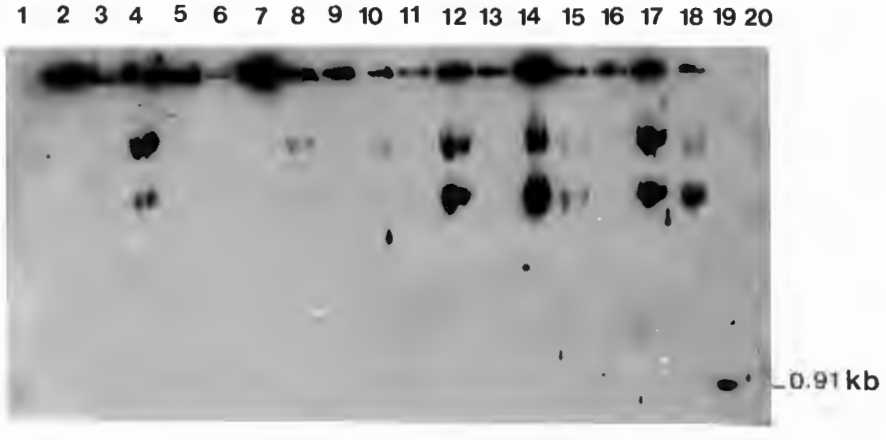
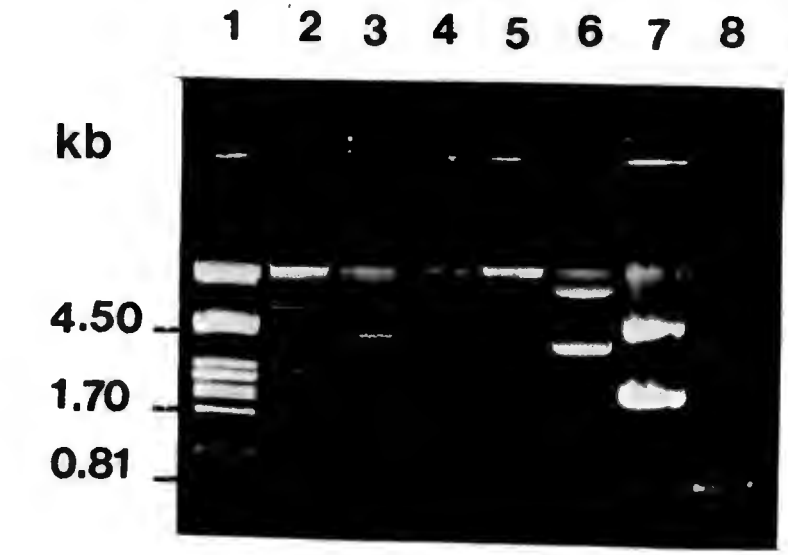


Figure 4.1. Agarose gel (a) and Southern blot (b) of each of 17 maxi-preped plasmid pools (200 clones/pool) of the *E. coli* genomic library. a) Samples run on a 0.8% agarose gel with lanes 2-18 showing pooled plasmid DNA. Lanes 1 and 20 contain λ DNA digested with *PstI*. 913-bp *envZ* PCR product is in lane 19. b) Southern blot analysis using a 913-bp *envZ* gene product from *E. coli* K12. Pool 13 (lane 14) contains a plasmid(s) harbouring the *E. coli* K12 *envZ* gene.

a)



b)

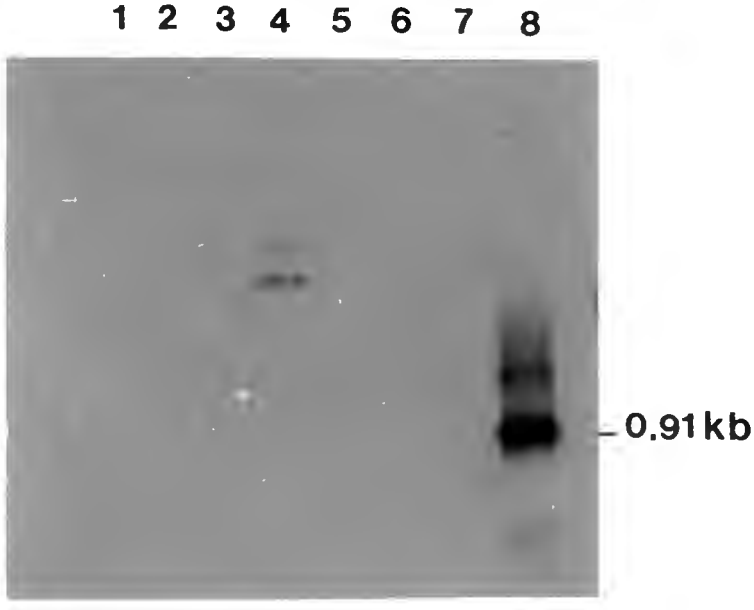


Figure 4.2. Agarose gel (a) and Southern blot (b) analysis of 6 mini-prepped plasmids occurring in the positive pool 13 (Figure 4.1). λ DNA digested with *PstI* in lane 1. Lane 2-7, miniprep plasmid DNA samples. Lane 8, 913-bp *envZ* positive control. Lane 4 contains the positive clone, pE18.

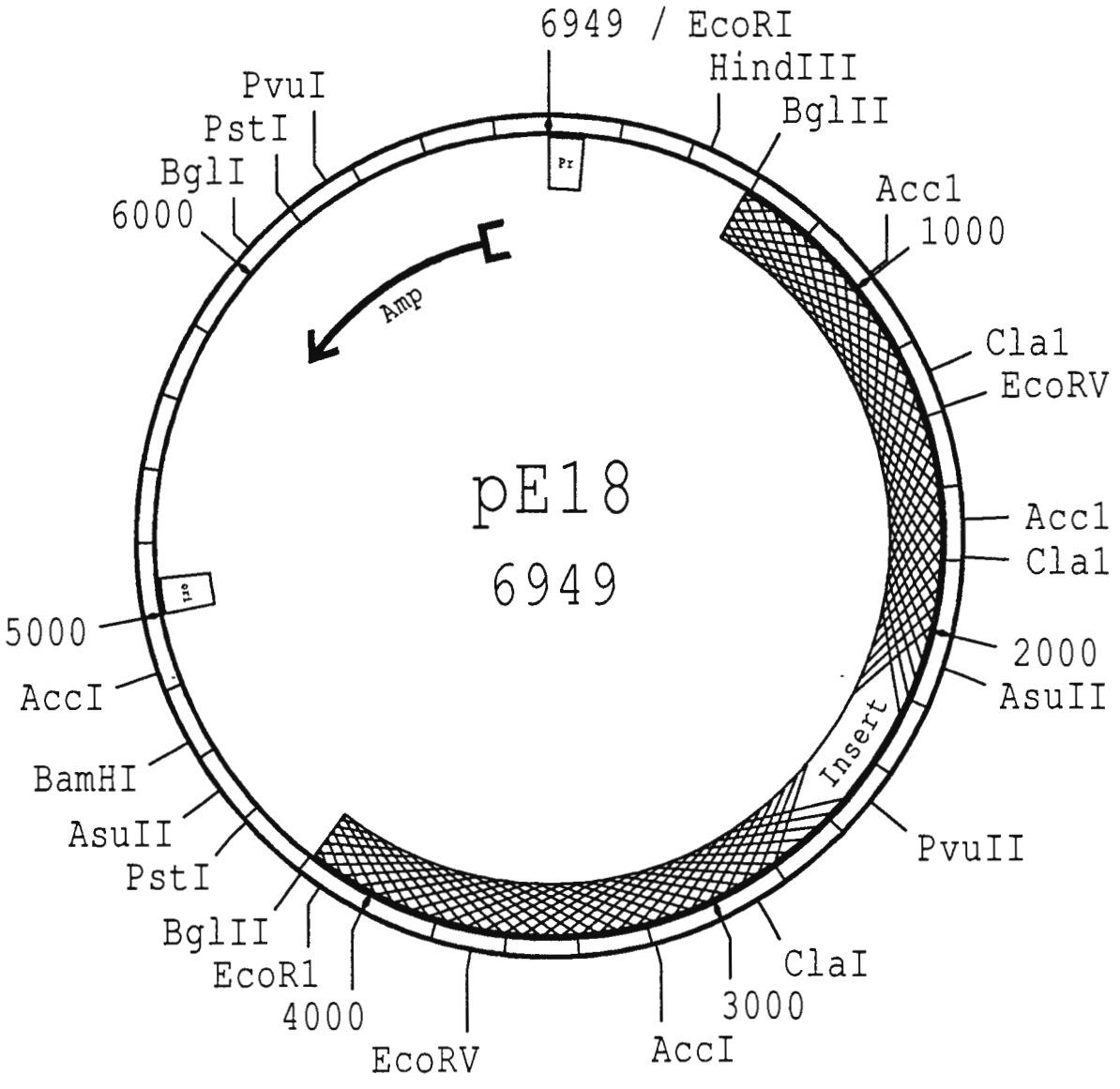
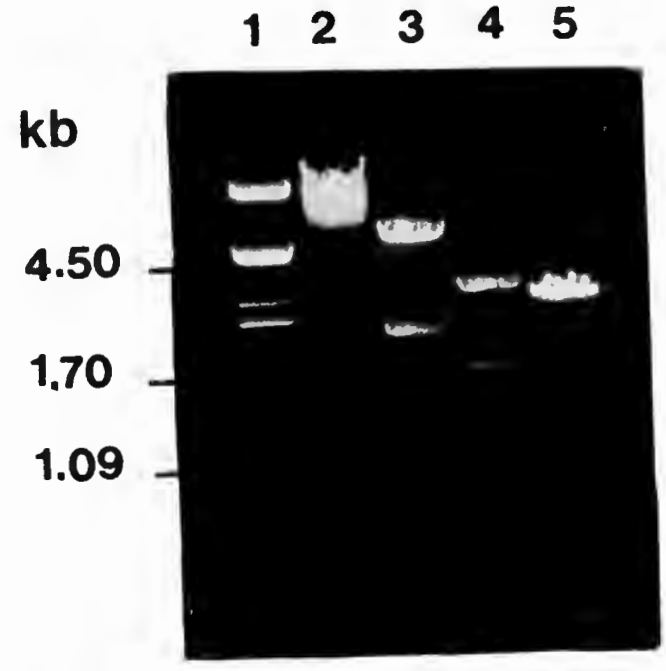


Figure 4.3. Restriction endonuclease map of pE18. The relative position of the *E. coli* K12 DNA insert, the B-lactamase gene (Amp), the λ promotor (pr), the origin of replication (ori) and the recognition sites of various restriction nucleases are shown.

a)



b)

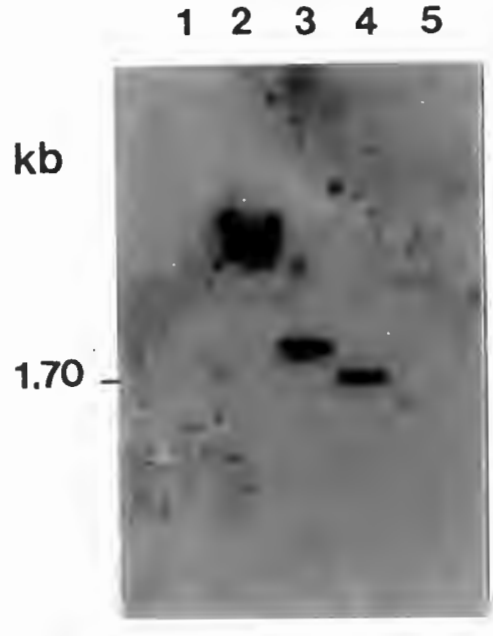


Figure 4.4. Agarose gel (a) and Southern blot (b) of restriction digests confirming the location of the *envZ* gene in pE18. Lane 1, λ DNA digested with *PstI*. pE18 digested with *ClaI*, *AsuII* and *ClaI/BglII* restriction endonucleases in lanes 2-4 respectively. Lane 5 pSK cut with *EcoRV*.

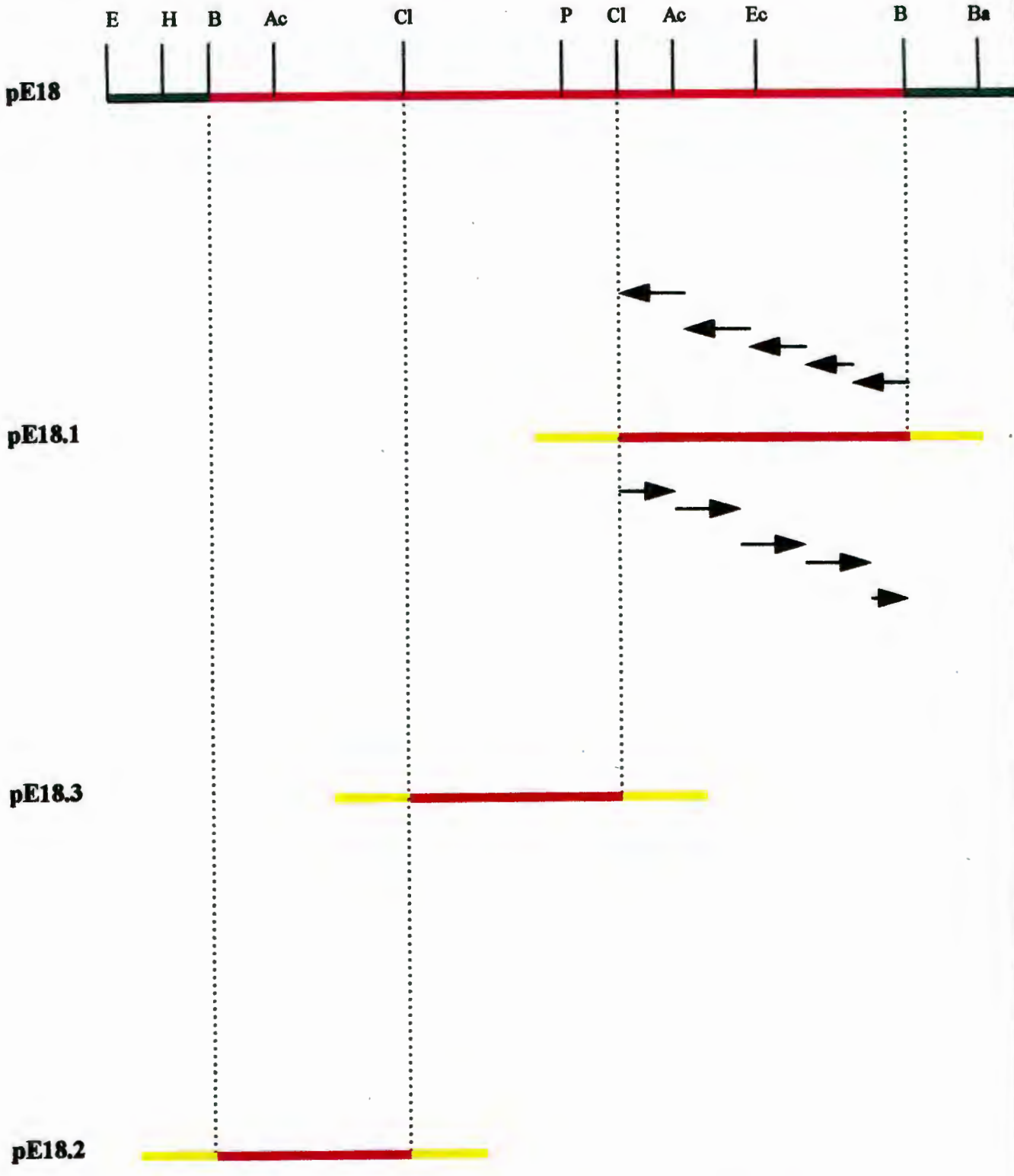
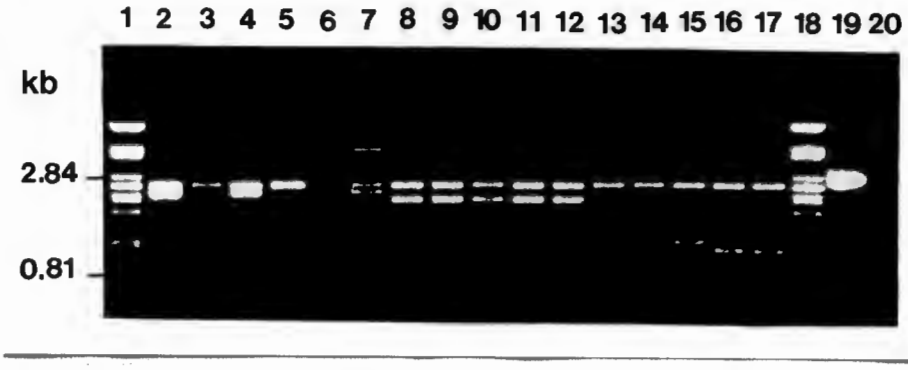


Figure 4.5. Sub-cloning and sequencing strategy of the *E. coli* K12 *envZ* gene. Red lines represent *E. coli* K12, green lines pEcoR251 DNA and yellow lines pSK DNA. pE18 contains a 3.6 kb K12 DNA cloned into the *BglII* site of pEcoR251. pSK vectors carrying subcloned fragments of pE18. pE18.1, pE18.2 and pE18.3 contain *ClaI/BglII* , *BglII/ClaI* and *ClaI/ClaI* fragments respectively. Restriction enzymes represented are *EcoRI* (E), *HindIII* (H), *BglII* (B), *AccI* (Ac), *ClaI* (Cl), *PvuII* (P), *EcoRV* (Ec), and *BamHI* (Ba). Arrows show nested deletions as a result of exonuclease III shortenings of pE18.1.

a)



b)

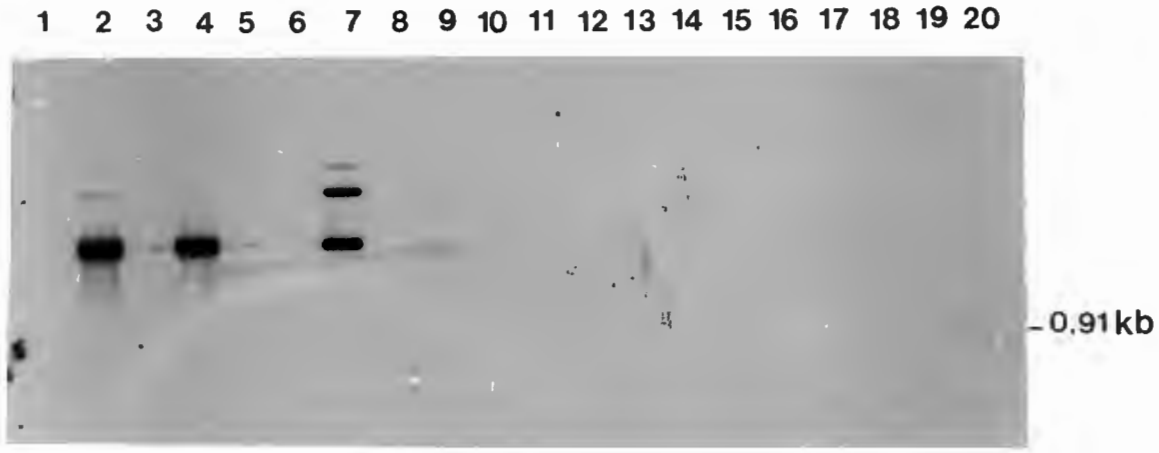


Figure 4.6. Agarose gel (a) and Southern blot (b) of possible subclones containing pE18.1, pE18.2 and pE18.3 mini-preps. The probe was a 913-bp PCR generated *envZ* gene product from *E. coli* K12. Lane 1 and 19, λ DNA digested with *PstI*. Lane 2-7 shows the pE18.1 subclones, lanes 8-12 show the pE18.2 subclones and lanes 13-17 show the pE18.3 subclones. Lane 18 contains the 913-bp PCR generated *envZ* gene.

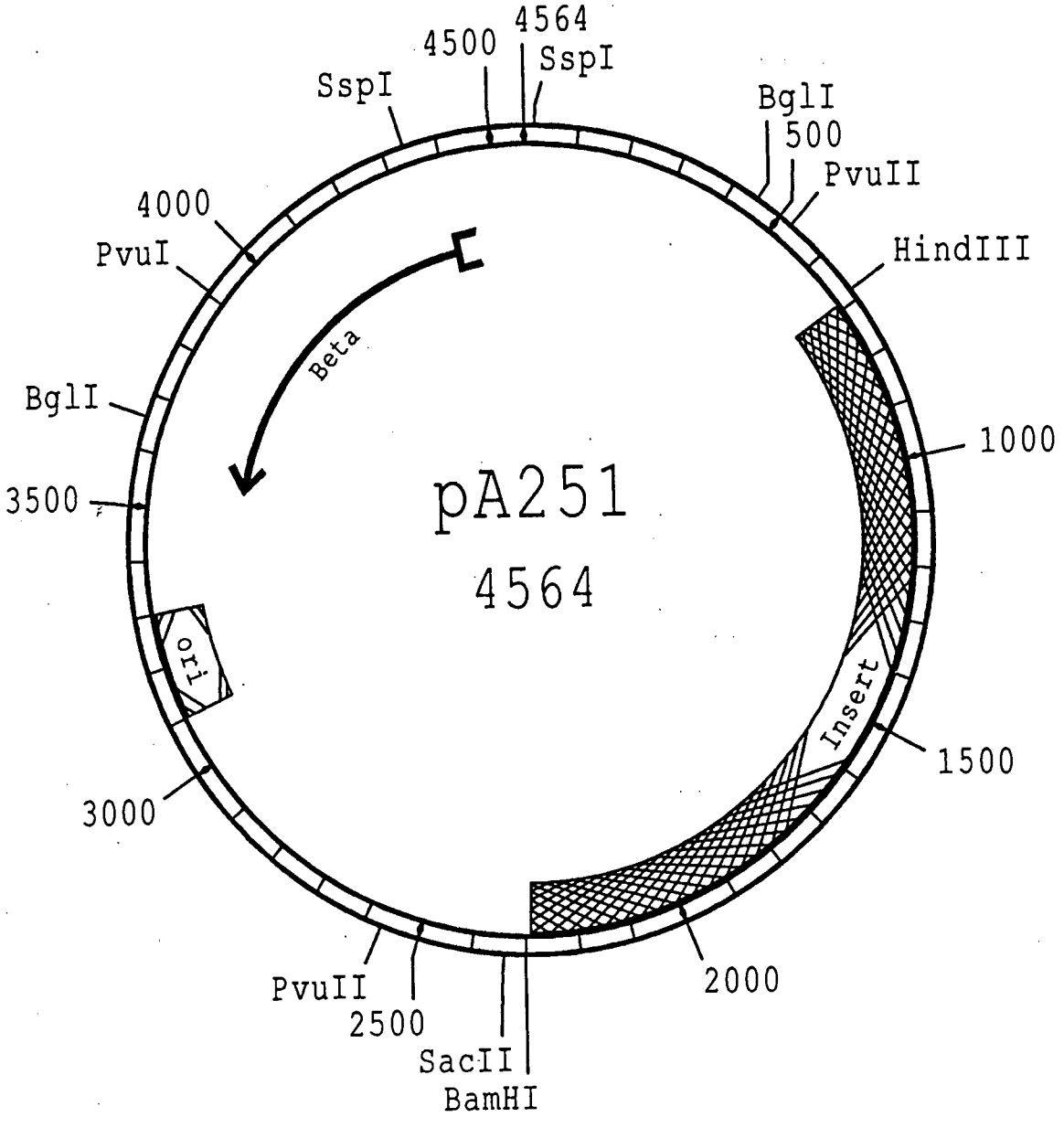


Figure 4.7 Restriction endonuclease map of plasmid pA251 containing the *ompR* gene from *E. coli* K12. The relative positions of the Beta lactamase gene (Beta), the origin of replication (ori) and the recognition sites of various restriction sites are shown.

Plasmids pE18.1 was shortened with exonuclease III to generate a range of nested deletions which were used for nucleotide sequencing of the *envZ* genes of *E. coli* K12 (Figure 4.4) in both the forward and reverse directions. End sequencing of pE18.2 and pE18.3 showed that pE18.1 contained the *envZ* gene from *E. coli* (data not shown). Similarly, pA251 was shortened (Adams pers. comm.) and used for sequencing of the *ompR* gene from *E. coli* K12.

4.4.3 Nucleotide sequence analysis of the *E. coli* K12 *ompB* operon

A number of overlapping fragments of the 1.7 kb and 1.6 kb inserts in pE18.1 and pA251 respectively were sequenced (Figure 4.5 and 4.7). A contiguous sequence of 958-bp was obtained for pA251 (Figure 4.8) by assembling the sequence of these fragments. Similarly, pE18.1 gave a 1792-bp contiguous sequence (Figure 4.9). Unfortunately, alignment of the sequence obtained from pE18.1 to the *envZ* gene sequence available from Genbank, revealed a section of 152-bp of the *envZ* gene near the 5' prime end had been truncated (Figure 4.9). However, the available sequenced for the *ompB* operon showed 99.4% identity over 1890-bp to the reported sequence data of the *E. coli ompB* operon from Genbank.

The RNA polymerase recognition site (at bp 15-27), the Pribnow box (at bp 39-43), and the ribosome recognition site (at bp 142-146) were also identified (Figure 4.8) for *ompR*. The *ompR* start codon is situated at 154-bp (Figure 4.8). The *envZ* gene ribosome binding site (at bp 857-861), ATG start codon (at bp 871), as well as the first 87-bp of the *envZ* gene were found on plasmid pA251. The ATG start codon is situated at the translation termination site of the *ompR* gene as described by Mizuno *et al.* (1982).

The stem loop structure characteristic of prokaryote transcription termination is however found in pE18.1. This is situated at bases 2223 to 2252 of the *ompB* operon.

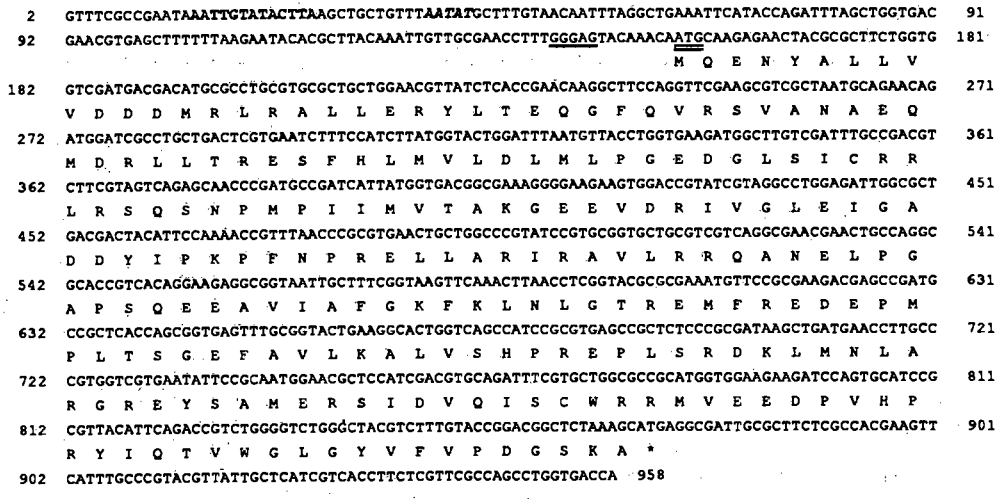


Figure 4.8. Nucleotide sequence of *E. coli* K12 *ompR* gene and adjacent regions. The derived amino acid sequence is presented. The RNA polymerase recognition site (bold), Pribnow Box (italics) and Ribosome binding site (underlined) can also be seen. The Start codon is double underlined and the termination codon is represented by an asterisk.

```
871   ATGAGGCGATTGCGCTTCTCGCCACGAAGTTCATTTGCCCGTACGTTATTGCTCATCGTCACCTTCTCGTTCGCCAGCCTGGTGACCAnn  960
      M  R  R  L  R  F  S  P  R  S  S  F  A  R  T  L  L  L  I  V  T  F  S  F  A  S  L  V  T
961   nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn  1050
      X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X
1051  nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnATCTACCGTGAGCTGGGGATCTCTCTCTACTCC  1138
      X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X  X   I  Y  R  E  L  G  I  S  L  Y  S
1139  AACGAGGCTGCCGAAGAGGCAGGTCTGCGTTGGGCGCAACACTATGAATTCTTAAGCCATCAGATGGCGCAGCAACTGGGCGGCCCGACG  1228
      N  E  A  A  E  E  A  G  L  R  W  A  Q  H  Y  E  F  L  S  H  Q  M  A  Q  Q  L  G  G  P  T
1229  GAAGTGCGCGTTGAGGTCAACAAAAGTTCGCCTGTCGTCTGGCTGAAAACCTGGCTGTCGCCCAATATCTGGGTACCCGTGCCGCTGACC  1318
      E  V  R  V  E  V  N  K  S  S  P  V  V  W  L  K  T  W  L  S  P  N  I  W  V  P  V  P  L  T
1319  GAAATTCATCAGGGCGATTTCTCTCCGCTGTTCCGCTATACGCTGGCGATTATGCTATTGGCGATAGGCGGGGCGTGGCTGTTTATTCGT  1408
      E  I  H  Q  G  D  F  S  P  L  F  R  Y  T  L  A  I  M  L  L  A  I  G  G  A  W  L  F  I  R
1409  ATCCAGAACCGACCGTTGGTCGATCTCGAACACGCAGCCTTGCAGGTTGGTAAAGGGATTATTCCGCCGCCGCTGCGTGAGTATGGCGCT  1498
      I  Q  N  R  P  L  V  D  L  E  H  A  A  L  Q  V  G  K  G  I  I  P  P  P  L  R  E  Y  G  A
1499  TCGGAGGTGCGTTCCGTTACCCGTGCCTTTAACCATATGGCGGCTGGTGTTAAGCAACTGGCGGATGACCGCACGCTGCTGATGGCGGGG  1588
      S  E  V  R  S  V  T  R  A  F  N  H  M  A  A  G  V  K  Q  L  A  D  D  R  T  L  L  M  A  G
1589  GTAAGTCACGACTTGCGCACGCCGCTGACGCGTATTCGCCTGGCGACTGAGATGATGAGCGAGCAGGATGGCTATCTGGCAGAATCGATC  1678
      V  S  H  D  L  R  T  P  L  T  R  I  R  L  A  T  E  M  M  S  E  Q  D  G  Y  L  A  E  S  I
1679  AATAAAGATATCGAAGAGTGCAACGCCATCATTGAGCAGTTTATCGACTACCTGCGCACCGGGCAGGAGATGCCGATGGAAATGGCGGAT  1768
      N  K  D  I  E  E  C  N  A  I  I  E  Q  F  I  D  Y  L  R  T  G  Q  E  M  P  M  E  M  A  D
1769  CTTAATGCAGTACTCGGTGAGGTGATTGCTGCCGAAAGTGGCTATGAGCGGGAAATTGAAACCGCGCTTTACCCCGGCAGCATTGAAGTG  1858
      L  N  A  V  L  G  E  V  I  A  A  E  S  G  Y  E  R  E  I  E  T  A  L  Y  P  G  S  I  E  V
1859  AAAATGCACCCGCTGTCGATCAAACGCGCGGTGGCGAATATGGTGGTCAACGCCGCCCGTTATGGCAATGGCTGGATCAAAGTCAGCAGC  1948
      K  M  H  P  L  S  I  K  R  A  V  A  N  M  V  V  N  A  A  R  Y  G  N  G  W  I  K  V  S  S
1949  GGAACGGAGCCGAATCGCGCCTGGTTCCAGGTGGAAGATGACGGTCCGGGAATTTCGCCGGAACAACGTAAGCACCTGTTCCAGCCGTTT  2038
      G  T  E  P  N  R  A  W  F  Q  V  E  D  D  G  P  G  I  S  P  E  Q  R  K  H  L  F  Q  P  F
2039  GTCCGCGGCGACAGTGCGCGCACCATTAGCGGCACGGGATTAGGGCTGGCAATTGTGCAGCGTATCGTGGATAACCATAACGGGATGCTG  2128
      V  R  G  D  S  A  R  T  I  S  G  T  G  L  G  L  A  I  V  Q  R  I  V  D  N  H  N  G  M  L
2129  GAGCTTGGCACCAGCGAGCGGGGCGGGCTTTCCATTCGCGCCTGGCTGCCAGTGCCGGTAACGCGGGCGCAGGGCACGACAAAAGAAGGG  2218
      E  L  G  T  S  E  R  G  G  L  S  I  R  A  W  L  P  V  P  V  T  R  A  Q  G  T  T  K  E  G
2219  TAAATAAACGGGAGGCGAAGGTGCCTCCCGTTTTGCTTTCTATAAGATACTGGATAGATATTCTCCAGCTTCAAATCATTACAGTTTCGG  2308
      *
2309  ACCAGCCGCTACCAGCGCGGCACCCGCAGGGGTGTCGGTGTATTTATCGAAGTTGTCGATAAACAGTTTCGCCAGGGTTTCGGCTTTTTC  2398
2399  CTGCCACTGTTCCGGAGAAGCGTAGGTGTTACGCGGATCGAGAATCTTCGTGTCTACGCCCGGCAGTTCGGTTGGGATCGCCAGGTTAAA  2488
2489  CATCGGCAGAGTGAAGGTTTCTGCATTATCCACGAACCGTTGAGGATGGCGTCGGATAATGGCGCGGGTATCTTTAATCGAGATACGTTT  2578
2579  GCCTGTGCCGTTCCAGCCAGTGTTAACCAGATAAGCCTGCGCGCCCGCCGCCTGCATACGTTTCACCAGCACTTCTGCGTACTGAGTCGG  2668
2669  GTGCAGCGACAGGAATGCCGCGCCGAAGCAAGCGGAGAAGGTTGGCGTCGGTTCGGTGATGCCACGCTCAGTACCGGCCAGTTTGGCGGT  2758
2759  GAAGCCAGAGAGGAAGTGATACTGGGGTTTGATCGGCAGTCAGGCGAGAAACCGGCGGCAACACGCCGAAAGCATCAGCAGTCAGGAAGA  2848
2849  TAACCTTAGTCGCGTGGCCCGCTTTGGAAACCGGCTTAACAATGTTATCG  2898
```

Figure 4.9. Partial nucleotide sequence of *E. coli* K12 *envZ* gene. The derived amino acid sequence is also presented. The start codon (double underline) and the termination codon (asterisk) is also represented. The stem loop structure for transcription termination has also been underlined and bold.

a)

```
                101                                                 150
EC              ---------a -at-a----- --c------- ---------- ----------
K12             .......... .......... .........-- ---------- ----------
ST              ---------g -cc-g----- --a------- gggttg-cg- -t---------
Consensus       AACGTGATC- T--C-ACAGA AT-AATAATG TTTCGCCGAA TAAATTGTAT

                151                                                 200
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              -t-------- -----a---- ---------- --------c- ----------
Consensus       ACTTAAGCTG CTGTTTAATA TGCTTTGTAA CAATTTAGGC TGAAATTCAT

                201                                                 250
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ------a--t ---------t c--------- ---------- -----a----
Consensus       ACCAGATTTA GCTGGTGACG AACGTGAGCT TTTTTAAGAA TACACGCTTA

                251                                                 300
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- -------gc-
ST              ---t------ ---------- --------g- ---------- ---t--t---
Consensus       CAAATTGTTG CGAACCTTTG GGAGTACAAA CAATGCAAGA GAACTACAAG

                     301                                            350
EC              ---------- ---------- ---------- ---------- ----------
K12             c--------- ---------- ---------- ---------- ----------
ST              ---------- -t-------- t-----t--- --g-----a- ----------
Consensus       ATTCTGGTGG TCGATGACGA CATGCGCCTG CGTGCGCTGC TGGAACGTTA

                351                                                 400
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ---g-----g --g------- ---------- ---------c --t--g----
Consensus       TCTCACCGAA CAAGGCTTCC AGGTTCGAAG CGTCGCTAAT GCAGAACAGA

                401                                                 450
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              -------t-- ------c--- ---------- ----c----- ----------
Consensus       TGGATCGCCT GCTGACTCGT GAATCTTTCC ATCTTATGGT ACTGGATTTA

               451                                                  500
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ---c-g--a- ---------- tc-------- --t--t--c- -g--------
Consensus       ATGTTACCTG GTGAAGATGG CTTGTCGATT TGCCGACGTC TTCGTAGTCA

                501                                                 550
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              a--t--t--a ---------- -------c-- ------g--t -----g--t-
Consensus       GAGCAACCCG ATGCCGATCA TTATGGTGAC GGCGAAAGGG GAAGAAGTGG
```

```
                551                                                   600
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ---------- c--g-----a --c-----c- -t-------- ---t------
Consensus       ACCGTATCGT AGGCCTGGAG ATTGGCGCTG ACGACTACAT TCCAAAACCG

                601                                                   650
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ---------- -c--g---t- ---g-----t --gc-c---t -a--------
Consensus       TTTAACCCGC GTGAACTGCT GGCCCGTATC CGTGCGGTGC TGCGTCGTCA

                651                                                   700
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ---a------ -----c---- -g-----g-- ---------c --t--c--g-
Consensus       GGCGAACGAA CTGCCAGGCG CACCGTCACA GGAAGAGGCG GTAATTGCTT

                701                                                   750
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ---------- t-----g--- ---------- ----g----- ---t-----t
Consensus       TCGGTAAGTT CAAACTTAAC CTCGGTACGC GCGAAATGTT CCGCGAAGAC

                751                                                   800
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- --.------- ---------- ---------- ----------
ST              --a------- ----g----- ---g------ ---------- -a--gt-a--
Consensus       GAGCCGATGC CGCTCACCAG CGGTGAGTTT GCGGTACTGA AGGCACTGGT

                801                                                   850
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ---------- --c------- ----t----- ---------- --t--g----
Consensus       CAGCCATCCG CGTGAGCCGC TCTCCCGCGA TAAGCTGATG AACCTTGCCC

                851                                                   900
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ----c--c-- g--------g ---------- ---------- c-----c--c
Consensus       GTGGTCGTGA ATATTCCGCA ATGGAACGCT CCATCGACGT GCAGATTTCG

                901                                                   950
EC              --t------- ---------- ---------- ---------- ----------
K12             t--tg----- ---------- ---------- -t-------- ----------
ST              ---------- -t-------- ---------g --a------- ----t-----
Consensus       CGCCTGCGCC GCATGGTGGA AGAAGATCCA GCGCATCCGC GTTACATTCA

                951                                                  1000
EC              ---------- ---------- ---------- ---------- ----------
K12             ---------- ---------- ---------- ---------- ----------
ST              ---------- --c------- ---------- ---------t ----------
Consensus       GACCGTCTGG GGTCTGGGCT ACGTCTTTGT ACCGGACGGC TCTAAAGCAT
```

```
            1001                                                1050
EC          ---------- ---------- ---------- ---------- ----------
K12         ---------- ---------- ---------- ---------- ----------
ST          -------a-- --------a- -g-------- ---------c ---c-g----
Consensus   GAGGCGATTG CGCTTCTCGC CACGAAGTTC ATTTGCCCGT ACGTTATTGC

            1051                                                1100
EC          ---------- ---------- ---------- -------t-- t---------
K12         ---------- ---ctc---- ---------- ----c-.... ..........
ST          ---------- ---------- -t-------- -------c-- c---------
Cosensus    TCATCGTCAC CTTGCTGTTC GCCAGCCTGG TGACGAC-TA -CTGGTGGTG
```

b)

```
            1201                                                1250
EC          -------g-- t-----t--c --t--c--t- ---------- ----------
ST          -------a-- c-----g--t --g--t--c- ----a----- t--c------
K12         .......... .......... .......... ....------ ----------
Consensus   CGCAGTT-GT -GTGCC-CC- GC-TT-CG-C GGGAGATCTA CCGTGAGCTG

            1251                                                1300
EC          ---------- ---------- ---------- ---------- ----------
ST          ---------- -----a---- ---a--c--- --------c- -gt-------
K12         ---------- ---------- ---------- ---------- ----------
Consensus   GGGATCTCTC TCTACTCCAA CGAGGCTGCC GAAGAGGCAG GTCTGCGTTG

            1301                                                1350
EC          ---------- ---------- ---------- ---------- ----------
ST          ---------- --c------- -------c-- ---------- ---t-a----
K12         ---------- ---------- ---------- ---------- ----------
Consensus   GGCGCAACAC TATGAATTCT TAAGCCATCA GATGGCGCAG CAACTGGGCG

            1351                                                1400
EC          ---------- ---------- ---------- ---------- ----------
ST          ---------- ---------- ---------- ----c----- c-----g---
K12         ---------- ---------- ---------- ---------- ----------
Consensus   GCCCGACGGA AGTGCGCGTT GAGGTCAACA AAAGTTCGCC TGTCGTCTGG

            1401                                                1450
EC          ---------- ---------- ---------- ---------- ----------
ST          --c------- ---------- ---------- --g------- ----------
K12         ---------- ---------- ---------- ----c----- ----------
Consensus   CTGAAAACCT GGCTGTCGCC CAATATCTGG GTACGCGTGC CGCTGACCGA

            1451                                                1500
EC          ---------- ---------- ---------- ---------- ----------
ST          g--------- --------t- -------t-- ---t--c--- --------c-
K12         ---------- ---------- ---------- ---------- ----------
Consensus   AATTCATCAG GGCGATTTCT CTCCGCTGTT CCGCTATACG CTGGCGATTA

            1501                                                1550
EC          ---------- ---------- ---------- ---------- ----------
ST          ----cc---- ---------c ---------- ---------- a-----t---
K12         ---------- ---------- ---------- ---------- ----------
Consensus   TGCTATTGGC GATAGGCGGG GCGTGGCTGT TTATTCGTAT CCAGAACCGA
```

```
                1551                                                1600
EC              ---------- ---------- ---------- ---------- ----------
ST              -----a--g- ----t----- t--c------ -----a--g- -g--a-----
K12             ---------- ---------- ---------- ---------- ----------
Consensus       CCGTTGGTCG ATCTCGAACA CGCAGCCTTG CAGGTTGGTA AAGGGATTAT

                1601                                                1650
EC              ---------- ---------- ---------- ---------- ----------
ST              ---------- ----tg--a- -------c-- t--a-----c --t--g----
K12             ---------- ---------- ---------- ---------- ----------
Consensus       TCCGCCGCCG CTGCGTGAGT ATGGCGCTTC GGAGGTGCGT TCCGTTACCC

                1651                                                1700
EC              ---------- ---------- ---------- ---------- ----------
ST              -g--g----- ---------a --c--c--g- -----t---- c--------t
K12             ---------- ---------- ---------- ---------- ----------
Consensus       GTGCCTTTAA CCATATGGCG GCTGGTGTTA AGCAACTGGC GGATGACCGC

                1701                                                1750
EC              ---------- ---------- ---------- ---------- ----------
ST              -----at--- -------c-- c--c------ ---------- --t----c--
K12             ---------- ---------- ---------- ---------- ----------
Consensus       ACGCTGCTGA TGGCGGGGGT AAGTCACGAC TTGCGCACGC CGCTGACGCG

                1751                                                1800
EC              ---------- ---------- ---------- ---------- ----------
ST              ------t--- -----g---- -----g---- -g-a--c--t -----c--g-
K12             ---------- ---------- ---------- ---------- ----------
Consensus       TATTCGCCTG GCGACTGAGA TGATGAGCGA GCAGGATGGC TATCTGGCAG

                1801                                                1850
EC              ---------- ---------- ---------- ---------- ----------
ST              -g-------- ------c--- --------t- -------t-- c--a------
K12             ---------- ---------- ---------- ---------- ----------
Consensus       AATCGATCAA TAAAGATATC GAAGAGTGCA ACGCCATCAT TGAGCAGTTT

                1851                                                1900
EC              ---------- ---------- ---------- ---------- ----------
ST              --t-----t- ----t----- t-----a--- --a-----g- ----------
K12             ---------- ---------- ---------- ---------- ----------
Consensus       ATCGACTACC TGCGCACCGG GCAGGAGATG CCGATGGAAA TGGCGGATCT

                1901                                                1950
EC              ---------- ---------- ---------- ---------- ----------
ST              c---t-c--g --g--c---- -------g-- g-----c--- --------t-
K12             ---------- ---------- ---------- ---------- ----------
Consensus       TAATGCAGTA CTCGGTGAGG TGATTGCTGC CGAAAGTGGC TATGAGCGGG

                1951                                                2000
EC              ---------- ---------- ---------- ---------- ----------
ST              -g---a-c-- t------c-g g-a------- -cc-g----- ----------
K12             ---------- ---------- ---------- ---------- ----------
Consensus       AAATTGAAAC CGCGCTTTAC CCCGGCAGCA TTGAAGTGAA AATGCACCCG
```

```
            2001                                                2050
EC          ---------- ---------- ---------- ---------- ----------
ST          --c-----t- -g-------- ---------- --------t- -t-----c--
K12         ---------- ---------- ---------- ---------- ----------
Consensus   CTGTCGATCA AACGCGCGGT GGCGAATATG GTGGTCAACG CCGCCCGTTA

            2051                                                2100
EC          ---------- ---------- ---------- ---------- ----------
ST          ------c--- -----t--g- ---------- c--c---t-- c---------
K12         ---------- ---------- ---------- ---------- ----------
Consensus   TGGCAATGGC TGGATCAAAG TCAGCAGCGG AACGGAGCCG AATCGCGCCT

            2101                                                2150
EC          ---------- ---------- ---------- --g------- ----------
ST          ----t----- a--------- --g-----c- --aa------ g--g-----a
K12         ---------- ---------- ---------- --t------- ----------
Consensus   GGTTCCAGGT GGAAGATGAC GGTCCGGGAA TT-CGCCGGA ACAACGTAAG

            2151                                                2200
EC          ---------- ---------- ---------- ---------- ----------
ST          --t------- ----t----- g--t------ --c--c--t- g--cc-----
K12         ---------- ---------- ---------- ---------- ----------
Consensus   CACCTGTTCC AGCCGTTTGT CCGCGGCGAC AGTGCGCGCA CCATTAGCGG

            2201                                                2250
EC          ---------- ---------- ---------- ---------- ----------
ST          ---a--gc-g --------g- ---------- c--ta-c--- ----------
K12         ---------- ---------- ---------- ---------- ----------
Consensus   CACGGGATTA GGGCTGGCAA TTGTGCAGCG TATCGTGGAT AACCATAACG

            2251                                                2300
EC          ---------- ---------- ---------- ---------- ----------
ST          ---------- -a-------- --------t- ----at-g-- g---------
K12         ---------- ---------- ---------- ---------- ----------
Consensus   GGATGCTGGA GCTTGGCACC AGCGAGCGGG GCGGGCTTTC CATTCGCGCC

            2301                                                2350
EC          ---------- ---------- ---------- ---------- ----------
ST          -----a--g- -t--t--gg- t--c-tc--- --g------- ----g-ca--
K12         ---------- ---------- ---------- ---------- ----------
Consensus   TGGCTGCCAG TGCCGGTAAC GCGGGCGCAG GGCACGACAA AAGAAGGGTA

            2351                                                2400
EC          ---------- ---------- ---------- ---------- ----------
ST          -ga--gg-a- gtatt-ccc- c---tt-.-- c-caacg-cg g-t--cg-c-
K12         ---------- ---------- ---------- ---------- ----------
Consensus   AATAAACGGG AGGCGAAGGT GCCTCCCGTT TTGCTTTCTA TAAGATACTG

            2401                                                2450
EC          ---------- ---------- ---------- ---------- ----------
ST          ttc-a-gccg -catccggc- tgt--c---- --c-----t- -g--gcta--
K12         ---------- ---------- ---------- ---------- ----------
Consensus   GATAGATATT CTCCAGCTTC AAATCATTAC AGTTTCGGAC CAGCCGCTAC
```

```
            2451                                                  2500
EC          ---------- ---------- ---------- ---------- ----------
ST          -----.tt-g --t--c---- -a--c--a-- c--c------ --c--a--g-
K12         ---------- ---------- ---------- ---------- ----------
Consensus   CAGCGCGGCA CCCGCAGGGG TGTCGGTGTA TTTATCGAAG TTGTCGATAA

            2501                                                  2550
EC          ---------- ---------- ---------- ---------- ----------
ST          ---------- ---c-cggt- ---------- ----t----- ---c--c---
K12         ---------- ---------- ---------- ---------- ----------
Consensus   ACAGTTTCGC CAGGGTTTCG GCTTTTTCCT GCCACTGTTC CGGAGAAGCG

            2551                                                  2600
EC          --cc------ ---------- ---------- ---------- ----------
ST          ---------- ----g----- ---g-gg--a --c-----t- -------a--
K12         ---------- ---------- ---------- ---------- ----------
Consensus   TAGGTGTTAC GCGGATCGAG AATCTTCGTG TCTACGCCCG GCAGTTCGGT

            2601                                                  2650
EC          ---------- ---------- ---------- ---------- ----------
ST          c--------- --a-cg---- g-------cg ---------c --g-----a-
K12         ---------- ---------- ---------- ---------- ----------
Consensus   TGGGATCGCC AGGTTAAACA TCGGCAGAGT GAAGGTTTCT GCATTATCCA

            2651                                                  2700
EC          .--------- ---------- ---------- ---------- ----------
ST          ga-------- c--a------ ---------t -----c---- ----g--g--
K12         .--------- ---------- ---------- ---------- ----------
Consensus   -CGAACCGTT GAGGATGGCG TCGGATAATG GCGCGGGTAT CTTTAATCGA

            2701                                                  2750
EC          ---------- ---------- ---------- ------.... ..........
ST          ---------- --g-----a- -------g-- ---------- ----------
K12         ---------- ---------- ---------- ---------- ----------
Consensus   GATACGTTTG CCTGTGCCGT TCCAGCCAGT GTTAACCAGA TAAGCCTGCG
```

Figure 4.10 Sequence alignment of *E. coli* K12 (EC), *S. typhimurium* (ST), and *E. coli* K12 (this study) *ompB* operon. A) Shows sequence alignment of the *ompR* gene and B) shows sequence alignment of *envZ*. A derived consensus sequence is shown below the alignment. Nucleotides identical to the consensus are indicated by dashes (-) and nucleotides differing from the consensus are shown in small lettering. The respective base pair variations between the *ompB* operon of the two *E. coli* K12 strains are in bold. Areas were no sequence was alighned are indicated by a dot (.).

Sequence alignment of the *ompB* operon of *E. coli, S. typhimurium* and *E. coli* K12 as determined in this study shows the high sequence identity between these organisms (Figure 4.10). The respective base pair variations between our strain and the reported strain of *E. coli* K12 can also be seen.

The absence of the 152-bp fragment from the *envZ* gene will pose a problem with regards to homologous recombination studies in *E. chrysanthemi* as this area can not be phenotypically characterised. It is however, hoped that the isolation of the *ompB* operon from *E. coli* K12 can now be used as a tool in recombinational mutagenesis studies in *E. chrysanthemi* for all other areas of the *ompB* operon.

4.4.4 A Possible Homologous Recombination Mutagenesis Strategy for *E. chrysanthemi*

Recombinational mutagenesis could be achieved by cloning fragments of both the *envZ* and *ompR* gene into a suicide vector, such as pGP704 (Miller and Mekalanos, 1988). The constructs can then be transformed into *E. coli* SY327 and SM10 (Miller and Mekalanos, 1988). SY327 is only useful for initial cloning experiments as it is unable to mobilize pGP704. SM10 is not as transformable but has the necessary *trans* acting factors for the mobilisation of pGP704 derivatives. These clones can then be mated with *E. chrysanthemi* and homologous recombinant mutants can be screened. The mutants can further be screened by growning them in media of different osmolarities and the effects on the expression of the major OMP of *E. chrysanthemi* can then be determined. The phenotypes will either be a lack of Omp3 expression ($Omp3^-$) or definitive Omp3 expression ($Omp3^+$). Similarly, the same could occur for Omp1 ($Omp1^+$ or $Omp1^-$). An array of Omp1 and Omp3 expression mutants can then be isolated. All these mutations will lie within the *ompR* and *envZ* gene homologues of *E. chrysanthemi.*

Many types of *E. coli ompR* mutants have been isolated and characterised at the molecular level in terms of their *invivo* osmoregulatory phenotypes, and also the

invitro biochemical properties of their gene products (Hall and Silhavy, 1981; Nara *et al.*, 1986; Mizuno *et al.*, 1988; Aiba *et al.*, 1989; Kanamaru *et al.*, 1989; Stock *et al.*, 1989; Kanamaru *et al.*, 1990; Nakashima *et al.*, 1991; Bowrin *et al.*, 1992; Parkinson and Kofoid, 1992). For this reason, OmpR has been devided into a N-terminal imput domain (amino acid residues 1-179) and a C-terminal output domain (amino acid residues 180-339). The N-terminal half of the OmpR molecule contains the site involved in phosphorylation by EnvZ, whereas the C-terminal half possesses a DNA binding site for the OmpF and OmpC promotor DNA (Kato *et al.*, 1989). By constructing clones in suicide vectors of these regions, the *ompR* homologue in *E. chrysanthemi* can be targeted. The expected mutants would have $Omp1^{-}$ $Omp3^{-}$ as OmpR DNA binding site would be inactivated. Similarly, the loss of the phosphorylation site in N-terminal region of *ompR* homologue of *E. chrysanthemi* would result in $Omp1^{+}$ $Omp3^{-}$ phenotypes. This would be a result of the loss of the ability of OmpR to be phosphorylated. This would in turn directly affect OmpR's ability to bind to the Omp3 promotor since OmpR-P would hypothetically, only bind to the Omp3 promotor. Omp1 would be produced constitutively irrespective of medium osmolarity as only unphosphorylated OmpR would be present.

Other areas of the *ompR* homologue in *E. chrysanthemi* can also be targeted. For example, the acid pocket, where mutations in this region for *E. coli* have resulted in the the loss of the phoshorylation site of *ompR* (Brissette *et al.*, 1991). Depending on the nature and location of the mutation there is either a constitutive or loss in activity of OmpR.

envZ gene mutations in *E. coli* have been found to be pleotropic, affecting the production of OmpC and OmpF as well as other outermembrane proteins and periplasmic proteins (Hall and Silhavy, 1981; Wandersman *et al.*, 1980). EnvZ can essentially be devided into two domains by two membrane spanning segments. These two membrane spanning segments devide the EnvZ protein into a N-terminal receptor domain located in the periplasm and a C-terminal signaling domain protruding into the cytoplasm. These two domains are also seperated by a linker emanating from the second transmembrane segment (Figure 1.4).

Mutations in the receptor domain (amino acid resudues 42-162) (Figure 4.9) have been extensively studied for *E. coli* (Aiba *et al.*, 1989; Forst *et al.*, 1989; Tokishita *et al.*, 1991). These mutations result in substantial autokinase acivity, thus corresponding to a induced signaling behaviour for *E. coli*. Unfortunately this area of the *envZ* gene obtained in this study has been truncated and will have to be re-isolated from *E. coli* for mutagenic studies in *E. chrysanthemi*.

Homologous recombination mutagenesis can be targeted towards the linker region, C-terminal signaling domain and the transmembrane spanning regions of the *envZ* gene homologue in *E. chrysanthemi*. Linker mutations for *E. coli* (amino acid residues 181-220) have shown that the protein gets locked in an "OFF" state (Tokishita *et al.*, 1992). This ultimately results in constitutive production of OmpF and the total repression of OmpC. Similarly, if this area was mutated for the *envZ* homologue in *E. chrysanthemi* there would be constitutive production of Omp1 and the total repression of Omp3.

Mutations in the two transmembrane spanning segments (TM1 {amino acid residues 14-42} and TM2 {amino acid residues 162-180}) of *E. coli* have also been characterised (Tokishita and Mizuno, 1994). Mutations in TM1 have resulted in defects in its phosphatase activity, wheras defects in TM2 have resulted in defects in the EnvZ kinase activity. Consequently they exhibit different phenotypes, $OmpF^{-}$ OmpC-constitutive and $OmpF^{(+/-)}$ $OmpC^{(+/-)}$, were +/- refers to a lowering in both OmpC and OmpF expression respectively (Tokishita and Mizuno, 1994). Similarly mutations in the homologous *E. chrysanthemi* TM1 and TM1 regions of the EnvZ protein could be carried out with expected similar results.

Lastly, mutations in the C-terminal signaling domain (amino acid residues 221-451) have also been well characterised for *E. coli envZ* gene (Aiba *et al.*, 1989). Mutations in this region result in the inactivation or loss of the autophosphorylation activity resulting in the phenotype $OmpF^{-}$ OmpC-constsitutive. This is due to the reduced phosphatase activity of the mutant EnvZ. This area could also be targetd for recombinational mutagenesis in *E. chrysanthemi envZ* homologue.

Selective inactivation of the *envZ* and *ompR* genes could thus be used to study the physiological affects on both enviromental sensing as well as pathogenicity of *E. chrysanthemi*.

CHAPTER 5

General Discussion, Conclusions and Future Research Perspectives

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5.1 General Discussion and Conclusion

Expression of the major outer membrane proteins under different osmotic potentials has been studied in many Gram-negative organisms (Stock *et al.*, 1989; Csonka, 1989; Csonka and Hanson, 1991; Parkinson and Kofoid, 1992; Parkinson; 1993). All these studies have shown that there is a two component signal transduction system consisting of a sensor protein and response regulator. An example being the EnvZ sensor protein and its cognate response regulator, OmpR, of *E. coli*. EnvZ and OmpR are situated in the cytoplasmic membrane and cytoplasm respectively. These two proteins govern the expression of two major outer membrane proteins, OmpF and OmpC. Under conditions of high osmotic potential, OmpC expression is favoured over OmpF, and *visa versa*.

Since a similar system had been identified for *S. typhimurium*, and sequence alignment with *E. coli* showed high sequence identity (85%), indicative of a high degree of conservation, a similar system was sought in *E. chrysanthemi*. It has also been well established that *E. chrysanthemi* is sensitive to desiccation and thus would benefit in having similar signal transduction system to monitor osmotic potential or water activity and respond accordingly. The isolation of the major outer membrane proteins of *E. chrysanthemi* yielded three major OMP, namely Omp1 (37.5 kd), Omp2 (35.5 kd) and Omp3 (34.5 kd). Both culture growth phase and growth temperature had no effect on the expression of these major OMP. However, two additionally expressed OMP were visible once the culture reached mid log phase. These additionally expressed OMP were thought to be produced in response to iron shortage in the growth medium. The expression of high molecular weight OMP and their association with iron deficient growth conditions has been reported by Expert and Toussaint (1985). Temperatures used in this study did not result in differential expression of the major OMP, but higher growth temperatures (> 37°C) may however prove otherwise.

The effect of increased osmotic potential in the growth medium showed a marked increase in the expression of Omp3 with the subsequent repression in Omp1 in *E. chrysanthemi*. These two proteins may thus be the equivalent of the OmpF and OmpC proteins of *E. coli* respectively. The level of Omp2 in the outer membrane of *E. chrysanthemi* remained

this protein is similarly unaffected by medium osmolarity. These results lend evidence towards a signal transduction system, similar to the OmpB system of *E. coli*, being present in *E. chrysanthemi*.

A number of induced OMP have also been documented and these are usually associated with a nutrient deficiency. The PhoE porin of *E. coli* (Stock *et al.*, 1989), is expressed in response to phosphate deficiency. Induced OMPs have also been found to enhance the uptake of a specific nutrient that may be in abundance in the growth medium, such as LamB of *E. coli*, specific for the uptake of maltose and maltodextrins (Heine *et al.*, 1988). Since *E. chrysanthemi* produces pectate lyase (Chatterjee and Starr, 1980), of which one responsible for the breakdown of pectin, similar induced outer membrane proteins were sought which may be specific for the uptake of these breakdown products. The products of pectin degradation include mono- and di-galacturonic acid. When growth media was supplemented with polygalacturonic acid (PGA), a induced OMP was observed. This protein was designated OmpG (27 kd), and may be responsible for the selective uptake of mono- and digalacturonic acid.

As there is a high degree of sequence identity between the *envZ* gene of *E. coli* and *S. typhimurium*, primers were designed from areas of high sequence identity and used to amplify a 913-bp fragment of the *envZ* gene from *E. coli* K12. Southern Blot analysis of *E. chrysanthemi* genomic DNA using the PCR generated 913-bp DNA fragment from *E. coli* as the probe, indicated that an *envZ* homologue was present in *E. chrysanthemi*. PCR reactions using *E. chrysanthemi* genomic DNA as the template yielded no product. This merely indicated that there was sufficient sequence variation within the region of the primers in the *envZ* homologue of *E. chrysanthemi*.

The cloning and characterization of the *E. coli ompB* operon was conducted in order to perform homologous recombination mutagenesis studies in *E. chrysanthemi*. A genomic library was generated for *E. coli* K12 and a single clone was identified with the *envZ* gene. This plasmid, pE18, was mapped, and the portion carrying the *envZ* gene was subcloned

(plasmid pE18.1) for sequence analysis. Plasmid pA251 carrying the *ompR* gene was kindly

(plasmid pE18.1) for sequence analysis. Plasmid pA251 carrying the *ompR* gene was kindly donated by Adams (pers. comm.).

Sequencing of pA251 yielded a 958-bp contiguous sequence containing the *ompR* gene of *E. coli*. A 1792-bp contiguous sequence containing the *E. coli envZ* gene from pE18.1 was also obtained. Sequence alignment of the *E. coli* K12 sequence obtained from Genbank and that of our sequence for *E. coli* K12 showed minor base pair variations. These variations could be strain specific. The *ompB* operon of *E. coli* can now be used for homologous recombination studies in *E. chrysanthemi*.

In conclusion, it is clear that a signal transduction system is present in *E. chrysanthemi*. The isolation and sequence analysis of the *ompB* operon of *E. coli* will thus facilitate the homologous recombination mutagenesis of the *E. chrysanthemi ompB* homologue.

5.2 Future Research Perspectives

Since the *ompB* operon of *E. coli* has been fully characterized, specific areas of the *ompB* operon in *E. chrysanthemi* can be targeted for homologous recombination studies. A possible strategy using the suicide vector pGP704 has been devised. This will enable the targeting of specific regions of the *ompR* and *envZ* gene homologues of *E. chrysanthemi*. Resulting phenotypic characteristics relating to the expression of Omp1 and Omp3 can be then be identified. In this way one can obtain a fuller understanding of the function of the *ompB* homologue in *E. chrysanthemi*.

It is also hoped that the *ompB* homologue from *E. chrysanthemi* will eventually be isolated and sequenced so site specific mutagenesis can be carried out to identify the importance of specific amino acid replacements on the signal transduction system in *E. chrysanthemi*.

Other possibilities include the isolation and characterized of the induced *ompG* gene, which codes for the OmpG outer membrane protein in *E. chrysanthemi*. The effects of mutations in both *ompB* operon and the *ompG* gene will also be studied relative to the virulence and pathogenicity of *E. chrysanthemi*.

APPENDIX A

Methods

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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 500 µl 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 (50mM glucose, 25mM 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 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, the tube was inverted to drain it and the pellet was resuspended in 4.2 ml Tris-EDTA (TE) buffer, pH 8.0. 4.4 g of cesium chloride (CsCl) was added and dissolved and 400 µl of ethidium bromide (EtBr) (10 mg/ml) was added.
9. The tube was centrifuged at 15 000 rpm for 15 min to precipitate any remaining protein debris.
10. The refractive index of the solution was adjusted to 1.394.
11. The sample was sealed in a Beckman Quickseal ultracentrifuge tube (5ml) and centrifuged for a minimum of 6 h at 55 000 rpm in a Beckman Vti 65 rotor at 15°C.
12. 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 wave length UV light (350 nm).
13. The EtBr was extracted at least three times using equal volumes of salt saturated isopropanol.
14. 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.
15. The pellet was washed with 1 ml of 70% ethanol at 12 000 rpm for 10 min.
16. The DNA was resuspended in 200 µl TE, pH 8 and the concentration of DNA was determined spectrophotometrically as described in A1.

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 them 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. After 5 min on ice, the cellular debris was collected by centrifugation at 10 000g for 5 min.
6. 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.
7. 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. Alkaline phosphatase treatment of plasmid DNA

Alkaline phosphatase treatment of plasmid DNA was performed according to Sambrook *et al.* (1989).

1. Restriction endonucleases in typical 20 µl plasmid digests were inactivated by heating the sample for 10 min at 65°C.

2. The sample was made up to 100 µl with distilled water and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and the tube was shaken well.
3. The Eppendorf tube was centrifuged for 5 min at 6 000rpm. The upper aqueous phase was removed, and any remaining phenol was extracted from this phase with one volume of chloroform:isoamylalcohol (24:1).
4. DNA was precipitated from the aqueous phase with two and a half volumes of 100% ethanol and one tenth volume of 4M LiCl.
5. The DNA was pelleted and washed with 70% ethanol before being resuspended in 44 µl of distilled water.
6. Calf intestinal alkaline phosphatase (2 µl) and 5 µl of the appropriate buffer was added to the DNA, and the solution was incubated for one hour at 37°C.
7. The reaction was stopped by heating the sample to 65°C for 10 min.
8. DNA was precipitated from the solution as outlined in step 4.

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 0.25 M 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 0.4 M NaOH and placed over a glass bridge so that the wick ends were touching the bottom of a reservoir tank containing 0.4 M 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).

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

2 X 15 min washes with 100 ml wash buffer.

5. The membrane was equilibrated for 5 min in buffer 3.
6. The AMPPD^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

1. Plasmid DNA (12ug) 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 run 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. Minipreparation 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 labeling 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.* (1989) 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, 1992)

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 minutes.
4. Determine OD_{595}. generate standard curve and determine concentration of your unknown.

A16. Construction of T-vectors

Procedure of Clark (1988) was used.

1. Digest plasmid with a blunt ended restriction exonuclease (eg. *EcoRV*).
2. Ethanol precipitate cut DNA as in A2.
3. Resuspend in 15 µl TE.
4. Incubate with Taq Polymerase (1 unit/µg plasmid DNA/20 µl volume), Standard Buffer (Buffer A), and 220 µg/ml BSA, in the presence of 2mM dTTP for two hours at 70°C.
5. Phenol extract as in A1. and precipitate as before.
6. Resuspend in 20 µl TE Buffer (pH 8)

APPENDIX B

Buffers and solutions

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Buffers and solutions

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APPENDIX B

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

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

Minimal Media

Na_2HPO_4	1.42 g
KH_2PO_4	0.27 g
NH_4NO_3	0.40 g
$MgSO_4 7H_2O$	0.24 g
Glycerol	0.3%
Distilled water to	1 L

B16. Antibiotics and media additives

	Concentration	Stock
Ampicillin	100 µl/ml	100 mg/ml in water

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.

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 1N 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 1 N 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% Seperating 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.

APPENDIX C

Plasmid maps

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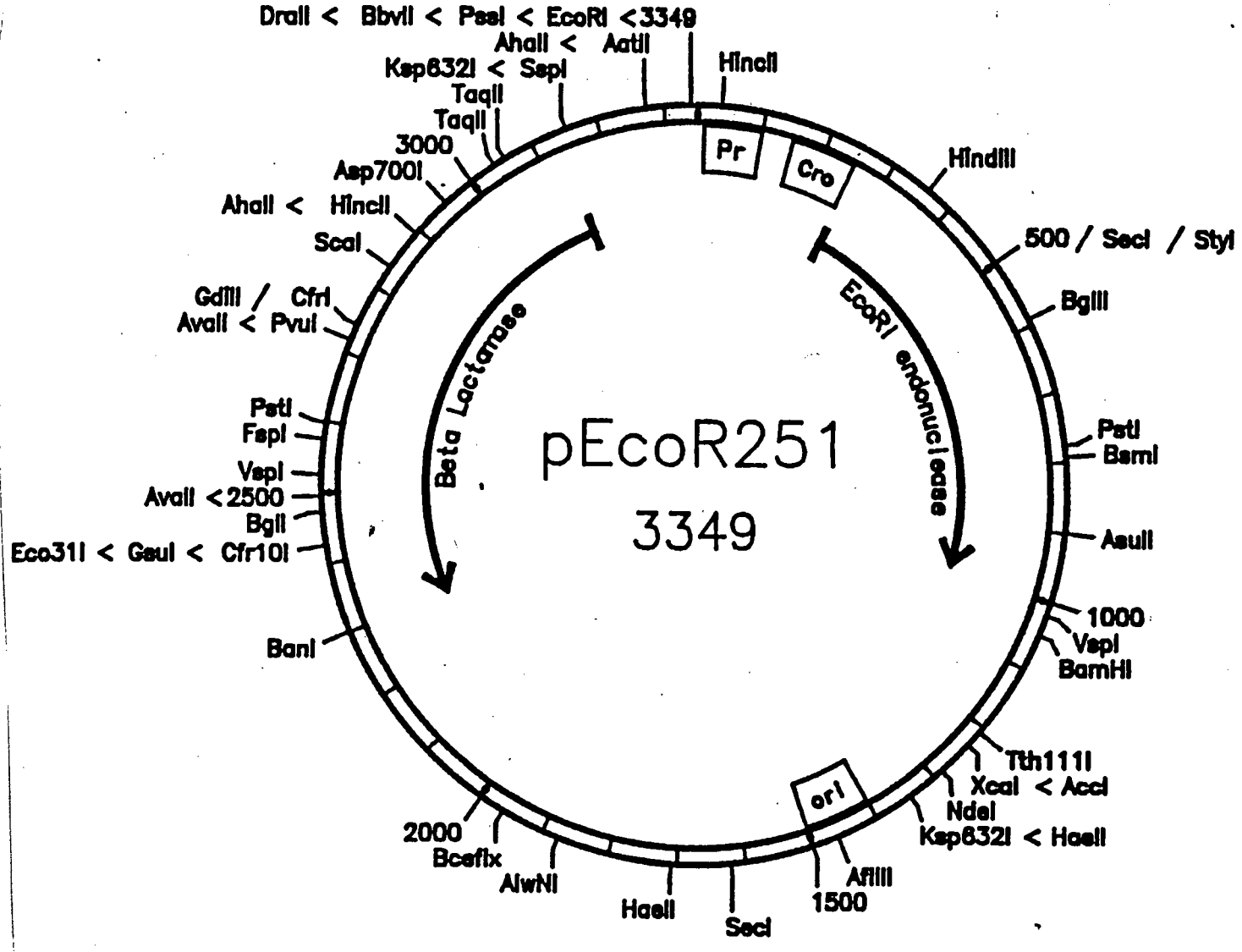
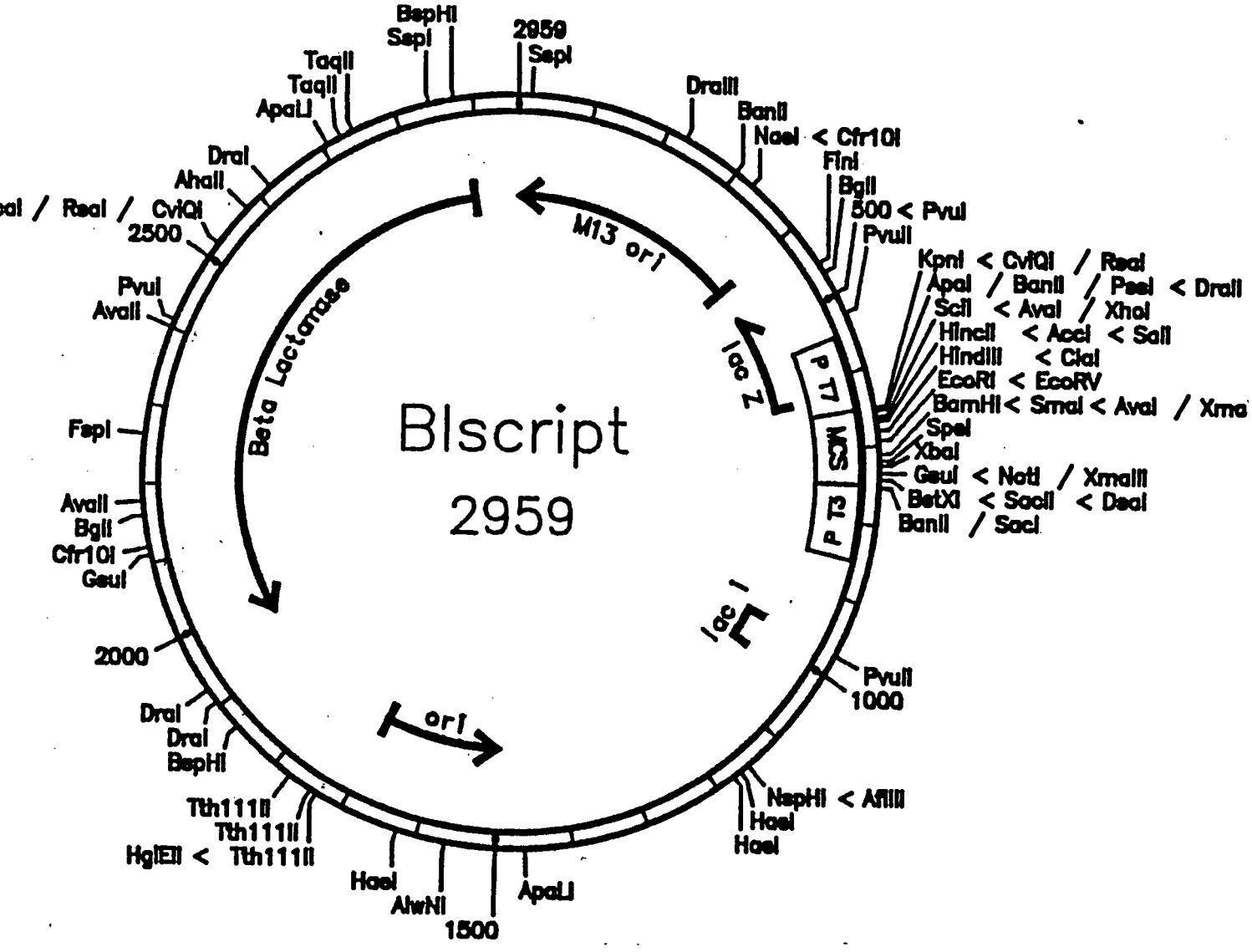


C1. Plasmid map of pEcoR251. DNA is cloned into the gene coding for the restriction endonuclease *EcoRI*. This inactivates the gene thus preventing cell death through the action of the *EcoRI* protein.



C2. Plasmid map of pSK (Stratagene) showing the relative positions of the M13 origin of replication, the plasmid's origin of replication (ori), the β-lactamase gene, the lac I promoter (lac i)and the *lac Z'* gene (lacZ), the lambda phage T7 (P T7) and T3 (P T3) promoters, and the multiple cloning site (mcs).

ABBREVIATIONS

A	adenine/adenosine
Amp	ampicillin
ATP	adenosine 5'-triphosphate
bp	base pair
C	cytidine/cytosine
cDNA	complementary DNA
°C	degrees centigrade
CsCl	caesium chloride
DIG	digoxigenin
DMSO	dimethyl sulphxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
g	gram
G	guanosine
GCG	Genetics Computer Group
h	hour
kb	kilobase
kd	kilodalton
L	litre
LB	Luria Bertani broth
M	molar
min	minute
ml	milliliter
mM	millimolar
mol	mole
mRNA	messenger RNA
nm	nanometer
OMP	outer membrane protein
PCR	polymerase chain reaction

RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	solium dodecyl sulphate
Sp	spectinomycin
T	thymidine
TE	tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
U	uridine
uv	ultraviolet
V	volts
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside
α-	alpha
β-	beta
λ-	lambda
μ-	micro

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