Temperature regulation of the *Escherichia coli* K5 capsule gene cluster

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Abstract

Expression of *E. coli* group 2 capsular polysaccharides is temperature regulated with capsules only being expressed at temperatures above 20°C. Using the K5 capsule gene cluster as a model system, it has been shown that the nucleoid associated protein H-NS plays a unique dual role in regulating capsule gene expression. At 37°C H-NS is required for maximal expression of the capsule genes whereas at 20°C H-NS acts to repress expression. Transposon mutagenesis has identified an additional regulatory factor involved in gene expression, a tyrosine phosphorylated GTPase, BipA. BipA was also shown to play a dual role in transcription.

The role of the Integration Host Factor in activation of the region genes at 37°C was also investigated using IPCRM. In addition potential *cis*-acting elements were identified in both capsule promoters, suggesting a role for these sequences in the coordinate regulation of transcription. The results presented here indicate that a complex regulatory network involving a number of global regulators exists for the control of expression of group 2 capsules in *E. coli*.

Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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Index of Abbreviations

ABC	ATP binding cassette
ATP	adenosine triphosphate
BPI	bacterial/permeability inducing protein
CMP-KDO	cytidine 5'-monophosphate, 2-keto-3-deoxyoctonate
CRP	cAMP receptor protein
CTAB	hexadecyltrimethyl-ammonium bromide
CTD	carboxy terminal domain
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
ECA	enterobacterial common antigen
EF-G	elongation factor G
EPEC	Enteropathogenic *E. coli*
GaINAc	N-acetyl galactosamine
GlcA	glucuronic acid
GlcNAc	N-acetyl glucosamine
GTP	guanosine triphosphate
IHF	integration host factor
IPCRM	inverse PCR mutagenesis
IVET	*in vivo* expression technology
KDO	2-keto-3-deoxyoctonate
LPS	lipopolysaccharide
NTD	amino terminal domain
PCR	polymerase chain reaction
PMN	polymorphonuclear leucocytes
RNA	ribonucleic acid
RNAP	RNA polymerase
SDS	sodium dodecyl sulphate
STM	signature tagged mutagenesis

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Chapter 1

Introduction - Part I

1.1 General features of bacterial polysaccharides

Polysaccharides are highly hydrated polymers composed of repeating monosaccharide units linked by glycosidic bonds. They form a diverse group of molecules as a consequence of the vast variety of existing monosaccharides. Further diversity is achieved through branching of the polysaccharide chain and substitution of polysaccharide components with organic and inorganic molecules, for example phosphate and pyruvate. Capsular polysaccharides can differ in their mode of attachment to the cell surface ranging from a loose association with the cell surface, as with slime polysaccharides, to the covalent linkages observed with lipopolysaccharide (LPS) molecules in Gram negative bacteria and cell-wall teichoic acids in Gram positive bacteria.

Bacterial polysaccharides are generally located on the outer surface of the cell and may mediate direct interactions between the cell and the environment. For this reason they have been assigned a number of functions.

1.2 Functions of bacterial polysaccharides

1.2.1 Prevention of desiccation

Due to the high water content of many polysaccharides (Costerton *et al.*, 1981), the presence of a hydrated gel around the bacterial surface may protect the cell from desiccation (Roberson & Firestone, 1992, Ophir & Gutnick, 1994). This can aid the cell in transmission and survival. In the case of *Escherichia coli*, *Acinetobacter calcoaceticus* and *Erwinia stewartii* it has been shown that mucoid isolates are more resistant to drying than isogenic non-mucoid strains (Ophir & Gutnick, 1994). In addition, desiccation has been found to induce colanic acid polysaccharide biosynthesis in *E. coli* (Ophir & Gutnick, 1994).

1.2.2 Bacteria-plant interactions

Bacterial polysaccharides have been implicated in establishing symbiotic relationships with plants. Several bacterial polysaccharides have been associated with the interactions of *Rhizobium meliloti* with leguminous plants (Gray & Rolfe, 1990; Noel, 1992). *Rhizobium meliloti* interacts with specific legume plants to form nitrogen-fixing nodules. It produces a major polysaccharide called succinoglycan that is required for invasion and development of the nitrogen fixing nodule. *Rhizobium meliloti* also secretes oligosaccharides that may be involved in nodulation by inducing changes in root hairs that facilitate entry of the bacteria (Gray & Rolfe, 1990).

Polysaccharides are also important for survival of plant pathogens. In the case of *Erwinia amylovora* the polysaccharide capsule is essential for growth within the plant and may confer some degree of resistance to the defence responses of the plant (Bugert & Geider, 1995)

1.2.3 Role in adhesion

The main role of polysaccharides in adhesion is as components of biofilm formation. Bacterial biofilms are surface-associated sessile bacterial communities composed of exopolysaccharide surrounded microcolonies (Costerton *et al.*, 1995). Capsular polysaccharides and slime mediate the adherence of bacteria to various surfaces and subsequently facilitate biofilm formation (Costerton *et al.*, 1987). The polysaccharides within the biofilm can then facilitate the binding of other microorganisms, which combined with cell division can help promote biofilm growth (Costerton *et al.*, 1981; 1987). Biofilms adhere to a variety of surfaces both inside and outside the host, including tooth enamel, medical devices, catheters, prostheses, industrial machinery and water systems. In the host biofilms display increased resistance to antimicrobial agents and host defences and have been implicated as the root of many persistent and chronic bacterial infections (Costerton *et al.*, 1999). In the urinary tract, notable biofilm-associated infections include prostatitis, chronic cystitis and catheter–associated infections. Biofilm-dependent infections can have serious consequences for immunocomprimised patients (Costerton *et al.*, 1995). Outside the host biofilms can lead to fouling of industrial surfaces and water pipes (Costerton *et al.*, 1987).

In contrast, certain capsular polysaccharides have been found to prevent adhesion of bacteria with surfaces. For example, a cell-surface polysaccharide in *Proteus mirabilis* can facilitate the migration of swarm cells by reducing surface friction (Gygi *et al.*, 1995)

1.2.4 Role in virulence

Many bacteria associated with invasive disease including *E. coli*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Salmonella typhi* and *Klebsiella* spp. produce LPS and capsular polysaccharides that allow them to resist host defence mechanisms and hence are regarded important in virulence (Cross, 1990). Capsular polysaccharides are likely to protect and maintain viability of the bacterial cell against immediate host defences, after which other virulence factors may be expressed to allow the bacteria to survive and persist. For example, the *E. coli* K1 capsule, a virulence factor associated with *E. coli* isolates responsible for neo-natal meningitis, has been found to help maintain bacterial viability during invasion of the blood-brain barrier but is not necessary for the subsequent invasion of bacteria into brain endothelial cells (Hoffman *et al.*, 1999).

An important mechanism by which invasive bacteria can resist non-specific host defences is through the interactions of polysaccharide capsules with the complement system. In the absence of specific antibody, expression of a capsule can alter activation of the complement system via the alternative pathway and of C3b-mediated opsonophagocytosis by polymorphonuclear leukocytes (PMN's)(Cross, 1990).

Activation of the alternative pathway begins with the non-specific binding of C3b to the bacterial cell surface. Bound C3b then interacts with additional serum factors (B, D & properdin) to form the convertase C3bBb. This can activate the complement amplification loop and lead to the binding of more C3, which can act as a ligand of C3b receptors on PMN's or macrophages. It can also trigger the products of the terminal complement sequence C5-C9, which can ultimately form the membrane attack complex on the outer membrane of bacteria, leading to bacteriolysis and death (Frank *et al.*, 1987). The expression of capsular polysaccharides by invasive bacteria can interfere at different stages in this process.

Capsular polysaccharides may prevent complement mediated killing by acting as a permeability barrier to complement components, masking underlying cell surface structures which would otherwise be potent activators of the alternative pathway (Howard & Glynn, 1971; Horwitz & Silverstein, 1980). *E. coli* causing extra-intestinal infections express cell surface structures, for example capsules and LPS, in a limited number of serotype combinations. It is likely that certain combinations of polysaccharides on the cell surface define the capability of particular isolates to confer resistance to complement mediated killing (Kim *et al.*, 1986).

Some capsular polysaccharides are poor activators of the alternative pathway of complement. Those containing sialic acid, for example, *E. coli* K1 and group B *Streptococci*, have the ability to bind the complement component Factor H, a major regulatory protein of the alternative pathway that binds to C3b (Moxon and Kroll, 1990). Bound Factor H can act as a cofactor to promote the binding of Factor I to C3b to form iC3b, which breaks the amplification loop of the complement cascade. Other capsules, for example certain Pneumococci serotypes have a low affinity for factor B, this inhibits the formation of C3 convertase thereby increasing H-C3b formation which can again break the amplification loop (Moxon & Kroll, 1990; Van Dijk *et al.*, 1979).

Capsular polysaccharides may also confer resistance to complement-mediated opsonophagocytosis, by steric mechanisms. The capsule can act as a mechanical barrier masking the underlying C3b deposited on the cell surface preventing recognition of C3b by phagocytic cells (Brown *et al.*, 1983; Winkelstein, 1981). Most capsular polysaccharides are hydrophilic and hence have a net negative charge on the bacterial surface. The hydrophilic properties of capsules are thought to have an antiphagocytic effect by reducing the surface tension at the interface between the phagocytic cell and the bacterium. This could impair the efficiency of phagocytic ingestion and also confer resistance to complement-mediated opsonophagocytosis (Moxon & Kroll, 1990; Horwitz & Silverstein, 1980)

Certain polysaccharides may confer resistance to specific host immunity as a consequence of the structural similarities between capsular polysaccharides and polysaccharides encountered on the host tissue. For this reason, certain polysaccharides are poorly immunogenic and give a poor antibody response. These include the *E. coli* K1 capsular polysaccharide, a poly-α-2,8-sialic acid which has the

same structure as the carbohydrate terminus of the embryonic neural cell adhesion molecule n-CAM (Finne *et al*, 1983) and the *E. coli* K5 capsular polysaccharide, a heteropolysaccharide consisting of 4-β-glucuronic acid and 4-α-N-acetyl glucosamine, which is similar in structure to the first polymeric intermediate of heparin, desulpho-heparin (Vann *et al*, 1981)(Table 1.2.).

In addition to the roles outlined for evading host immune responses, other roles have recently been suggested for capsular polysaccharides. In *E. coli* it has been speculated that the K5 capsular polysaccharide, a group 2 antigen, may play a role in the colonisation of the large intestine and subsequently increase the likelihood of bacterial translocation from the intestine (Herias *et al.*, 1997). More recently, the capsular polysaccharide components of *Mycobacterium tuberculosis* have been found to promote adhesion to and penetration of host cells, suggesting the adhesive capacity of capsular polysaccharides may have a number of implications for pathogenicity (Daffe & Etienne, 1999).

1.3 Polysaccharides of *Escherichia coli*

The number and diversity of polysaccharides a cell produces ranges from species to species, however in the case of *E. coli* there are essentially four types of polysaccharides: lipopolysaccharide (O antigens), capsular polysaccharide (K antigens), enterobacterial common antigen (ECA) and colanic acid. The O and K antigens are serotype-specific antigens with over 173 O antigens and over 80 K antigens described on the surface of *E. coli.*

Lipopolysaccharide (LPS) is an important component of the outer membrane of gram-negative bacteria (Whitfield & Valvano, 1993). Lipopolysaccharides are complex cell surface glycolipids consisting of three regions; lipid A, core oligosaccharide and the O-specific polysaccharide chain or O antigen (Whitfield & Valvano, 1993). The lipid A moiety in the outer membrane is linked to the O antigen via the core oligosaccharide. LPS differs from most capsular polysaccharides which form an envelope, or capsule around the cell and are anchored via either the phospholipid or core-lipid A. The O antigen extends from the surface of the cell and in some cases can be masked by the K antigen. Both K and O antigens can vary in composition and length (Whitfield & Valvano, 1993).

ECA is a family specific surface antigen produced by all members of the Enterobacteriaceae except *Erwinia chrysanthemi*. Unlike O and K antigens, ECA has an identical structure in all species tested so far. It is located in the outer leaflet of the outer membrane and is a heteropolymer of amino sugars, linked to a L-glycerophosphatidyl residue. In some cases it can be bound to the complete lipopolysaccharide core (Kuhn *et al*, 1988). The role of ECA is currently unknown.

Colanic acid (or M antigen) is a mucoid exopolysaccharide produced by most *E. coli* strains and many species of the family Enterobacteriaceae including *Klebsiella* and *Salmonella* spp. (Whitfield and Valvano, 1993). It is not serotype specific and because it appears to be loosely attached to cells, in the past it has been designated as a slime polysaccharide rather than a capsule. Colanic acid is a high molecular weight polymer of glucose, galactose, fucose and glucuronic acid, substituted with acetyl and pyruvate residues. It does not appear to be involved in pathogenesis and its precise function is not clear. Colanic acid synthesis is stimulated by low temperature and desiccation suggesting that it functions outside the host, as opposed to other capsular polysaccharides which are thought to function within the host (Ophir & Gutnick, 1994). Recently it has been shown that colanic acid is up-regulated in bacterial biofilms and is a critical factor in the formation of the complex three-dimensional structure and depth of *E. coli* biofilms (Prigent-Combaret *et al.*, 1999; Danese *et al.*, 2000).

1.4 Capsular polysaccharides of *Escherichia coli*

Over 80 capsular polysaccharides (K antigens) have been described on the surface of E. *coli* (Ørskov & Ørskov, 1992). The capsular polysaccharides of *E. coli* were originally divided into three groups, group I comprising of subgroups IA and IB, group II and group III sharing similar characteristics to group II (Jann & Jann, 1997). These groups were based on serological characteristics and a variety of biochemical and genetic criteria. Upon further analysis a new and expanded classification system based solely on genetic and biosynthetic criteria has been established consisting of four separate groups (Whitfield & Roberts, 1999). This new system eliminates the use of serotyping as a means of classification of *E. coli* capsular polysaccharides (Table 1.1.). *E. coli* isolates generally express only one type of capsular polysaccharide, however certain K antigens can be co-expressed with

Table 1.1. Classification of *E. coli* capsules (Whitfield & Roberts, 1999)

Characteristic	Group			
	1	2	3	4
Former K-antigen group	IA	II	I/II or III	IB (O-antigen capsules)
Co-expressed with O serogroups	Limited range (O8,O9,O20,O101)	Many	Many	Often O8,O9 but sometimes none
Co-expressed with colanic acid	No	Yes	Yes	Yes
Thermostability	Yes	No	No	Yes
Terminal lipid moiety	Lipid A-core in K_{LPS}; unknown for capsular K-antigen	α-Glycerophosphate	α-Glycerophosphate?	Lipid A-core in K_{LPS}; unknown for capsular K antigen
Direction of chain growth	Reducing terminus	Non-reducing terminus	Non-reducing terminus?	Reducing terminus
Polymerisation system	Wzy-dependent	Processive	Processive	Wzy dependent
Trans-plasma membrane export	Wzx (PST)	ABC-2 exporter	ABC-2 exporter?	Wzx (PST)
Translocation proteins	Wza, Wzc	KpsD, KpsE (KpsF?)	KpsD, KpsE?	Unknown
Elevated levels of CMP-KDO synthetase	No	Yes	No	No
Genetic locus	*cps* near *his* and *rfb*	*kps* near *serA*	*kps* near *serA*	*rfb* near *his*
Thermoregulated (i.e. not expressed below 20 °C)	No	Yes	No	No
Positively regulated by the Rcs system	Yes	No	No	No
Model system	Serotype K30	Serotypes K1, K5	Serotypes K10, K54	Serotypes K40, O111
Similar to	*Klebsiella*, *Erwinia*	*Neisseria*, *Haemophilus*	*Neisseria*, *Haemophilus*	Many genera

Table 1.2. Repeating units of typical K antigens

Group	K antigen	Structure
1	K30	2)-Man-(1,3)-Gal-(1 3 1 β-GlcA-(1,3)-Gal
2	K1	8)-α-NeuNAc-(2,
	K5	4)-β-GlcA-(1,4)-α-GlcNAc-(1,
3	K10	3)-α-Rha-(1,3)-β-QuiNMal-(1 | OAc
	K54	3)-β-GlcA-(1,3)-α-Rha-(1, | CO.NH threonine (serine)
4	K40	4)-β-GlcA-(1,4)-α-GlcNAc-(1,6)-α-GlcNAc-(1 | CO.NH (serine)

Abbreviations: Gal, galactose; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNAc, N-acetylneuraminic acid; Oac, O-acetyl; QuiNMal, 4,6-dideoxy-4-malonylaminglucose; Rha, rhamnose.

For original references see Jann & Jann, 1990

colanic acid (Ørskov *et al.*, 1977; Keenleyside *et al.*, 1993). It is important to note that *E. coli* K-12 laboratory strains do not express the K12 antigen as the K-12 designation is unrelated to capsule type.

1.4.1 Group 1 capsular polysaccharides

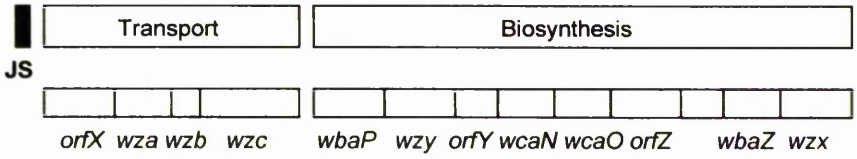
Group 1 capsular polysaccharides are generally associated with isolates of *E. coli* causing enteric disease. This group originally comprised subgroups IA and IB however under the new classification system group 1 represents group IA only (Table 1.1.). Of the large number of K antigens structurally defined in *E. coli*, 16 show characteristics typical of group 1 capsules (Jann & Jann, 1997). *E. coli* strains with group 1 capsules express their K antigen on the cell surface in two different forms. Firstly, a low-molecular-weight form termed K_{LPS}, which comprises K-antigenic oligosaccharides linked to a lipid-A-core and resembles a lipopolysaccharide (LPS) linked O antigen. The second form is a high-molecular-weight capsular K antigen, which is associated with the cell surface. The mode of attachment of the high molecular weight K antigen is not yet known, however LPS is not involved (MacLachlan *et al.*, 1993).

Group 1 polysaccharides are acidic polysaccharides and resemble the capsules of *Klebsiella* spp. on the basis of structure, genetics and patterns of expression. For example, the *E. coli* K28 and K55 antigens are identical to the K54 and K5 antigens of *Klebsiella* respectively (Jann & Jann, 1992). Group 1 K antigens can be expressed at all growth temperatures and are co-expressed with a limited range of neutral O antigens (Jann & Jann, 1990). They are not co-expressed with the exopolysaccharide colanic acid as it has been suggested that group 1 producing strains are actually unable to produce colanic acid. It is believed this is due to the lateral transfer of a large chromosomal segment from *Klebsiella pneumoniae* to *E. coli*, which appears to have replaced the colanic acid locus region in group 1 *E. coli* strains (Drummelsmith & Whitfield, 1999; Rahn *et al.*, 1999).

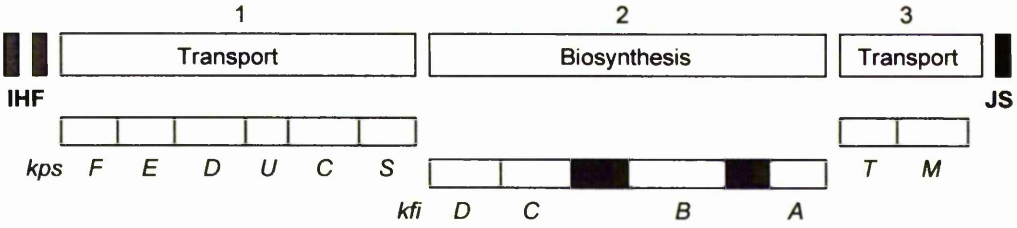
The genes for production of the group 1 K_{LPS} and capsular K antigen are encoded at the *cps* locus found near the *his* and *rfb* gene clusters on the *E. coli* chromosome (Whitfield *et al.*, 1989). The K30 antigen gene cluster has been cloned and analysed and used as a prototype for group 1 capsules in *E. coli*. The K30 gene cluster is a 16 kb locus containing twelve genes required for synthesis,

Figure 1.1. Genetic organisation of representative group 1, group 2 and group 3 capsule gene clusters. A representative from each group is shown, group I - K30 (Drummelsmith & Whitfield, 1999; Rahn *et al.*, 1999); Group 2 – K5 (Roberts, 1996; Simpson *et al.*, 1996; Stevens *et al.*, 1997); Group 3 – K10 (Pearce & Roberts, 1995; Clarke *et al.*, 1999). The larger boxes denote the defined functional regions. The smaller labeled boxes define specific genes that have been identified within each cluster. The hatched boxes in region 2 of the K5 capsule gene cluster define intergenic gaps. **JS** - JUMPstart sequence; **IHF** - Integration host factor consensus sequences

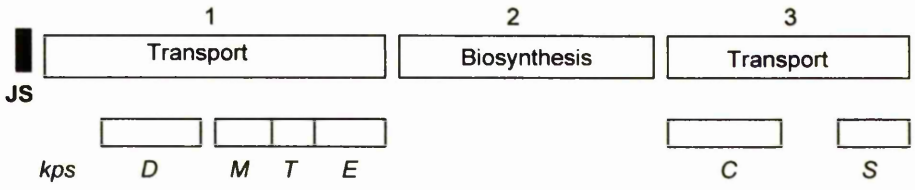
Group 1 - K30



Group 2 - K5



Group 3 - K10



polymerisation and translocation of the K30 antigen (Figure 1.1.). The genetic organisation of the K30 biosynthetic gene cluster is similar to that of the colanic acid gene cluster in *E. coli* and the *Klebsiella* K2 capsule (Stevenson *et al.*, 1996; Arakawa *et al.*, 1991). Functions have been identified for all but three open reading frames in the K30 gene cluster (Jayaratne *et al.*, 1994; Kido *et al.*, 1995; Drummelsmith *et al.*, 1997; Drummelsmith & Whitfield, 1999). Of the twelve genes in the K30 cluster, four genes (*wbaP*, *wcaN*, *wcaO*, *wbaZ*) encode glycosyltransferases which are required for synthesis of the K30 repeat unit. Gene *wzy* encodes the K30 repeat unit polymerase enzyme and gene *wzx* encodes a PST(1) exporter which transfers lipid-linked K30 repeat units across the plasma membrane to the periplasmic space (Drummelsmith & Whitfield, 1999).

Further analysis of the group 1 K30 prototype has revealed that group 1 K antigens are polymerised and exported by the Wzy-dependent system (Drummelsmith & Whitfield, 1999). The Wzy-dependent system was originally described for biosynthesis of certain *Salmonella* O antigens (Whitfield, 1989). To summarise, repeat units are assembled on a carrier lipid such as undecaprenyl phosphate, through the action of glycosyltransferase enzymes. The lipid-linked repeat units are then transferred across the plasma membrane by the actions of the Wzx protein by an unknown mechanism. The polymerase enzyme, Wzy, then catalyses polymerisation of the polysaccharide at the periplasmic face of the plasma membrane. In the case of the K30 antigen, WbaP, a member of a family of UDP-hexose:undecaprenylphosphate hexose-1-P transferase enzymes is used as the initiating glycosyltransferase (Drummelsmith & Whitfield, 1999). Polymerisation by the Wzy-dependent system occurs at the reducing terminus of the polysaccharide, one repeat unit at a time.

After analysis of a number of group 1 capsule gene clusters a conserved region of genes, *orfX-wza-wzb-wzc*, was identified (Rahn *et al.*, 1999)(Figure 1.1.). In the K30 antigen gene cluster *wza* and *wzc* gene products are known to be involved in translocation and surface assembly of the K30 antigen, suggesting that group 1 K antigens may have a conserved pathway for translocation and surface assembly (Drummelsmith & Whitfield, 1999; Rahn *et al.*, 1999). The Wza protein has recently been found to a play a major role in capsule translocation. Wza is a surface exposed outer membrane lipoprotein which belongs to the outer membrane auxiliary (OMA) protein family, members of which are found widely distributed in gram negative

bacteria. It has recently been postulated that Wza forms an outer membrane pore through which the K30 capsular antigen is translocated. This is the first evidence of a potential mechanism for translocation of high molecular weight polysaccharide across the outer membrane (Drummelsmith & Whitfield, 2000).

Group 1 capsule gene clusters are regulated by the Rcs regulatory protein network (Jayaratne *et al.*, 1993). The Rcs system is a group of regulatory proteins known to regulate colanic acid synthesis in *E. coli* (Gottesman & Stout, 1991). RcsA is a positive regulator of this system and is believed to interact with the promoter-operator region of the capsule genes.

Upstream of the K30 antigen gene cluster a characteristic JUMPstart sequence has been identified (Rahn *et al.*, 1999). The JUMPstart sequence is a 39bp sequence which is also present upstream of many polysaccharide biosynthesis genes in enteric bacteria (Hobbs & Reeves, 1994; Arakawa *et al.*, 1995). It contains the *ops* (operon polarity suppressor) motif and is thought to play a role in antitermination, eliminating transcriptional polarity in large transcriptional units (Nieto *et al.*, 1996; Bailey *et al.*, 1997). The JUMPstart sequence is also located upstream of group 2 capsule gene clusters and O antigen gene clusters in *E. coli* (Bailey *et al.*, 1997; Stevens *et al.*, 1997; Marolda & Valvano, 1998). It has been suggested that the JUMPstart sequence may function in conjunction with RfaH to inhibit transcriptional termination at a stem-loop structure found immediately downstream of the translocation-surface assembly region of the K30 antigen gene cluster (Rahn *et al.*, 1999). RfaH is a regulatory protein known to activate the transcription of several virulence and fertility genes in enteric bacteria. Examples include regulating expression of lipopolysaccharide biosynthesis genes in *E. coli* and *Salmonella typhimurium* (Pradel & Schnaitman, 1991; Brazas *et al.*, 1991), α-haemolysin toxin in *E. coli* (Bailey *et al.*, 1992) and F-factor sex pilus (Beutin & Achtman., 1979). RfaH has also been implicated in the expression of group 2 and group 3 capsules (Stevens *et al.*, 1994; Clarke *et al.*, 1999).

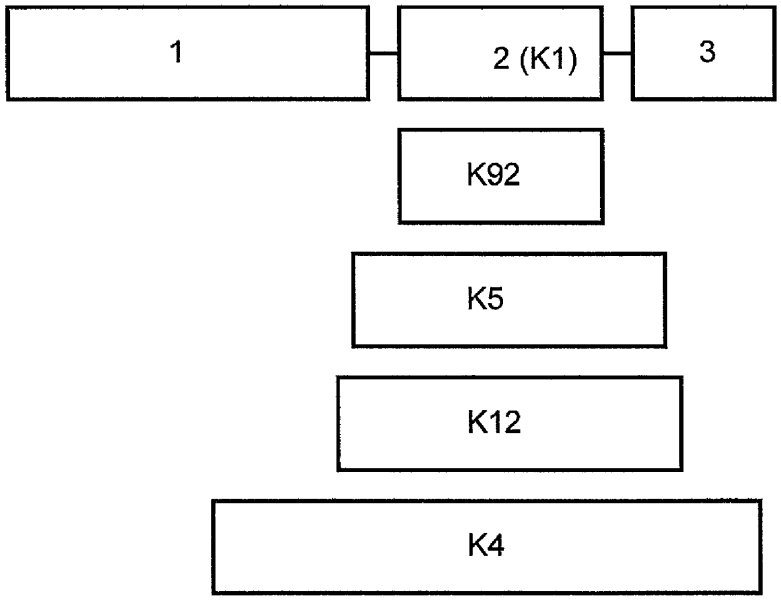
1.4.2 Group 2 capsular polysaccharides

Group 2 K antigens (the focus of this study) are associated with *E. coli* which cause serious invasive disease, including neonatal meningitis, septicaemia and pyelonephritis (Ørskov & Ørskov, 1992). They exhibit higher charge densities than

the group 1 K antigens and may contain hexuronic acids, N-acetylneuraminic acid or KDO as acid components (Jann & Jann, 1992). Group 2 K antigens can be coexpressed with many O antigens in contrast to the limited number expressed with the group 1 K antigens. They resemble the capsules of *H. influenzae* and *N. meningitis* on the basis of structure, mode of attachment to the cell surface, assembly and capsule gene organisation (Jann & Jann, 1990). *E. coli* isolates expressing group 2 K antigens have an α-glycerophosphatidic acid moiety attached to the reducing terminus of the polysaccharide via a phosphodiester linkage. This acts as a membrane anchor that links the K antigen to the cell surface (Schmidt & Jann, 1982; Jann & Jann, 1990). In certain *E. coli*, KDO acts as a "linker" between the lipid and the reducing terminus of the polysaccharide (Roberts, 1996; Jann & Jann, 1997). Hence, *E. coli* isolates expressing group 2 K antigens have elevated levels of the enzyme CMP-KDO synthetase at capsule permissive temperatures. In *E. coli* group 2 antigens containing polysialic acid, for example the K1 and K92 antigens the KDO linker is absent. The reason for this difference is not known.

The gene clusters for production of group 2 K antigens are located near to the *serA* locus on the *E. coli* chromosome (Boulnois and Roberts, 1990; Vimr, 1991) and have been a focus of molecular research, with the K1, K4, K5, K7, K12 and K92 antigen gene clusters having been cloned and analysed (Silver *et al.*, 1981; Roberts *et al.*, 1986; Roberts *et al.*, 1988; Vimr *et al.*, 1989; Drake *et al.*, 1990)(Figure 1.1.). As a result of this work, it has been revealed that the group 2 K antigens have a conserved genetic organisation, which consists of three functional regions (Roberts *et al.*, 1988)(Figure 1.2.). Region 2 is the central serotype-specific region which encodes enzymes for synthesis and polymerisation of the K antigen (Petit *et al.*, 1995; Sieberth *et al.*, 1995). Some group 2 K antigens contain sugars which are not commonly found in *E. coli*, in such circumstances region 2 also encodes additional enzymes required for the biosynthesis of the relevant nucleotide sugar precursors. For example, the K5 antigen region 2 encodes a UDP-Glc dehydrogenase enzyme for the synthesis of UDP-Glucuronic acid (GlcA) a component sugar of the K5 polysaccharide (Petit *et al.*, 1995, Sieberth *et al.*, 1995). Region 2 is flanked by regions 1 and 3 which are strongly conserved between different group 2 K antigen gene clusters (Roberts *et al.*, 1988; Roberts, 1995)(Figure 1.2). Region 1 contains six genes (*kpsFEDUCS*) and region 3 contains two genes (*kpsMT*). They encode proteins necessary for polymer translocation from its

Figure 1.2. Schematic representation of the organisation of *E. coli* group 2 capsule gene clusters (Roberts, 1996). The K1 capsule gene cluster is shown with the three functional regions. The boxes labelled K92, K5, K4 and K12 represent the serotype specific region 2s that are inserted between the conserved regions 1 and 3



cytoplasmic site of synthesis, through the two membranes of the gram negative cell envelope to the cell surface. Conservation of these regions suggests a common pathway of export exists for group 2 capsules (Roberts, 1995).

Export of group 2 capsules is not fully understood. The two proteins of region 3 KpsM and KpsT are known to form an inner membrane polysaccharide export system (Smith *et al.*, 1990; Pavelka *et al.*, 1991). Homologues of KpsM and KpsT have been found in other encapsulated bacteria and are thought to have an analogous role in polysaccharide export in other species. Examples include, BexA/B of *H. influenzae* type b (Kroll *et al.*, 1990) and CtrC/D of *N. meningitidis* group B (Frosch *et al.*, 1991). Together, KpsM and KpsT belong to a large superfamily of transport proteins called ATP-binding cassette (ABC-2) transporters or traffic ATPases (Ames *et al.*, 1992; Higgins, 1992). ABC transporters are involved in a wide range of import and export pathways in both prokaryotes and eukaryotes (Doige *et al.*, 1993). They typically consist of a hydrophobic integral inner-membrane protein and a hydrophilic peripheral inner-membrane protein containing an ATP-binding domain, KpsM and KpsT respectively (Smith *et al.*, 1990; Doige *et al.*, 1993).

An important feature of the Group 2 K antigens is that they are under temperature regulation at the level of transcription with capsule gene expression at 37°C but not at 20°C (Ørskov *et al.*, 1984; Simpson *et al.*, 1996). This contrasts with other capsule groups whose genes are all constitutively expressed. It suggests that group 2 K antigens may be required by invasive bacteria upon entering the host where they encounter elevated temperatures. Environmental regulation is a common feature of virulence gene expression and can allow the bacteria to adapt to varying ecological niche encountered within the host during infection. The control of group 2 capsule gene expression is complex involving RfaH and IHF (Stevens *et al.*, 1994; Stevens *et al.*, 1997) and will be discussed in section 1.5.5.

1.4.3 Group 3 capsular polysaccharides

Group 3 K antigens have the same general characteristics as those of group 2, with group 3 capsule genes also located near *serA* on the *E. coli* chromosome (Table 1.1).(Ørskov and Nyman, 1974). Genetic analysis of the K10 and K54 capsule gene clusters (prototypes from this group) revealed that group 3 capsule gene clusters also appear to have a conserved genetic organisation (Pearce and

Roberts, 1995)(Figure 1.1.). A central serotype-specific region is flanked by group 3 capsule specific sequences in a similar manner to the group 2 capsule gene clusters. Analysis of the *E. coli* K10 capsule gene cluster revealed two regions (region 1 and 3) conserved between different group 3 capsule gene clusters. These regions are thought to be involved in group 3 capsule export, the details of which are not fully understood. However, region 1 and region 3 of the K10 and K54 capsule gene clusters encode homologues of group 2 capsule region 1 and 3 proteins (*kpsDEMT*)(Clarke *et al.*, 1999). Complementation studies have demonstrated that group 2 *kpsD* and *kpsE* mutations were complemented by clones from the K10 and K54 gene clusters. Therefore, despite little sequence homology it appears that there are functionally conserved steps in the export of group 2 and group 3 capsular polysaccharides (Pearce and Roberts, 1995). This suggests that an ABC-2 transporter-dependent system similar to that of group 2 capsules may function in the export of group 3 capsules. In the group 3 K10 capsule gene cluster grouping of these export genes differs from the group 2 gene clusters, with *kpsD* and *kpsE* found flanking *kpsM* and *kpsT* in region 1, not region 3 (Clarke *et al.*, 1999)(Figure 1.1.).

Unlike group 2 K antigens, group 3 K antigens are expressed at all growth temperatures and do not have elevated levels of CMP-KDO synthetase (Ørskov *et al.*, 1984; Finke *et al.*, 1990; Pearce & Roberts, 1995). It has been demonstrated that like the group 2 capsules, the K10 capsule gene cluster is RfaH regulated. A JUMPstart motif has been located upstream of the K10 region 1 and it has been speculated that RfaH may allow readthrough transcription from region 1 into region 2 (Clarke *et al.*, 1999).

1.4.4 Group 4 capsular polysaccharides

Group 4 has only recently been formed and is composed of former group IB K antigens and surface polysaccharides known as O-antigen capsules (Table 1.1.). The group IB K antigens were originally designated as capsules as they masked neutral O8 and O9 antigens coexpressed on the same cell in agglutination reactions. In the absence of O8 and O9 antigens the K antigen was reclassified as an O antigen (Jann & Jann, 1990). For example, K87=O32; K85=O141; K9=O104 (Whitfield *et al.*, 1984). These K antigens were distinguished from other group 1 K

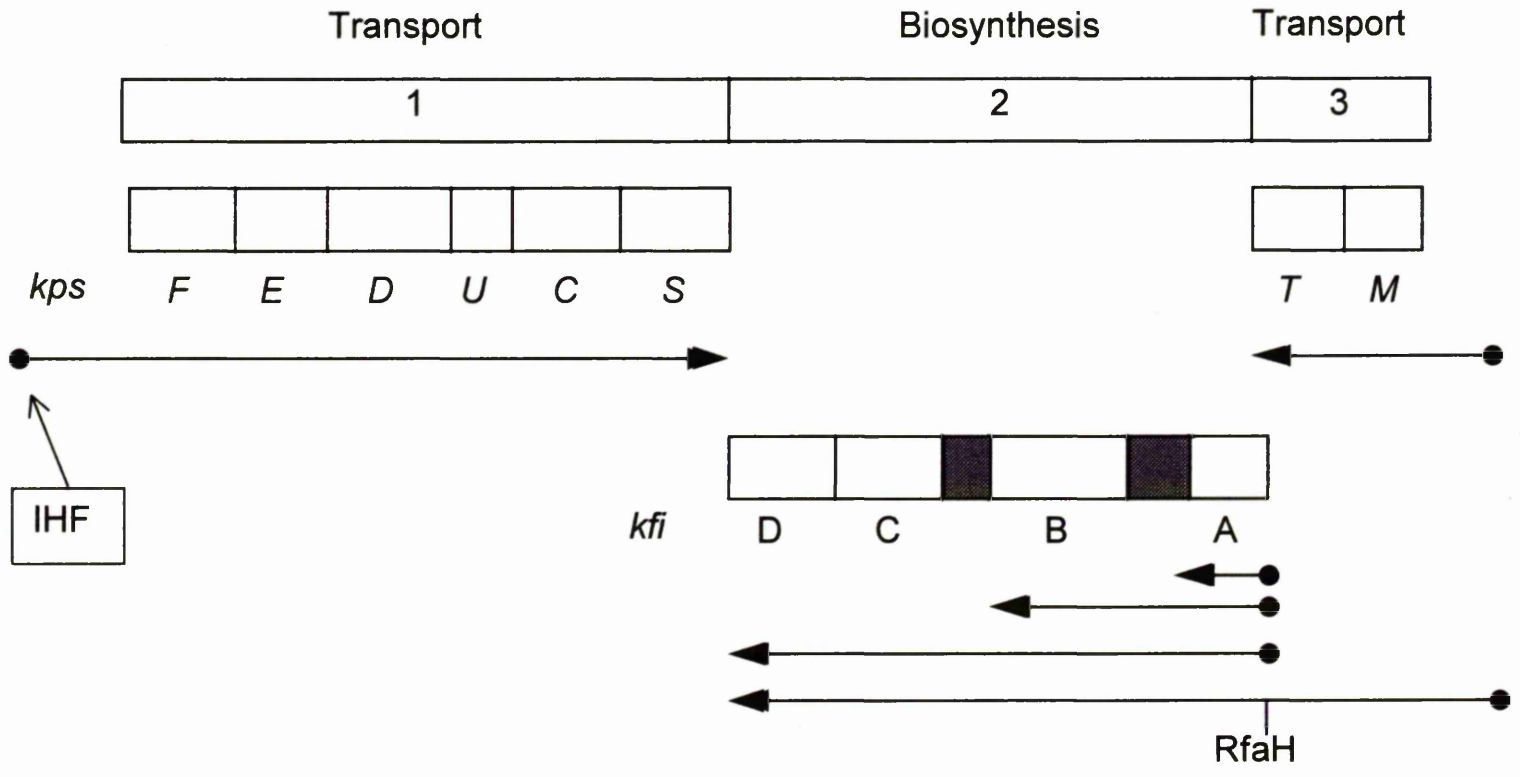
antigens on the presence of amino sugars as a component of their repeat structure (Jann & Jann, 1997). In some cases, for example the K40 antigen, the repeat unit was also substituted with amino acids in a similar manner to the O antigens of *Proteus* spp. However, it now appears that the surface expression as smooth LPS and as unlinked O-antigen capsule of group IB K antigens, such as K40, has led to the formation of a new group of capsular polysaccharides (Amor & Whitfield, 1997; Whitfield & Roberts, 1999). The group IB K antigens are assigned to group 4 on the basis of a number of biosynthetic and regulatory criteria.

Other polysaccharides assigned to group 4 include the O-antigen capsules. First described by Goldman *et al.*,(1982) the O-antigen capsules exist as O-antigen in a LPS-unlinked capsular form (Goldman *et al.*, 1982). The detection of O-antigen capsules may have been overlooked as O antigens were generally expected to be lipid A core linked. Examples include O26, O55, O100 and O111.

The K40 and O111 capsules have been used as group 4 capsule prototypes. Like the group 1 capsules, the K40 and O111 antigens also appear to be polymerised and exported by the Wzy-dependent system (Amor & Whitfield, 1997; Wang *et al.*, 1998). However, the main difference separating the two systems is the identity of the initiating glycosyltransferase. In the case of K40 and O111, WecA is used (Amor & Whitfield, 1997; Wang *et al.*, 1998). WecA transfers either GlcNAc-1-P or GalNAc-1-P to undecaprenyl phosphate in the initiating reaction of the polymerisation process.

The K40 prototype biosynthesis gene cluster is located near the *his* region on the *E. coli* chromosome, the same site as that of *E. coli* O antigen (*rfb*) clusters. It consists of six open reading frames (*orf1-6*). Predicted functions of *orf1-6* gene products include homologues of components of the Wzy-dependent polymerisation and export system and putative glycosyltransferases (Amor & Whitfield, 1997). The region upstream of the K40 capsule gene cluster shows no features required for regulation by the Rcs regulatory system suggesting this system does not play a role in the expression of Group 4 capsules. However, the K40 biosynthesis capsule genes are preceded by a JUMPstart sequence, 200 bp upstream of *orf1*, suggesting a potential role for RfaH in group 4 capsule gene regulation (Amor & Whitfield, 1997).

Figure 1.3. Transcriptional organisation of the *Escherichia coli* K5 capsule gene cluster (Roberts, 1996; Simpson *et al.*, 1996; Stevens *et al.*, 1997). Boxes labelled 1-3 represent the three functional regions described in the text. Region 1 and 3 genes are designated *kps* whilst K5-specific (region 2) genes are designated *kfi*. Arrows indicate the direction of transcription.



1.5 Genetics and biochemistry of group 2 capsule production in *E. coli*

1.5.1 The *E. coli* K5 capsule gene cluster

The K5 capsule gene cluster has been used as a paradigm for group 2 capsule gene clusters in *E. coli* and other bacterial species. The K5 capsule is composed of two alternating monosaccharides and its structure has been determined as -GlcA-(β1-4)-GlcNAc-(α1-4)- (Table 1.2.)(Vann *et al.*, 1981). The K5 capsule gene cluster has now been completely sequenced and provides a useful foundation for in-depth genetic and molecular analysis. The *E. coli* K5 capsule gene cluster can be seen in Figure 1.3.

1.5.2 Region 1 of the *E. coli* K5 capsule gene cluster

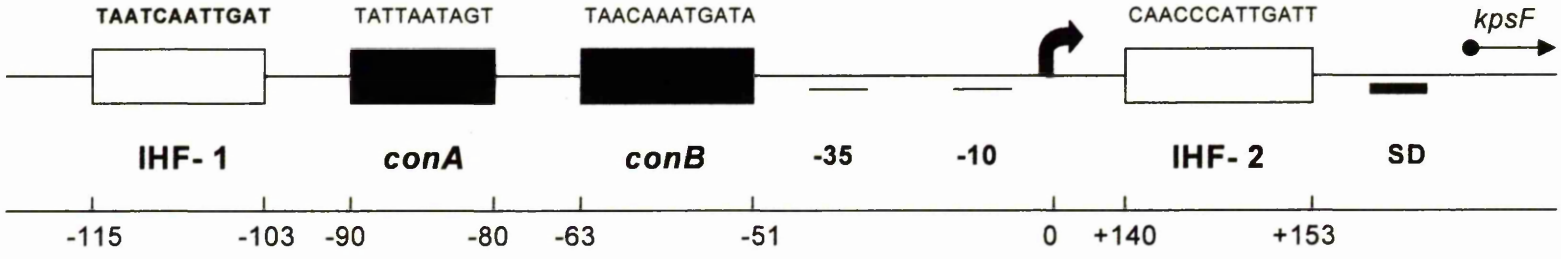
Region 1 consists of six genes, *kpsF, E, D, U, C* and *S* (Figure 1.3.). Functions have been assigned to the majority of the region 1 gene products. The KpsE and KpsD proteins are located in the periplasm and are believed to be involved in the translocation of polysaccharide across the periplasmic space and onto the cell surface (Bronner *et al.*, 1993; Rosenow *et al.*, 1995a; Wunder *et al.*, 1994). Mutations in either *kpsC* or *kpsS* result in the accumulation of cytoplasmic K5 polymer, which lacks a phosphatidyl KDO at its reducing end (Bronner *et al.*, 1993). This suggests that KpsC and KpsS play a role in the attachment of 2-keto-3-deoxyoctonate (KDO) to phosphatidic acid and the subsequent ligation of phosphatidyl-KDO to the reducing terminus of the polysaccharide (Roberts, 1995; 1996). The KpsU protein is a capsule specific cytoplasmic cytidine 5'-monophosphate CMP-KDO synthetase enzyme that generates CMP-KDO for polysaccharide biosynthesis (Pazzani *et al.*, 1993a; Rosenow *et al.*, 1995b). The KpsU and KpsC proteins appear to be translationally coupled suggesting they may interact together in some form of complex (Pazzani *et al.*, 1993b). The KpsF protein is not essential for capsule expression and its role is unclear. It is has been speculated that it may play a role in the export of capsular polysaccharide (Simpson *et al.*, 1996; Cieslewicz & Vimr, 1997).

Region 1 is organised as a single transcriptional unit arising from a promoter located 223bp upstream of the first region 1 gene *kpsF* (Simpson *et al.*, 1996)(Figure 1.3.). Transcription from the region 1 promoter generates an 8.0kb polycistronic transcript, which is processed to yield a separate, stable 1.3kb *kpsS* transcript. This may allow differential expression of the KpsS protein (Simpson *et al.*, 1996). A Rho-dependent transcriptional terminator has been found in the *kpsF* gene, which may be required during times of physiological stress to prevent the synthesis of untranslated region 1 transcripts (Simpson *et al.*, 1996). The region 1 promoter resembles a typical *E. coli* σ^{70} promoter with both -10 and -35 consensus sequences present (Figure 1.4.). It is flanked by integration host factor consensus binding sites (IHF-1 & IHF-2) positioned at –103 to –115bp 5' (IHF-1) and +140 to +153bp 3' (IHF-2) to the transcription initiation site (Simpson *et al.*, 1996). Integration host factor is a DNA binding protein which regulates a number of operons in *E. coli* and is known to play a regulatory role in region 1 transcription (Freundlich *et al.*, 1992; Simpson *et al.*, 1996). The mechanism of regulation however is not fully understood. The region 1 promoter has been shown to be temperature regulated at the level of transcription with gene expression at 37°C but not at 20°C (Simpson *et al.*, 1996). Analysis of the region 1 promoter revealed no alternative sigma factor binding sites.

1.5.3 Region 2 of the *E. coli* K5 capsule gene cluster

Region 2 of the *E. coli* K5 capsule gene cluster is 8.0kb and consists of four genes, *kfi A-D* (Petit *et al.*, 1995)(Figure 1.3.). Products of the region 2 genes are thought to be essential for biosynthesis of the K5 polysaccharide as mutations in these genes abolish any detectable K5 capsule production (Petit *et al.*, 1995). Of the proteins encoded by region 2, KfiC appears to be a K5 transferase enzyme which add the glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) residues to the non-reducing end of the K5 polysaccharide chain (Petit *et al.*, 1995; Sieberth *et al.*, 1995). The KfiD protein is a UDP-glucose dehydrogenase which catalyses the formation of UDP-glucuronic acid for incorporation into the K5 antigen (Petit *et al.*,

Figure 1.4. Schematic representation of the region 1 promoter (Roberts, 1996; Simpson *et al.*, 1996; Stevens *et al.*, 1997). Shaded arrow indicates transcription initiation site; conA & conB represent AT-rich regions conserved in the region 1 and 3 promoters; IHF-1 and IHF-2 represent integration host factor consensus sequences; Number line indicates position of relevant sequences with respect to the transcription initiation site.



1995; Sieberth *et al.*, 1995). It has been demonstrated that both KfiC and KfiD are associated with the inner membrane (Rigg *et al.*, 1998). The functions of the KfiA and KfiB proteins are not known. However, it has been shown that the KfiA protein is also associated with the inner membrane and it has been suggested that KfiA may play a role in the initiation of the polymerisation of the K5 polysaccharide (Rigg *et al.*, 1998).

There have been three promoters detected within the K5 region 2 (Petit *et al.*, 1995)(Figure 1.3.). The region 2 promoters are expressed at both 37°C and 20°C, in contrast to the region 1 and region 3 promoters, which are temperature regulated. Expression at 20°C however does not generate any detectable K5 polysaccharide (I.S. Roberts unpublished; Roberts, 1996)

Expression of the region 2 genes requires the *rfaH* gene (Stevens *et al.*, 1994). RfaH acts as an anti-terminator and allows readthrough transcription to proceed from region 3 into region 2 (Stevens *et al.*, 1997). In the absence of RfaH, transcription originating from the three region 2 promoters is at levels insufficient to generate a K5 capsule (Stevens *et al.*, 1997). As a consequence of transcription of the region 2 genes arising from the region 3 promoter, region 2 is therefore also temperature regulated.

1.5.4 Region 3 of the *E. coli* K5 capsule gene cluster

Region 3 consists of two genes, *kpsM* and *kpsT* (Figure 1.3.). KpsM and KpsT appear to be translationally coupled allowing balanced expression of both proteins. The proteins encoded by region 3 are involved in the export of K5 polysaccharide across the cytoplasmic membrane. The KpsM and KpsT proteins are conserved in group 2 capsules and are members of a family of ATP-binding cassette (ABC-2) transporters and comprise an inner-membrane polysaccharide export system (Smith *et al.*, 1990; Pavelka *et al.*, 1991; Bliss & Silver, 1996).

The region 3 genes are organised as a single transcriptional unit (Bliss & Silver, 1996; Roberts, 1996)(Figure 1.3.). Transcription arises from a promoter located 741bp upstream of the initiation codon of the region 3 gene, *kpsM*. This promoter resembles a standard *E. coli* σ^{70} promoter with a -10 consensus sequence but no -35 consensus sequence (Stevens *et al.*, 1997). The region 3 promoter is

temperature regulated at the level of transcription with gene expression at 37°C but not at 20°C. Analysis of the promoter revealed no alternative sigma factor binding sites or integration host factor consensus binding sequences.

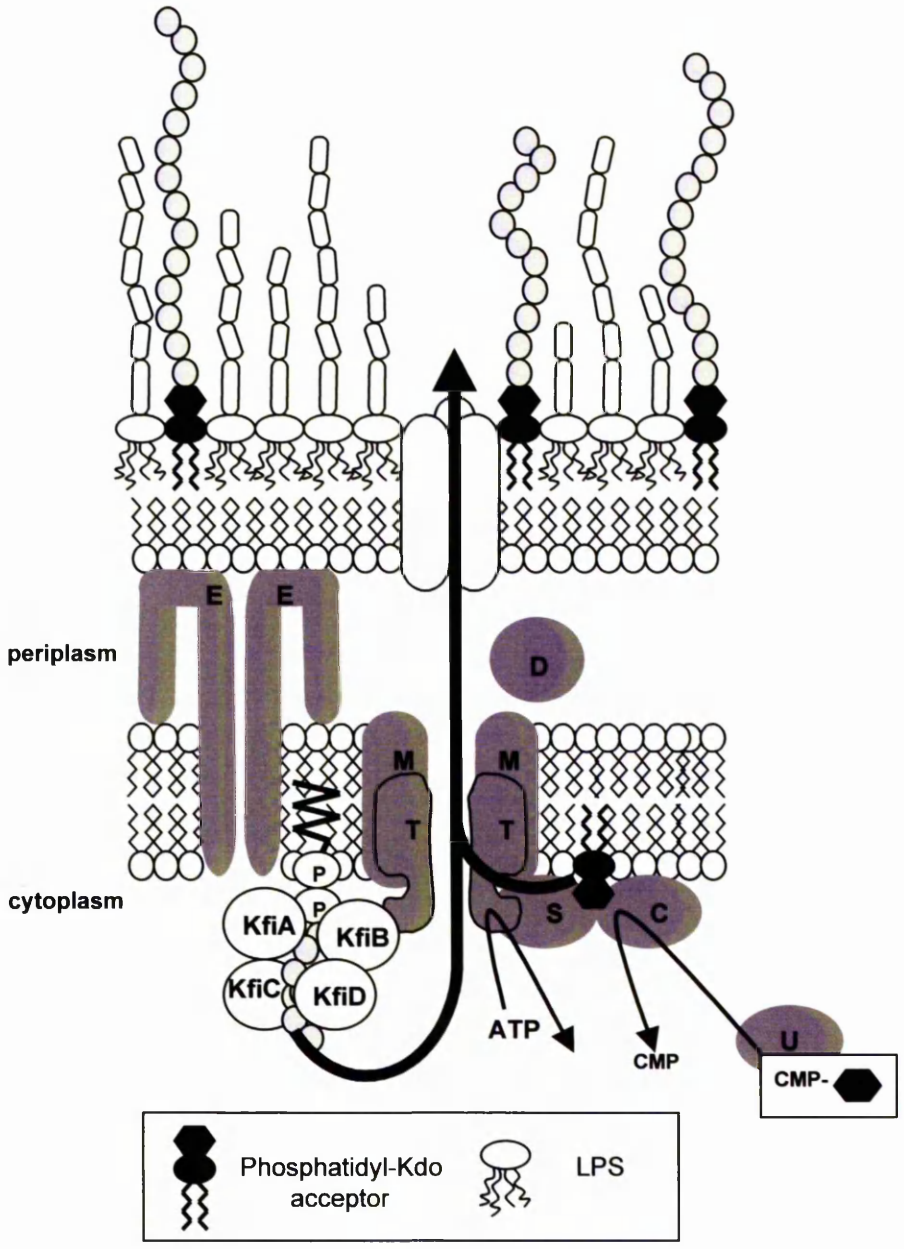
1.5.5 Regulation of the *E. coli* K5 capsule gene cluster

The mechanism of temperature regulation of the K5 capsule gene cluster is not clear. The role of certain known regulatory proteins in K5 capsule gene expression has been analysed however, only RfaH and IHF were found to have a regulatory role (Stevens *et al.*, 1994; Simpson *et al.*, 1996). The Rcs protein regulatory network, which is known to regulate other capsule gene clusters in *E. coli* does not act to regulate the K5 capsule genes (Stevens *et al.*, 1994). After sequence alignment of the region 1 and 3 sequences it became apparent that two AT-rich regions (conA & conB) were conserved in both the region 1 and region 3 promoters (Figure 1.4.). It is possible that these sequences may represent *cis*-acting regulatory sites which could facilitate the binding of regulatory proteins, suggesting that perhaps the region 1 and region 3 promoters may be co-ordinately regulated.

1.5.6 Model for expression of the *E. coli* K5 antigen

A model for expression of the *E. coli* K5 antigen has been proposed (Roberts, 1995; Whitfield & Roberts, 1999). Assembly of the K5 capsule takes place on the cytoplasmic face of the inner membrane (Figure 1.5.). It begins with elongation of the polysaccharide by the KfiC protein. The KpsMT ABC-2 transporter then delivers the nascent polysaccharide across the inner membrane. This process is thought to involve a hetero-oligomeric membrane-bound complex on the inner membrane, consisting of KfiABCD, KpsCMST (Rigg *et al.*, 1998). These proteins act in concert to polymerise then translocate the K5 polysaccharide (Figure 1.5.). KfiABCD are required for polymerisation, KfiC transfers glucuronic acid and N-acetylglucosamine residues to the non reducing end of the polysaccharide and KfiD supplies the UDP-glucuronic acid for K5 antigen biosynthesis. The roles of the KfiA and KfiB proteins in polymerisation are still not clear.

Figure 1.5. Model for assembly of the K5 polysaccharide. (Whitfield & Roberts, 1999) Proteins involved in polymer synthesis (KfiABCD) interact with a 'scaffold' comprising KpsCMST to form a biosynthesis-export-translocation complex on the inner membrane. The polymer is shown growing on an undecaprenyl pyrophosphate carrier before being transferred to phophatidyl-Kdo.



KpsCMST are required for formation and stabilisation of the biosynthetic/export complex on the inner membrane (Rigg *et al.*, 1998). KpsT provides energy derived from ATP hydrolysis to KpsM, which mediates the transport process. The exact roles of KpsS and KpsC in this process are not known.

The mechanism of translocation of the K5 antigen from the periplasm, across the outer membrane and assembled on the cell surface is not fully understood. It is thought to occur at Bayer junctions where the inner and outer membranes appear to meet and is mediated by the KpsE and KpsD proteins (Bayer & Thurow, 1977). It is possible that KpsE and KpsD may be linked to the biosynthetic/export complex located on the inner membrane and form a multiprotein capsule assembly complex (Figure 1.5.). Secondary structure analysis of KpsE suggests that it is similar to members of the membrane fusion protein family (Bliss & Silver, 1996; Dinh *et al.*, 1994). These proteins are believed to interact with ABC-type transport proteins (and others) and perhaps outer membrane proteins to facilitate substrate transport of large molecules. This suggests a potential interaction between KpsE and the KpsMT ABC-2 transporter in the transport of the K5 polysaccharide. The KpsE protein has a large periplasmic domain of 300 amino acids and is anchored to the inner membrane via an N-terminal transmembrane domain. It is possible the large periplasmic domain may interact with the outer membrane. The KpsD protein is also involved in translocation of the K5 polysaccharide and may play a role in recruiting porins to the capsule assembly complex to allow translocation across the outer membrane (Roberts, 1996) (Figure 1.5.).

1.6 Biochemistry & genetics of capsular polysaccharides from other gram-negative bacteria

Organisation of the group 2 K antigen gene clusters shows a marked similarity to the gene clusters of *H. influenzae* type b and *N. meningitidis* group B (Figure 1.6.). Analysis of capsule proteins from these species has revealed homologues of group 2 proteins required for transport and cell surface expression of the polysaccharide. The ABC-transporter proteins are conserved in all three systems (KpsM/T, BexA/B, CtrA/D), suggesting a common pathway for capsule polysaccharide export in gram negative bacteria. The conservation of protein

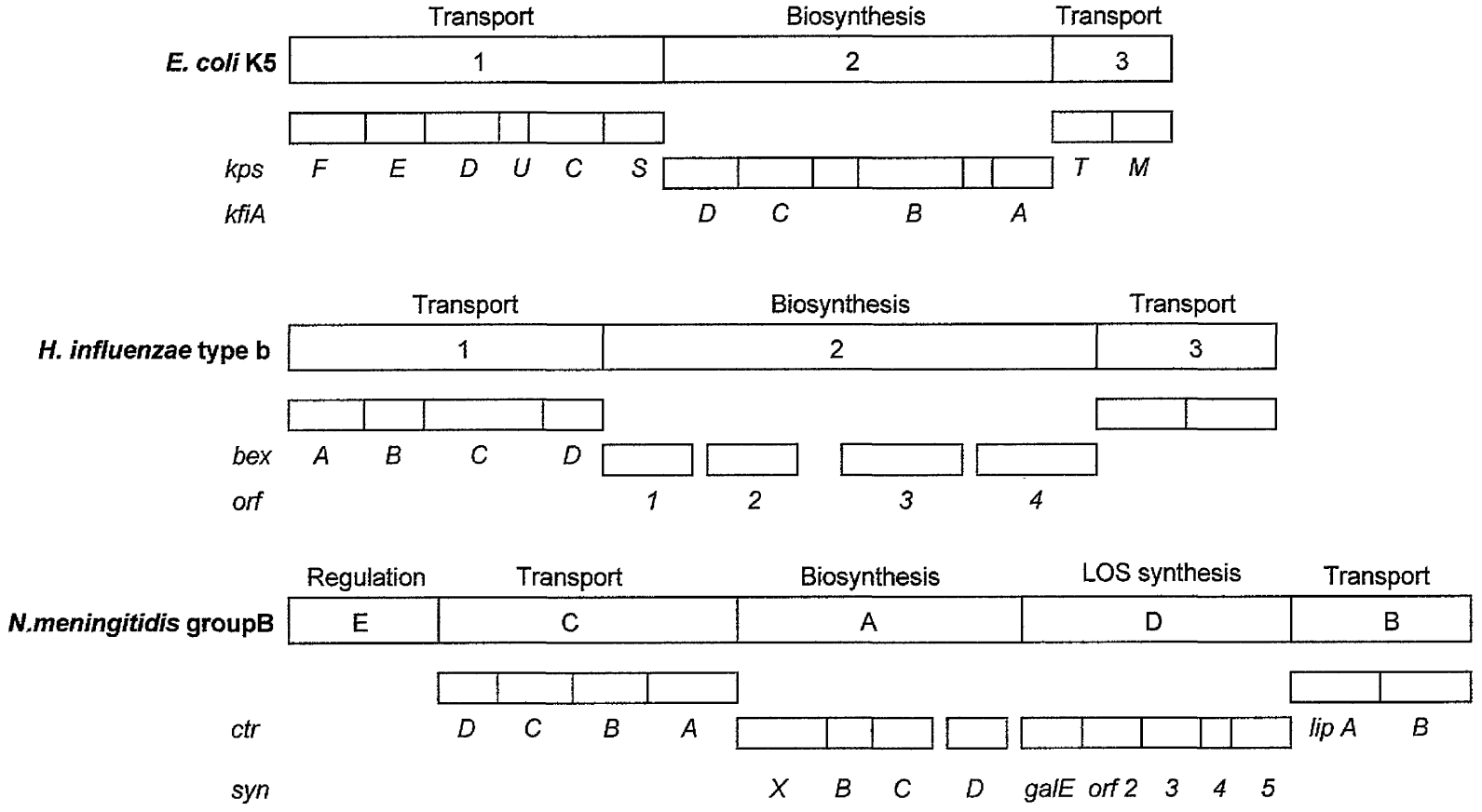
function and gene organisation in these species suggests they have a common ancestral origin. The general features of the *H. influenzae* type b and *N. meningitidis* group B gene clusters are outlined below.

1.6.1 Neisseria meningitidis

N. meningitidis produces at least eight different capsular serotypes. The group B serotype is identical in structure to the *E. coli* K1 antigen (Table 1.2.). The *N. meningitidis* group B gene cluster is composed of five distinct regions (A, B, C, D, and E)(Figure 1.6.)(Frosch *et al.*, 1989). Region A is the central region encoding four genes (*synX-D*) involved in capsular polysaccharide biosynthesis (Edwards *et al.*, 1994). Adjacent to region A, region C contains four genes *ctrA-D* organised in a single operon. It has been suggested that regions C and A share a common promoter region and may be co-ordinately regulated. It is thought this could be an important regulatory control point in meningococcal gene clusters (Swartley *et al.*, 1996). The products of region C are thought to be responsible for the transport of the polysaccharide across the inner and outer membrane to the cell surface. CtrA and CtrB together form an ABC transporter and play a role in export of the group B polysaccharide (Frosch *et al.*, 1989; 1991).

Region B of the *N. meningitidis* gene cluster encodes two proteins LipA and LipB which show homology to KpsC and KpsS from the *E. coli* group 2 gene cluster. LipA and LipB are proposed to add the phospholipid-anchoring group onto the reducing ends of the capsular polysaccharide chain (Frosch & Muller, 1993). Region D encodes four genes, *galE* that encodes an enzyme involved in the biosynthesis of lipooligosaccharides, and *rfbBCD* that encode enzymes involved in rhamnose biosynthesis for LPS production (Hammerschmidt *et al.*, 1994). The role of region D and E in capsule production is not fully understood. It was originally reported that region D and E were involved in capsule regulation as deletions in these regions resulted in increased levels of capsule production when expressed in *E. coli* (Frosch *et al.*, 1989). However, other authors did not observe similar results with region D deletions in a meningococcal host (Hammerschmidt *et al.*, 1994). The role of region D in capsule regulation is not yet elucidated. Regions analogous to region D and E were not found in *E. coli* group 2 gene clusters.

Figure 1.6. Genetic organisation of *Haemophilus influenzae* type b and *Neisseria meningitidis* group B capsule gene clusters (Kroll *et al.*, 1989; Frosch *et al.*, 1989). The organisation of the *E. coli* K5 capsule gene cluster is shown for comparison (Roberts, 1996). The larger boxes denote the defined functional regions. The smaller labeled boxes define specific genes that have been identified within each cluster



1.6.2 Haemophilus influenzae

H. influenzae produces six serologically distinct capsular polysaccharides (type a-f). Type b isolates are commonly associated with invasive disease in humans (Robbins *et al.*, 1980). The *H. influenzae* type b *cap* gene cluster displays a conserved genetic organisation similar to the *E. coli* group 2 gene clusters (Kroll *et al.*, 1989)(Figure 1.6.). It consists of three functional regions (1, 2, and 3). Regions 1 and 3 are common to all *H. influenzae* serotypes and encode proteins involved in export of the polysaccharide to the cell surface (Kroll *et al.*, 1989). Region 2 is the central serotype-specific region that encodes enzymes responsible for biosynthesis of the capsule (Van Eldere *et al.*, 1995). Region 1 contains four genes *bexABCD*. BexA and BexB comprise an ABC transporter system that plays a role in the export of type b polysaccharide (Kroll *et al.*, 1990)

In some type b strains duplication of the capsule gene cluster arises. In such isolates the gene cluster only contains one intact copy of *bexA* that is positioned in the bridge region between the two directly repeated copies (Kroll *et al.*, 1988; 1990). These strains can be unstable and one copy of the gene cluster is often lost due to homologous recombination. This can lead to loss of the complete *bexA* gene and prevent capsule production (Kroll *et al.*, 1988)

1.7 Genetic diversity of capsular polysaccharides

The lateral transfer of virulence genes is not uncommon in Gram negative bacteria and is often mediated by homologous recombination. Transposable elements flanking virulence determinants may contribute to gene transfer and in some cases may play a role in gene regulation. Lysogenic bacteriophage have also been found to carry virulence determinants and can be responsible for insertion of virulence genes into a host chromosome (Cheetham & Katz, 1995). Such events allow the acquisition of a variety of new virulence determinants from other species. It has been suggested that *E. coli* group 2 capsule diversity has arisen through the acquisition of new region 2 determinants from other bacteria (Boulnois & Jann, 1989). Transfer of different region 2 sequences is thought to occur by homologous recombination and may involve homologous sequences flanking region 2 (Roberts,

1996). Sequences identified in the group 3 K10 gene cluster include, IS elements IS*3* and IS*110* and an ØR73-like prophage. It has been suggested that the ØR73-like prophage may have played a role in the acquisition of the group 3 capsule gene cluster by integrating at a site within a group 2 capsule gene cluster (Clarke *et al.*, 1999).

Introduction - Part II

1.8 Regulation of virulence gene expression

Pathogenic bacteria encounter a wide variety of changing environmental conditions both in the environment and during the infection cycle. To survive within the host they must express specific gene products which will allow them to persist, multiply and resist host defence mechanisms. Depending on the stage of infection there will be marked differences in the conditions experienced. For this reason many bacteria have evolved complex systems to regulate virulence gene expression in response to different environmental signals (Mekalanos, 1992). This involves the activation of genes encoding products which enhance growth and survival, and the downregulation of gene products which may be a liability in certain situations. Regulation of virulence genes is a common feature of pathogenic bacteria as virulence factors generally have little known use to bacteria outside the host. Bacteria therefore exercise such regulatory systems to overcome the high energy expenditure costs of unnecessary expression of virulence genes. Expressing the appropriate genes in the appropriate environment.

1.9 Environmental control of virulence gene expression

The environmental signals that activate virulence gene expression in a variety of animal pathogens are generally simple physical and chemical factors, for example: temperature, osmolarity, O_2, pH, nitrogen compounds, nutrient availability and inorganic ion concentrations. Table 1.3 outlines examples of such environmental signals to demonstrate the variety of ever-changing conditions experienced within the host. Many pathogens have the ability to respond to more than one environmental signal and can use an array of stimuli to sense their location and express the appropriate genes. Examples of regulatory networks utilising a variety of environmental stimuli are vast some of which are discussed in section 1.10.3.

Table 1.3. Environmental signals implicated in virulence gene regulation in diverse bacterial species (Mahan *et al.*, 1996).

Organism	Environmental signal(s)	Adaptation response
Agrobacterium tumefaciens	Phenolic compounds, monosaccharides Opines	Crown gall tumour formation Ti plasmid transfer
Bacillus anthracis	CO_2	Capsule and toxin production
Bordetella pertussis	Temperature, SO_4, nicotinic acid	Activation and repression of virulence factors, including toxin and filamentous haemagglutinin
Corynebacterium diphtheriae	Iron	Toxin production
Escherichia coli	Iron Stress NO, superoxide Carbon source pH, oxygen, lysine	Shiga-like toxin, aerobactin, α-haemolysin Pilus expression Oxidative stress response, macrophage survival Pili Polyamine production
Klebsiella pneumoniae	N_2 O_2 , N_2	Urease production Nitrogen fixation
Listeria monocytogenes	Temperature	Virulence factors, listeriolysin O
Neisseria gonorrhoeae	Stress	Pili production
Porphyromonas gingivalis	Temperature	Fimbriae production
Pseudomonas aeruginosa	Enterobactin, iron Osmolarity Iron Homoserine lactone	Ferric enterobactin receptor Alginate synthesis Exotoxin A Elastin-specific protease
Salmonella typhimurium	pH, oxygen, growth phase, starvation pH Iron Growth phase, starvation Carbon source Osmolarity Oxygen	Host cell invasion, macrophage survival, virulence Acid tolerance response Enterobactin Plasmid-encoded virulence genes (spv) involved in systemic survival Pili production, OmpD production Porin expression Invasion, PrgH, OrgA expression
Salmonella typhi	Osmolarity	Porin expression/Vi capsule production
Shigella flexneri	Osmolarity Temperature Growth phase	Porin expression, Ipa expression Plasmid-encoded host cell invasion genes Extracellular proteins
Streptococci	CO_2	M-protein expression
Vibrio cholerae	pH, osmolarity, temperature Iron	Virulence factor expression, including toxin and pili Outer membrane proteins
Yersinia spp.	Temperature, Ca^{2+}	Yops

1.9.1 Temperature

Many virulence genes are regulated in response to changes in growth temperature. As the host temperature is relatively constant and higher than the ambient environment, this cue can provide an efficient signal for many animal pathogens. This allows the bacterium to discriminate between the host and the outside world. Hence, it is not surprising that many genes required to produce disease are specifically expressed at 37°C. For example, Uropathogenic strains of *E. coli* often produce pyelonephritis-associated pili or Pap to enhance colonisation of the urinary tract during infection. Expression of *pap* genes is regulated by temperature with gene expression at 37°C but not below 25°C (Goransson *et al.*, 1990). In *Shigella flexneri* the primary environmental cue for virulence gene expression is temperature with virulence genes associated with invasive disease expressed at 37°C but not at 30°C (Dorman & Porter, 1998). In the case of *Yersinia* spp., the Yop virulon is expressed optimally at 37°C. This virulon encodes a network of proteins responsible for the survival and multiplication of *Yersinia* cells in host tissues during infection (Cornelis & Wolf-Watz, 1997). As mentioned in Part I, the *E. coli* K5 capsular polysaccharide is regulated by temperature however, like many other systems details of the regulatory mechanism have not been elucidated.

It is important to note that not all virulence genes are expressed optimally at 37°C. For example, in *Y. enterocolitica* the Inv protein which facilitates passage of *yersiniae* through the intestinal epithelium is expressed maximally at 26°C (Isberg *et al.*, 1988; Straley & Perry, 1995).

1.9.2 Osmolarity

Osmolarity is another important environmental cue known to control virulence in several organisms. Osmolarity is important in the expression of cholera toxin, Tcp pili and other virulence determinants of *V. cholerae*. Optimum expression of these genes occurs in the osmolarity range of host tissues (Miller & Mekalanos, 1988). The transmembrane binding protein ToxR is involved in this process and together with the membrane protein ToxS it is capable of transcriptional activation of the cholera toxin gene (*ctx*) promoter (DiRita & Mekalanos, 1991; Miller *et al.*, 1987).

High osmolarity has also been found to stimulate *Salmonella* invasion; the regulatory osmo-sensor OmpR is a critical virulence factor involved in this process (Galan, 1996; Dorman *et al.*, 1989).

Temperature and osmolarity are two chosen examples of a variety of environmental factors, many of these can be seen in Table 1.3. The complexity and diversity of these regulatory systems can differ greatly. Certain bacteria have been found to regulate virulence gene expression in response to cell density (Salmond *et al.*, 1995; Fuqua *et al.*, 1996). This system is known as "quorum sensing" and relies on the accumulation of specific signalling molecules called autoinducers (AI) as a measure of cell density. When autoinducer levels reach a critical concentration in the surrounding environment transcription of density-dependent genes is initiated. This regulatory system is mediated by two genes, one encoding the AI (LuxI/signal generator) and the other encoding an AI receptor (LuxR/response regulator) (Salmond *et al.*, 1995; Fuqua *et al.*, 1996). In *P. aeruginosa* quorum sensing controls the production and secretion of virulence associated factors including the production of extracellular proteases (Chapon-Herve *et al.*, 1997; Holden *et al.*, 1999). It has been suggested that a similar cell-cell signalling mechanism may be responsible for differential gene expression in biofilms (Prigent-Combaret *et al.*, 1999)

More recently it has been suggested that contact between the bacterial cell and host cells can activate gene transcription, and may play an important regulatory role in pathogenesis (Pettersson *et al.*, 1996; Zhang & Normark, 1996). The attachment of *E. coli* to host cells via P-pili in the urinary tract has been found to activate transcription of the *airS* gene. AirS is a sensor-activator protein which is involved in the control of iron-acquisition systems (Zhang & Normark, 1996). In certain genera, for example, *Salmonella*, *Shigella*, *Yersinia*, contact with host cells can stimulate protein secretion (Galan, 1996). A novel protein-secretion system required for the specific export of virulence determinants has been found to be involved in this process (Salmond & Reeves, 1993) This system is known as the type III or contact-dependent secretory pathway. Contact with the host can lead to either increased virulence gene expression or delivery of preformed virulence proteins to the host cell. In the case of *Salmonella* contact with host cells stimulates the secretion of *Salmonella* invasion proteins (Zierler & Galan, 1995).

1.10 Transcriptional control of virulence gene expression

1.10.1 Regulation of transcription initiation

Transcription initiation can be regulated in a variety of different ways and generally involves numerous different regulatory factors. Regulation of initiation can be dependent on RNA polymerase displaying promoter selectivity. Many promoters utilise nucleoid associated proteins that alter promoter architecture to promote initiation of transcription. A large number of promoters require the interaction of transcription activators or repressors to regulate transcription initiation. But generally transcription initiation arises through complex combinations of different regulatory factors. Some of the regulatory factors required for virulence gene regulation are outlined below.

1.10.1.1 DNA topology

Many of the environmental cues mentioned in the previous section, for example, temperature and osmolarity, can alter the supercoiling of DNA. Such alterations combined with effects of regulatory proteins have been shown to affect virulence gene expression (Dorman, 1995; Drlica, 1992). Regulatory proteins control transcription by altering the architecture of the DNA in the promoter region. Open complex formation and transcription will therefore depend on the degree of change in DNA topology (Dorman, 1995; Perez-Martin *et al.*, 1994; Goosen & van de Putte, 1991). The binding of other regulatory factors will also depend on the structure of the DNA in the promoter region and is therefore also affected by changes in DNA topology.

1.10.1.2 RNA polymerase

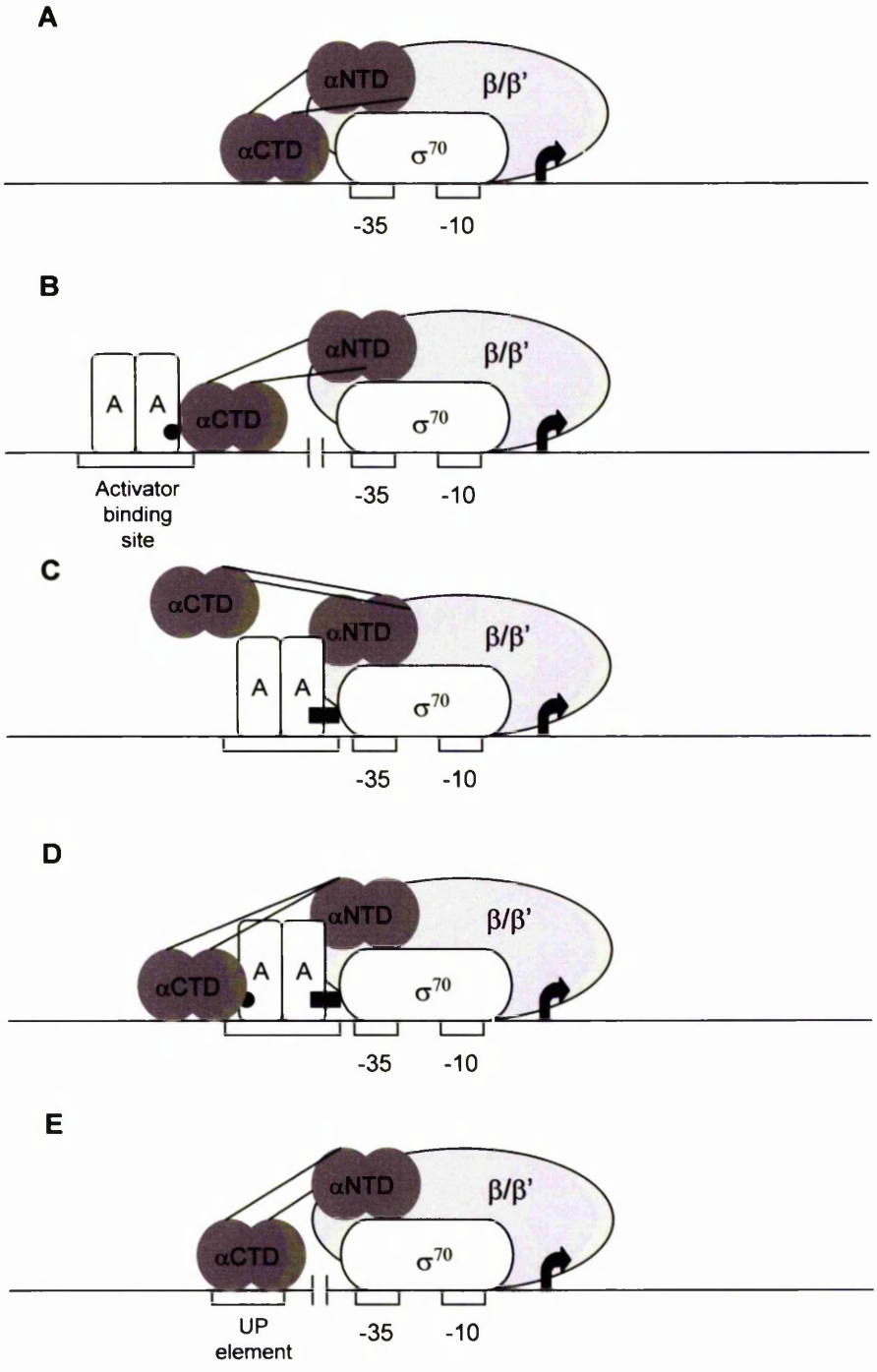
In *E. coli*, the major RNA polymerase holoenzyme (RNAP) responsible for transcription initiation is a multisubunit enzyme composed of $\alpha_2\beta\beta'\sigma^{70}$. The β subunit carries the catalytic site of RNA polymerisation while the β' subunit allows the RNAP to bind non-specifically to DNA. The sigma (σ) subunit of RNAP is an

important determinant of promoter recognition. Bacteria produce a series of interchangeable sigma factors that constitute the first level of regulation of transcription initiation (Helmann & Chamberlin, 1988). Each sigma factor directs the core RNA polymerase to a distinct set of promoters and is responsible for transcription of specific sets of genes. In *E. coli* six different species of σ subunit have been identified, of which the σ^{70} is by far the most abundant and most commonly used. σ^{70} is utilised for transcription of a large number of *E. coli* genes both pathogenic and commensal. It is also a target for transcription activators in gene regulation. σ^{32} (σ^{H}) and σ^{24} (σ^{E}) are used to transcribe genes for heat shock proteins under stress conditions. σ^{54} (σ^{N}) is required for transcription of nitrogen-regulated genes. σ^{38} (σ^{S}) is important in the expression of stationary-phase-specific genes and finally, σ^{28} (σ^{F}) is needed for transcription of a set of genes coding for the formation of flagella (Gross *et al.*, 1992).

At some promoters anti-σ factors are utilised as an additional mechanism of regulating transcription initiation. Anti-σ factors are antagonists of σ-factors and can be cytoplasmic or inner membrane bound. They bind and reversibly inactivate σ-factors preventing promoter recognition. Anti-σ factors regulate a wide range of cellular processes including bacteriophage growth, sporulation, stress response, flagellar biosynthesis and virulence (Hughes & Mathee, 1998).

The α subunit of RNAP consists of two independently folded domains connected by a flexible interdomain linker (Blatter *et al.*, 1994). The amino terminal domain (αNTD) plays a key role in RNAP assembly whereas the carboxy terminal domain (αCTD) has a more diverse role with three known functions (Busby & Ebright, 1994). Firstly it serves as an initiator for RNAP assembly, secondly it participates in promoter recognition through direct sequence specific α-DNA interactions and thirdly it is the target for transcription regulation by a large set of transcription activating proteins (Figure 1.7.)

Figure 1.7. Model for the role of the RNA polymerase in promoter recognition and transcriptional activation (Blatter *et al.*, 1994; Rhodius & Busby, 1998). (A) RNAP docking at an activator–independent promoter. (B) Activator A contacting αCTD at an activator-dependent promoter. (C) Activator contacting σ^{70} at an activator-dependent promoter. (D) Activator contacting both αCTD and σ^{70} at an activator-dependent promoter. (E) RNAP αCTD contacting an upstream element (UP-element). The different RNAP subunits are illustrated, docked at a promoter. α subunits denoted as two domains, amino terminal αNTD and carboxy terminal αCTD joined by a flexible linker. Specific surfaces of RNAP σ^{70} contact the –10 and –35 hexamers; black circles indicate contact between activator and αCTD; black rectangles indicate contact between activator and σ^{70}; arrow indicates direction of transcription.



1.10.1.3 Role of RNA polymerase in transcription activation

The RNAP holoenzyme is a vital component of the transcription apparatus; hence it is an obvious target for regulating transcription initiation. The RNAP can interact with specific DNA sequences or transcription activators to promote initiation of transcription (Figure 1.7.). Bacterial promoters contain a number of conserved sequences that are recognised by different subunits of the RNAP holoenzyme. Specific surfaces of the RNAP σ^{70} subunit contact conserved sequences in the promoter region known as the –10 and –35 hexamer motifs (Gross *et al.*, 1992). In some cases an upstream extension of the –10 motif is also contacted by the σ^{70} subunit to promote transcription initiation (Barne *et al.*, 1997). At some promoters, transcription initiation is dependent on interactions between the carboxy-terminal domain of the α subunit of RNAP (αCTD) and a third promoter sequence recognition element known as the UP element (Ross *et al.*, 1993). UP elements are typically 20bp AT-rich sequences located upstream of the –35 hexamer. The most extensively characterised UP element is an AT-rich sequence at the ribosomal RNA promoter *rrnB* P1 that stimulates promoter activity at least 30 fold (Newlands *et al.*, 1991; Ross *et al.*, 1993; Rao *et al.*, 1994). UP elements have been located in a number of *E. coli* promoters and can function with holoenzymes containing different sigma factors (Ross *et al.*, 1993; van Ulsen *et al.*, 1997). αCTD-UP element binding results in tighter RNAP-promoter association and may promote changes in RNAP conformation and RNAP-promoter organisation (Blatter *et al.*, 1994; Ebright & Busby, 1995).

Promoters may contain one or more of these conserved sequences in a variety of combinations. In some cases interaction between RNAP and these recognition elements is sufficient to promote transcription initiation, however many bacterial promoters also require the action of transcription activators.

Many transcription activators interact with RNAP through direct protein-protein contacts to stimulate transcription initiation. In *E. coli* a number of activator contact sites have been found in the RNA polymerase enzyme, with different activators interacting with different contact sites (Ishihama, 1993). Many transcription activators function through interactions with the αCTD of RNAP. Such interactions are essential to recruit the RNAP to promoter DNA and are

thought to increase the affinity of RNAP for the promoter (Ebright & Busby, 1995). The αCTD is connected to αNTD by a flexible linker, this allows αCTD to contact activators bound at various positions located upstream of the –35 region, provided the activator binding site is located on the same face of the DNA. (Blatter *et al.*, 1994; Jeon *et al.*, 1997). Hence, the location of binding elements for activators that interact with αCTD is generally variable. A well-characterised transcription factor that interacts directly with αCTD is the *E. coli* cAMP-receptor protein (CRP). CRP operates at more than 100 promoters in *E. coli* and is regulated by cAMP levels within the cell (Kolb *et al.*, 1993). A surface exposed loop of CRP (activating region 1, AR1) interacts with the αCTD subunit of RNAP (Busby & Ebright, 1997).

Activators can also function through interactions with a specific region of the σ^{70} subunit of RNAP, generally those that bind in the –35 region of the promoter. For example, transcription of genes belonging to the phosphate (*pho*) regulon in *E. coli* require the specific activator protein PhoB which interacts with the σ^{70} subunit of RNAP to promote transcription (Kim *et al.*, 1995). Interactions with RNAP are not limited to the αCTD and region 4 σ^{70}, activators also contact other regions of RNAP (Ishihama, 1997). The DnaA protein can initiate transcription from the λ P_R promoter through interactions with the RNAP β subunit (Rhodius & Busby, 1998). A DNA binding protein (SSB) from bacteriophage N4 has been found to interact with RNAP β' subunit to activate transcription from the N4 late promoters (Miller *et al.*, 1997).

Transcription activators have been found to make more than one contact with RNAP. The regulatory protein FNR interacts with both region 4 of RNAP σ^{70} and αCTD to activate transcription at a number of target promoters in *E. coli* (Busby & Ebright, 1997; Williams *et al.*, 1997). Certain promoters can be coregulated by different transcription activators making independent interactions with two different contact sites in RNAP (Scott *et al.*, 1995; Hochschild & Joung, 1997).

1.10.1.4 Nucleoid associated proteins

E. coli produces various small DNA binding proteins that can bind large quantities of DNA and contribute to the structure of bacterial nucleoid (Drlica & Rouviere-Yaniv, 1987; Schmid, 1990; Schneider *et al.*, 1997). Collectively these

proteins are referred to as nucleoid associated or histone-like proteins and include HU, H-NS, Integration host factor (IHF) and Factor for inversion stimulation (Fis). In addition, these proteins can affect other cellular processes including site specific DNA recombination and DNA replication. They also play a major role in regulation of gene expression (Drlica & Rouviere-Yaniv, 1987; Schmid, 1990; Finkel & Johnson, 1992). Individual systems are not restricted to the control of one nucleoid associated protein as in some cases two or more of these proteins may act in concert, for example, Fis and IHF cooperate in promoting site specific recombination of bacteriophage λ DNA (Thompson *et al.*, 1987; Finkel & Johnson, 1992). In other cases they can play opposing roles, for example Fis and H-NS have antagonistic affects on *hns* transcription (Falconi *et al.*, 1996).

1.10.1.4.1 IHF

Integration host factor is a site specific DNA binding protein involved in numerous cellular processes (Friedman, 1988). IHF was first discovered as a bacterial protein required for site specific recombination of bacteriophage lambda DNA but is now known to be involved in transposition, DNA replication and phage chromosome packaging. It can also act as a transcription factor in the control of gene expression and IHF binding sites are found upstream of many σ^{54} dependant promoters (Goosen & van de Putte, 1991). The role of IHF in cellular processes is typically as an accessory factor exerting a differing degree of control depending on the event. This can range from a 2-fold influence as in promoter activation to a 10,000-fold effect as in lambda recombination (Peacock *et al.*, 1984). IHF binds and severely bends its target DNA into a virtual U-turn (Rice *et al.*, 1996; Rice, 1997).

IHF is a 20kDa heterodimeric protein (αβ) encoded by two unlinked genes *himA* encoding the α subunit and *himD* (*hip*) encoding the β subunit. It recognises an asymmetrical binding site approximately 30 to 35bp long, which can be divided into two domains. The 3' domain is significantly conserved and has a defined degenerate core consensus sequence of WATCAANNNNTTR (W=A or T, R=A or G, N = any nucleotide)(Friedman, 1988). The 5' domain is a degenerate AT-rich region with no obvious conserved pattern. The 5' domain is not always necessary for function of IHF with only a subset of known IHF binding sites containing this

AT-rich element (Goodrich *et al.*, 1990; Hales *et al.*, 1994). Its presence is thought to enhance IHF binding (Hales *et al.*, 1994; Goodman *et al.*, 1999). IHF is involved in the regulation of type I fimbriae in *E. coli* and more recently in alginate production in *P. aeruginosa* (Delic-Attree *et al.*, 1996).

1.10.1.4.2 H-NS and H-NS-related proteins

H-NS is a major component of bacterial nucleoid capable of condensing and packaging DNA. It influences a variety of cellular processes such as recombination and replication and also has a major role in modulation of expression of a large number of genes, mostly by negatively affecting transcription (Atlung and Ingmer, 1997). It is not clear however how H-NS achieves this. H-NS is a small 15.5 kDa histone-like protein encoded by the *hns* gene. The *hns* gene is regulated by environmental factors such as cold shock and entry into stationary phase and is subject to negative autoregulation (La Teana *et al.*, 1991; Dersch *et al.*, 1993; Ueguchi *et al.*, 1993). The H-NS protein consists of two domains that are joined by a flexible linker. The flexible linker allows each domain to function and fold independently within the context of the full length protein (Smyth *et al.*, 2000). The carboxy terminal is the DNA binding domain and the amino terminal is the domain responsible for mediating H-NS oligomerization (Ueguchi *et al.*, 1996; Dorman *et al.*, 1999; Smyth *et al.*, 2000). The oligomerization state of H-NS is thought to influence its biological capabilities, including its ability to recognise and bend curved DNA (Spurio *et al.*, 1997). H-NS has been implicated as a regulator of a number of virulence genes, for example the thermoregulation of Pap-pili in uropathogenic *E. coli* and the thermo-osmotic regulation of virulence gene expression in *Shigella flexneri* (Porter and Dorman, 1994; Van der Woude *et al.*, 1995). H-NS homologues have been found in a number of other species including *S. typhimurium*, *Yersinia* spp and *V. cholerae* (Tendeng *et al.*, 2000). A paralogue of H-NS has been described in *E. coli* termed StpA. In amino acid sequence StpA is 58% identical to H-NS. The relationship between H-NS and StpA is the subject of intensive research. They both have overlapping and distinct roles in the cell and experimental analysis has shown that H-NS and StpA can form heteromeric complexes *in vivo* (Williams *et al.*, 1996; Free *et al.*, 1998). However the role of StpA in virulence gene regulation is not well established.

A new class of H-NS related DNA binding proteins, thought to be involved in down regulating virulence gene expression, has been identified in different enterobacteria (Mikulskis & Cornelis, 1994). Members of this group include YmoA of *Y. enterocolitica* and Hha of *E. coli* (Mikulskis & Cornelis, 1994). Hha regulates expression of virulence genes such as Vir antigen and haemolysin in response to environmental stimuli in *E. coli* (Mourino *et al.*, 1996). In the case of haemolysin, Hha is thought to interact with H-NS to modulate thermo-osmotic regulation of the haemolysin operon (Nieto *et al.*, 1997; Nieto *et al.*, 2000).

1.10.1.4.3 Fis (Factor for Inversion Stimulation)

The Fis protein has been shown to be involved in several different biological processes. These include DNA inversion reactions mediated by the Hin family of recombinases, integration and excision of bacteriophage λ DNA, regulation of gene transcription including stable RNA operon genes (Nilsson *et al.*, 1990; Ross *et al.*, 1990) and regulation of DNA replication initiation at *oriC* (Filutowicz *et al.*, 1992; Wold *et al.*, 1996). Fis consists of two functionally distinct regions (Koch *et al.*, 1991; Osuna *et al.*, 1991). The carboxy terminal is the DNA binding domain which contains a helix-turn-helix motif typical of many DNA binding proteins and the amino terminal region is required for promoting DNA inversion. Upon binding to DNA Fis induces bending of the DNA duplex which is a critical feature of its ability to affect biological processes. Fis is a sequence specific DNA binding protein however the consensus sequence it recognises is largely degenerate (Finkel & Johnson, 1992). Fis consensus sequences have been located upstream of a number of rRNA and tRNA operons in *E. coli* (Verbeek *et al.*, 1990). Three Fis binding sites have been located in the Upstream Activating Region found upstream of the rRNA rrnB operon. Binding of Fis to this region is known to stimulate transcription (Nilsson *et al.*, 1990; Ross *et al.*, 1990). It has been suggested that Fis may activate transcription by enhancing binding of the RNA polymerase to the promoter (Nilsson *et al.*, 1990; Ross *et al.*, 1990).

1.10.1.5 Transcriptional activators

In bacteria there are a vast number of known regulatory proteins that share significant homology, possess similar functions and form families of regulator proteins. Many of these are involved in the regulation of virulence genes. A well documented example is the AraC/XylS family of transcriptional regulators (Gallegos *et al.*, 1997). These regulators are positive activators characterised by a conserved helix–turn-helix (HTH) DNA binding motif typical of DNA binding proteins. Members of this group generally function in one of three main areas, carbon metabolism, stress response and pathogenesis. Regarding pathogenesis, AraC-like proteins regulate the production of virulence factors in both animal and plant pathogens. These are generally animal pathogens colonising the gastrointestinal tract however AraC-like regulators have also been found in those pathogens colonising the respiratory and urinary tract. AraC-like virulence regulators include CfaD, BfpT, and Rns from *E. coli*, Caf1R, VirF and LcrF from *Y. pestis*, InvF from *S. typhimurium* and MxiE and VirF from *Shigella*. Virulence factors typically regulated by AraC-like regulators include adhesive structures such as fimbriae and pili (CfaD, BfpT, Rns), capsules (CafR) and invasins (InvF, MxiE, VirF). AraC-like activator genes are found both plasmid and chromosomally encoded and all exert regulatory effects in response to specific environmental stimuli, including temperature, osmolarity and Ca^{2+} concentration (Gallegos *et al.*, 1997). The *E. coli* CfaD is an AraC like transcriptional activator of the *cfaABCDE* operon required for the production of CFA/I fimbriae. CFA/I fimbriae allow enterotoxigenic strains of *E. coli* to adhere to the intestinal epithelium (Jordi, 1992).

The regulatory effects of AraC-like regulators are not limited to individual virulence factors. They can influence the expression of more than one virulence factor. The VirF protein of *Yersinia* spp regulates the expression of multiple virulence factors, including secreted Yop proteins (Cornelis *et al.*, 1989). Likewise, VirF of *Shigella* spp. also regulates multiple virulence factors including those required for invasion and spreading within host cells (Dorman & Porter, 1998).

Another well known group of regulatory proteins is the LysR-type regulators (Schell, 1993). LysR-type regulatory proteins are one of the most common types of regulator found in bacteria. They are responsible for the transcriptional activation of a diverse array of target genes. Members include LysR,

CysB, and IlvY of *E. coli*, MetR and SpvR of *S. typhimurium* and NodD of Rhizobium. LysR-type proteins are characterised as sequence related, coinducer-responsive, transcriptional activators that share several regulatory properties including a predicted helix-turn-helix (HTH) DNA binding motif in the amino terminal region of the protein. To activate gene expression some LysR-type regulators require binding of a coinducer molecule that alters properties of the LysR-type protein (Schell, 1993). In *S. typhimurium* the LysR type regulator SpvR works in concert with IHF and σ^S to activate the *Salmonella* plasmid-encoded virulence genes (*spv*) (Coynault *et al.*, 1992; Marshall *et al.*, 1999).

1.10.1.6 Transcriptional repressors

Many regulatory proteins can act as transcriptional repressors. Repressor proteins generally prevent transcription initiation of specific genes by binding in the promoter region under unfavourable environmental conditions. When the surrounding conditions change and require a particular gene to be expressed the repressor protein releases the promoter DNA and transcription initiation can proceed. Repressor proteins also function by binding to transcriptional activators or inducer molecules preventing them from activating transcription. As with transcriptional activators families of transcriptional repressors have been described in gram negative bacteria, these include the GalR-LacI-type transcriptional repressors (Nguyen & Saier, 1995). The GalR-LacI type repressors are a family of proteins regulating transcription of inducible genes in bacteria. Most members of this family require effectors to modulate regulation generally in the form of carbohydrate molecules (Weickert & Adhya, 1992)

Some regulatory factors have dual roles in gene regulation and can act as both repressors and activators. The FNR protein which is essential for oxygen-related gene expression has been shown to have both activator and repressor properties. It activates genes required for survival under low oxygen conditions and represses certain genes not required for anaerobic conditions (Spiro & Guest, 1990).

Nucleoid associated proteins can also act as repressors. H-NS has been implicated as a repressor of virulence genes in many prokaryotic systems including thermoregulation of Pap–pili in uropathogenic *E. coli* and virulence gene regulation in *Shigella* (Goransson *et al.*, 1990; Dorman *et al.*, 1990; Tobe *et al.*, 1993). In

uropathogenic *E. coli* H-NS blocks methylation of *pap* DNA, a process essential to *pap* regulation. This prevents transcription of the *pap* operon (White-Zieglier *et al.*, 1998).

1.10.1.7 Two-component systems

Two component regulatory systems represent a widespread mechanism by which prokaryotes can sense their environment and respond accordingly (Hoch, 2000). Two-component elements are composed of two regulatory proteins, a signal sensor protein and a response regulator protein that together can modulate gene expression. The transmembrane sensor protein can respond to a specific stimulus by autophosphorylating at a histidine residue and then relaying the phosphate group to the transcriptional regulator protein. The phosphorylated regulator protein is then able to direct transcription from specific sets of target genes (Hoch, 2000).

Many virulence factors are regulated by two-component regulatory systems where environmental stimuli encountered during infection lead to alterations of virulence gene expression. Environmental cues known to activate two-component regulatory systems include pH, temperature, osmolarity and ion concentration. A well characterised example is the *phoP/phoQ* system of *S. typhimurium* (Garcia Vescovi *et al.*, 1994). The PhoP/PhoQ system regulates a number of virulence genes located on the *Salmonella* pathogenicity island (SPI-2). These include genes required for survival within host macrophage cells and a type III secretion system. Environmental regulation of SPI-2 gene expression also involves two other two-component regulatory systems, SsrA/SsrB, which is encoded within the island and is modulated by PhoP/PhoQ, and OmpR/EnvZ, which is thought to regulate the type III secretion system of SPI-2 (Lee *et al.*, 2000). Similarly the PhoP/PhoQ two-component regulatory system of *Y. pestis* has been found to play a key role in regulating virulence genes (Oyston *et al.*, 2000). In *B. pertussis* the BvgA/BvgS two-component system is responsible for activation and repression of specific sets of virulence genes (Miller *et al.*, 1989). Pertussis toxin, adenylate cyclase toxin and the filamentous haemagglutinin adhesin are all regulated by this system (Uhl & Miller, 1995).

1.10.2 Regulation of transcription termination

Gene expression is not always regulated at transcription initiation, in some systems regulatory factors have been found that alter transcription elongation and termination. In prokaryotes a group of RNAP-associated proteins which act as specific elongation factors have been identified. This group can be divided into two subgroups, the non-processive transcription antitermination factors and the processive elongation factors.

1.10.2.1 Non-processive antitermination factors

Members of this group regulate a number of different operons encoding different metabolic activities. These proteins have been termed antiterminator proteins and include BglG and SacY (Henkin, 1996; Rutberg, 1997). They can prevent transcription termination by using a mechanism similar to that of transcription attenuation, where they bind a specific RNA sequence to prevent the RNA from forming a transcription termination structure. This allows RNA polymerase to continue transcription elongation beyond this sequence.

1.10.2.2 Processive elongation factors

This group includes the bacteriophage λ proteins N and Q, and the RfaH protein. N and Q proteins act by modifying RNAP altering its resistance to transcription termination (Friedman & Court, 1995; Mogridge *et al.*, 1995). The RfaH protein works in concert with a short conserved sequence element (ops element) to modify transcription terminators allowing the RNAP to process RNA and continue transcription elongation (Bailey *et al.*, 1997; Stevens *et al.*, 1997).

1.10.3 Examples of virulence gene regulation

Having outlined a variety of regulatory factors contributing to virulence gene regulation, it must be noted that typically many regulatory systems utilise many of these factors in complex integrated regulatory cascades. Within these systems regulatory factors can interact with RNA polymerase, promoter DNA and each

other to regulate gene expression. In the next section two regulatory systems are described as an example of the complexity of transcriptional gene regulation.

1.10.3.1 Virulence gene regulation in *Shigella flexneri*

S. flexneri is the causative agent of bacillary dysentery in humans. The first stage of this disease is bacterial invasion of the colon, followed by bacterial spread through host cells. Invasion and survival in host cells requires a large number of virulence genes many of which are located on the 230kb *Shigella* virulence plasmid. Additional regulatory genes are also found on the chromosome (Dorman & Porter, 1998).

The invasion proteins are encoded by the plasmid located *ipa* genes. These proteins are secreted into the extracellular medium during infection by a type III secretion system encoded by the plasmid encoded *mxi* and *spa* genes. All of these genes are modulated by two regulatory proteins VirF and VirB (Adler *et al.*, 1989). VirF is an AraC-like transcriptional regulator containing a characteristic helix-turn-helix DNA binding motif (Gallegos *et al.*, 1997). VirF plays a key role in *Shigella* virulence gene expression and is required to initiate a cascade of regulatory events. VirB is a regulatory protein, which is believed to bind DNA. Activation of the *virB* gene by VirF leads to subsequent activation of the *ipa*, *mxi* and *spa* genes (Dorman & Porter, 1998). Additional virulence factors located on the *Shigella* virulence plasmid are encoded by the *icsA* (*virG*) and *virA*. genes. These proteins have been found to play a role in intercellular spreading. The *icsA* gene is regulated directly by the VirF protein and is also post-transcriptionally regulated by the plasmid encoded VirK protein (Dorman & Porter, 1998).

The *Shigella* virulence genes are regulated by several environmental cues, the primary stimulus being a temperature of 37°C, others include osmotic stress and pH 7.4 (Dorman & Porter, 1998). The nucleoid associated protein H-NS controls the temperature regulation of *Shigella* virulence genes by repressing transcription of VirF below 37°C (Dorman *et al.*, 1990; Falconi *et al.*, 1998). H-NS mainly acts at the virB promoter however, the *virF* promoter undergoes a conformational change which facilitates H-NS mediated temperature regulation (Falconi *et al.*, 1998). Another nucleoid-associated protein involved in virulence gene regulation is IHF. IHF acts

as a positive regulator of the two regulatory genes *virB* and *virF* (Porter & Dorman, 1997).

Two-component regulatory systems also play an important role in *Shigella* virulence gene expression. The OmpR/EnvZ two-component system has been found to regulate virulence gene expression in *Shigella* (Bernardini *et al.*, 1990) and the pH control of virulence genes is mediated by the chromosomal encoded CpxR/CpxA system (Porter & Dorman, 1997).

1.10.4 Regulation of alginate biosynthesis in *Pseudomonas aeruginosa*

Mucoid *P. aeruginosa* strains producing alginate are usually associated with chronic colonization of the respiratory tract in cystic fibrosis patients. The alginate exopolysaccharide is an important virulence factor with antiphagocytic & adherence properties (Govan & Deretic, 1996). Mucoid isolates arise through mutations that inactivate the anti-σ factor MucA (Martin *et al.*, 1993). MucA inhibits the sigma factor AlgU (a σ^E- like sigma factor) which is responsible for transcription initiation of a number of genes in the alginate operon (Schurr *et al.*, 1996). Inactivation of MucA relieves AlgU from repression and causes conversion to mucoidy. (Schurr *et al.*, 1996).

The AlgD protein, a GDP-mannose dehydrogenase, is a key enzyme, required for alginate biosynthesis (Tatnell *et al.*, 1994). Regulation of the *algD* promoter is controlled at a number levels. The *algD* promoter is activated by several environmental cues, these include high osmolarity, nitrogen & phosphate starvation and ethanol-induced membrane perturbation (Berry *et al.*, 1989). Further regulation of *algD* is exerted by a variety of different regulatory proteins. These include AlgB, AlgR and AlgZ, all of which have been shown to activate transcription of *algD* (Wozniak & Ohman, 1994; Baynham & Wozniak, 1996). AlgR and AlgB are transcriptional activators and show homology to two-component system response regulators (Wozniak & Ohman, 1994). AlgR binds to three distinct sites in the *algD* promoter, two of which are a considerable distance upstream from the transcriptional start site (Mohr *et al.*, 1990). The AlgB binding site has not yet been identified. AlgZ is a ribbon-helix-helix DNA binding protein thought to bind to the *algD* promoter (Yu *et al.*, 1997). It has been suggested that both AlgZ and AlgR may

be involved in direct contact with RNAP in activating transcription of the *algD* gene (Baynham *et al.*, 1999).

The *algD* promoter is also under control of at least two histone-like proteins IHF and AlgP (AlgR3) (Kato *et al.*, 1990; Delic-Attree *et al.*, 1997). IHF has been found to play a key role in activation of *algD* and IHF binding sites are located in the *algD* promoter. It is thought that IHF bends the *algD* promoter DNA allowing interaction between AlgR and RNAP (Delic-Attree *et al.*, 1997).

An additional regulator involved in *algD* expression is the CysB protein. CysB is a LysR-type transcriptional activator, which binds to the *algD* promoter and activates *algD* expression. More recently a novel gene called *phpA* has been found to play a role in transcription of *algD*. This gene has homology to a leucine aminopeptidase of *E. coli* and its exact role in gene regulation is unknown (Woolwine & Wozniak, 1999). Therefore, it can be seen that in the case of alginate gene regulation an array of different regulatory factors are required to allow optimal alginate production.

1.11. Aims & Objectives

The aim of this study was to elucidate the mechanism of temperature regulation of the *E. coli* K5 capsule gene cluster. The main objectives set were to investigate the role of previously identified promoter sequences found in the region 1 and region 3 promoters. This study also investigated the role of specific gene regulators known to be involved in transcriptional regulation. In addition this study aimed to identify novel transcriptional regulators involved in regulation of the *E. coli* K5 capsule gene cluster using random transposon mutagenesis.

Chapter 2

Materials and Methods

2.1. Bacterial strains and plasmids

The bacterial strains used in this study are listed in Table 2.1. and those generated have been listed in Table 2.2. The plasmids used in this study are listed in Table 2.3. and those generated have been listed in Table 2.4.

2.2. Media and growth conditions

All strains were routinely grown in sterilised Luria broth (L-broth) (Tryptone 10g/l, NaCl 10g/l, Yeast extract 5g/l, made up to 1 l with distilled water. For L-agar 15g/l of bacteriological agar was added) unless otherwise stated. Sterilisation was performed by autoclaving at 121°C for 15 minutes. Antibiotics were added when necessary at the following concentrations; ampicillin (Ap), 100μg/ml; chloramphenicol (Cm), 25μg/ml; kanamycin (Km), 50μg/ml; spectinomycin, 50μg/ml; streptomycin (Sm), 25μg/ml.

2.3. Transformation of *E. coli.*

2.3.1. Preparation of competent cells

Competent cells were prepared as described by Sambrook *et al.*, (1989). Bacteria were grown to mid-logarithmic phase and collected by centrifugation at 2750 x *g* (4°C) for 10 min. Cells were resuspended in 4ml ice-cold 10mM NaCl and recentrifuged immediately at 2750 x g (4°C) for 5 min. Cells were then resuspended in 4ml ice-cold 100mM $CaCl_2$ and placed on ice for 30 min. Finally, the competent cells were centrifuged at 1800 x g (4°C) for 5 min, resuspended in 1ml 100mM $CaCl_2$ and stored on ice until required.

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

Strain	Genotype	Source
DH5α	*supE*44 Δ*lacU*169 (Ø80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*1 *relA*1	Hanahan (1983)
MC4100*hns*::kan	*araD*139 Δ(*argF-lac*)U196 *rpsL*150 *relA*1 *deoC*1 *ptsF*25 *rbsR flb* B5301	J. Hinton
PA360	F⁻ *serA*1 *rpsL*9 *thr*1 *leuB*6 *hisG*1 *argH*1 *thi*1 *rfbD*1 *malT*1 (λ^R) *fhuA*2 *lacY*1 *gal*6 *galP*63 *xyl*7 *mtlA*2 *supE*44	M. Stevens
XAC	Su⁻ *lac pro ava argE thi rif* (*rpoB*)	D. Pickard
XL1-Blue	*supE*44 *hsd*R17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*⁻ F' [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(tet^r)]	Bullock *et al.*, (1987)
MS101	PA360 *serA*$^+$ K5 *kps*$^+$	Stevens, (1995)
MS108	MS101 *kfiA*::*luxAB*	Stevens, (1995)
MS120	MS101 *kpsT*::*luxAB*	Stevens, (1995)
MS150	MS101 Δ(*arg-lacZ*)	Simpson, (1996)
MS150*himA*::cat	MS150*himA*::cat Cm^R	Stevens, (1995)

Table 2.2. *E. coli* strains generated in this study.

Strain	Genotype
MS108*hns*::kan	*kfiA*::*luxAB hns*::kan
MS108*bipA*::Tn*5*	*kfiA*::*luxAB bipA*::Tn*5*
MS120*hns*::kan	*kpsT*::*luxAB hns*::kan
MS120*bipA*::Tn*5*	*kpsT*::*luxAB bipA*::Tn*5*
MS152	MS150 *hns*::kan
MS153	MS150 *bipA*::Tn*5*
XAC.7	XAC*bipA*::Tn*5*

Table 2.3. Plasmids used in this study.

Plasmid	Genotype	Reference / Source
pBluescript	high copy number cloning vector, Ap^R	Stratagene
pCB192	*lacZ* promoter probe vector, Ap^R	Schneider & Beck, (1986)
pDSHcH	1.1-kb *Hin*cII-*Hin*dIII fragment containing the region 1 promoter cloned into pCB192	Simpson *et al.*, (1996)
pDS200	11-kb *Eco*RI fragment encompassing region 1; cloned in pKK223-3	Simpson *et al.*, (1996)
pRT733	suicide vector carrying Tn*phoA*, Km^R, Ap^R	Taylor *et al.*, (1989)
pUC19	high copy number cloning vector, Ap^R	Yanisch-Perron *et al.*, (1985)

Table 2.4. Plasmids generated in this study.

Plasmid	Description
pSR2	1.1-kb *Hinc*II-*Hind*III fragment containing the region 1 promoter cloned into pUC19
pSR3A	pSR2 with conA deleted
pSR3B	pSR2 with conB deleted
pSR3AB	pSR2 with conA & conB deleted
pSR4A	1.1-kb *Hinc*II-*Hind*III fragment containing the region 1 promoter with conA deletion, cloned into pCB192
pSR4B	1.1-kb *Hinc*II-*Hind*III fragment containing the region 1 promoter with conB deletion, cloned into pCB192
pSR4AB	1.1-kb *Hinc*II-*Hind*III fragment containing the region 1 promoter with conA & conB deletions, cloned into pCB192
pIHF-1	pSR2 with consensus sequence IHF-1 deleted
pIHF-2	pSR2 with consensus sequence IHF-2 deleted
pCBIHF-1	1.1-kb *Hinc*II-*Hind*III fragment containing the region 1 promoter with IHF-1 deletion, cloned into pCB192
pCBIHF-2	1.1-kb *Hinc*II-*Hind*III fragment containing the region 1 promoter with IHF-2 deletion, cloned into pCB192
pTn5.7	5.9kb *BamH*I fragment containing the Tn*5* insertion in the *bipA* gene cloned into pBluescript

2.3.2. Transformation of plasmid DNA

Plasmid DNA (0.1-1μg) was mixed with 100μl competent cells and placed on ice for 1hr. The cells were then incubated at 42°C for 3 min. After adding 200μl L-broth the cells were incubated at 37°C for 1 hour. Aliquots of transformed cells were then plated onto selective media.

2.4. Transduction of *E. coli* using bacteriophage P1*vir*

Recipient bacteria were grown to late logarithmic phase (A_{600}~1.5) in 5ml L-broth and collected by centrifugation at 1500 *g* for 10 min. Cells were resuspended in 2.5ml of 10mM $MgSO_4$ containing 5mM $CaCl_2$. Aliquots of P1*vir* lysate (10, 50 & 100μl) were then mixed with 100 μl bacteria and incubated statically for 30 min at 30°C. Next, 100μl of 1M sodium citrate was added to each tube and mixed, followed by the addition of 1ml L-broth. Tubes were incubated for 1hr at 37°C to allow phenotypic expression. Citrate prevents reinfection of the cells by chelating divalent calcium ions needed for adsorbtion of the bacteriophage. Cells were collected by centrifugation (1500 *g* for 10 min), resuspended in L-broth containing 5mM citrate and plated onto selective medium.

2.4.1 Preparation of bacteriophage P1*vir* lysate

Bacteriophage P1*vir* lysates were prepared by the method described by Silhavy *et al.*, (1984). Donor bacteria were grown to early logarithmic phase (A_{600}~0.2) in 5ml L-broth containing 0.2% (w/v) glucose, 5mM $CaCl_2$. Next, 100μl of P1*vir* lysate (c. 10^9 pfu/ml) was added and incubation continued at 37°C until the cells lysed (~2-3 hr). Chloroform (100μl) was added, mixed by vortexing and the lysate transferred to 1.5ml microcentrifuge tubes. Cell debris was removed by centrifugation.

2.5. DNA manipulation

2.5.1. Routine manipulation

Restriction endonucleases were obtained from Boehringer Mannheim and used according to the manufacturer recommendations. Restriction digests were performed in 20µl volumes with 1 unit of enzyme, 1-5µg DNA and 1X dilution of the corresponding enzyme buffer.

DNA fragments were separated by agarose gel electrophoresis using 0.7% (w/v) agarose, performed at 100v in TAE buffer (0.5M Tris, 5.7 % acetic acid, 10mM EDTA pH 8.0) containing 0.5µg/ml ethidium bromide. 1kb DNA ladder was used as size markers. DNA was visualised under ultraviolet light. DNA extraction from agarose gels was carried out using the Qiaex II gel extraction kit (Qiagen) or the Geneclean III kit (Amersham Bio101) following the manufacturers protocol.

2.5.2. Phenol extraction and ethanol precipitation of DNA

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA and mixed to form an emulsion. After centrifugation at 11600 x g for 5 min in a microcentrifuge, the upper aqueous phase was removed and extraction with phenol:chloroform:isoamyl alcohol (25:24:1) repeated. DNA was precipitated by the addition of 0.1 volume 3M Sodium acetate (pH 5.2); 2 volumes ethanol and subsequent incubation at –20°C for > 30 min. The precipitate was collected by centrifugation at 11600 x g for 10 min. The pellet was washed once with 1 ml ice-cold 70% (v/v) ethanol, air dried and redissolved in sterile distilled water.

2.5.3. Ligation

Ligation of DNA fragments was performed in 20µl volumes with 1 unit of bacteriophage T4 DNA ligase and a corresponding aliquot of T4 DNA ligase buffer. For blunt end ligations an ATP-free buffer was used and ATP was added at a final concentration of 1mM. Reactions were incubated overnight at 16°C.

2.5.4. DNA end repair

Protruding 3' termini were removed using the 3'-5' exonuclease activity of bacteriophage T4 DNA polymerase. Reactions were carried out in a 20µl final volume, comprising of 1 unit of T4 DNA polymerase, 1µl of dNTP's at a concentration of 2mM and 0.2-5µg of DNA digested with the appropriate restriction enzyme. Reactions were incubated at room temperature for 15 min.

2.6. Procedures for extraction of DNA and RNA

2.6.1. Plasmid DNA - small scale extraction

Small scale extraction of plasmid DNA was performed by the alkaline lysis method described by Birnhoim and Doly (1979). Bacteria were grown to stationary phase and 1.5ml culture collected by centrifugation at 11600 x *g* for 2 min in a microcentrifuge. Cells were resuspended in 100µl ice-cold solution I (50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM Tris EDTA (pH 8.0)) and left on ice for 30 min. The cells were lysed by the addition of 200µl solution II (0.2M NaOH, 1 % sodium dodecyl sulphate (SDS)), mixed and placed on ice for 5 min. The lysed cells were then neutralised with 150µl solution III (3M Potassium acetate, 11.5% glacial acetic acid) and placed on ice for a further 5 min. Cell debris was then removed by centrifugation at 11600 x *g* for 5 min in a microcentrifuge and the supernatant was recovered. The remaining solution was phenol:chloroform extracted twice and ethanol precipitated. The resulting pellet was redissolved in 20µl sterile distilled water and stored at -20°C.

High purity plasmid DNA required for PCR reactions and sequencing was extracted using the Wizard SV Plus DNA Purification System (Promega) according to the manufacturer protocol.

2.6.2. Plasmid DNA - large scale extraction

Large scale extraction was performed using the Qiagen plasmid Midi-kit (Qiagen) according to the manufacturer protocol.

2.6.3. Chromosomal DNA extraction

Bacteria were grown to stationary phase and 1.5ml collected by centrifugation at 11600 x *g* for 2 min in a microcentrifuge. Cells were resuspended in 567µl TE buffer (10mM Tris, 1mM EDTA), 30µl 10 % SDS and 3µl proteinase K (20mg/ml) and incubated at 37°C for 1 hr to allow cell lysis. Next, 100µl 5M NaCl was added and mixed, followed by the addition of 10% Hexadecyltrimethyl-ammonium bromide (CTAB) (in 0.7M NaCl, preequilibrated to 65°C). Preparations were then incubated at 65°C for 10 min before extraction with an equal volume of chloroform:isoamyl alcohol (24:1). Having recovered the aqueous phase, the DNA was precipitated by the addition of 0.6 volumes of isopropanol. The precipitate was collected by centrifugation at 11600 x *g* for 10 min. The pellet was washed twice with 1ml ice-cold 70% ethanol and air dried. Finally the chromosomal DNA was redissolved in 50µl sterile distilled water.

2.6.4. Total RNA extraction

Bacteria were grown to mid-logarithmic phase and collected by centrifugation at 2750 x *g* (4°C) for 10 min. Pelleted cells were then resuspended in 400µl REB (2% SDS (w/v), 10% Sucrose (w/v), 200mM Sodium acetate (pH 5.2)). After mixing, 400µl boiling REB was added and preparations incubated at 100°C for 2 min. Total RNA and DNA was then extracted with an equal volume of Tris-buffered phenol, followed by further extractions with phenol:chloroform (25:24) and chloroform respectively. DNA and RNA were then precipitated with 2 volumes ethanol at –70°C for >1 hour. The precipitate was collected by centrifugation at 11600 x *g* for 10 min. and pellets redissolved in 400µl sterile distilled water. Solutions were incubated at 28°C for 20 min with 4µl 1M MgCl and 40 units RNase-free DNase I (Boehringer Mannheim) to remove DNA. Extractions with phenol, phenol:chloroform and chloroform were then repeated. RNA was precipitated by the addition of 0.1 volume 3M Sodium acetate (pH 5.2); 2.5 volumes ethanol and incubated at –70°C for >1 hour. The precipitate was collected by centrifugation at 11600 x *g* for 10 min. The pellet was washed once with 1ml ice-cold 70% ethanol and air dried. Finally the total RNA was redissolved in 50µl sterile distilled water. The RNA concentration was determined by measuring absorbance at

260nm (an absorbance of 1 at 260nm is equivalent to 40µg/ml RNA). All solutions used for extraction and dilution of RNA were treated with diethyl pyrocarbonate (DEPC) to inactivate ribonucleases. DEPC was added at 0.05% (v/v) and solutions incubated at 37°C for 12 to 18 hours prior to autoclaving.

2.7. Procedures for DNA hybridisation

2.7.1. Southern blotting

DNA was separated by agarose gel electrophoresis and resulting gels were soaked in 0.4M NaOH for 15min. DNA fragments were allowed to transfer overnight onto to Hybond N+™ (Amersham Life Sciences) using 0.4M NaOH as a transfer solution.

2.7.2. Preparation of a fluorescein labelled probe

Probes were labelled with fluorescein using the Gene Images random prime labelling module (Amersham Life Sciences) according to the manufacturer protocol.

2.7.3. Hybridisation conditions

Following transfer, membranes were washed in neutralisation solution for 15 min, DNA was fixed to the membrane by exposure to UV light for 4 min. Fixed membranes were incubated in hybridisation buffer for 1 hour to prehybridise. The labelled probe was then added and incubation continued overnight at 65°C with rotation. After hybridisation membranes were washed once in 1X SSC, 0.1% (w/v) SDS for 15 min at 65°C, then for a further 15 min at 65°C in 0.5X SSC, 0.1% (w/v) SDS.

2.7.4. Detection of fluorescein labelled probe

Detection of the fluorescein labelled probe was carried out using the Gene Images CDP-Star detection module (Amersham Life Sciences) according to the protocol supplied by the manufacturer.

2.8. Procedures for RNA hybridisation

2.8.1. Dot/slot blotting RNA

RNA samples were heated to 65°C for 5 min in three volumes of the following solution (500µl formamide; 162µl formaldehyde (37% solution); 100µl 10X MOPS buffer). After heating the samples were ice-chilled and 1 volume of cold 20X SSC was added. Samples were applied on to nylon membrane (Hybond N^+, Amersham Life Sciences) using the Bio-Rad Biodot-SF apparatus. The membrane was fixed by 2-5 min exposure on a UV-transilluminator.

2.8.2. Preparation of a radiolabelled DNA probe

Restriction endonuclease fragments or PCR products for use as probes were radiolabelled using random hexanucleotide primers as described by Feinberg and Vogelstein (1983). DNA fragments (10 to 100ng in 10µl sterile distilled water) were denatured by heating in a boiling water bath for 10 min. and then placed on ice. The following reaction components were then added to a microcentrifuge tube and made up to a final volume of 20µl with sterile distilled water: 25ng denatured DNA; 3µl dNTP mix (prepared by making a 1+1+1 mixture of 0.5mM dATP, dGTP and dTTP each); 2µl Hexanucleotide mix (Boehringer Mannheim) 10X concentration; 5µl 50µCi [α^{32}P] dCTP, 3000 Ci/mmol; 1µl 2U Klenow enzyme labelling grade. The reaction mixture was incubated at 37°C for 30 min. After incubation the reaction was stopped by heating to 65°C for 10 min.

2.8.3. Hybridisation conditions

Membranes were pre-hybridised in prehybridisation solution (5X SSPE; 5X Denhardts solution; 0.5% (w/v) SDS) for 1 hour at 65°C in a rotating oven. The labelled probe was denatured by heating to 100°C for 5 min and subsequently added to the prehybridisation solution. Membranes were incubated for at least 12 hours at 65°C.

Following hybridisation the membranes were washed twice in 2X SSPE; 0.1% (w/v) SDS at room temperature for 10 min, then washed in 1X SSPE, 0.1% (w/v) SDS at 65°C for 15 min. Finally the membranes were incubated in 0.1X SSPE, 0.1% (w/v) SDS at 65°C for 10 min. After the final wash membranes were wrapped in Saran Wrap and signals due to bound probe were detected and quantified using a FujiX BAS2000 Phosphoimager™ with Aida v2.0 software. Hybridisation using a 16S RNA gene radiolabelled probe was used to confirm the total loading of RNA onto the filter.

2.9. Deletion mutagenesis

2.9.1. Alkali denaturation of template DNA

Alkali denaturation was carried out according to the method described by Dorrell *et al.*, (1996). Plasmid DNA (10ng) was mixed with 10µl denaturing solution (1M NaOH, 1mM EDTA) in a volume of 40µl and then incubated at 37°C for 15 minutes. The reaction was neutralised by the addition of 5µl of 3M sodium acetate (pH 4.8). The denatured DNA was then precipitated by adding 150µl absolute ethanol and incubating at -20°C for 60 minutes. Precipitated DNA was collected by centrifugation at 11600 x g for 10 minutes in a microcentrifuge. The pellet was then washed in 150µl 70% ethanol and redissolved in 20µl H_20. Dilutions of denatured DNA were then used as template for inverse PCR mutagenesis.

2.9.2. Inverse PCR Mutagenesis (IPCRM)

IPCRM was performed using a modified version of the protocol described by Wren *et al.*, (1994). This method uses plasmid DNA as a circular template to delete specific regions of DNA. Primers are designed in opposite orientations from the region to be deleted and after amplification, the resulting PCR product is self ligated and transformed back into *E. coli*. Primers used for this purpose and binding locations can be seen in Table 2.5. and Figure 2.1. respectively. In this study no restriction sites were incorporated into the primers, as a blunt end self ligation was carried out. This was due to the replacement of Taq DNA polymerase in the original protocol with Pfu DNA polymerase. Reactions were carried out in a 50µl final volume. The reactions were incubated at 94°C for 2 min in a programmable

heat block (Hybaid, Teddington, UK), then for a further 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C. At the end of 40 cycles the reaction mixtures were incubated at 72°C for 8 min. Resulting PCR products were ethanol precipitated and digested with *Dpn*I to remove template DNA (Gatlin *et al.*, 1995) before self ligation and transformation into *E. coli* XL-1 Blue.

2.10. Transposon mutagenesis

A modified lambda phage (λ467) containing Tn*5* was used to generate pools of random transposon mutants. This was performed using the method described by de Bruijn & Lupski (1984). *E. coli* XAC Su^- cells were grown up to an A_{600} of 0.6 to 0.8 in L-broth containing 0.2% (w/v) maltose. Assuming an A_{600} 0.8 culture to contain approximately 5 x 10^8cells/ml, 4 ml aliquots of culture were infected with λ467 (Tn*5*) at a ratio of 1:10, 1:1 and 10:1 cells:phage. The mixture was incubated at 37°C for 1 hour and aliquots were plated out onto L-agar containing kanamycin to select for Tn*5*. Colonies which grew after overnight incubation at 37°C were assumed to contain Tn*5* insertions into the chromosome

2.11. β-galactosidase assay

β-galactosidase activity was measured as described by Miller (1972). Strains to be tested were grown to mid-logarithmic phase and the absorbance at 600nm was recorded. All reaction components and cell cultures were stored on ice. To each reaction tube 100 µl cell culture, 900µl Z buffer, 40µl chloroform and 20µl 0.1% sodium dodecyl sulphate (SDS) were added. The reaction mix was then vortexed and equilibrated to 37°C. To begin the reaction 200µl of o-nitrophenyl-β-D-galactopylanoside (4mg/ml in Z buffer) was added and the time taken for a yellow colour to develop was recorded. The reaction was stopped by the addition of 500µl 1M Na_2CO_3. Absorbance readings of the reaction samples at 550nm and 420nm were taken and the units of β-galactosidase activity were calculated using the following formula. Assays were performed in triplicate and repeated at least four times.

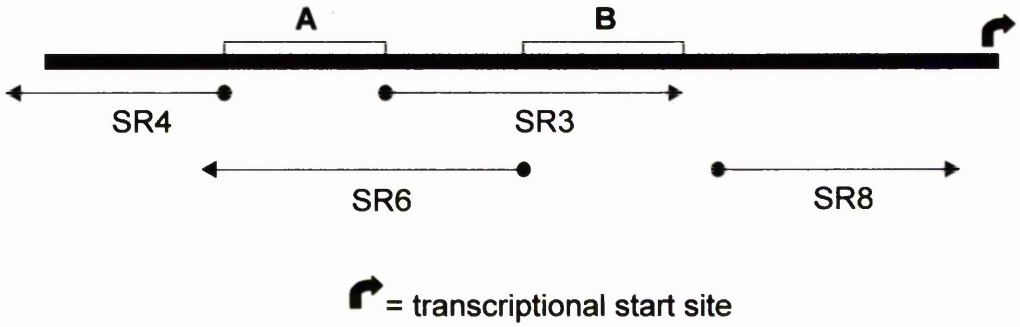
Units of β-galactosidase activity = $1000.\frac{A_{420} - (1.75.A_{550})}{t.v.A_{600}}$

(t = time of the reaction in minutes, v = volume of culture used in the assay in ml)

Table 2.5. PCR primers used in this study

Primer	Sequence (5' to 3')
SR3	CAAGTAGGAAACATTTTAACAAATGATA
SR4	CCAAAAACAATTTATCAATTGATTATTTTC
SR6	AAATGTTTCCTACTTGACTATTAATAC
SR7	GTCAGTCCATGCTTATATGCAGG
SR8	CCTAAATTCCTTGTTCATAATGTAGGA
SR9	GCGAACAGAGGTAATTAGATATGG
HNS-1	ACTACAATGAGCGAAGCAC
HNS-2	GATTATTGCTTGATCAGGAAATCG
IHF-1F	AAATTGTTTTTGGTATTAATAGTCAAG
IHF-1R	TTTTCTTGTAAAAAAAGAACGTATGA
IHF-2F	TAGCATAAATAAATTATAGTGGGTT
IHF-2R	TAACAAAATTTTTAATGAATATAAAACCAT
Tn5-3	GCCGCACGATGAAGAGCAGAAGT

Figure 2.1. Schematic representation of the region upstream of the region 1 promoter transcriptional start site indicating binding locations of IPCRM primers. The solid line represents the region upstream of the region 1 promoter transcriptional start site labelled with a solid arrow. Boxes labelled A and B represent conserved regions conA and conB respectively. Arrows labelled SR3-SR8 indicate IPCRM primers SR3-SR8 binding positions. Primer sequences can be found in Table 2.5.



2.12. Luciferase assay

Strains under test were grown to mid logarithmic phase at 20°C or 37°C in L-broth. To each 100µl sample, 10 µl of a 1% (v/v) solution of n-decanal (in absolute ethanol) was added, mixed for 1 second and the relative light units (rlu) produced measured over 5 seconds using an LKB Wallac 1200 luminometer. Measurements were performed in triplicate and repeated at least four times.

2.13. DNA sequencing

Automated DNA sequencing was carried out using the ABI PRISM™ BigDye terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corporation) according to the manufacturers recommendations. Cycle sequencing reactions were carried out in a 20µl final volume, comprising of 8µl Terminator Ready reaction mix, 0.3-0.5µg double-stranded template DNA and 3.2pmole primer SR7 -(5'-GTCAGTCCATGCTTATATGCAGG- 3'), made up to 20µl with distilled water. Reactions were incubated in a thermal cycler (Hybaid) for 25 cycles of 96°C for 30 s, 50°C for 15 s, 60°C for 4 min. Extension products were purified by ethanol precipitation and sequences were analysed on an ABI PRISM™ 377 DNA sequencer.

2.14. Assay of capsular polysaccharide production

2.14.1. Preparation of K5-specific bacteriophage lysates

K5 bacteriophage lysates were prepared by the method of Silhavy *et al.*, (1984). L-broth (5 ml) containing 0.2% (w/v) glucose and 5mM $CaCl_2$ was inoculated with 50µl of a donor strain overnight culture and incubated for 30 min at 37°C with aeration. Next, 100µl of a K5 phage lysate (~5 x 10^8 phage/ml) was added and incubation continued at 37°C with aeration for 2-3 hr until the cells lyse. After the cells have lysed 100µl chloroform was added, mixed and cell debris was collected by centrifugation at 11600 x *g* for 10 min in a microcentrifuge. The

supernatants were then transferred to fresh 1.5 ml microcentrifuge tubes and stored at 4°C.

2.14.2. Assay for sensitivity to K5-specific phage

E. coli strains were grown to an OD_{600} 0.5 in 10ml L-broth. Cells were collected by centrifugation at 2750 g for 10 min (4°C) and resuspended in 10mM $MgSO_4$. The K5 phage was diluted in phage dilution buffer (Neat, 10^{-3}, 10^{-6}, 10^{-9}). An aliquot of each dilution (100µl) was then incubated with 100µl cells for 20 min at room temperature. Next, 3ml of molten soft top agar (~ 40-45°C) (5g/l NaCl, 10g/l Trypticase peptone, 7.5g/l bacteriological agar) was added to each tube and poured onto L-agar plates. Plates were incubated at 37°C overnight and were then observed for plaques representing lysis due to K5 phage infection.

2.14.3. Measurement of extracellular K5 polysaccharide production

Extracellular K5 polysaccharide produced was quantified using the carbazole assay described by Bitter & Muir (1962). Purified K5 polysaccharide was used as a standard. An aliquot (5ml) of sulphuric acid reagent (0.025M sodium tetraborate.H_2O in sulphuric acid) was placed in tubes cooled to 4°C. Next, 1ml of sample was layered onto the acid, the tubes stoppered and the reaction mixed gently and then vigorously with constant cooling. The tubes were heated for 10 min in a boiling water bath and cooled to room temperature. This was followed by the addition of 200µl 0.125% carbazole in abs. ethanol or methanol. The reaction was mixed and heated in a boiling water bath for 15 min. After cooling to room temperature the absorbance was read at 530nm.

Chapter 3

Results

Involvement of BipA in expression of *E. coli* K5 capsular polysaccharide

3.1 Construction and identification of regulatory mutants

The temperature regulation of *E. coli* K5 capsule gene expression is thought to be the result of interactions of a complex regulatory circuit. Previous work has shown that IHF and RfaH are both involved in this process (Section 1.5.2; Section 1.5.3). However, little is known about other regulatory proteins which may facilitate temperature regulation. In this study a transposon system was adopted to identify genes involved in *E. coli* K5 temperature regulation. In many cases transposons have been used to generate random mutations in the host chromosome such mutations can then be screened to analyse the effects they have on expression of particular genes. The modified λ phage 467 carrying Tn*5* was used in this study to generate a pool of random transposon mutants; this was achieved by infecting *E. coli* strain XAC with λ467 (as described in section 2.10). A promoter probe vector containing the region 1 promoter upstream of a promoterless *lacZ* gene (pDSHcH) was introduced into the mutant population and regulatory mutants were selected on L-agar plates containing ampicillin, kanamycin and X-gal. Mutants with transposon insertions altering region 1 promoter activity were identified as colonies showing reduced β-galactosidase activity when grown at 37°C. Approximately 10,000 mutants were screened in this way and isolates of particular interest were further analysed. One particular isolate XAC.7 pDSHcH was identified as showing reduced β–galactosidase activity in contrast to the control strain (XAC pDSHcH) and was chosen for further analysis. Vector pDSHcH from this strain was extracted and reintroduced into XAC, where β–galactosidase assays were performed to ensure no point mutations within the plasmid had altered expression of the *lacZ* gene.

To determine the identity of the gene containing the Tn*5* insertion Southern blot analysis was utilised. Chromosomal DNA was digested using *BamH*I and *Sma*I and probed with a 3.3kb *Hind*III fragment spanning the central region of Tn*5*.

DNA flanking the Tn*5* insertion was localised to a 5.9kb *BamH*I chromosomal DNA fragment (Figure 3.1.). Size selected (5.9kb) *BamH*I digested chromosomal DNA was cloned into the *Bam*HI site of pBluescript and clones containing the Tn*5* insertion were selected on L-agar plates supplemented with kanamycin and X-gal. The orientation of the clones was verified by digestion with *Hind*III to produce two restriction fragments 4.2kb and 4.9kb (Figure 3.2.). Of the six resulting kanamycin resistant colonies clone pTn*5*.7 was chosen for nucleotide sequencing using a primer specific to the end of Tn*5* (Tn*5*-3 5'-GCCGCACGATGAAGAGCAGAAGT-3') (Figure 3.2.). Sequencing revealed that the transposon had inserted into the *E. coli bipA* gene (Figure 3.3.). BipA (also known as TypA) is a tyrosine phosphorylated GTPase which is known to play a role in the pathogenesis of enteropathogenic *E. coli* (EPEC) (Farris *et al.*, 1998; Freestone *et al.*, 1998).

3.2 Role of BipA in the expression of the *E. coli* K5 capsule genes

3.2.1 Region 1

To investigate the role of BipA in expression of the region 1 genes the *bipA*::Tn*5* mutation was introduced into strain MS150 (MS101 Δ*argF-lac*, $K5^+$) by transduction using bacteriophage P1*vir* to generate strain MS153 (MS150*bipA*::Tn*5*). To confirm transfer of the *bipA* mutation Southern blots were performed using a 3.3kb *Hind*III fragment from Tn*5* as a probe (Figure 3.4.).

The promoter probe vector pDSHcH was introduced into MS153 and region 1 promoter activity was measured at 37°C and 20°C (Figure 3.5.). At 37°C the *bipA* mutation reduced β-galactosidase activity by five fold but at 20°C it had no effect suggesting that BipA may contribute to maximal transcription from the region 1 promoter at 37°C.

Figure 3.1. Southern blot analysis of the Tn*5* insertion and flanking chromosomal DNA in strain XAC.7 (A) Diagram analysis of Tn*5* insertion. Restriction endonuclease cleavage sites used to analyse Tn*5* insertion and sizes of restriction fragments are shown. (B) Southern blot analysis of Tn*5* insertion. Chromosomal DNA from XAC.7 was digested with *BamH*I, *Sma*I and both *BamH*I and *Sma*I and probed with a 3.3 kb Tn*5* specific probe

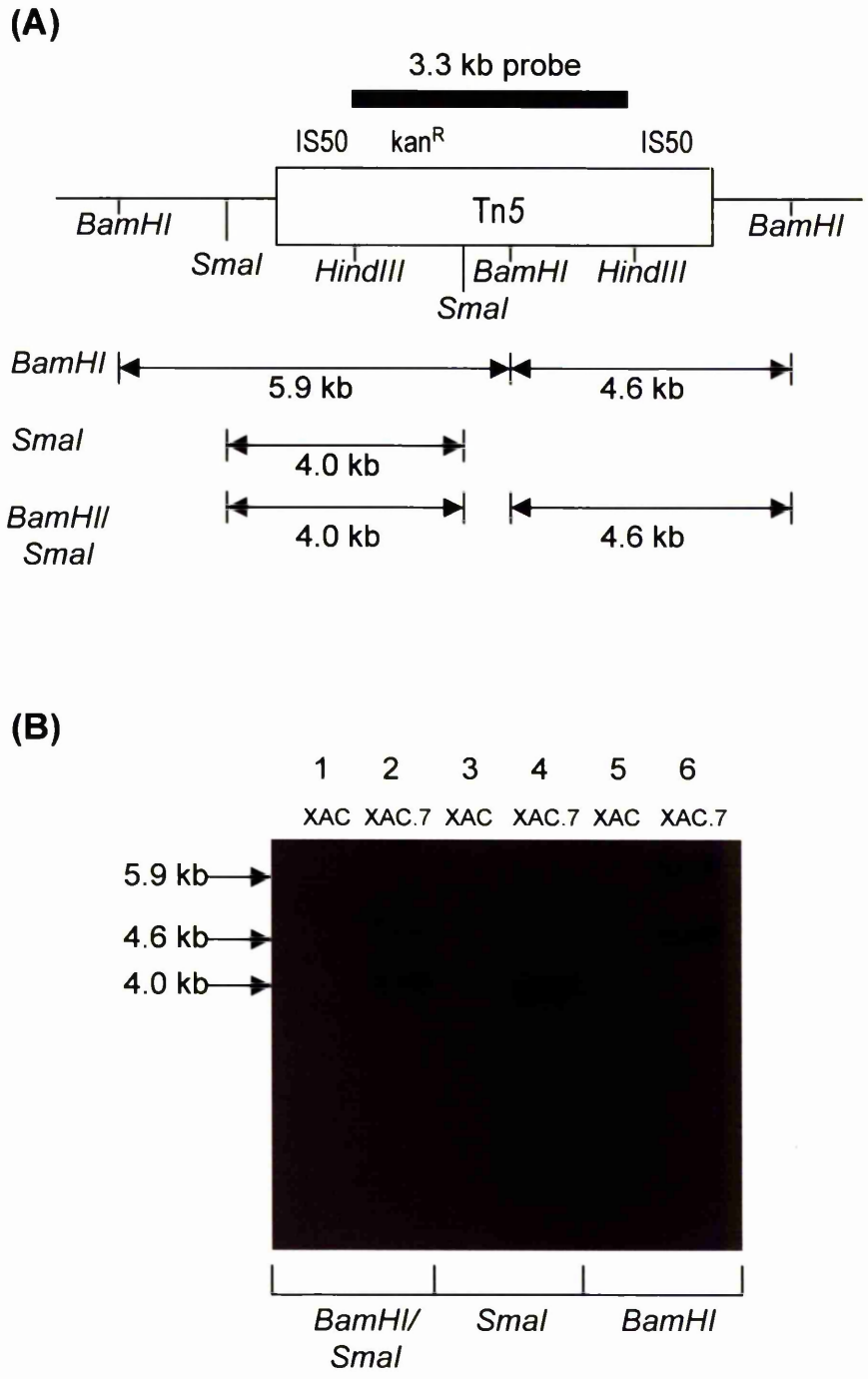
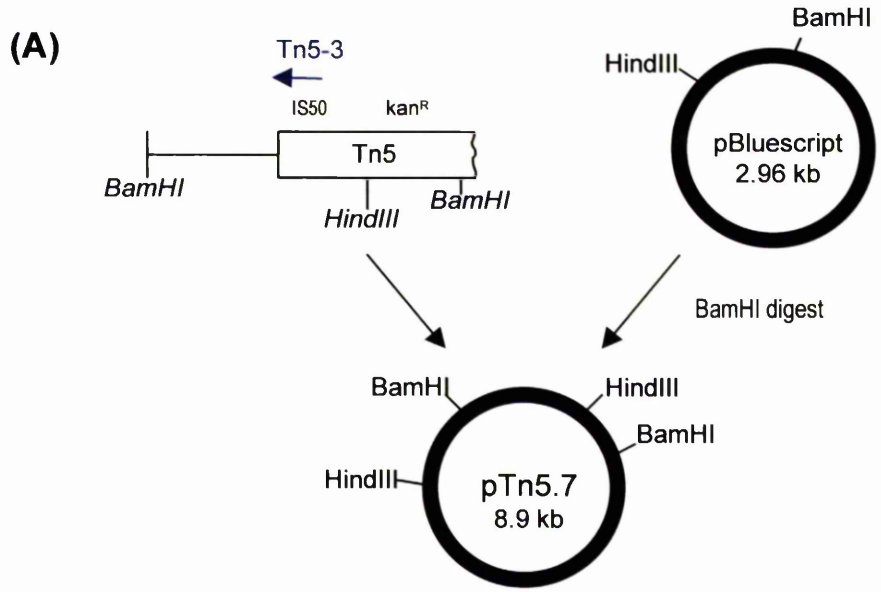


Figure 3.2. *Hind*III digest of pTn*5*.7

(A) Construction of pTn*5*.7. Restriction endonuclease cleavage sites used to construct pTn*5*.7 are shown. Binding location of primer Tn*5*-3 used for nucleotide sequencing of DNA flanking Tn*5* shown. (B) Lane 1, 1kb ladder; Lane 2, pTn*5*.7 DNA digested with *Hind*III



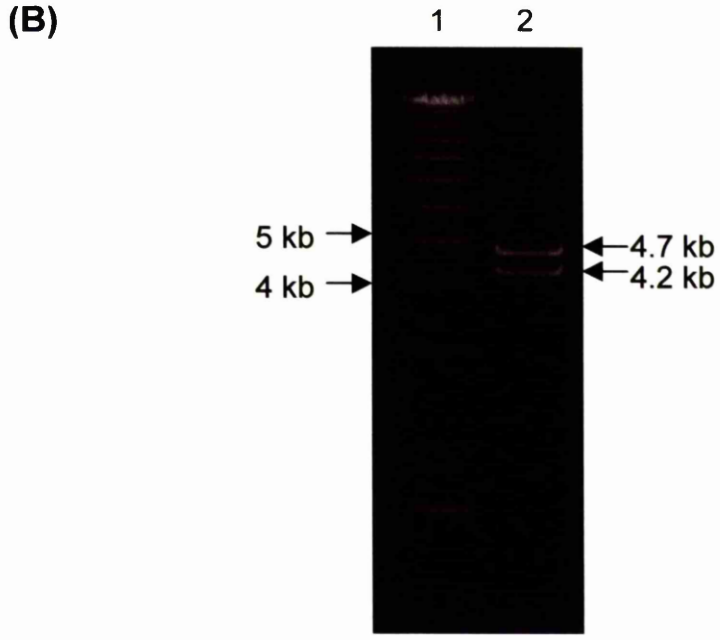


Figure 3.3. Multiple alignment of nucleotide sequence of the Tn5 insertion target with homologous sequences. Top line of alignment shows sequence data (1-522bp) obtained from nucleotide sequencing of pTn5.7 using primer Tn5-3 which binds to the IS*50* terminal end of Tn*5*. Middle and bottom lines of the alignment show sequences identified as showing signifcant homology to the pTn5.7 sequence using a BLAST alignemnt (Altschul *et al.*, 1997). Code preceeding sequences indicates Genbank accession number (AF05833 = *E. coli* tyrosine phosphorylate protein A (*typA*); AJ278218 = *E.coli bipA* gene for the BipA GTPase). The numbers at either end of the sequences denote the position of the nucleotides in the sequence entries.

```
pTn5.7       1 cgtttgcgacacgcaaaacgttgaagcgctgccggcactctccgttgatg 50
AF058333   861 cgtttgcgacacgcaaaacgttgaagcgctgccggcactctccgttgatg 910
AJ278218  1236 cgtttgcgacacgcaaaacgttgaagcgctgccggcactctccgttgatg 1285

pTn5.7      51 agccgaccgtttctatgttcttctgcgttaacacctcgccgttctgcggt 100
AF058333   911 agccgaccgtttctatgttcttctgcgttaacacctcgccgttctgcggt 960
AJ278218  1286 agccgaccgtttctatgttcttctgcgttaacacctcgccgttctgcggt 1335

pTn5.7     101 aaagaaggtaagttcgtaacgtctcgtcagatcctggatcgtctgaacaa 150
AF058333   961 aaagaaggtaagttcgtaacgtctcgtcagatcctggatcgtctgaacaa 1010
AJ278218  1336 aaagaaggtaagttcgtaacgtctcgtcagatcctggatcgtctgaacaa 1385

pTn5.7     151 agaactggtacacaacgttgcgctgcgcgtagaagaaaccgaagacgccg 200
AF058333  1011 agaactggtacacaacgttgcgctgcgcgtagaagaaaccgaagacgccg 1060
AJ278218  1386 agaactggtacacaacgttgcgctacgcgtagaagaaaccgaagacgccg 1435

pTn5.7     201 atgcgttccgcgtttctggtcgtggcgaactgcacctgtctgttcttatc 250
AF058333  1061 atgcgttccgcgtttctggtcgtggcgaactgcacctgtctgttcttatc 1110
AJ278218  1436 atgcgttccgcgtttctggtcgtggcgaactgcacctgtctgttcttatt 1485

pTn5.7     251 gaaaacatgcgtcgtgaaggtttcgaactggcggtatcccgtccgaaagt 300
AF058333  1111 gaaaacatgcgtcgtgaaggtttcgaactggcggtatcccgtccgaaagt 1160
AJ278218  1486 gaaaacatgcgtcgtgaaggtttcgaactggcggtatcccgtccgaaagt 1535

pTn5.7     301 aatcttccgtgaaatcgacggtcgtaaacaagagccgtatgaaaacgtga 350
AF058333  1161 aatcttccgtgaaatcgacggtcgtaaacaagagccgtatgaaaacgtga 1210
AJ278218  1536 aatcttccgtgaaatcgacggtcgtgacgtagagccgtatgaaaacgtga 1585

pTn5.7     351 cgctggacgttgaacaacagcatcagggttctgtgatgcaggcgctgggc 400
AF058333  1211 cgctggacgttgaacaacagcatcagggttctgtgatgcaggcgctgggc 1260
AJ278218  1586 cgctggacgttgaagaacagcatcagggttctgtgatgcaggcgctgggc 1635

pTn5.7     401 gaacgtaaaggcgacctgaaaaacatgaatccagacggtaaaggccgcgt 450
AF058333  1261 gaacgtaaaggcgacctgaaaaacatgaatccagacggtaaaggccgcgt 1310
AJ278218  1636 gaacgtaaaggcgacctgaaaaacatgaatccagacggtaaaggccgcgt 1685

pTn5.7     451 acgtctcgactacgtgatcccaagccgtggtctgattggcttccgttctg 500
AF058333  1311 acgtctcgactacgtgatcccaagccgtggtctgattggcttccgttctg 1360
AJ278218  1686 acgtctcgactacgtgatcccaagccgtggtctgattggcttccgttctg 1735

pTn5.7     501 agttcatgaccatgacttctgg 522
AF058333  1361 agttcatgaccatgacttctgg 1382
AJ278218  1736 agttcatgaccatgacttctgg 1757
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Figure 3.4. Southern blot analysis of strain MS153 (MS150*bipA*::Tn*5*). Chromosomal DNA was extracted from strains MS150(*bipA*$^+$), MS153(*bipA*::Tn*5*) and XAC.7(*bipA*::Tn*5*) and digested with *BamH*I. To confirm transfer of the *bipA*::Tn*5* mutation digested DNA was probed with a 3.3kb Tn*5* specific probe. Arrows indicate bands representing hybridisation of probe to 5.9kb and 4.6kb chromosomal fragments. Lane 1, MS150 *BamH*I digested chromosomal DNA; Lane 2, MS153 *BamH*I digested chromosomal DNA; Lane 3, XAC.7 chromosomal DNA.

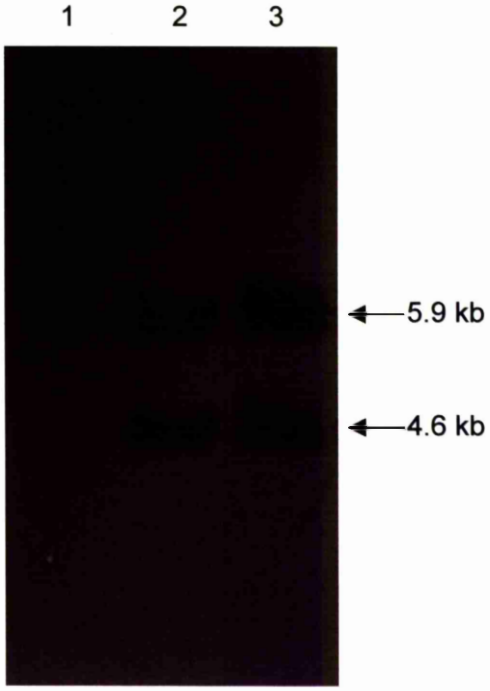
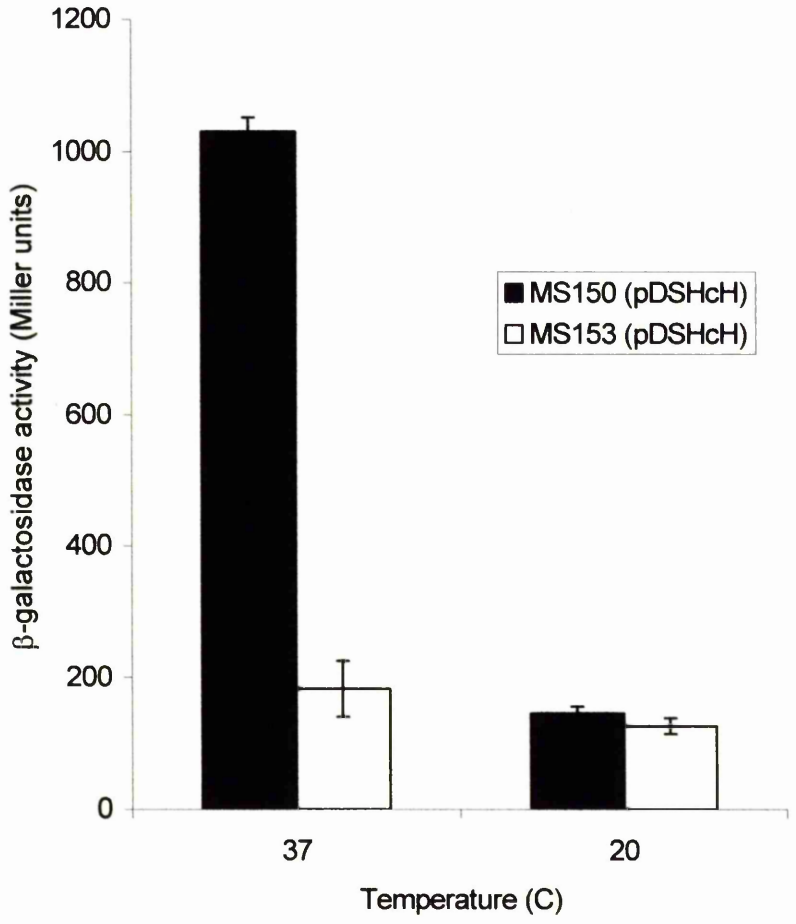


Figure 3.5. The effect of the *bipA* mutation on region 1 promoter activity at 37°C and 20°C. Strains MS150 and MS153(*bipA*::Tn*5*) carrying plasmid pDSHcH (Region 1 promoter::*lacZ*) were grown to OD_{600} 0.4 in Luria broth at 37°C and 20°C. Promoter activity was measured as *lacZ* expression using a β-galactosidase actvity assay. Shaded bars represent activity of MS150 pDSHcH and plain bars represent activity of MS153 pDSHcH. β-galactosidase actvity assays were performed in triplicate and repeated at least 4 times. Error bars represent standard error of the mean.



3.2.2 Region 2 and 3

To analyse the role of BipA in expression of the region 2 and region 3 genes the *bipA* mutation was introduced into strains MS120 (MS101 *kpsT*::*luxAB*) and MS108 (MS101 *kfiA*::*luxAB*) by transduction using bacteriophage P1*vir*. MS108 and MS120 contain *luxAB* fusions to the region 2 and region 3 genes respectively. In the presence of n-decanal the *lux* genes produce a blue-green bioluminescence that can be detected in a luminometer and therefore can be used as a measure of capsule gene expression. Transfer of the *bipA* mutation was verified by Southern hybridisation using a 3.3kb *Hind*III fragment from Tn*5* as a probe (data not shown). To determine if transcription of *kpsT* and *kfiA* was altered by the *bipA* mutation strains MS120*bipA*::Tn*5* and MS108*bipA*::Tn*5* were grown at 37°C and 20°C and luciferase assays were performed (Figure 3.6.; Figure 3.7.). At 37°C expression of *kpsT* in strain MS120*bipA*::Tn*5* was reduced by 50% (Figure 3.7.). The *bipA* mutation had similar effects on expression of the *kfiA* gene at 37°C with gene expression also reduced by 50%. At 20°C the *bipA* mutation appeared to have no significant effects on expression of the *kfiA* gene (Figure 3.6.. However there was a small but significant decrease in the expression of *kpsT* at this temperature (Figure 3.7). This data suggests that BipA also plays a role in facilitating maximal expression of the region 2 and region 3 genes at 37°C. Surprisingly this data also suggests BipA may play a regulatory role in the expression of the region 3 genes at 20°C.

To further assess the role of the *bipA* mutation in expression of the K5 capsule gene cluster uronic acid assays and K5 phage sensitivity assays were performed using strains MS150 and MS153 grown at 37°C and 20°C (Figure 3.8.). Phage assays were performed using a K5 specific phage which can only infect cells producing the K5 capsular polysaccharide. At 37°C MS153 gave plaques of similar diameter to the control strain (MS150) however the plaques obtained with this strain MS153 did not show clear complete lysis (data not shown).

The carbazole assay was used to quantify the production of the *E. coli* K5 capsular component uronic acid using the K5 polysaccharide as a standard. At 37°C uronic acid production in the *bipA* mutant was two fold less than that of the control

Figure 3.6. The effect of the *bipA* mutation on the temperature dependent expression of the region 2 *kfiA::luxAB* gene fusion. Strains MS108 (*kfiA::luxAB*) and MS108 (*kfiA::luxAB*; *bipA*::Tn*5*) were grown to OD_{600} 0.4 in Luria broth at 37°C and 20°C. *kfiA::luxAB* expression was measured by luciferase assay. Shaded bars represent activity of MS108 and plain bars represent activity of MS108 *bipA*::Tn*5*. Luciferase assays were performed in triplicate and repeated at least four times. Error bars represent the standard error of the mean.

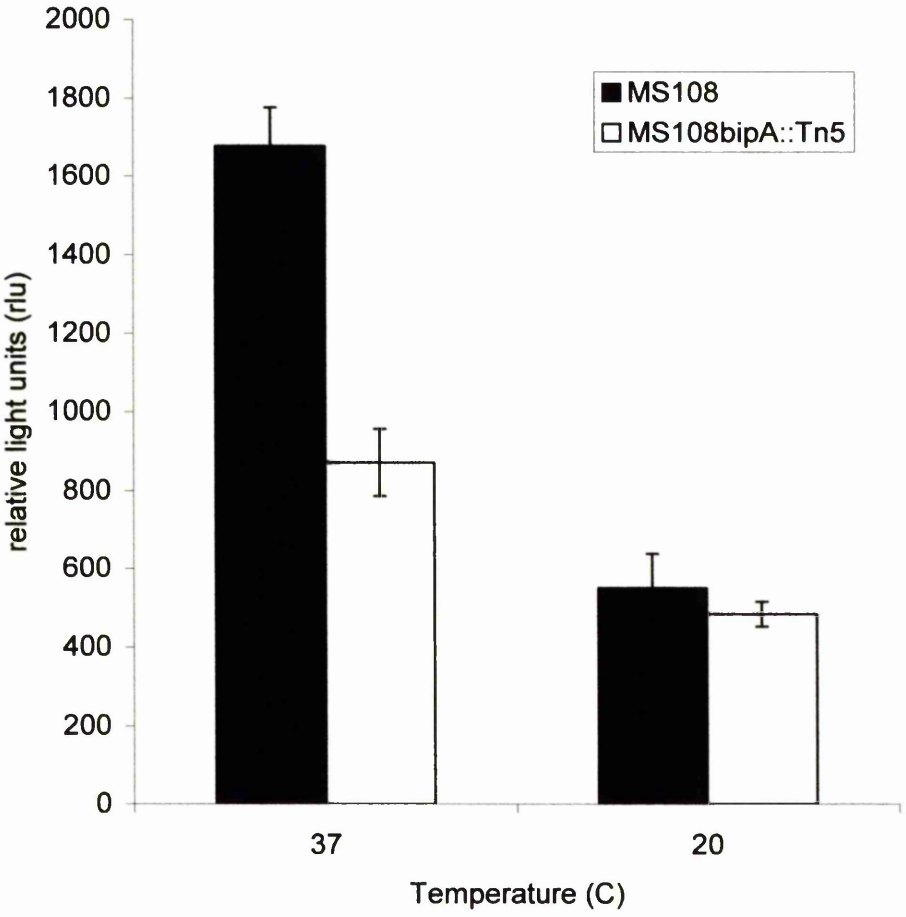


Figure 3.7. The effect of the *bipA* mutation on the temperature dependent expression of the region 3 *kpsT*::*luxAB* gene fusion. Strains MS120 (*kpsT*::*luxAB*) and MS120 (*kpsT*::*luxAB*; *bipA*::Tn*5*) were grown to OD_{600} 0.4 in Luria broth at 37°C and 20°C. *kpsT*::*luxAB* expression was measured by luciferase assay. Shaded bars represent activity of strain MS120 and plain bars represent activity of MS120 *bipA*::Tn*5*. Luciferase assays were performed in triplicate and repeated at least four times. Error bars represent standard error of the mean.

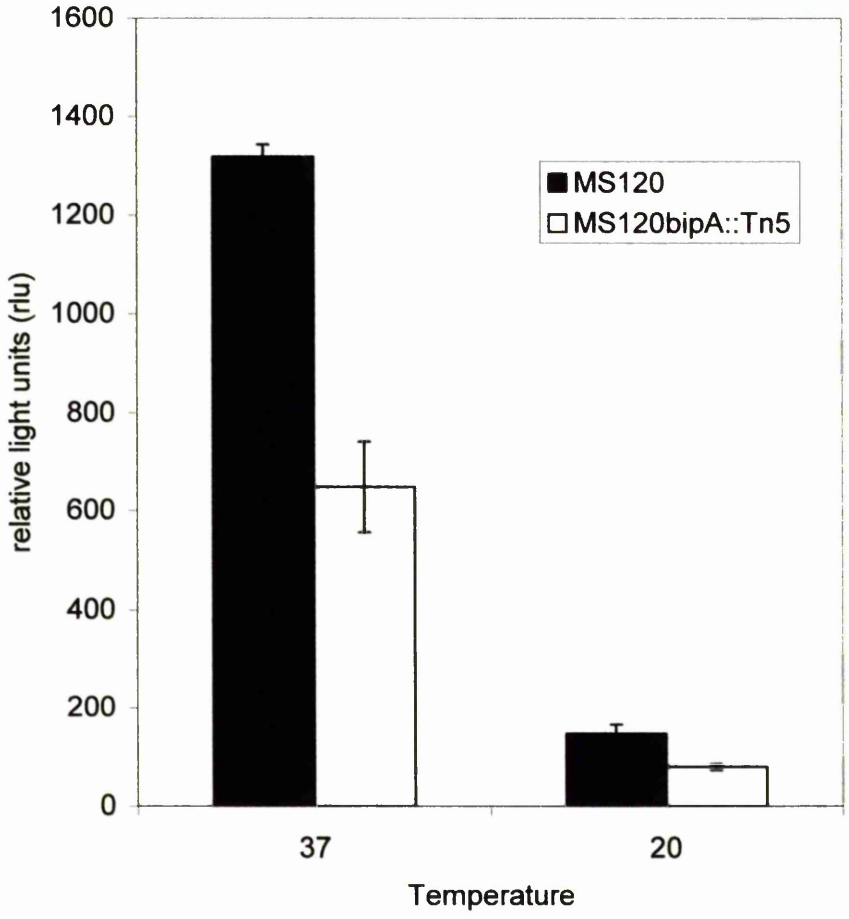
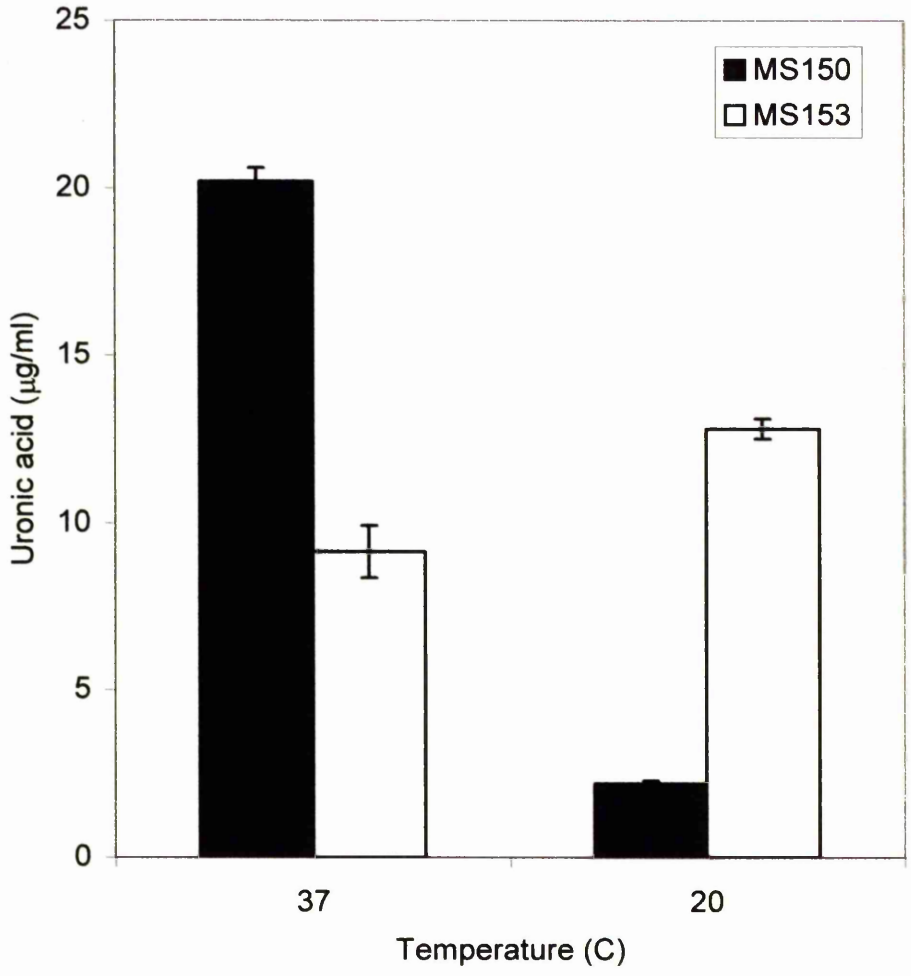


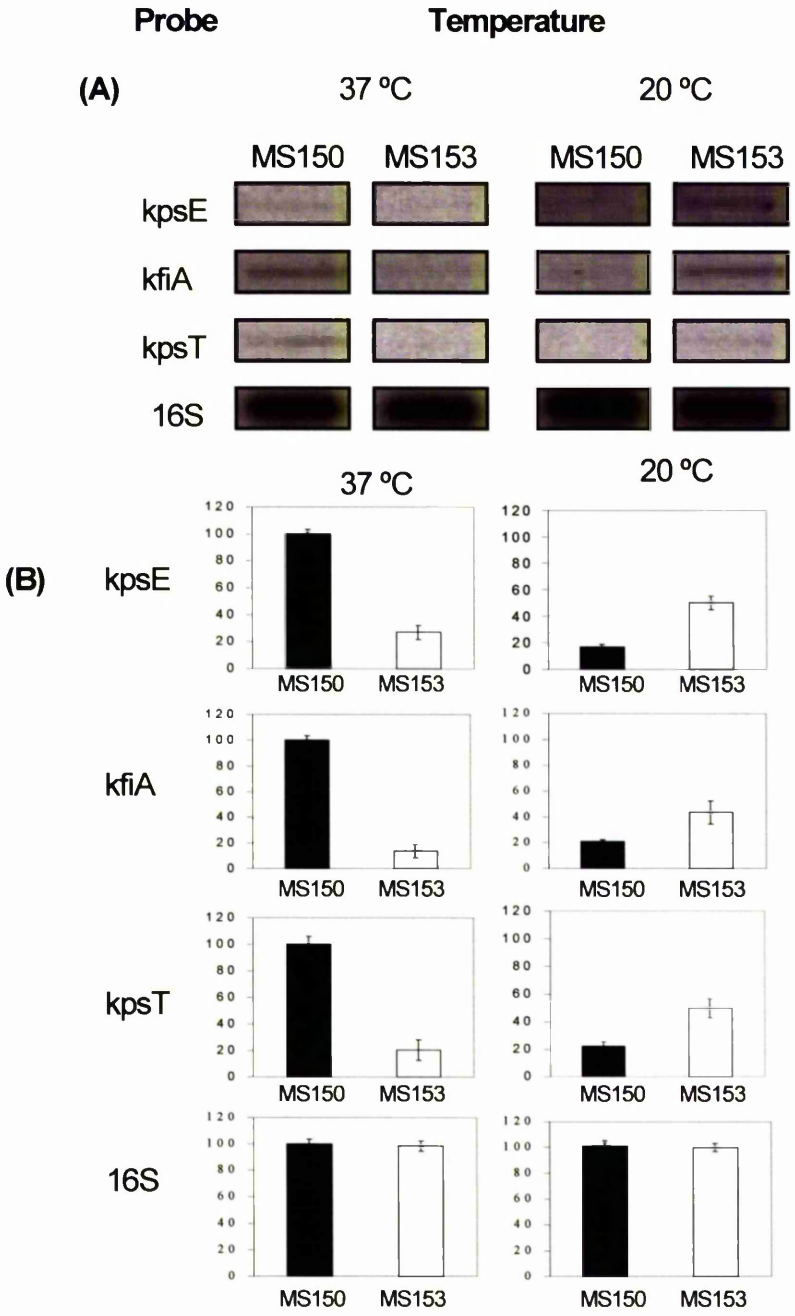
Figure 3.8. The effect of the *bipA* mutation on extracellular K5 polysaccharide production at 37°C and 20°C. Strains MS150 (K5$^+$) and MS153 (K5$^+$, *bipA*::Tn*5*) were grown to OD_{600} 0.8 in Luria broth at 37°C and 20°C. Extracellular K5 polysaccharide production was measured as uronic acid production using the carbazole assay (Bitter & Muir, 1962). Shaded bars represent polysaccharide production by strain MS150 and plain bars represent polysaccharide production by strain MS153. Measurements were performed in triplicate and repeated at least four times. Error bars represent standard error of the mean.



strain (MS150) (Figure 3.8). However at 20°C an increase in uronic acid production was observed suggesting possible increased production of K5 polysaccharide at this temperature a result not seen with the luminescence and β–galactosidase data, with the exception of the MS120*bipA*::Tn*5* luminescence results (Figure 3.7, 3.8.) This data suggests that BipA may be involved in regulation of the capsule genes at both 37°C and 20°C.

Due to potential problems associated with use of the *luxAB* genes of *Vibrio harveyii* (Forsberg *et al.*, 1994) and multicopy promoter probe vectors quantitative RNA slot blot hybridisation was used to verify the data generated using these methods. Total RNA was extracted from strains MS150 and MS153 grown at 37°C and 20°C. Samples were immobilised on a nylon membrane and then probed with radioactively labelled probes from region 1 (*kpsE*), 2 (*kfiA*) and 3 (*kpsT*) (as described in section 2.8.). A 16S RNA probe was used as a control to confirm equal loading. RNA signals were detected and quantified using a Phosphoimager (Figure 3.9.). This data showed that the *bipA* mutation reduced expression of the capsule genes at 37°C. The greatest reduction was seen in the expression of the *kfiA* gene which was reduced seven fold, with expression of the *kpsT* gene reduced five fold and *kpsE* gene reduced four fold. At 20°C an increase in capsule gene expression was seen in the *bipA* mutant with expression of *kpsE*, *kfiA* and *kpsT* at least two fold higher when compared with the control strain (MS150). This data supports the notion that BipA is required for maximal expression of the capsule genes at 37°C but also suggests that BipA may be additionally required for thermoregulation of the capsule genes at 20°C. The RNA slot blot results and carbazole assay data obtained at 20°C are not supported by the luminescence data obtained with strain MS120*bipA*::Tn*5*. It is unclear why the *kpsT*::*luxAB* fusion has yielded such contradictory data and clarification would require further analysis using alternative region 3 gene fusions. Suggestions raised on the involvement of BipA in the regulation of the region 3 genes at 20°C have therefore been based on the strength of the carbazole assay and RNA slot blot hybridisation data.

Figure 3.9. Slot blot hybridisation (A) Slot blot hybridisation, total RNA extracted from strains MS150 and MS153 grown at 37°C and 20°C. 50µg total RNA samples were applied to the membrane and probed with ^{32}P labelled K5 specific probes *kpsE*; *kfiA*; *kpsT*; 16S. (B) Band intensity of RNA slot blot hybridisation. Band intensity analysed and quantified using Aida v2.0 software.



3.3 Discussion

To investigate the temperature regulation of the K5 capsule genes transposon mutagenesis was used to generate regulatory mutants. Insertion of the transposon into the *E. coli bipA* gene was found to affect transcription of both the region 1 and region 3 promoters at 37°C and 20°C. This data suggests that BipA plays an important role in capsule regulation. The data obtained at 37°C suggests BipA plays a role in maximising expression of the region 1 and 3 promoters at this temperature. As sequence analysis of BipA has revealed no characteristic DNA binding motifs or sequence homology to known DNA binding or transcriptional activating proteins, it is unlikely that the BipA protein acts directly on the region 1 or region 3 promoter (Plunkett *et al.*, 1993). It is perhaps more likely to expect BipA to mediate its effect indirectly via other as yet unknown regulatory factors. Analysis of protein patterns in a *bipA* mutant by Freestone and co-workers revealed that BipA is responsible for the synthesis or modification of 30+ proteins in stationary phase as well as 12 other proteins in exponential phase (Freestone *et al.*, 1998). This data suggests that BipA has a global role in regulation in the cell and therefore may regulate the activity of proteins required for maximising capsule gene expression at 37°C. BipA homologues have also been found in a number of other pathogenic bacterial species including *H. influenzae*, *Helicobacter pylori*, *P. aeruginosa*, *N. meningitidis*, *V. cholerae* and *Campylobacter jejuni* (Plunkett *et al.*, 1993). Although no roles have been assigned to BipA in these species the widespread distribution of such homologues may indicate that BipA may have a common function in pathogenic bacteria and play an important role in the cell.

The experimental data obtained at 20°C suggests that BipA plays a role in the thermoregulation of the capsule genes. At this temperature transcription of the capsule genes was significantly increased in a *bipA* mutant suggesting inactivation of BipA alleviates repression of capsule gene expression. It is likely that BipA indirectly acts to repress transcription from the capsule promoters.

To summarise the data it would seem that BipA plays a regulatory role at both capsule permissive and non-permissive temperatures. It is not clear however how BipA mediates such control and further detailed studies will be required to establish the precise nature of this mechanism. As the BipA protein was only recently described its function in the cell is not fully understood. Sequence analysis

of BipA shows that it is a tyrosine phosphorylated GTPase with sequence similarity to ribosome binding GTPases including elongation factor G (EF-G) and Tet(O)/Tet(M) tetracycline resistance proteins. Homology with EF-G is particularly conserved in the amino terminal end of the protein where GTP binding motifs have also been located (Plunkett *et al.*, 1993). Further sequence analysis has shown that the *bipA* sequence obtained from pathogenic isolates differs in 18 base pairs from the sequence of *bipA* from K-12 strains (Plunkett *et al.*, 1993). These differences result in six different amino acid residues, five of which are located in the last 25 residues of the BipA protein (Farris *et al.*, 1998). As such differences only arise in pathogenic strains it is possible they are related to a potential role for TypA in virulence. Recent work has suggested that BipA (also known as TypA) is a new class of virulence regulatory molecule (Qi *et al.*, 1995; Farris *et al.*, 1998). In enteropathogenic *E. coli* BipA has been implicated in virulence regulation where it co-ordinately regulates several processes related to EPEC infection, for example the formation of actin rich pedestals (pseudopods) in host epithelial cells during infection. Formation of these cytoskeletal structures was not activated in a *bipA* mutant suggesting BipA is required for EPEC directed organisation of the cytoskeleton in host cells (Farris *et al.*, 1998).

BipA is also known to confer resistance to host defence proteins and peptides. In *S. typhimurium* a BipA homologue was found to be involved in resistance to the bactericidal/permeability-inducing protein (BPI) a cationic host defence component produced by human granulocytes (Qi *et al.*, 1995). Upon exposure to BPI *bipA* was induced more than seven fold. Resistance to BPI was also demonstrated in EPEC strains (Farris *et al.*, 1998). The mechanism by which BipA confers bacterial resistance to host defence proteins and peptides is not yet known.

Flagella mediated cell motility in EPEC strains is also controlled by BipA. Export of the major structural subunit of the bacterial flagellum is negatively regulated by BipA and motility in cells lacking BipA was markedly upregulated. This suggests BipA positively and negatively influences cell motility.

In addition to the regulatory targets of BipA identified in EPEC other authors have demonstrated pleiotropic effects of inactivation of *bipA*. Through analysis of alterations in global protein production it was revealed that a number of proteins were affected by the inactivation of *bipA*. Production of the carbon

starvation-inducible protein Csp15, universal stress protein A (UspA) and DNA binding gene regulator H-NS were all altered in a *bipA* mutant (Freestone *et al.*, 1998). The role of BipA in controlling the production and modification of such proteins is yet to be determined.

A novel characteristic of the BipA protein is that it is phosphorylated on tyrosine residues, till recently tyrosine phosphorylation was thought to be restricted to eukaryotes. However, it has since become apparent that tyrosine phosphorylation is present in a number of bacterial species (Kennelly & Potts, 1996). In eukaryotes tyrosine phosphorylation is an important component of host cell signalling mechanisms (Hamawy *et al.*, 1995), whereas its role in prokaryotes is not as well understood. To date tyrosine phosphorylation of BipA has only been demonstrated in pathogenic isolates of *E. coli* and not K-12 strains, however the BipA protein is still produced in these strains. It has been suggested that the phosphorylation capabilities of BipA may be associated with its role in pathogenesis. Analysis of EPEC BipA revealed a protein factor associated with the particulate fraction of the EPEC strain which markedly stimulated phosphorylation. The identity of the factor remains to be determined however it may represent a potential regulatory molecule acting in concert with BipA. Many bacterial GTPases are known to require additional factors to catalyse the uptake and release of guanine nucleotide (March, 1992). Further observations revealed that EPEC BipA phosphorylation can lead to an increased rate of nucleotide hydrolysis, suggesting phosphorylation of BipA may cooperate with the GTPase function of the protein.

From the observations made so far in this study it would seem that BipA potentially represents an important component of the K5 capsule gene regulatory cascade. Details of this regulatory circuit are far from complete and it is likely that other regulatory proteins contribute to transcriptional regulation of the region 1 and region 3 promoters. Analysis of other regulatory proteins involved in capsule expression will be the subject of the next two chapters.

Chapter 4

Results

Involvement of Integration Host Factor and promoter elements in expression of the region 1 *E. coli* K5 capsule genes

4.1 Introduction

The architecture of the promoter DNA is a very important feature of transcription initiation. The correct positioning of promoter elements is essential to allow regulatory proteins to interact with one another and the RNA polymerase enzyme. Transcriptional regulation in *E. coli* is diverse and many promoter elements have been identified in varying positions in the promoter region. Some promoters contain binding elements for a number of different proteins in addition to being targets for non-specific regulatory binding proteins.

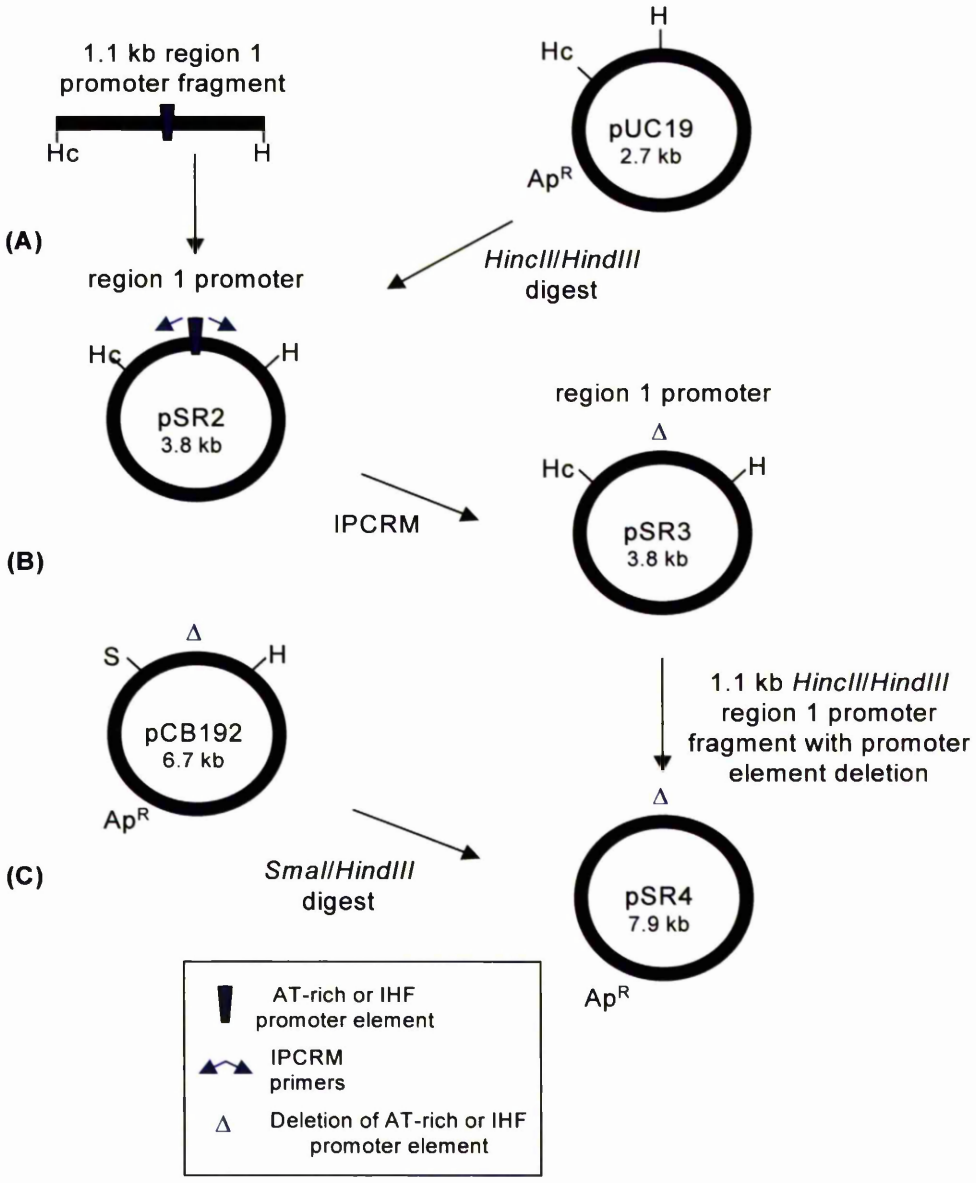
Analysis of the *E. coli* K5 region 1 promoter has revealed potential promoter elements involved in transcriptional regulation of this promoter (Figure 1.4). IHF binding sites have been identified at positions –103 to -115 bp (IHF-1) and +140 to +153 bp (IHF-2) relative to the transcription start site (Figure 1 4). Two AT-rich elements (conA & conB) have been found to be conserved in both the region 1 and region 3 promoters and are located at positions –80 to –90 bp (conA) and –51 to –63 bp (conB) upstream of the transcription start site. The roles of these sites in regulation of the region 1 promoter was analysed using Inverse PCR deletion mutagenesis.

4.2 Role of AT rich elements in region 1 promoter activity

4.2.1 Construction and analysis of AT-rich deletions

A template for IPCRM was made by ligating a 1.1kb *Hind*III/*Hin*cII fragment containing the region 1 promoter, from plasmid pDS200, into the *Hind*III and *Hin*dI sites of pUC19 to generate pSR2 (Figure 4.1.). Ligation products were

Figure 4.1. Construction of AT-rich element deletion plasmids. **(A)** A 1.1kb *Hinc*II/*Hind*III region 1 promoter fragment encompassing promoter elements conA, conB, IHF-1 and IHF-2 was ligated into *Hinc*II/*Hind*III digested high copy number vector pUC19 to generate pSR2. **(B)** Construct pSR2 was used as a template for IPCRM to yield PCR products with specific deletions dictated by the choice of IPCRM primer pair used in the amplification reaction. PCR products were self-ligated to generate deletion constructs pSR3A, pSR3B and pSR3AB. **(C)** The 1.1kb *Hinc*II/*Hind*III region 1 promoter fragment containing the specific deletions was excised from pSR3A, pSR3B and pSR3AB and ligated into *Sma*I/*Hind*III digested promoter probe vector pCB192 to generate constructs pSR4A, pSR4B and pSR4AB. Restriction endonuclease cleavage sites used to construct the plasmids are shown and abbreviated as follows S, *Sma*I; H, *Hind*III; Hc, *Hinc*II. Plasmids are not drawn to scale.



transformed into DH5α and successful ligations were selected by blue/white screening on L-agar supplemented with ampicillin and X-gal. Ligation of the promoter fragment into pUC19 was confirmed by digestion with *Hind*III and *Hinc*II to give a 1.1kb fragment representing the region 1 promoter and a 2.7kb fragment representing the pUC19 vector.

pSR2 was used as a template for IPCRM to make three separate deletions. IPCRM primers SR4 5'-CCAAAAACAATTTATCAATTGATTATTTTC-3' and SR3 5'-CAAGTAGGAAACATTTTAACAAATGATA-3' were used to delete conA, IPCRM primers SR6 5'-AAATGTTTCCTACTTGACTATTAATAC-3' and SR8 5'-CCTAAATTCCTTGTTCATAATGTAGGA-3' were used to delete conB and primers SR4 and SR8 were used to make a deletion encompassing both conA and conB (For primer binding sites see Figure 2.1.). PCR products of 3.8kb were amplified and digested with *Dpn*I to remove template DNA, then transformed into strain XLI-Blue and selected on L-agar supplemented with ampicillin and X-gal (Figure 4.2.). To confirm deletion of the AT-rich elements plasmid DNA was extracted from XLI-Blue and sequenced using primer SR7 5'-GTCAGTCCATGCTTATATGCAGG-3'. Plasmids harbouring the correct deletions were named pSR3A, pSR3B and pSR3AB corresponding to the deletion of conA, conB and both conA & conB respectively (Figure 4.3.). To analyse the effects of the deletions on promoter activity the 1.1kb *Hind*III/*Hinc*II fragment containing the region 1 promoter with specified deletions was ligated into the *Hind*III/*Sma*I sites of the promoter probe vector pCB192 (Schneider & Beck, 1986). pCB192 is the derivative promoter probe vector of plasmid pDSHcH which was used as a positive control (Simpson, 1996). Ligation products were transformed into strain MS150 and successful ligations selected for on L-agar supplemented with ampicillin and X-gal. Ligation of the promoter fragment was verified by digestion with *Eco*RV which digests the plasmid into two fragments of sizes 5.4 kb and 2.5 kb (Figure 4.4.). Plasmid derivatives were termed pSR4A, pSR4B and pSR4AB corresponding to the AT-rich element deletion they contained (Figure 4.3.).

Strains harbouring the deletion plasmids were grown at 37°C and 20°C and β-galactosidase assays performed (Figure 4.5.). At 37°C deletion of conA, conB and both conA and conB significantly reduced transcription of the region 1 promoter. The greatest reduction was seen with the deletion of conA which reduced promoter activity 4 fold,

Figure 4.2. Electrophoretic analysis of IPCRM amplification product. IPCRM amplification of plasmid pSR2 (3.8kb) was performed using primers SR3 and SR4 to generate a 3.8kb product with a conA deletion (Lane 2). Post amplification PCR product shown was self ligated to yield plasmid pSR3A. Lane 1 contains the 1kb DNA ladder, adjacent arrows indicate sizes of DNA ladder fragments in kilobases.

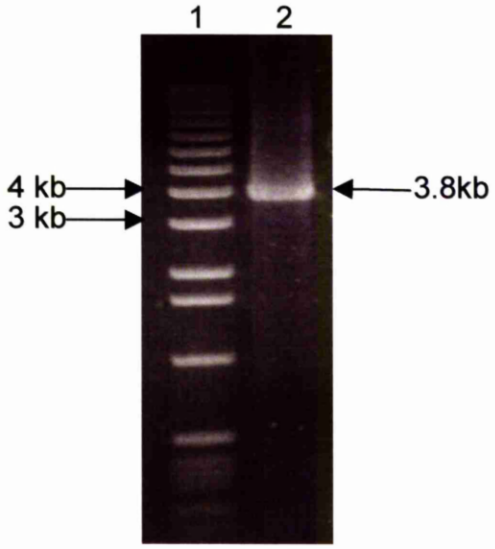


Figure 4.3. AT-rich promoter element deletion constructs. Sequence of region 1 promoter from – 95bp to + 7bp relative to the transcription start site of constructs pDSHcH, pSR4A, pSR4B and pSR4AB shown. AT-rich regions conA and conB are highlighted in red and deleted regions indicated by dotted lines. IPCRM primers used to construct deletions shown in blue. –35 and –10 hexamers underlined. Nucleotide sequences of the region 1 promoter were derived from double stranded plasmid templates (pSR3A, pSR3B & pSR3AB) using primer SR7 5'-GTCAGTCCATGCTTATATGCAGG 3' which anneals at position +108 to +86bp downstream of the transcription start site.

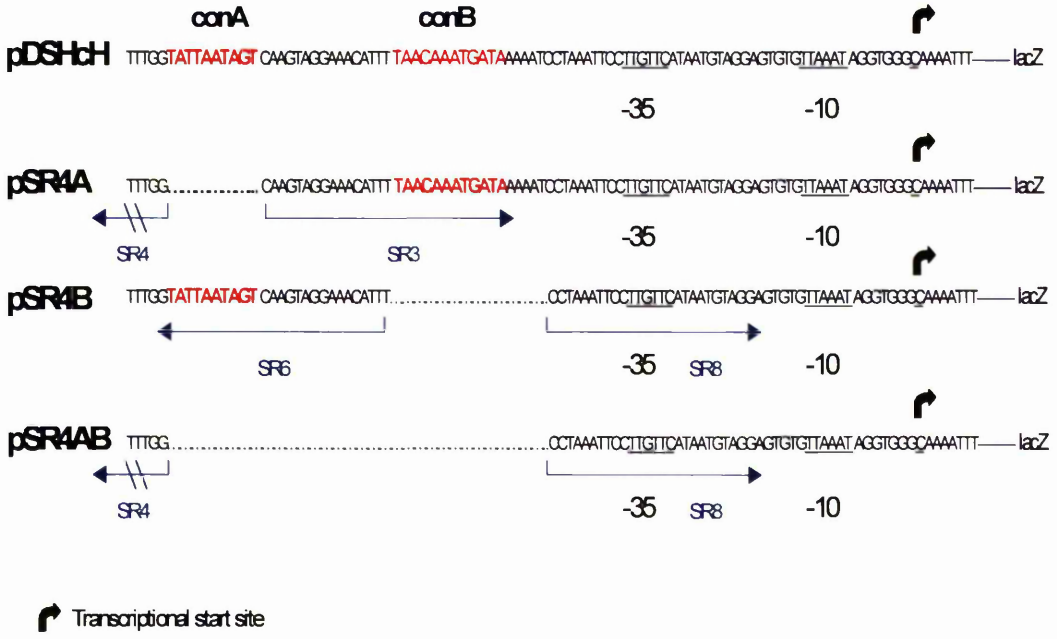


Figure 4.4 Electrophoretic analysis of restriction digest of construct pSR4A. A *Hinc*II/*Hind*III 1.1kb region 1 promoter fragment with conA deleted was ligated into promoter probe vector pCB192 to generate construct pSR4A. Digestion of pSR4A with endonuclease *Eco*RV was used to confirm size and correct ligation of the 1.1kb promoter fragment. *Eco*RV cleaves pSR4A twice to yield two fragments of sizes 5.4kb and 2.5kb (Lane 2) indicated by arrows on right hand side. Lane 1 contains the 1kb DNA ladder, adjacent arrows indicate sizes of DNA ladder fragments in kilobases.

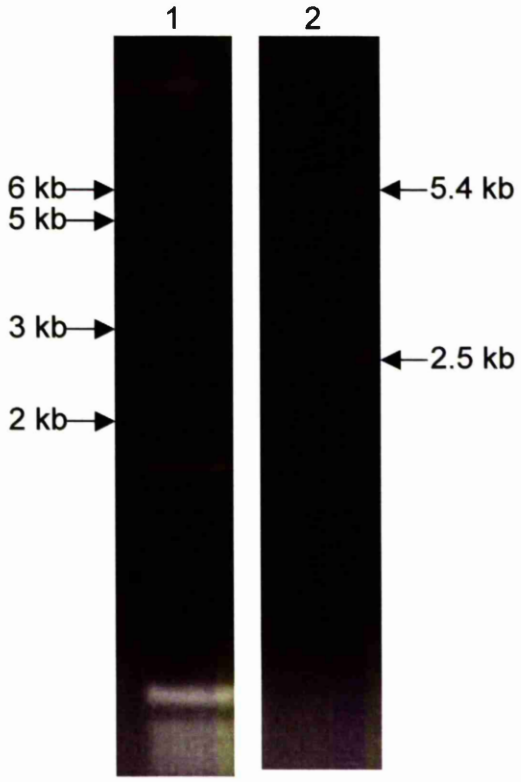
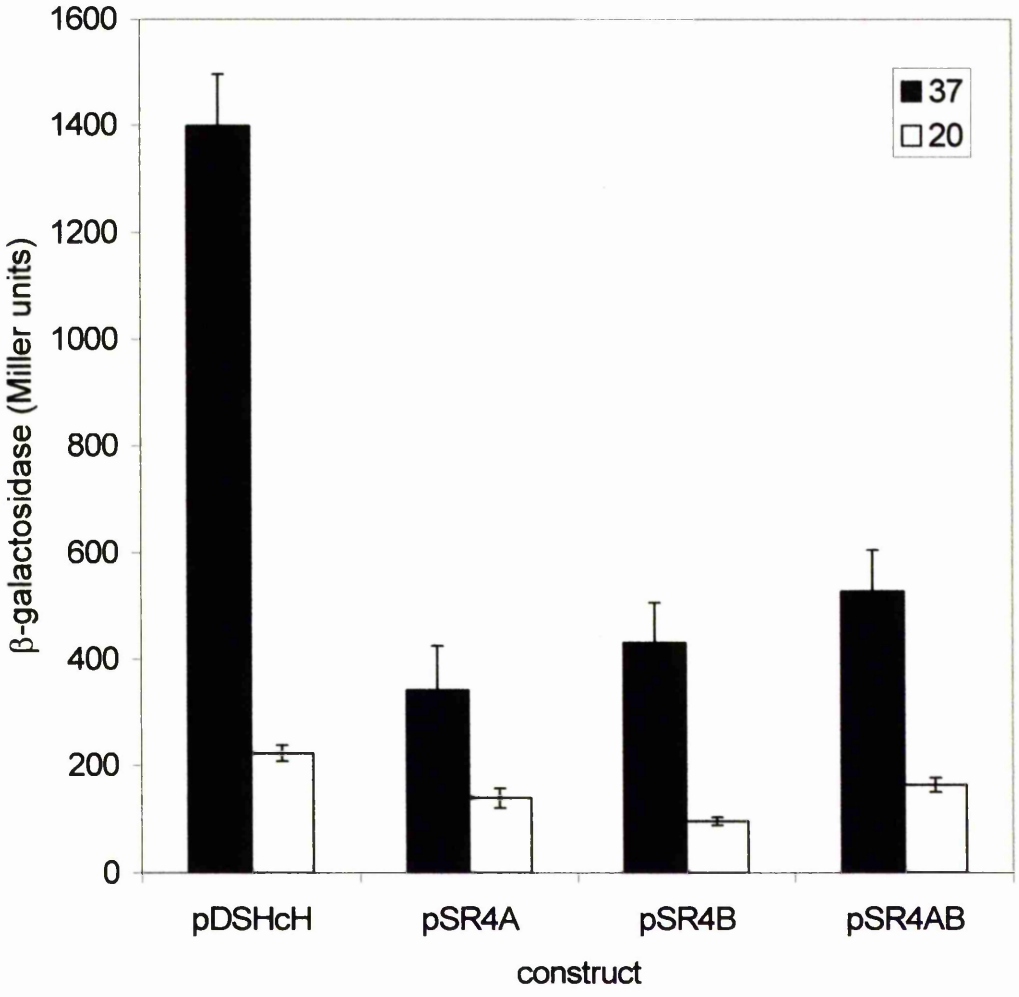


Figure 4.5. Region 1 promoter activity of conA and conB deletion constructs at 37°C and 20°C. Strain MS150 carrying plasmids pDSHcH (Region 1 promoter::*lacZ*), pSR4A (pDSHcH ΔconA), pSR4B (pDSHcH ΔconB) and pSR4AB (pDSHcH ΔconA;ΔconB) was grown to OD_{600} 0.4 in Luria broth at 37°C and 20°C. Promoter activity was measured as *lacZ* expression using a β-galactosidase actvity assay. Shaded bars represent activity at 37°C and plain bars represent activity at 20°C. β-galactosidase actvity assays were performed in triplicate and repeated at least 4 times. Error bars represent standard error of the mean.



deletion of conB reduced promoter activity about 3 fold and deletion of both conA and conB reduced promoter activity over 2 fold. At 20°C promoter activity in strains carrying the deletion constructs was also slightly reduced.

4.3 Role of IHF elements in region 1 promoter activity

4.3.1 Construction and analysis of IHF deletions

The IHF binding elements IHF-1 and IHF-2 were deleted in the same way as the AT rich element using the IPCRM and cloning protocol outlined in Figure 4.1. IPCRM primers IHF-IF 5'-AAATTGTTTTTGGTATTAATAGTCAAG-3' and IHF-1R 5'-TTTTCTTGTAAAAAAAGAACGTATGA-3' were used to delete IHF-1 and IPCRM primers IHF-2F 5'-TAGCATAAATAAATTATAGTGGGTT-3' and IHF-2R 5'-TAACAAAATTTTTAATGAATATAAAACCAT-3' were used to delete IHF-2. pSR2 (section 4.2.1.) was used as a template for IPCRM. IPCRM 3.8kb deletion products were digested with *Dpn*I then transformed into strain XLI-blue and selected on L-agar supplemented with ampicillin and X-gal. Deletion of IHF-1 and IHF-2 was confirmed by sequencing using primer SR7 and primer SR9, respectively (data not shown).

IPCRM deletion products were named pIHF-1 and pIHF-2 corresponding to the deletion of sites IHF-1 and IHF-2 respectively. Promoter fragments containing the specified deletions were ligated into the *Sma*I/*Hind*III sites of pCB192 to generate constructs pCBIHF-1 and pCBIHF-2 (Figure 4.6.). Strains harbouring the IHF deletion plasmids pCBIHF-1 and pCBIHF-2 were subjected to β-galactosidase assays when grown at 37°C and 20°C to analyse the effects on region 1 promoter activity (Figure 4.7.). Deletion of the IHF binding sites significantly reduced transcription of the region 1 promoter by about 2 fold at 37°C.

4.4 Role of IHF in production of extracellular K5 polysaccharide

To determine the effects of IHF on the production of extracellular polysaccharide an IHF mutant strain deficient in production of one of the IHF subunits (MS150*himA*::cat) was grown at 37°C and 20°C and carbazole

Figure 4.6. IHF consensus element deletion constructs. Sequence of region 1 promoter from -170 to +165 relative to the transcription start site of constructs pDSHcH, pCBIHF-1 and pCBIHF-2 shown. IHF consensus binding sequences are highlighted in red and deleted regions denoted by dotted lines. IPCRM primers used to construct deletions shown in blue. –35 and –10 hexamers are underlined. Nucleotide sequences of the region 1 promoter were derived from double stranded plasmid templates (pIHF-1 & pIHF-2) using primer SR7 5'-GTCAGTCCATGCTTATATGCAGG-3' for IHF-1 and primer SR9 5'-GCGAACAGAGGTAATTAGATATGG 3' for IHF-2. SR7 anneals at position +108 to +86 bp downstream of the transcription start site and SR9 anneals at position +1193 to +1170. Arrow indicates transcription start site.

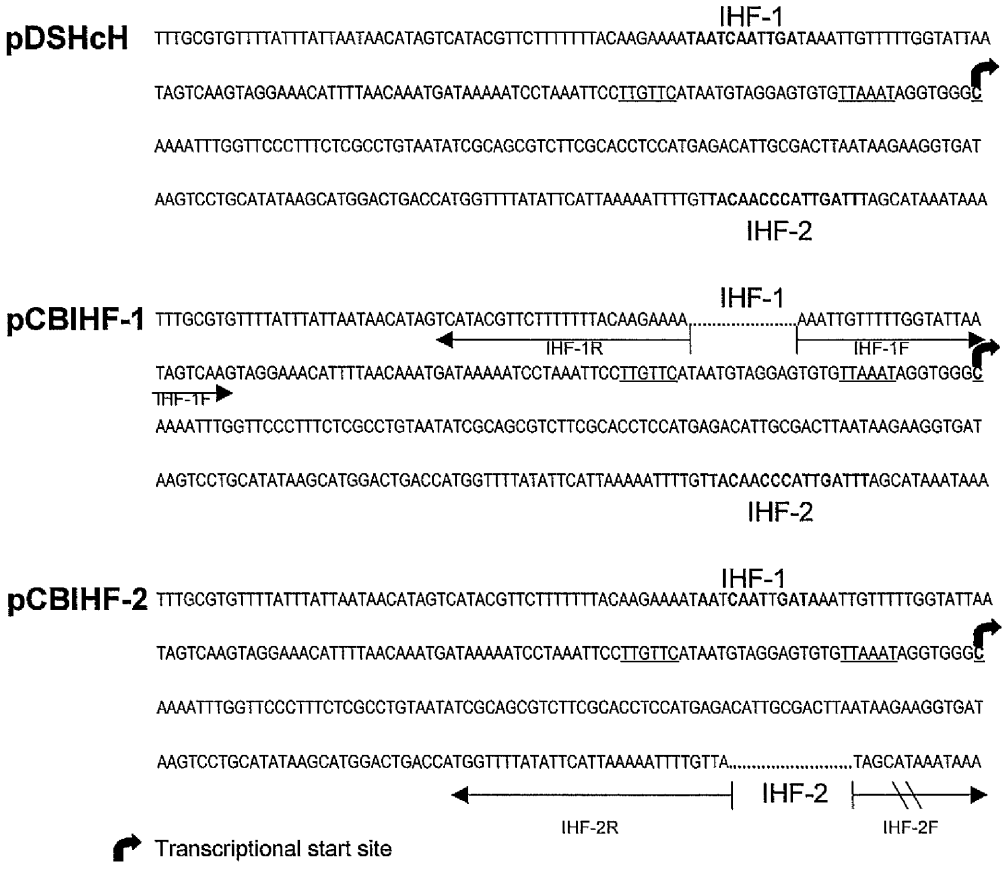
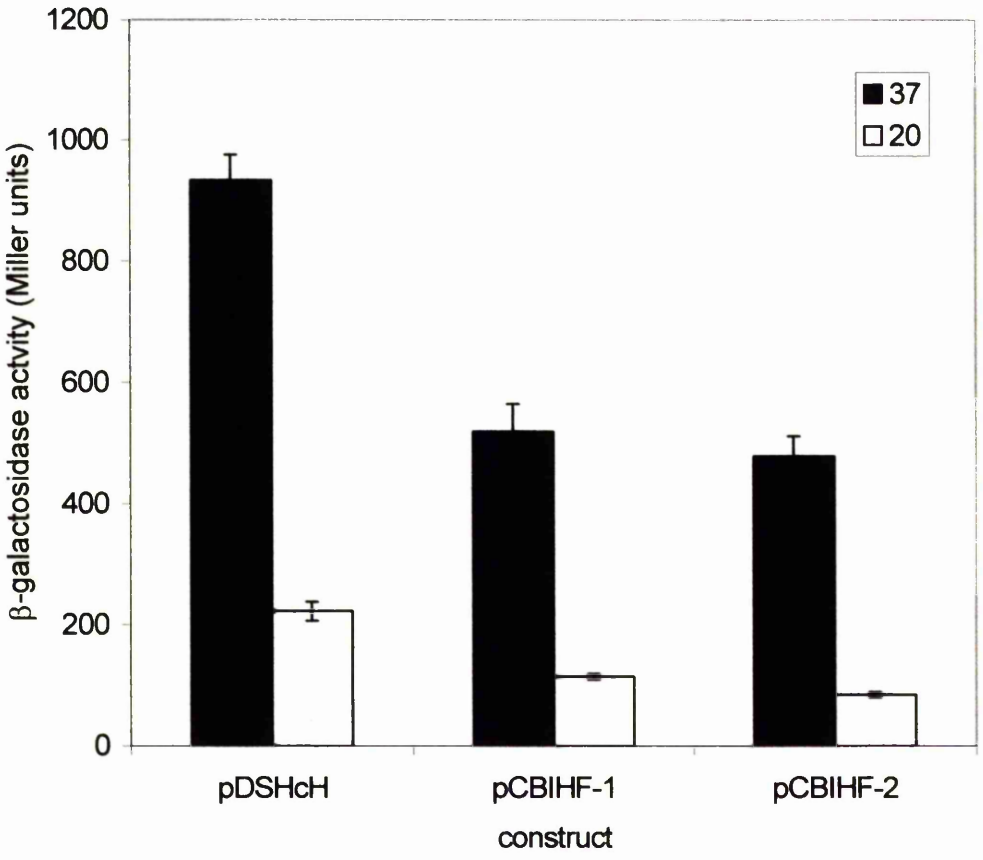


Figure 4.7. Region 1 promoter activity of IHF-1 and IHF-2 deletion constructs at 37°C and 20°C. Strain MS150 carrying plasmids pDSHcH (Region 1 promoter::*lacZ*), pCBIHF-1 (pDSHcH ΔIHF-1) and pCBIHF-2 (pDSHcH ΔIHF-2) was grown to OD_{600nm} 0.4 in Luria broth at 37°C and 20°C. Promoter activity was measured as *lacZ* expression using a β-galactosidase actvity assay. Shaded bars represent activity at 37°C and plain bars represent activity at 20°C. β-galactosidase actvity assays were performed in triplicate and repeated at least 4 times. Error bars represent standard error of the mean.



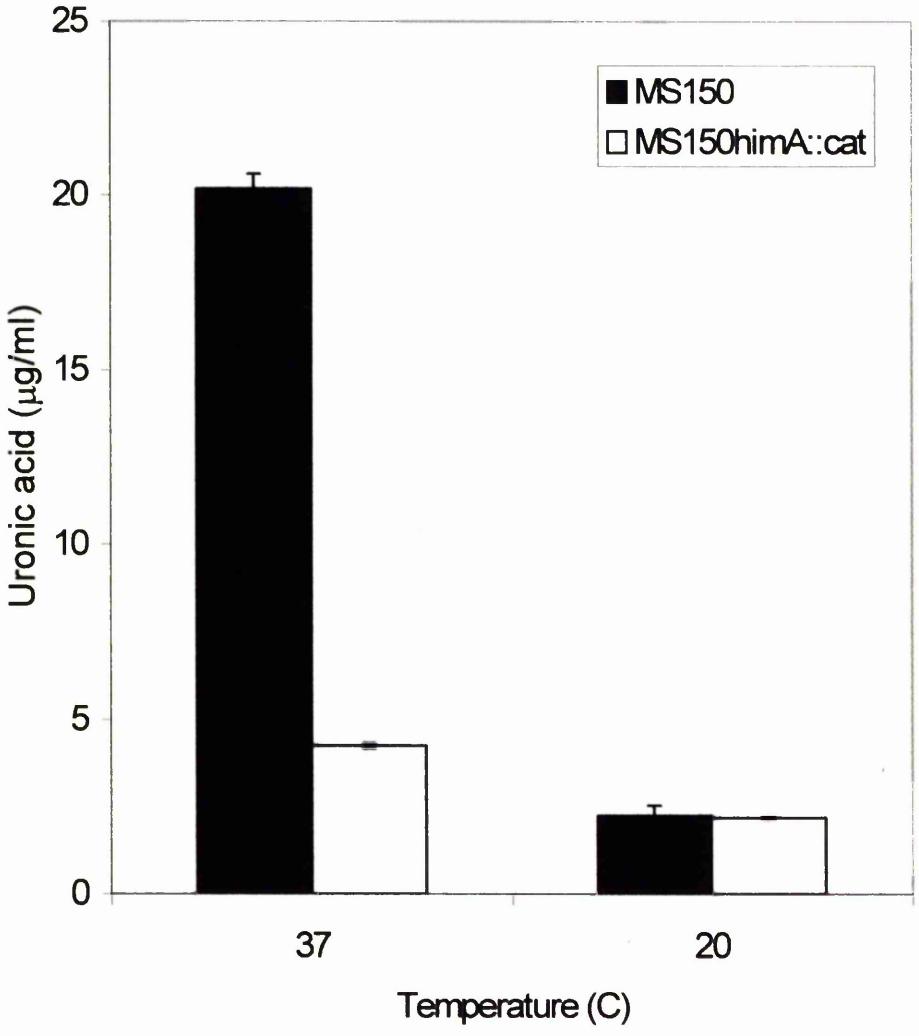
assays performed (Figure 4.8.). Strain MS150 was used as a control strain and purified K5 polysaccharide used as a standard. At 37°C the IHF mutant strain MS150*himA*::cat reduced production of extracellular polysaccharide containing uronic acid residues by over 4 fold. At 20°C there was no significant difference in production between strains MS150 and MS150*himA*::cat.

4.5 Discussion

4.5.1 Role of AT-rich elements conA and conB on transcription of the region 1 promoter

Previously two AT-rich elements (conA & conB) were identified in both the region 1 and 3 promoters. To analyse the role of conA and conB in the region 1 promoter PCR mutagenesis was used to delete these regions and effects on promoter activity were assayed at 37°C and 20°C. Deletion of conA, conB and both conA and conB had significant effects on promoter activity at 37°C suggesting they play an important role in regulation of transcription from the region 1 promoter. AT-rich UP elements that bind RNA polymerase to activate transcription have been located upstream of the transcriptional start sites in a number of *E. coli* operons (Ross *et al.*, 1993; van Ulsen *et al.*, 1997a). It is unlikely however that conA and conB represent UP elements, firstly these sites do not show any homology to the UP element consensus sequence and secondly the location of conA and conB is 5' to the expected location of UP elements (Ross *et al.*, 1993; Estrem *et al.*, 1998). It has been suggested that conA and conB could represent *cis*-acting regulatory sites that may facilitate the binding of regulatory factors acting on the region 1 promoter, this would be consistent with the findings of this study. It is most likely that conA and conB represent such *cis*-acting regions and are required for maximal activity of the region 1 promoter

Figure 4.8. The effect of the *himA* mutation on extracellular K5 polysaccharide production at 37°C and 20°C. Strains MS150 (K5+) and MS150*himA*::*cat* (K5+) were grown to OD_{600} 0.8 in Luria broth at 37°C and 20°C. Extracellular K5 polysaccharide production was measured as uronic acid production using the carbazole assay (Bitter & Muir, 1962). Shaded bars represent polysaccharide production by strain MS150 and plain bars represent polysaccharide production by strain MS150*himA*::*cat*. Measurements were performed in triplicate and repeated at least four times. Error bars represent standard error of the mean.



4.5.2 Role of IHF on transcription of the region 1 promoter

To investigate the role of IHF on transcription of the region 1 promoter capsule production was analysed in an IHF mutant at 37°C and 20°C. The data obtained at 37°C suggests that IHF plays a role in maximising expression of the region 1 promoter at this temperature. This supports previous observations whereby expression of the KpsE protein was reduced in a *himA* mutant. (Simpson *et al.*, 1996)

IHF is a DNA bending protein involved in transcriptional regulation (Friedman, 1988). It is believed that IHF modulates promoter DNA architecture to facilitate or inhibit promoter activity. It has been shown that IHF can bend DNA up to 180° in a virtual U-turn. With respect to the region 1 promoter it is likely that IHF bends the promoter DNA to facilitate maximal transcription at this promoter. IHF is known to regulate a number of virulence genes both positively and negatively often acting in concert with other transcription factors. For example, IHF positively regulates the expression of a number of virulence genes in *Shigella flexneri.* It is required for maximal expression of the AraC-like regulator VirF and virulence gene regulator VirB (Dorman & Porter, 1998). In the case of VirB IHF acts antagonistically with H-NS to promote gene expression. (Dorman & Porter, 1997). Both VirF and VirB are required for expression of *Shigella* invasion genes located on the *Shigella* virulence plasmid (Dorman & Porter, 1998).

IHF is also involved in the regulatory cascade of the *S. typhimurium* virulence plasmid genes (Marshall *et al.*, 1999). IHF is believed to play a role in organising the DNA structure in the vicinity of the *spv*R gene promoter. The SpvR protein, a LysR-like transcription factor, then subsequently controls the transcription of the *Salmonella* plasmid virulence genes. (Marshall *et al.*, 1999).

Transcriptional regulation by IHF generally involves binding of IHF to a specific asymmetric site on promoter DNA. Promoters controlled by IHF normally contain one or more of these target sequences. To investigate the functional importance of these sites located in the region 1 promoter deletions of IHF-1 and IHF-2 were made and promoter activity analysed at 37°C and 20°C. The data obtained at 37°C suggests that IHF binding sites IHF-1 and IHF-2 are required for maximal expression of the region 1 promoter at this temperature. This was

supported by further observations from our laboratory using gel-retardation assays that have demonstrated binding of IHF protein to the region 1 promoter. (Rowe *et al.*, 2000).

The organisation of the IHF-1 and IHF-2 binding sites in the region 1 promoter is unusual but not unknown for bacterial promoters. IHF consensus sequences are generally located upstream of the transcription start site however the IHF-2 site is located 140bp downstream of the transcription start site. The role of IHF at this site is not clear. In the case of the *Pseudomonas aeruginosa algD* promoter expressing alginate biosynthesis proteins, two IHF binding sites have also been identified. Site 1 upstream of the transcription start site and site 2 downstream of the transcription start site. Mutational analysis has shown that both sites are required for full activation of the *algD* promoter (Wozniak, 1994). It was suggested that IHF binding at site 2 may facilitate interactions between a factor bound to a 3' enhancer and RNA polymerase. Further analysis of the *algD* promoter revealed that IHF binding to site 2 induces a strong looping–out of DNA. In addition, a DNA binding protein was found specifically bound to the 3' region downstream of site 2. This was later identified as CysB, a LysR-like transcription factor (Delic-Attree *et al.*, 1997). It has been suggested that IHF and CysB may interact with the same region of DNA in the *algD* promoter to promote activation of transcription (Delic-Attree *et al.*, 1997).

In the *E. coli pst* promoter which controls expression of components of the inorganic phosphate transport system, organisation of the IHF regulatory elements is similar to that of the *algD* promoter with one IHF binding site upstream of the transcriptional start site and the other downstream. (Spira & Yagil, 1999) The IHF binding site located downstream from the transcription start has been shown to bind IHF in gel-mobility assays and IHF is known to activate transcription of this promoter. (Spira & Yagil, 1999) In this case however it was suggested that IHF transiently binds to the downstream IHF consensus site, dissociating shortly after the initiation of transcription to prevent blocking the RNAP during the beginning of transcription (Spira & Yagil, 1999).

The precise dynamics of DNA bending enforced by IHF binding downstream of the transcription start site is still unclear. In *Caulobacter* species activation of the flagellar gene *flaN* requires an enhancer element located 3' to the transcription start site and IHF which binds upstream of the enhancer element

(Gober & Shapiro, 1992). Location of these sites suggests IHF induced bending of promoter DNA facilitates interaction of the enhancer with other promoter factors. In the case of the region 1 promoter this may suggest that sequences 3' to IHF-2 and potentially in the 5' end of the *kpsF* gene are important for the maximal expression of the region 1 promoter at 37°C.

It is also important to note that the reduction of capsule expression in a *himA* mutant at 37°C was greater than the reduction in activity of promoters with IHF deletions at 37°C. This suggests that IHF may also regulate other proteins involved in the regulation of capsule expression. Further experiments will be necessary to assess the potential regulatory targets of IHF.

In addition to BipA, IHF appears to represent another component of the K5 capsule gene regulatory cascade, that plays an important role in maximising transcription from the region 1 promoter at 37°C. It is likely that IHF through modulation of promoter geometry facilitates the binding of other regulatory factors to the promoter region leading to transcriptional activation at 37°C. This process may involve the interaction of IHF with other regulatory proteins.

Chapter 5

Results

Involvement of H-NS in the temperature dependent expression of the *E. coli* K5 capsular polysaccharide

5.1 Role of H-NS in transcription of the region 1 K5 capsule genes

To investigate the role of H-NS in expression of the region 1 genes the *hns*::*kan* mutation from strain MC4100*hns*::*kan* (J.Hinton) was introduced into strain MS150 (MS101Δ*argF-lacZ*, $K5^+$) by transduction using bacteriophage P1*vir* to generate strain MS152 (MS150*hns*::*kan*). To confirm transfer of the *hns*::*kan* mutation PCR was performed using primers hns-1 5'-ACTACAATGAGCGAAGCAC-3' and hns-2 5'-GATTATTGCTTGATCAGGAAATCG-3' which amplify the *hns* gene. A PCR product of 0.4kb was amplified using chromosomal DNA from MS150 whereas a product of 1.7kb was amplified using chromosomal DNA from strain MS152, indicating that the native copy of *hns* had been replaced by the *hns*::*kan* mutation (Figure 5.1.). This was also confirmed by Southern blot analysis using a 0.4 kb *hns* probe (data not shown).

Plasmid pDSHcH was introduced into strains MS150 and MS152 and β-galactosidase assays were performed at 37°C and 20°C. Transcription of the region 1 promoter was reduced greater than 2 fold when cells were grown at 37°C, suggesting H-NS may play a role in maximising region 1 transcription at this temperature (Figure 5.2.). At 20°C, however a small but significant increase ($p=0.05$) in promoter activity was observed. This data suggests that H-NS may be additionally involved in the thermoregulation of the region 1 promoter at 20°C (Figure 5.2.).

Figure 5.1. Amplification of the *hns* mutation from strain MS152. The *hns* gene (0.4kb) was amplified by PCR from chromosomal DNA extracted from strains MS150 (K5$^+$ *hns*$^+$)(Lane 2) and MS152 (MS150 K5$^+$ *hns*::kan)(Lane 3) to confirm transfer of the *hns*::kan mutation (1.7kb). *hns* specific forward and reverse primers hns-1 and hns-2 respectively were used in the amplification reaction. Right hand side arrows indicate size of amplification products in kilobases. Lane 1 contains the 1kb DNA ladder, adjacent arrows indicate sizes of DNA ladder fragments in kilobases.

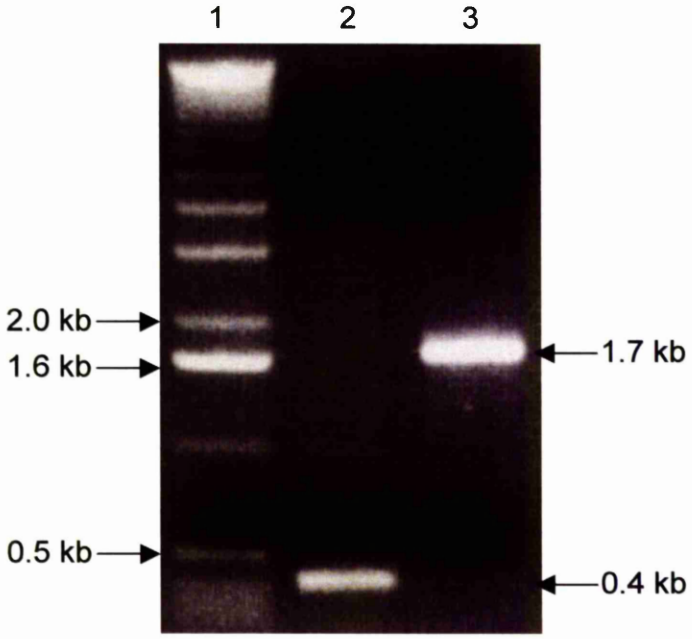
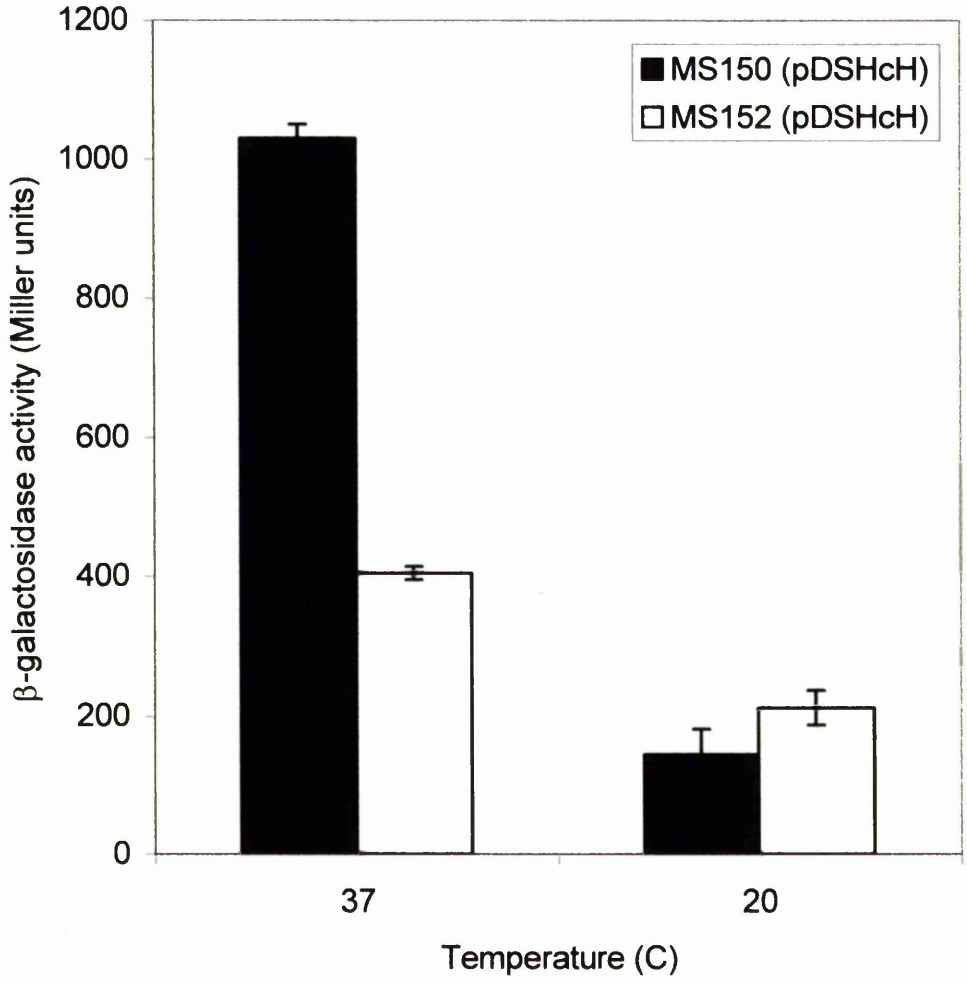


Figure 5.2. The effect of the *hns* mutation on region 1 promoter activity at 37°C and 20°C. Strains MS150 and MS152 (*hns*::kan) carrying plasmid pDSHcH (Region 1 promoter::*lacZ*) were grown to OD_{600} 0.4 in Luria broth at 37°C and 20°C. Promoter activity was measured as *lacZ* expression using a β-galactosidase actvity assay. Shaded bars represent activity of MS150 pDSHcH and plain bars represent activity of MS152 pDSHcH. β-galactosidase actvity assays were performed in triplicate and repeated at least 4 times. Error bars represent standard error of the mean



5.2 Role of H-NS in transcription of the region 2 and 3 K5 capsule genes

To determine the role of H-NS in expression of the region 2 and 3 genes the *hns*::*kan* mutation from MC4100*hns*::*kan* was introduced into strains MS108 (MS101*kfiA*::*luxAB*) and MS120 (MS101*kpsT*::*luxAB*) by transduction using bacteriophage P1*vir* to generate strains MS108*hns*::*kan* and MS120*hns*::*kan*. To verify replacement of *hns* with the *hns*::*kan* mutation PCR was performed using primers hns-1 and hns-2. A PCR product of 0.4kb representing the hns gene was amplified from MS150 chromosomal DNA whereas a 1.7kb product was amplified from MS152 chromosomal DNA. Strains MS108*hns*::*kan* and MS120*hns*::*kan* were grown at 37°C and 20°C and luciferase assays performed (as described in section 2.12) (Figure 5.3.; Figure 5.4.). Transcription of *kpsT*::*luxAB* was found to be reduced almost 2 fold at 37°C. However transcription of the *kfiA* gene was not significantly reduced at 37°C, suggesting H-NS plays a role in maximising transcription of the region 3 genes at 37°C (Figure 5.3.;Figure 5.4.). At 20°C transcription of both *kpsE* and *kfiA* was significantly increased compared with the control strain. The greatest increase was seen in the expression of the *kfiA* gene which was elevated 7 fold, whereas *kpsT* transcription was only increased 3 fold.

To further analyse the role of H-NS on expression of the K5 capsule gene cluster uronic acid assays were performed using strains MS150 and MS152 grown at 37°C and 20°C (Figure 5.5.). At 37°C the *hns* mutation reduced uronic acid production by 50%. This data suggests H-NS plays a role in maximising transcription at 37°C. At 20°C however, uronic acid production was increased almost six fold suggesting like BipA, H-NS may be required in the thermoregulation of the capsule genes.

As data generated from *lux* gene fusions and multicopy promoter probe vectors can sometimes be unreliable quantitative RNA slot blots were performed to support these experiments. Strains MS150 and MS153 were grown at 37°C and 20°C and total RNA extracted prepared RNA samples were applied to a nylon

Figure 5.3. The effect of the *hns* mutation on the temperature dependent expression of the region 2 *kfiA::luxAB* gene fusion. Strains MS108 (*kfiA::luxAB*) and MS108 (*kfiA::luxAB*; *hns*::kan) were grown to OD_{600} 0.4 in Luria broth at 37°C and 20°C. *kfiA::luxAB* expression was measured by luciferase assay. Shaded bars represent activity of MS108 and plain bars represent activity of MS108 *hns*::kan. Luciferase assays were performed in triplicate and repeated at least four times. Error bars represent the standard error of the mean.

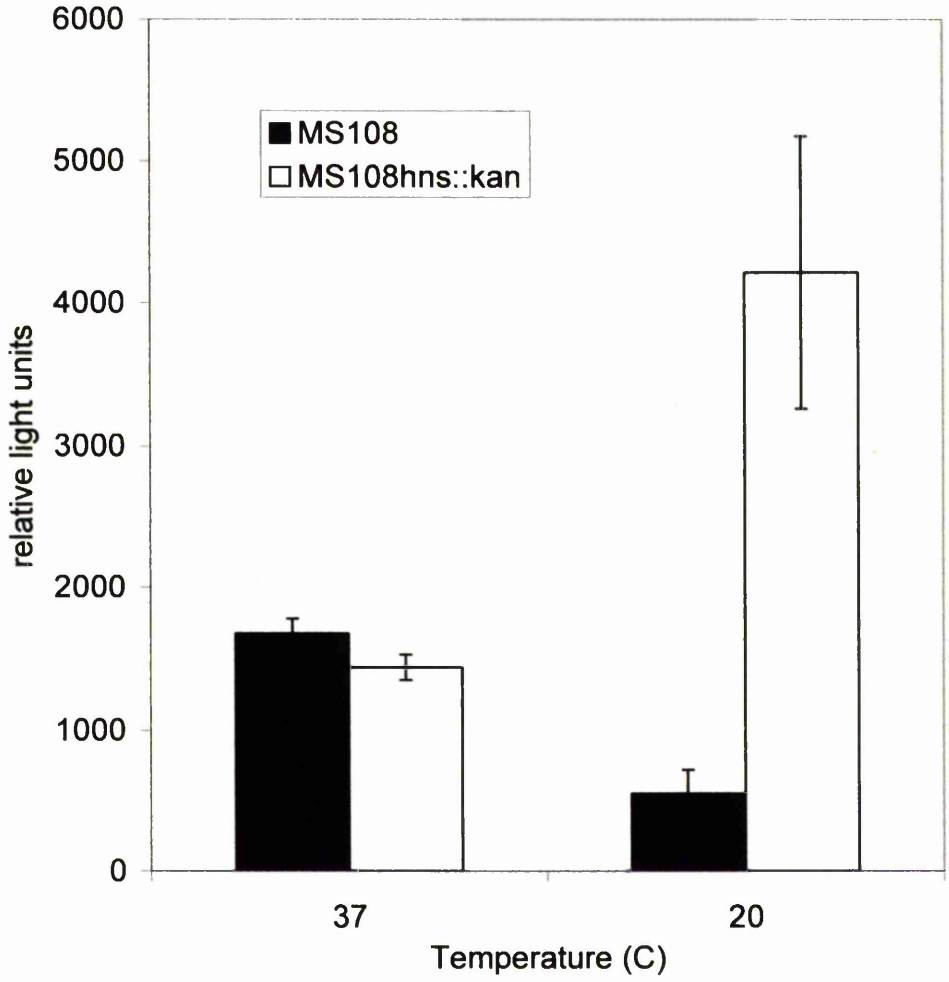


Figure 5.4. The effect of the *hns* mutation on the temperature dependent expression of the region 3 *kpsT*::*luxAB* gene fusion. Strains MS120 (*kpsT*::*luxAB*) and MS120 (*kpsT*::*luxAB*; *hns*::kan) were grown to OD_{600} 0.4 in Luria broth at 37°C and 20°C. *kpsT*::*luxAB* expression was measured by luciferase assay. Shaded bars represent activity of strain MS120 and plain bars represent activity of MS120 *hns*::kan. Luciferase assays were performed in triplicate and repeated at least four times. Error bars represent standard error of the mean

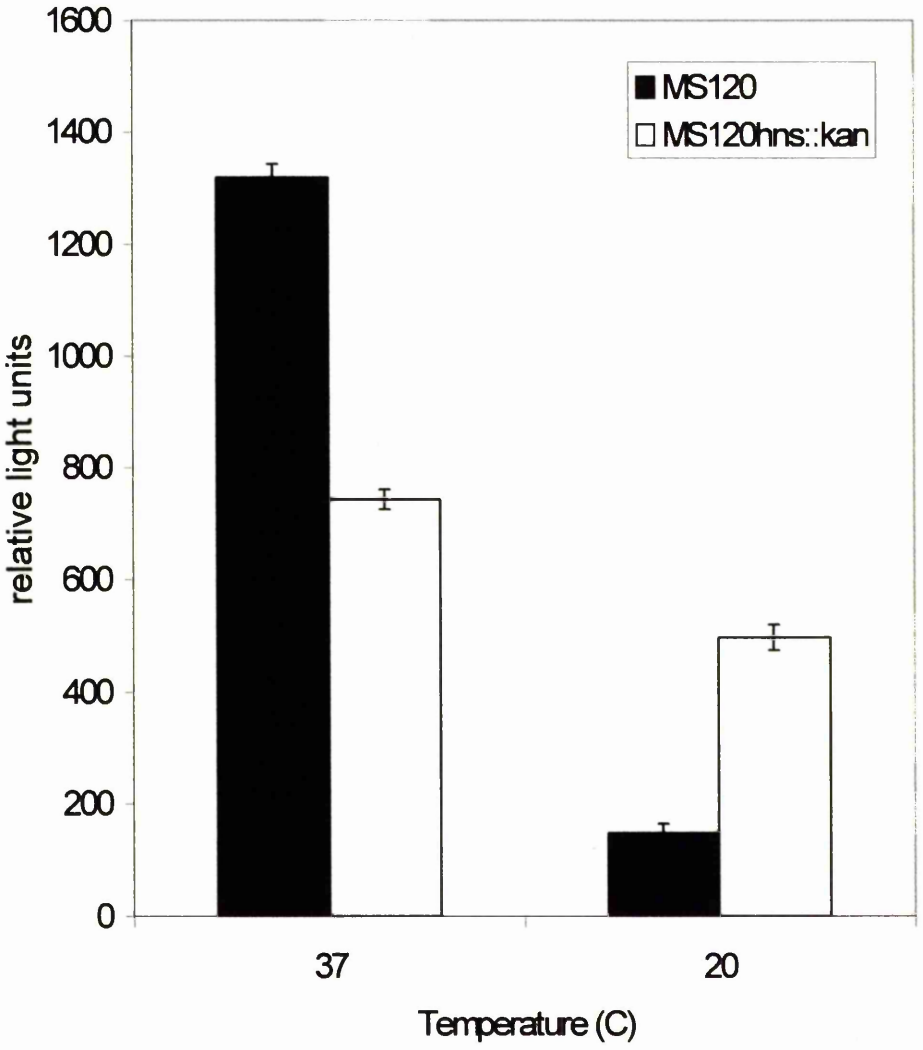
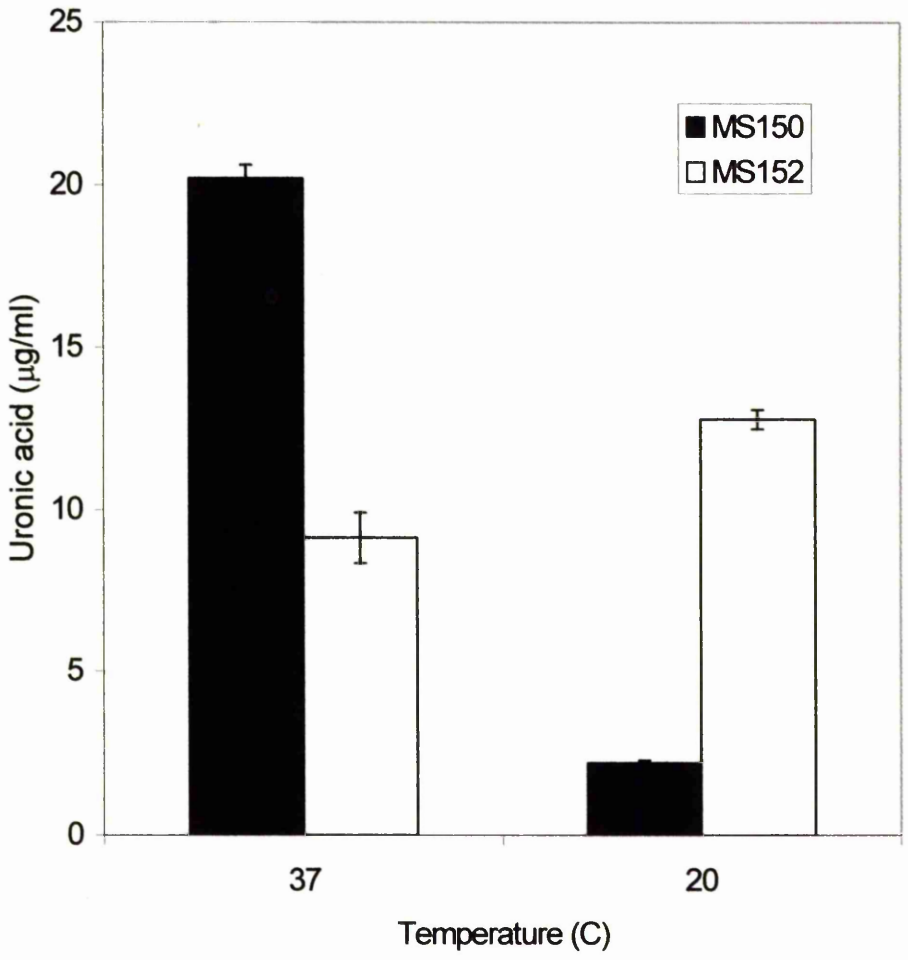


Figure 5.5. The effect of the *hns* mutation on extracellular K5 polysaccharide production at 37°C and 20°C. Strains MS150 (K5$^+$) and MS152 (K5$^+$, *hns*::kan) were grown to OD_{600} 0.8 in Luria broth at 37°C and 20°C. Extracellular K5 polysaccharide production was measured as uronic acid production using the carbazole assay (Bitter & Muir, 1962). Shaded bars represent polysaccharide production of strain MS150 and plain bars represent polysaccharide production of strain MS152. Measurements were performed in triplicate and repeated at least four times. Error bars represent standard error of the mean.



membrane and hybridised with radiolabelled probes from region 1 (*kpsE*), region 2 (*kfiA*) and region 3 (*kpsM*) (as described in section 2.8). A 16S RNA probe was used as a control to confirm equal loading. RNA signals were detected and quantified using a Phosphoimager (Figure 5.6.). Analysis of data from the slot blot hybridisation showed that the *hns* mutation reduced transcription of *kpsE*, *kfiA* and *kpsM* two fold at 37°C. At 20°C capsule expression was increased in the *hns* mutant. The greatest increase was seen in the expression of the *kfiA* gene which was increased over three fold, with expression of *kpsE* and *kpsM* increased two fold. This data confirms the β-galactosidase and *lux* experiments and firmly suggests that H-NS is required for maximal expression of the capsule genes at 37°C and thermoregulation of the capsule genes at 20°C

5.3 Discussion

5.3.1 Role of H-NS in the expression of the K5 capsule gene cluster

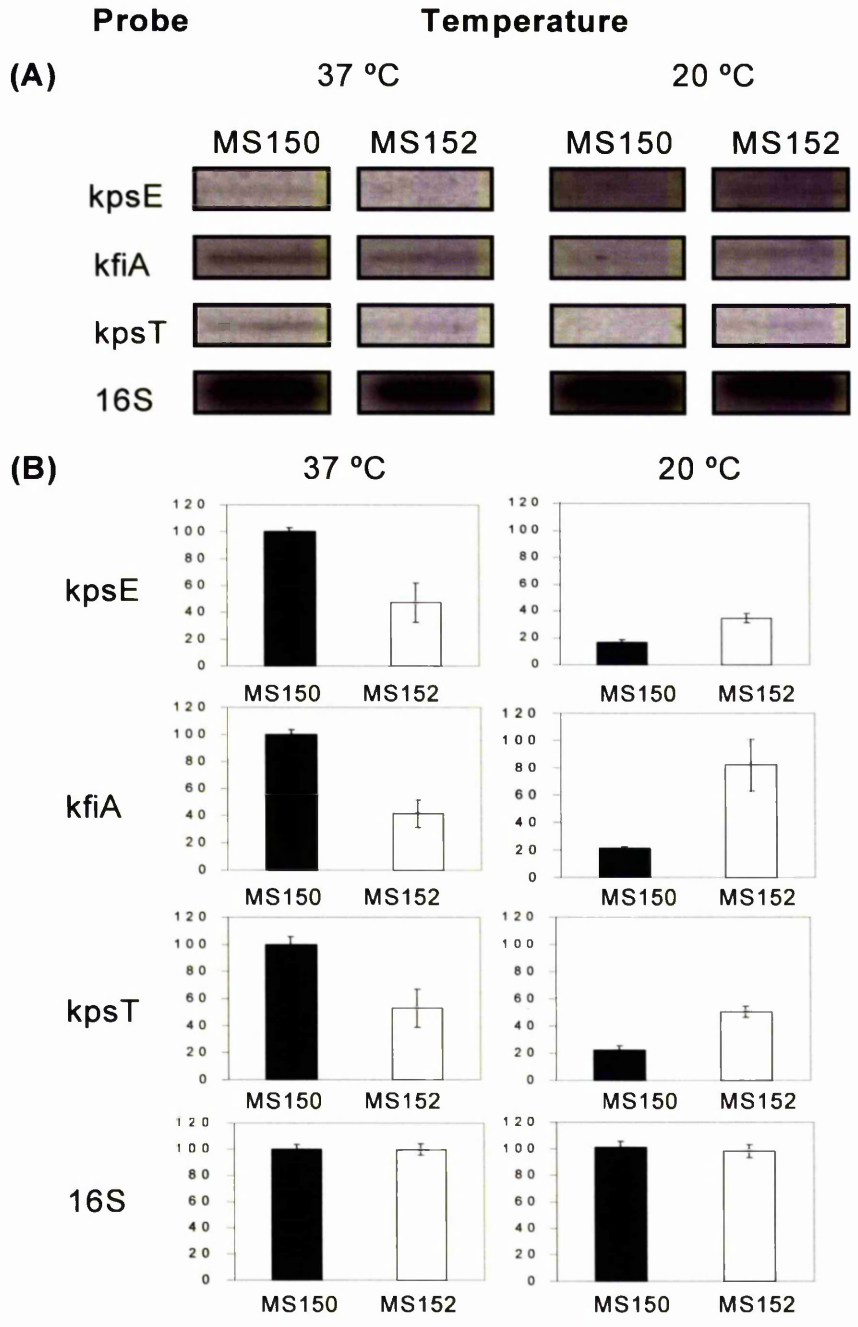
To investigate the role of H-NS in regulation of the K5 capsule genes expression of capsule genes from regions 1, 2 and 3 was analysed at 37°C and 20°C. The data generated suggests that H-NS plays an important role in capsule regulation at both temperatures.

It was previously reported that the H-NS protein did not play a role in capsule gene expression, however by repeating these experiments using a null *hns* mutation the data in this study indicates that H-NS does indeed play a role in capsule expression (Stevens *et al.*, 1994). The original experiments were performed using the *hns205* mutation in strain GM230 (Hinton *et al.*, 1992) which expresses a truncated H-NS protein (Hinton, J. personal communication). It is thought that the truncated protein may retain some H-NS activity which may explain why the role of H-NS was previously overlooked.

The data obtained with the region 1 promoter and the genes it expresses suggests that H-NS is required to maintain thermoregulation of the region 1 genes at 20°C. H-NS is known to influence the regulation of many genes in *E. coli* (Atlung & Ingmer, 1997). Typically H-NS exerts a negative influence on gene expression repressing transcription from certain promoters in undesirable circumstances for

Figure 5.6. Slot blot hybridisation

(A) Slot blot hybridisation, total RNA extracted from strains MS150 and MS152 grown at 37°C and 20°C. 50µg total RNA samples were applied to the membrane and probed with ^{32}P labelled K5 specific probes *kpsE*; *kfiA*; *kpsT*; 16S. (B) Band intensity of RNA slot blot hybridisation. Band intensity analysed and quantified using Aida v2.0 software



example, low temperature. H-NS can act directly by binding at target promoters thereby modulating promoter dynamics, or indirectly by controlling genes encoding other regulatory factors. In *Shigella flexneri*, H-NS is a component of the virulence regulatory cascade controlling expression of plasmid encoded virulence genes (Dorman & Porter, 1998). At low-temperature and low osmolarity H-NS binds to the *virB* promoter repressing expression of the VirB transcriptional activator, potentially by blocking the action of RNA polymerase (Tobe *et al.*, 1993). Under more favourable environmental conditions H-NS dependent repression is relieved enabling VirB to activate the *Shigella* virulence genes. Additionally H-NS represses expression of the regulatory gene *virF* at low temperatures and it has been suggested that temperature-induced conformational changes control this process by mediating H-NS binding to the *virF* promoter (Falconi *et al.*, 1998).

Thermoregulation of the *pap* pili in *E. coli* also requires the H-NS protein, with H-NS binding to the promoter region of the *pap* genes at 25°C. The presence of H-NS prevents methylation of *pap* DNA which is essential to *pap* regulation. When the temperature is elevated to 37°C repression of gene expression is lifted (Goransson *et al.*, 1990).

More recently a H-NS homologue in *V. cholerae* has been found to negatively modulate expression of several genes within the ToxR virulence gene regulon (Nye *et al.*, 2000). Genes encoding cholera toxin, toxin coregulated pilus and the regulatory protein ToxT are all contained within this region and hence controlled by H-NS (Nye *et al.*, 2000).

The data generated in this study also suggests H-NS is additionally required for maximal transcription from the region 1 promoter at 37°C. This is an unusual result as H-NS generally acts as a repressor of virulence gene expression rather than a transcriptional activator. While H-NS activating gene expression is unusual it is not unknown in bacterial systems. For example, H-NS makes a positive contribution to the expression of the maltose operon in *E. coli* (Johansson *et al.*, 1998). H-NS is thought to influence gene expression of MalT a regulatory protein required for activation of the maltose regulon (Johansson *et al.*, 1998). It is still unclear whether or not H-NS activates gene expression of *malT* by direct interaction with promoter DNA or indirectly via other regulatory factors.

With respect to the region 1 promoter the mechanism by which H-NS exerts its regulatory control remains to be determined. Further studies are required

to establish whether H-NS binds directly to the region 1 promoter or perhaps to promoters of genes whose expression is required for capsule regulation. In other systems H-NS has been shown to act antagonistically with other regulatory proteins to influence transcription. For example, regulation of Cfa/I fimbriae of enterotoxigenic *E. coli* requires the AraC-like transcription activator CfaD. One of the main functions of CfaD at the *cfa* promoter is to overcome the negative influence of H-NS (Jordi *et al.*, 1992). At the haemolysin operon of *E. coli*, H-NS interacts in a nucleoprotein complex with another regulatory DNA-binding protein Hha (Nieto *et al.*, 2000). Both proteins are thought to participate in thermo-osmotic regulation of haemolysin genes by binding to a regulatory region upstream of the haemolysin operon (Nieto *et al.*, 2000). H-NS also appears to have an antagonistic relationship with IHF at the *virB* promoter in *Shigella flexneri* (Dorman & Porter, 1998). The relationship between these two proteins at the region 1 promoter is not yet known.

The results obtained with the region 3 promoter and the genes it expresses mirrors the data obtained with the region 1 promoter in that H-NS appears to have a dual role in capsule regulation. At 37°C it maximises transcription and at 20°C it is involved in thermoregulation. The observations made in this study therefore indicate that H-NS co-ordinately regulates the region 1 and region 3 promoters at both 37°C and 20°C. The mechanism by which H-NS and the other regulatory proteins identified in this study exert their effects on the capsule promoters and each other remains to be determined and will be discussed in the next chapter.

Chapter 6

Discussion

Through analysis of capsule gene expression at 37°C and 20°C a number of regulatory factors have been identified that influence regulation of the *E. coli* K5 capsule genes. The architectural DNA binding proteins IHF and H-NS appear to play a role in transcriptional activation at 37°C, with H-NS also exerting affects on thermoregulation at 20°C. BipA, a tyrosine phosphorylated GTPase appears to play a dual role in capsule regulation and finally AT-rich sequences conA and conB have been identified as potential *cis*-acting regulatory elements required for maximal transcription of the region 1 promoter at 37°C. However, despite findings that a number of regulatory proteins control regulation of the capsule gene promoters little is known about nucleo-protein complexes formed at the promoter itself. Binding of IHF to the region 1 promoter has been demonstrated (Rowe *et al.*, 2000) but how IHF-induced conformational changes of promoter DNA facilitate binding of other proteins to this region is unclear. Binding of IHF to binding site IHF-1 would induce DNA bending bringing upstream sequences into contact with perhaps the RNA polymerase or other regulatory proteins. This suggests regions upstream of IHF-1 may also be important in promoter regulation. Binding of IHF to binding site IHF-2 would induce conformational changes in downstream regions suggesting potential enhancer regions may exist downstream of IHF-2. Recent studies have suggested that at some promoters IHF plays a direct role in transcription activation with no requirement for additional activators (Fyfe & Davies, 1998). However, at the region 1 promoter gel mobility shift assays performed using promoter fragments showed binding of an unknown factor(s) in addition to IHF specific binding (Hodgson, N. & Roberts, I.S., unpublished results). One possible explanation for this observation may be H-NS directly binding to the region 1 promoter or alternatively binding of a transcriptional activator.

In this study AT-rich sites conA and conB were found to play a role in expression of the region 1 promoter. Upstream AT-rich regions have been shown to be important for transcription activation of a number of virulence genes. For example, the bundle forming pilin subunit gene (*bfpA*) in enteropathogenic *E. coli*

and the type 4 pilin subunit gene (*tcpA*) in *V. cholerae* are both transcribed from σ^{70} dependent promoters with upstream AT-rich regions (Brown & Taylor, 1995; Tobe *et al.*, 1996). Interestingly both promoters are also subject to positive regulation by activator proteins belonging to the AraC family of transcriptional activators (BfpT in *E. coli* & ToxT in *V. cholerae*).

Analysis of conA and conB sites in the region 1 promoter suggests they do not function as UP elements due to their upstream location and therefore do not interact with RNA polymerase. However, it is possible that these sites bind regulatory factors which themselves interact with RNA polymerase. Genetic evidence has shown that a large number of transcriptional activators induce transcription through interactions with RNA polymerase. These include members of transcription activator families for example AraC-like regulators (AraC, MelR, Ada) and LysR–like regulators (CysB) and other well characterised activator proteins for example CAP and FNR (Ishihama, 1992; Ishihama, 1993). Interactions are not confined to a specific region of RNA polymerase with different activation targets for different proteins mapping to different segments of RNA polymerase (Ishihama, 1992; Ishihama, 1993). It has been suggested that IHF can interact with the RNA polymerase enzyme to activate transcription (Giladi *et al.*, 1992). However, there is some controversy over whether IHF actually activates transcription through protein-protein contacts with RNA polymerase or by an indirect mechanism (van Ulsen *et al.*, 1997a, b). Recent analysis of transcription activation by the IHF protein has concluded that it is most likely that IHF activates transcription by an indirect mechanism (Engelhorn & Geiselmann, 1998). With respect to the region 1 promoter the location of IHF sites appear to be incorrectly spaced to allow any interactions with RNA polymerase to occur.

Much of this work has been concerned with analysis of the region 1 promoter, with only limited analysis of the region 3 promoter. It is known that IHF does not appear to play a role in transcription of the region 3 genes and no IHF binding sites have been located in the region 3 promoter (Simpson *et al.*, 1996). However, AT-rich sites conA and conB have been identified in the region 3 promoter and similar experiments will need to be performed to determine if these sites have a similar role in expression of the region 3 genes. In addition to further analysis of region 3 regulation it would be interesting to determine if the regulation of other group 2 capsules are also dependent on the regulatory factors investigated

in this study. Due to the conserved genetic organisation of group 2 capsule genes it would be expected that they are regulated by similar regulatory factors. Little is known about the regulation of the *E. coli* K1 capsule genes and analysis of these regulatory mutants in a K1 background would be useful to determine their role in K1 gene expression. H-NS and IHF have not been reported to control the expression of other groups of *E. coli* capsular polysaccharides, however H-NS has been implicated in the expression of colanic acid (Sledjeski & Gottesman, 1995).

The role of BipA in capsule gene regulation is intriguing. The sequence similarity of BipA to GTPases, for example EF-G, suggests that BipA may be a new class of regulatory molecule acting at the level of the ribosome (Plunkett *et al.*, 1993; Farris *et al.*, 1998). This provides yet another example of processes exploited by bacterial systems to control protein and gene expression, emphasising the evolutionary diversity of bacterial pathogens. Further detailed experiments must assess the targets of BipA and the global role BipA plays in the cell. It would be interesting to see what role BipA takes in the infection process of *E. coli* K5 based on the results obtained with EPEC and *Salmonella* strains and the regulatory stimuli controlling the action of BipA.

It is unlikely that the regulatory factors identified in this study constitute the complete regulatory circuit required to control *E. coli* K5 capsule gene expression. Further screening using the transposon mutagenesis technique utilised in this study would be useful to try to identify other proteins which may be involved in capsule regulation. The regulatory proteins investigated in this study were all observed in response to changes in temperature; however, it is possible that temperature is not the sole stimuli required to activate capsule expression. Other conditions present in the host during infection may regulate capsule expression or expression of proteins involved with regulation of the capsule genes. Future studies must also assess the actual environmental signals and regulators that modulate the capsule genes *in vivo*. Within the host multiple regulatory pathways are integrated to co-ordinate the appropriate expression of virulence factors at specific sites, such conditions may be difficult to reproduce *in vitro*. *In vitro* assays like those used in this study are valuable tools required to further our knowledge of bacterial regulation and virulence. However, a common drawback of these systems is that they cannot always replicate the complex environment encountered within the host. Two recently developed methodologies permit *in vivo* examination of gene expression and may be possible

candidates to assist in the search for additional regulatory proteins involved in capsule regulation. Firstly, Signature Tagged Mutagenesis (STM) provides a means of identifying genes in bacteria which are involved in the infection process (Hensel *et al.*, 1995). STM has been successfully used to identify virulence genes in a number of bacteria including *S. typhimurium* (Hensel *et al.*, 1995), *V. cholerae* (Chiang & Mekalanos, 1998) and *Y. enterocolitica* (Darwin & Miller, 1999). Regulatory proteins required for virulence were identified from all three species. However success of STM is dependent on finding an efficient transposon delivery system and an appropriate animal model. Recently STM was successfully utilised to identify genes that *E. coli* K1 requires to colonise the host (Martindale *et al.*, 2000). Genes identified included those affecting the synthesis of cell surface structures, membrane transporters and transcriptional regulators (Martindale *et al.*, 2000).

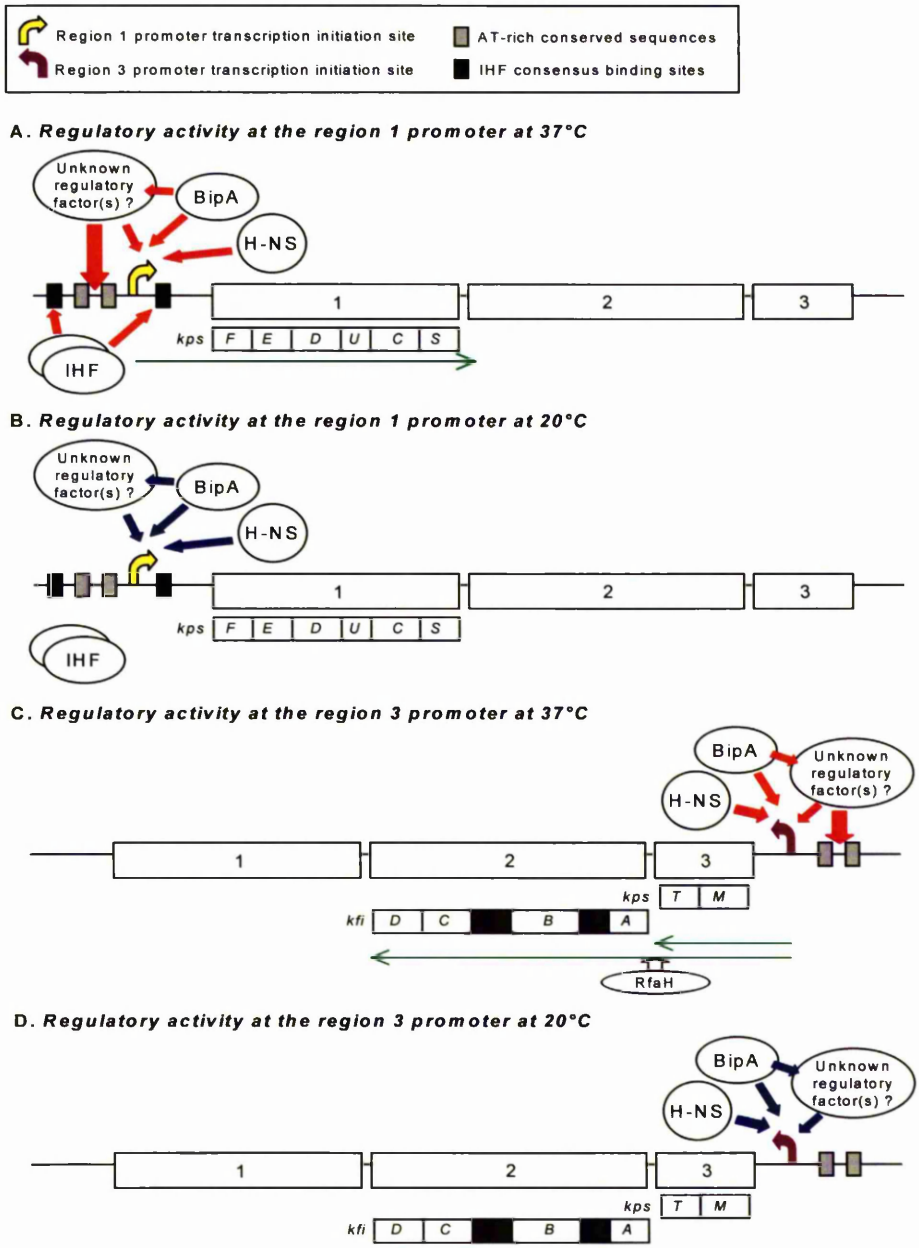
The second system which could be used to identify potential regulatory proteins is *in vivo* expression technology (IVET) (Mahan *et al.*, 1993). IVET does not rely on the reproduction of environmental signals *in vitro* but rather depends on the induction of genes in the host. However like STM, success is dependent on finding an appropriate animal model (Mahan *et al.*, 1993).

Over recent years the importance of RNA polymerase in promoter interactions has become more apparent. Recently, Ishihama and co-workers reported the use of modified RNA polymerase derivatives to investigate promoter interactions (Murakami *et al.*, 1997; Ozoline *et al.*, 1998). Experiments included the use of fluorescein labelled RNA polymerase and RNA polymerase αCTD conjugated to a chemical nuclease, Fe-BABE. These systems were primarily developed to monitor the presence or absence of an UP element in a given promoter (Murakami *et al.*, 1997; Ozoline *et al.*, 1998). However, experiments based on this concept of monitoring RNA polymerase interactions may prove useful to aid analysis of the region 1 promoter.

To conclude, clearly the regulation of the *E. coli* K5 capsule genes is a complex regulatory circuit subject to multiple regulatory inputs (Figure 6.1.). A possible model of regulation may involve regulatory factors such as an AraC-like regulator or an activator interacting with RNAP, binding the AT-rich sequences upstream of the region 1 and region 3 promoters to activate transcription at 37°C. This activation event would be assisted by changes in promoter architecture facilitated by the IHF and HNS proteins. This whole process would be mediated by other global transcriptional regulators exerting their control indirectly by binding to the promoter regions of proteins directly involved with the capsule gene promoters, thereby moderating their expression in a capsule permissive environment. The BipA/TypA protein may feed into this process by altering regulatory proteins from an inactive to active state. Additional transcriptional repressors may act in a non-permissive capsule environment to ensure there is no expression of the capsule genes. This regulatory cascade could then in turn be responsive to various environmental stimuli in addition to temperature that would be encountered during infection of the host.

Certain components in this circuit have been identified but much more information is required to accurately define the interactions of these factors with promoter DNA, regulatory factors and each other. It will then be possible to build a detailed framework of the regulatory cascades operating to express the *E. coli* K5 polysaccharide.

Figure 6.1. Schematic representation of the potential regulatory components active at the *E. coli* K5 capsule gene cluster promoters. Boxes labelled 1,2 and 3 correspond to regions 1, 2 and 3 of the *E. coli* K5 capsule gene cluster. Arrows shaded red indicate regulatory components activating transcription; Arrows shaded blue indicate regulatory components repressing transcription. Horizontal green arrows indicate presence and direction of transcription. Regulatory proteins acting both directly and indirectly to activate and repress transcription of the region 1 and 3 promoters at 37°C(A,C) and 20°C (B,D) are shown. Where regulatory sites of action have not been confirmed arrows are directed to the transcription initiation site



Chapter 7

References

Adler, B., Sasakawa, C., Tobe, T., Makino, S., Komatsu, K. and Yoshikawa, M. (1989) A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. *Molecular Microbiology* **3,** 627-35.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402.

Ames, G.F., Mimura, C.S., Holbrook, S.R., Shyamala, V. (1992) Traffic ATPases: a superfamily of transport proteins operating from *Escherichia coli* to humans. *Advances in Enzymology* **65**, 1-47.

Amor, P.A. & Whitfield, C. (1997) Molecular and functional analysis of genes required for expression of group IB K antigens in *Escherichia coli*: characterization of the *his*-region containing gene clusters for multiple cell-surface polysaccharides. *Molecular Microbiology* **26**, 145-61.

Arakawa, Y., Ohta, M., Wacharotayankun, R., Mori, M., Kido, N., Ito, H., Komatsu, T., Sugiyama, T., Kato, N. (1991) Biosynthesis of *Klebsiella* K2 capsular polysaccharide in *Escherichia coli* HB101 requires the functions of *rmpA* and the chromosomal *cps* gene cluster of the virulent strain *Klebsiella pneumoniae* Chedid (O1:K2). *Infection and Immunity* **59**, 2043-50.

Arakawa, Y., Wacharotayankun, R., Nagatsuka, T., Ito, H., Kato, N. and Ohta, M. (1995) Genomic organisation of the *Klebisiella pneumoniae cps* region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. *Journal of Bacteriology* **177**, 1788-1796.

Atlung, T. and Ingmer, H. (1997) H-NS a modulator of environmentally regulated gene expression. *Molecular Microbiology* **24**, 7-17.

Bailey, M., Koronakis, V., Schmoll, T. and Hughes, C. (1992) *Escherichia coli* HlyT protein, a transcriptional activator of haemolysin synthesis and secretion, is encoded by the *rfaH* (*sfrB*) locus required for expression of sex factor and lipopolysaccharide genes. *Molecular Microbiology* **6**, 1003-1012.

Bailey, M.J., Hughes, C., Koronakis, V. (1997) RfaH and the *ops* element, components of a novel system controlling bacterial transcription elongation. *Molecular Microbiology* **26**, 845-51.

Barne, K.A., Brown, J.A., Busby, S.J. and Minchin, S.D. (1997) Region 2.5 of the *Escherichia coli* RNA polymerase sigma 70 subunit is responsible for the recognition of the 'extended-10' motif at promoters. *EMBO J* **16**, 4034-4040.

Bayer, M. and Thurow, H. (1977) Polysaccharide capsule of *Escherichia coli*: Microscope study of its size, structure and sites of synthesis. *Journal of Bacteriology* **130**, 911-936.

Baynham, P.J. and Wozniak, D.J. (1996) Identification and characterization of AlgZ, an AlgT-dependent DNA-binding protein required for *Pseudomonas aeruginosa algD* transcription. *Molecular Microbiology* **22**, 97-108.

Baynham, P.J., Brown, A.L., Hall, L.L. and Wozniak, D.J. (1999) *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and *algD* transcriptional activation. *Molecular Microbiology* **33**, 1069-80.

Bernardini, M.L., Fontaine, A. and Sansonetti, P.J. (1990) The two-component regulatory system *ompR-envZ* controls the virulence of *Shigella flexneri*. *Journal of Bacteriology* **172**, 6274-81.

Berry, A., De Vault, J.D. and Chakrabarty, A.M. (1989) High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. *Journal of Bacteriology* **171**, 2312-7.

Beutin, L. and Achtman, M. (1979) Two *Escherichia coli* chromosomal cistrons, *sfrA* and *sfrB*, which are needed for expression of F-factor *tra* functions. *Journal of Bacteriology* **139**, 730-737.

Birnhoim, H. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **1**, 1513-1523.

Bitter, T. & Muir, H.M. (1962) A modified uronic acid carbazole reaction. *Analytical Biochemistry* 4, 330-334.

Blatter, E.E., Ross, W., Tang, H., Gourse, R.L. and Ebright, R.H. (1994) Domain organization of RNA polymerase α subunit: C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. *Cell* **78**, 889-896.

Bliss, J.M. and Silver, R.P. (1996) Coating the surface: a model for expression of capsular polysialic acid in *Escherichia coli* K1. *Molecular Microbiology* **21**, 221-231.

Boulnois, G. and Jann, K. (1989) Bacterial polysaccharide capsule synthesis, export and evolution of structural diversity. *Molecular Microbiology* **3**, 1819-1823.

Boulnois, G.J. and Roberts, I.S. (1990) Genetics of capsular polysaccharide production in bacteria. *Current Topics in Microbiology and Immunology* **150**, 1-18.

Brazas, R., Davie, E., Farewell, A. and Rothfield, L. (1991) Transcriptional organisation of the *rfaGBIJ* locus of *Salmonella typhimurium*. *Journal of Bacteriology* **173**, 6168-6173.

Bronner, D., Sieberth, V., Pazzani, C., Roberts, I.S., Boulnois, G.J., Jann, B. and Jann, K. (1993) Expression of the capsular K5 polysaccharide of *Escherichia coli*:

biochemical and electron microscopic analyses of mutants with defects in region 1 of the K5 gene cluster. *Journal of Bacteriology* **175**, 5984-5992.

Brown, E.J., Joiner, K.A., Garther, T.A., Hammer, C.H. and Frank, M.M. (1983) The interaction of C3b bound to *pneumococci* with factor H (beta 1H globulin), I (C3b/C4b inactivator), and properdin factor B of the human complement system. *Journal of Immunology* **131**, 409-415

Brown, R.C. and Taylor, R.K. (1995) Organization of *tcp*, *acf*, and *toxT* genes within a ToxT-dependent operon. *Molecular Microbiology* **16**, 425-39.

de Bruijn, F.J. & Lupski, J.R. (1984) The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids--a review. *Gene* **27**, 131-49.

Bullock, W.O., Fernandez, J.M. and Short, J.M. (1987) XL1-Blue: A high efficiency plasmid transforming *recA Escherichia coli* strains with beta-galactosidase selection. *BioTechniques* **5**, 376.

Busby, S. and Ebright, R.H. (1994) Promoter structure, promoter recognition, and transcription activation in prokaryotes. *Cell* **79**, 743-746.

Busby, S. & Ebright, R.H. (1997) Transcription activation at class II CAP-dependent promoters. *Molecular Microbiology* **23**, 853-9

Bugert, P. and Geider, K. (1995) Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwina amylovora*. *Molecular Microbiology* **15**, 917-933.

Chapon-Herve, V., Akrim, M., Latifi, A., Williams, P., Lazdunski, A., Bally, M. (1997) Regulation of the *xcp* secretion pathway by multiple quorum-sensing modulons in *Pseudomonas aeruginosa*. *Molecular Microbiology* **24**, 1169-78.

Cheetham, B.F. and Katz, M.E. (1995) A role for bacteriophages in the evolution and transfer of bacterial virulence determinants. *Molecular Microbiology*. **18**, 201-8.

Chiang, S.L. and Mekalanos, J.J. (1998) Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization. *Molecular Microbiology* **27**, 797-805.

Cieslewicz, M. & Vimr, E., (1997) Reduced polysialic acid capsule expression in *Escherichia coli* K1 mutants with chromosomal defects in *kpsF*. *Molecular Microbiology* **26,** 237-249.

Clarke, B.R., Pearce, R. and Roberts, I.S. (1999) Genetic organisation of the *Escherichia coli* K10 capsule gene cluster: identification and characterisation of two conserved regions in group III capsule gene clusters encoding polysaccharide transport functions. *Journal of Bacteriology* **181**, 2279-2285.

Cornelis, G.R and Wolf-Watz, H. (1997) The Yersinia Yop virulon: a bacterial system for subverting eukaryotic cells. *Molecular Microbiology* **23**, 861-7.

Cornelis, G.R., Bio, T., Lambert de Rouvroit, C., Michiels, T., Mulder, B., Sluiters, C., Sory, M.P., Van Bouchaute, M. and Vanooleghan, J.C. (1989) The Yersinia *yop* regulon. *Molecular Microbiology* **3**, 1455-9.

Costerton, J.W., Irvin, R.T. and Cheng, K.J. (1981) The bacterial glycocalyx in nature and disease. *Annual Review of Microbiology* **35**, 299-324.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) Bacterial biofilms in nature and disease. *Annual Review of Microbiology* **41**, 435-464.

Costerton, J.W., Levanowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995) Microbial biofilms. *Annual Review of Microbiology* **49**, 711-45.

Costerton, J.W, Stewart, P.S. and Greenberg, E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science.* **284**, 1318-22.

Coynault, C., Robbe-Saule, V., Popoff, M.Y. and Norel, F. (1992) Growth phase and SpvR regulation of transcription of *Salmonella typhimurium spvABC* virulence genes. *Microbial Pathogenesis* **13**, 133-43.

Cross, A. (1990) The biological significance of bacterial encapsulation. *Current Topics in Microbiology and Immunology* **150**, 87-95.

Daffe, M. and Etienne, G. (1999) The capsule of Mycobacterium tuberculosis and its implications for pathogenicity. *Tubercle and Lung Disease.***79**, 153-69.

Danese, P.N, Pratt, L.A. and Kolter, R. (2000) Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. *Journal of Bacteriology* **182**, 3593-6.

Darwin, A.J. and Miller, V.L. (1999) Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature-tagged transposon mutagenesis. *Molecular Microbiology* **32,** 51-62.

Delic-Attree, I., Toussaint, B., Froger, A., Willison, J.C. and Vignais, P.M. (1996) Isolation of an IHF-deficient mutant of a *Pseudomonas aeruginosa* mucoid isolate and evaluation of the role of IHF in *algD* gene expression. *Microbiology* **142**, 2785-2793.

Delic-Attree, I., Toussaint, B., Garin, J. and Vignais, P.M. (1997) Cloning, sequence and mutagenesis of the structural gene of *Pseudomonas aeruginosa* CysB, which can activate *algD* transcription. *Molecular Microbiology* **24**, 1275-1284.

Dersch, P., Schmidt, K. and Bremer, E. (1993) Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA-binding protein H-NS is subjected to growth-phase control and autoregulation. *Molecular Microbiology.* **8**, 875-89.

Dinh, T., Paulsen, I.T. and Saier, M.H. Jr. (1994) A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. *Journal of Bacteriology.* **176,** 3825-31.

DiRita, V.J. and Mekalanos, J.J. (1991) Periplasmic interaction between membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. *Cell* **64,** 29-37.

Doige, C.A. and Ferro-Luzzi Ames, G. (1993) ATP-dependent transport systems in bacteria and humans: relevance to cystic fibrosis and multidrug resistance. *Annual Review of Microbiology* **47,** 291-319.

Dorman, C., Lynch, A., Bhriain, N. and Higgins, C. (1989) DNA supercoiling in *Escherichia coli*: *topA* mutations can be supressed by DNA amplifications involving the *tolC* locus. *Molecular Microbiology* **3,** 531-540.

Dorman, C., Ni Bhriain, N. and Higgins, C. (1990) DNA supercoiling and environmental regulation of gene expression in *Shigella flexneri. Nature* **344,** 789-792.

Dorman, C. (1995) DNA topology and the global control of bacterial gene expression: implications for the regulation of virulence gene expression. *Microbiology* **141,** 1271-1280.

Dorman, C.J. and Porter, M.E. (1998) The *Shigella* virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. *Molecular Microbiology* **29,** 677-84.

Dorman, C.J., Hinton, J.C. and Free, A. (1999) Domain organization and oligomerization among H-NS-like nucleoid-associated proteins in bacteria. *Trends in Microbiology* **7,** 124-8.

Dorrell, N., Gyselman, V.G., Foynes, S., Li, S.R. and Wren, B.W. (1996) Improved efficiency of inverse PCR mutagenesis. *Biotechniques.* **21,** 604-08.

Drake, C., Roberts, I., Jann, B., Jann, K. and Boulnois, G. (1990) Molecular cloning and expression of the genes encoding the *Escherichia coli* K4 capsular polysaccharide, a fructose-substituted chondroitin. *FEMS Microbiology Letters* **66,** 227-230.

Drlica, K. (1992) Control of bacterial DNA supercoiling. *Molecular Microbiology* **6,** 425-433.

Drlica, K. and Rouviere-Yaniv, Y. (1987) Histone-like proteins of bacteria. *Microbiological Reviews* **51,** 301-319.

Drummelsmith, J., Amor, P.A. and Whitfield, C. (1997) Polymorphism, duplication, and IS1-mediated rearrangement in the chromosomal *his-rfb-gnd* region of *Escherichia coli* strains with group IA and capsular K antigens. *Journal of Bacteriology* **179,** 3232-8.

Drummelsmith, J. and Whitfield, C. (1999) Gene products required for surface expression of the capsular form of the group 1 K antigen in *Escherichia coli* (O9a:K30). *Molecular Microbiology* **31,** 1321-32.

Drummelsmith, J. and Whitfield, C. (2000) Translocation of group 1 capsular polysaccharide to the surface of *Escherichia coli* requires a multimeric complex in the outer membrane. *EMBO J.* **19**, 57-66.

Ebright, R.H. and Busby, S. (1995) The *Escherichia coli* RNA polymerase α subunit: structure and function. *Current Opinions in Genetics and Development* **5**, 197-203.

Edwards, U., Muller, A., Hammerschmidt, S., Gerady-Scahn, R. and Frosch, M. (1994) Molecular analysis of the biosynthesis pathway of the α2,8 polysialic acid capsule by *Neisseria meningitidis* serogroup B. *Molecular Microbiology* **14**, 141-150.

Engelhorn, M. and Geiselmann, J. (1998) Maximal transcriptional activation by the IHF protein of *Escherichia coli* depends on optimal DNA bending by the activator. *Molecular Microbiology* **30,** 431-41.

Estrem, S.T., Gaal, T., Ross, W. and Gourse, R.L. (1998) Identification of an UP element consensus sequence for bacterial promoters. *Proceedings of the National Academy of Science U S A.* **95**, 9761-6.

Falconi, M., Colonna, B., Prosseda, G., Micheli, G. and Gualerzi, C.O. (1998) Thermoregulation of *Shigella* and *Escherichia coli* EIEC pathogenicity. A temperature-dependent structural transition of DNA modulates accessibility of *virF* promoter to transcriptional repressor H-NS. *EMBO J.* **17,** 7033-43.

Falconi, M., Brandi, A., La Teana, A., Gualerzi, C.O. and Pon, C.L. (1996) Antagonistic involvement of FIS and H-NS proteins in the transcriptional control of *hns* expression. *Molecular Microbiology* **19**, 965-75.

Farris, M., Grant, A., Richardson, T.B. and O'Connor, C.O. (1998) BipA: a tyrosine-phosphorylated GTPase that mediates interactions between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells. *Molecular Microbiology* **28**, 265-79.

Feinberg, A. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6-13.

Filutowicz, M., Ross, W., Wild, J. and Gourse, R.L. (1992) Involvement of Fis protein in replication of the *Escherichia coli* chromosome. *Journal of Bacteriology* **174**, 398-407.

Finke, A., Jann, B. and Jann, K. (1990) CMP-KDO-synthetase activity in *Escherichia coli* expressing capsular polysaccharides. *FEMS Microbiology Letters* **69**, 129-134.

Finkel, S.E. and Johnson, R.C. (1992) The Fis protein: it's not just for DNA inversion anymore. *Molecular Microbiology* **6**, 3257-3265.

Finne, J., Leinonen, P.H. and Makela, P.H. (1983) Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. *Lancet.* **2**, 355-7.

Forsberg, A.J., Pavitt, G.D. and Higgins, C.F. (1994) Use of transcriptional fusions to monitor gene expression: a cautionary tale. *Journal of Bacteriology* **176**, 2128-32.

Frank, M., Joiner, K. and Hammer, C. (1987) The function of antibody and complement in the lysis of bacteria. *Review of Infectious Diseases* **9**, S537-S545.

Free, A., Williams, R.M. and Dorman, C.J. (1998) The StpA protein functions as a molecular adapter to mediate repression of the *bgl* operon by truncated H-NS in *Escherichia coli.Journal of Bacteriology* **180**, 994-7.

Freestone, P., Trinei, M., Clarke, S.C., Nystrom, T. and Norris, V. (1998) Tyrosine phosphorylation in *Escherichia coli. Journal of Molecular Biology* **279**, 1045-51.

Freundlich, M., Ramani, N., Mathew, E., Sirko, A. and Tsui, P. (1992) The role of integration host factor in gene expression in *Escherichia coli. Molecular Microbiology* **18**, 2557-2563.

Friedman, D. (1988) Integration host factor: a protein for all reasons. *Cell* **55**, 545-554.

Friedman, D. and Court, D. (1995) Transcription antitermination: the λ paradigm updated. *Molecular Microbiology* **18**, 191-200.

Frosch, M., Weisgerber, C. and Meyer, T. (1989) Molecular characterisation and expression in *Escherichia coli* of the gene complex encoding the polysaccharide capsule of *Neisseria meningitidis* group B. *Proceedings of the National Academy of Sciences USA* **84**, 1669-1673.

Frosch, M., Edwards, U., Bousset, K., Krauβe, B. and Weisgerber, C. (1991) Evidence for a common molecular origin of capsular gene loci in Gram-negative bacteria expressing group II capsular polysaccharides. *Molecular Microbiology* **5**, 1251-1263.

Frosch, M. and Muller, A. (1993) Phospholipid substitution of capsular polysaccharides and mechanisms of capsule formation in *Neisseria meningitidis. Molecular Microbiology* **8**, 483-493.

Fuqua, C., Winans, S.C. and Greenberg, E.P. (1996) Analysis of expression and thermoregulation of the *Yersinia pseudotuberculosis inv* gene with hybrid proteins. *Infection and Immunity* **56**, 2133-8.

Fyfe, J.A. and Davies, J.K. (1998) An AT-rich tract containing an integration host factor-binding domain and two UP-like elements enhances transcription from the *pilE*p1 promoter of *Neisseria gonorrhoeae. Journal of Bacteriology* **180**, 2152-9.

Galan, J.E. (1996) Molecular genetic basis of *Salmonella* entry into host cells. *Molecular Microbiology* **20**, 263-271.

Gallegos, M.T., Schleif, R., Bairoch, A., Hofmann, K. and Ramos, J.L. (1997) Arac/XylS family of transcriptional regulators. *Microbiology and Molecular Biology Reviews* **61,** 393-410.

Garcia Vescovi, E., Soncini, F.C. and Groisman, E.A. (1994) The role of the PhoP/PhoQ regulon in *Salmonella* virulence. *Research in Microbiology* **145**, 473-80.

Gatlin, J., Campbell, L.H., Schmidt, M.G. and Arrigo, S.J. (1995) Direct-Rapid (DR) mutagenesis of large plasmids using PCR. *BioTechniques* **19**, 559-564.

Giladi, H., Igarashi, K., Ishihama, A. and Oppenheim, A.B. (1992) Stimulation of the phage lambda pL promoter by integration host factor requires the carboxy terminus of the alpha-subunit of RNA polymerase. *Journal of Molecular Biology* **227**, 985-90.

Gober, J.W. and Shapiro, L. (1992) A developmentally regulated *Caulobacter flagellar* promoter is activated by 3' enhancer and IHF binding elements. *Molecular Biology of the Cell.* **3**, 913-26.

Goldman, R.C., White, D., Orskov, F., Orskov, I., Rick, P.D., Lewis, M.S., Bhattahrjee, A.K. and Leive, L. (1982) A surface polysaccharide of *Escherichia coli* O111 contains O-antigen and inhibits agglutination of cells by O-antiserum. *Journal of Bacteriology* **151**, 1210-21.

Goodman, S.D., Velten, N.J., Gao, O., Robinson, S. and Segall, A.M. (1999) *In vitro* selection of integration host factor binding sites. *Journal of Bacteriology* **181,** 3246-55.

Goodrich, J., Schwartz, M. and McClure, W. (1990) Searching for and predicting the activity of sites for DNA binding proteins: compilation and analysis of the binding sites for *Escherichia coli* integration host factor (IHF). *Nucleic Acids Research* **18**, 4993-5000.

Goosen, N. and van de Putte, P. (1995) The regulation of transcription initiation by integration host factor. *Molecular Microbiology* **16**, 1-7.

Goransson, M., Sonden, B., Nilsson, P., Dagberg, B., Forsman, K., Emanuelsson, K. and Uhiln, B.E. (1990) Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli. Nature* **344**, 682-5.

Gottesman, S. and Stout, V. (1991) Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. *Molecular Microbiology* **5**, 1599-606.

Govan, J.R. and Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia. Microbiological Reviews* **60**, 539-74.

Gray, J. and Rolfe, B. (1990) Exopolysaccharide production in *Rhizobium* and its role in invasion. *Molecular Microbiology* **4**, 1425-1431.

Gross, C., Lonetto, M. and Losick, R. (1992) Bacterial sigma factors. In *Transcriptional regulation*, pp. 129-176. Edited by K. Yamamoto and S. McKnight. Cold Spring Harbor Laboratory Press. Cold Spring Harbor.

Gygi, D., Rahmann, M., Lai, H-C., Carlson, R., Guard-Petter, J. and Hughes, C. (1995) A cell-surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis. Molecular Microbiology* **17**, 1167-1175.

Hales, L.M., Gumport, R.I. and Gardner, J.F. (1994) Determining the DNA sequence elements required for binding integration host factor to two different target sites. *Journal of Bacteriology* **176**, 2999-3006.

Hamawy, M.M. and Mergenhagen, S.E. and Siraganian, R. P. (1995) Protein tyrosine phosphorylation as a mechanism of signalling in mast cells and basophils. Cell Signal. 7, 535-44.

Hammerschmidt, S., Birkholz, C., Zahringer, U., Robertson, B., Van Putten, J., Ebeling, O. and Frosch, M. (1994) Contribution of genes from the capsule gene complex (*cps*) to lipooligosaccharide biosynthesis and serum resistance in *Neisseria meningitidis. Molecular Microbiology* **11**, 885-896.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**, 557.

Helmann, J.D. and Chamberlin, M.J. (1988) Structure and function of bacterial sigma factors. Annual Review of Biochemistry 57, 839-72.

Henkin, T.M. (1996) Control of transcription termination in prokaryotes. *Annual Review of Genetics* **30**, 35-57

Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E. and Holden, D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science.* **269,** 400-3.

Herias, M.V., Midtvedt, T., Hanson, L.Å. and Wold, A.E. (1997) *Escherichia coli* K5 capsule expression enhances colonization of the large intestine in the gnotobiotic rat. *Infection and Immunity* **65**, 531-536.

Higgins, D., Bleasby, A. and Fuchs, R. (1992), Clustal-V: improved software for multiple sequence alignment. *Computer Applications in Biosciences* **8**, 189-191.

Hinton, J.C., Santos, D.S., Seirafi, A., Hulton, C.S., Pavitt, G.D. and Higgins, C.F. (1992) Expression and mutational analysis of the nucleoid-associated protein H-NS of *Salmonella typhimurium. Molecular Microbiology* **6**, 2327-37.

Hobbs, M. and Reeves, P.R. (1994) The JUMPstart sequence : a 39bp element common to several polysaccharide gene clusters. *Molecular Microbiology* **12**, 855-856.

Hoch, J.A. (2000) Two-component and phosphorelay signal transduction. *Current Opinions in Microbiology* **3**, 165-70.

Hochschild, A. and Joung, J., (1997) Synergistic activation of transcription in *E. coli.* In *Nucleic Acids and Molecular Biology*, vol II. Edited by F. Eckstein, D. Lilley. Berlin: Springer; pp.101-114.

Hoffman, J.A., Wass, C., Stins, M.F. and Kim, K.S. (1999) The capsule supports survival but not traversal of *Escherichia coli* K1 across the blood barrier. *Infection and Immunity* **67**, 3566-70.

Holden, M.T., Ram Chhabra, S., de Nys, R., Stead, P., Bainton, N.J., Hill, P.J., Manefield, M., Kumar, N., Labatte, M., England, D., Rice, S., Givolsky, M., Salmond, G.P., Stewart, G.S., Bycroft, B.W., Kjelleberg, S. and Williams, P. (1999) Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. *Molecular Microbiology* **33,** 1254-66.

Horwitz, M. and Silverstein, S. (1980) Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and killing by human phagocytes. *Journal of Clinical Investigation* **65**, 82-94.

Howard, C.J. and Glynn, A.A. (1971) The virulence for mice of strains of *Escherichia coli* related to the effects of K antigens on their resistance to phagocytosis and killing by complement. *Immunology* **20**, 767-777.

Hughes, K.T. and Mathee, K. (1998) The anti-sigma factors. *Annual Review of Microbiology* **52**, 231-86.

Isberg, R.R., Swain, A. and Falkow, S. (1988) Analysis of expression and thermoregulation of the *Yersinia pseudotuberculosis* inv gene with hybrid proteins. *Infection and Immunity* **56,** 2133-8.

Ishihama, A. (1992) Role of the RNA polymerase alpha subunit in transcription activation. *Molecular Microbiology* **6**, 3283-8.

Ishihama, A. (1993) Protein-protein communication within the transcription apparatus. *Journal of Bacteriology* **175,** 2483-9.

Ishihama, A. (1997) Adaptation of gene expression in stationary phase bacteria. *Current Opinion in Genetics and Development.***7,** 582-8.

Jann, B. and Jann, K. (1990) Structure and biosynthesis of the capsular antigens of *Escherichia coli. Current Topics in Microbiology and Imuunology* **150**, 19-42.

Jann, K. and Jann, B. (1992) Capsules of *Escherichia coli*, expression and biological significance. *Canadian Journal of Microbiology* **38**, 705-710.

Jann, B. and Jann, K. (1997) Capsules of *Escherichia coli*. In *Escherichia coli: Mechanisms of Virulence*. Sussman, M. (ed). Cambridge: Cambridge University Press, pp. 113-143.

Jayaratne, P., Keenleyside, W.J., MacLachlan, P.R., Dodgson, C. and Whitfield, C. (1993) Characterization of *rcsB* and *rcsC* from *Escherichia coli* O9:K30:H12 and examination of the role of the *rcs* regulatory system in expression of group I capsular polysaccharides. *Journal of Bacteriology* **175**, 5384-5394.

Jayaratne, P., Bronner, D., MacLachlan, P.R., Dodgson, C., Kido, N. and Whitfield, C. (1994) Cloning and analysis of duplicated *rfbM* and *rfbK* genes involved in the formation of GDP-mannose in *Escherichia coli* O9:K30 and participation of *rfb* genes in the synthesis of the group I K30 capsular polysaccharide. *Journal of Bacteriology* **176,** 3126-39.

Jeon, Y.U., Yamazaki, T., Otomo, T., Ishihama, A. and Kyogoku, Y. (1997) Flexible linker in the RNA polymerase alpha subunit facilitates the independent motion of the C-terminal activator contact domain. *Journal of Molecular Biology* **267,** 953-62.

Johansson, J., Dagberg, B., Richet, E. and Uhlin, B.E. (1998) H-NS and StpA proteins stimulate expression of the maltose regulon *in Escherichia coli*. *Journal of Bacteriology* **180**, 6117-25.

Jordi, B.J. (1992) The mode of action of CfaD of *Escherichia coli* and VirF of *Shigella flexneri* and other members of the AraC family of positive regulators. *Molecular Microbiology* **6**, 3451.

Jordi, B.J., Dagberg, B., de Haan, L.A., Hamers, A.M., van der Zeijst, B.A., Gaastra, W. and Uhlin, B.E. (1992) The positive regulator CfaD overcomes the repression mediated by histone-like protein H-NS (H1) in the CFA/I fimbrial operon of *Escherichia coli EMBO J.* **11**, 2627-32.

Kato, J., Misra, T.K. and Chakrabarty, A.M. (1990) AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis *in Pseudomonas aeruginosa. Proceedings of the National Accademy of Science U S A.* **87**, 2887-91.

Keenleyside, W.J., Bronner, D., Jann, K., Jann, B. and Whitfield , C. (1993) Coexpression of colanic acid and serotype-specific capsular polysaccharides in *Escherichia coli* strains with group II K antigens. *Journal of Bacteriology* **175**, 6725-6730.

Kennelly, P.J. and Potts, M. (1996) Fancy meeting you here! A fresh look at "prokaryotic" protein phosphorylation. *Journal of Bacteriology* **178**, 4759-64.

Kido, N., Torgoy, V.I., Sugiyama, T., Uchiya, K., Sugihara, H., Komatsu, T., Kat, N. and Jann, K. (1995) Expression of the O9 polysaccharide of *Escherichia coli*: sequencing of the *E. coli* O9 rfb gene cluster, characterization of mannosyl

transferases, and evidence for an ATP-binding cassette transport system. *Journal of Bacteriology* **177,** 2178-87.

Kim, K.S., Kang, J.H. and Cross, A.S. (1986) The role of capsular antigens in serum resistance and *in vivo* virulence of *Escherichia coli. FEMS Microbiology Letters* **35,** 275-278.

Kim, S.K., Makino, K., Amemura, M., Nakata A. and Shingawa, H. (1995) Mutational analysis of the first helix of region 4.2 of the sigma 70 subunit of *Escherichia coli* RNA polymerase in transcriptional activation by activator protein PhoB. *Molecular and General Genetics* **24,** 1-8.

Koch, C., Ninnemann, O., Fuss, H. and Kahmann, R. (1991) The N-terminal part of the *E.coli* DNA binding protein FIS is essential for stimulating site-specific DNA inversion but is not required for specific DNA binding. *Nucleic Acids Research* **19,** 5915-22.

Kolb, A., Busby, S., Buc, H., Garges, S. and Adhya, S. (1993) Transcriptional regulation by cAMP and its receptor protein. *Annual Reviews in Biochemistry* **62,** 749-95.

Kroll, S. and Moxon, E. (1988) Capsulation and gene copy number at the *cap* locus of *Haemophilus influenzae* type b. *Journal of Bacteriology* **170,** 859-864.

Kroll, S., Hopkins, I. and Moxon, E. (1988) Capsule loss in *H. influenzae* type b occurs by recombination-mediated disruption of a gene essential for capsular polysaccharide export. *Cell* **53,** 347-356.

Kroll, S., Zamze, S., Loyonds, B. and Moxon, E (1989) Common organisation of chromosomal loci for production of different capsular polysaccharides in *Haemophilus influenzae. Journal of Bacteriology* **171** 3343-3347.

Kroll, S., Loyonds, B., Brophy, L. and Moxon, E (1990) The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsular polysaccharide export. *Molecular Microbiology* **4,** 1853-1862.

Kuhn, H-M., Meier-Dieter, U. and Mayer, H. (1988) ECA, the enterobacterial common antigen. *FEMS Microbiology Reviews* **54,** 195-222.

La Teana, A., Brandi, A., Falconi, M., Spurio, R., Pon, C.L. and Gualerzi, C.O. (1991) Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. *Proceedings of the National Academy of Science U S A.* **88,** 10907-11.

Lee, A.K., Detweiler, C.S. and Falkow, S. (2000) OmpR regulates the two-component system SsrA-SsrB in *Salmonella* pathogenicity island 2. *Journal of Bacteriology* **182,** 771-81.

MacLachlan, P., Keenleyside, W., Dodgson, C. and Whitfield, C. (1993) Formation of the K30 (group 1) capsule of *Escherichia coli* 09:K30 does not require attachment to lipopolysaccharide lipid A-core. *Journal of Bacteriology* **175,** 7515-7522.

Mahan, M.J., Slauch, J.M. and Mekalanos, J.J. (1993) Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 686-8.

Mahan, M.J., Slauch, J.M. and Mekalanos, J.J. (1996) Environmental regulation of virulence gene expression in *Escherichia*, *Salmonella* and *Shigella*, spp. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*. pp. 2803-2815. Edited by F.C. Neidhardt, R. CurtissIII, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasaruk, W.S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger.

March, P.E. (1992) Membrane-associated GTPases in bacteria. *Molecular Microbiology* **6**, 1253-7.

Marolda, C. L. and Valvano, M.A. (1998) Promoter region of the *Escherichia coli* O7-specific lipopolysaccharide gene cluster: structural and functional characterisation of an upstream untranslated mRNA sequence. *Journal of Bacteriology* **180**, 3070-3079

Marshall, D.G., Sheehan, B.J. and Dorman, C.J. (1999) A role for the leucine-responsive regulatory protein and integration host factor in the regulation of the *Salmonella* plasmid virulence (*spv*) locus in *Salmonella typhimurium*. *Molecular Microbiology*. **34**, 134-45.

Martin, D.W., Schurr, M.J., Mudd, M.H., Govan, J.R., Holloway, B.W. and Deretic, V. (1993) Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proceedings of the National Academy of Science U S A.* **90**, 8377-81.

Martindale, J., Stroud, D., Moxon, E.R. and Tang, C.M. (2000) Genetic analysis of *Escherichia coli* K1 gastrointestinal colonization. *Molecular Microbiology* **37**, 1293-1305.

Mekalanos, J. (1992) Environmental signals controlling expression of virulence determinants in bacteria. *Journal of Bacteriology* **174**, 1-7.

Mikulskis, A.V. and Cornelis, G.R. (1994) A new class of proteins regulating gene expression in enterobacteria. *Molecular Microbiology* **11**, 77-86.

Miller, A., Wood, D., Ebright, R.H. and Rothman-Denes, L.B. (1997) RNA polymerase beta' subunit: a target of DNA binding-independent activation. *Science.* **275**, 1655-7.

Miller, J. (1972) Assay of β-galactosidase. In *Experiments in molecular genetics*. Pp. 352-355. Cold Spring Harbor Laboratory Press. Cold Spring Harbor.

Miller, J., Mekalanos, J. and Falkow, S. (1989) Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* **243**, 916-921.

Miller, V.L., Taylor, R.K. and Mekalanos, J.J.(1987) Cholera toxin transcriptional activator *toxR* is a transmembrane DNA binding protein. *Cell* **48**, 271-279.

Miller, V.L. and Mekalanos, J.J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *Journal of Bacteriology* **170**, 2575-2583.

Mogridge, J., Mah, T.F. and Greenblatt, J. (1995) A protein-RNA interaction network facilitates the template-independent cooperative assembly on RNA polymerase of a stable antitermination complex containing the lambda N protein. *Genes and Development* **9**, 2831-45.

Mohr, C.D., Martin, D.W., Konyecsni, W.M., Govan, J.R., Lory, S. and Deretic, V. (1990) Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **172**, 6576-80.

Mourino, M., Madrid, C., Balsalobrie, C., Prenafeta, A., Munoa, F., Blanco, J., Blanco, M., Blanco J.E. and Jaurez, A. (1996) The Hha protein as a modulator of expression of virulence factors in *Escherichia coli*. *Infection and Immunity* **64**, 2881-4.

Moxon, E.R. and Kroll, J.S. (1990) The role of bacterial polysaccharide capsules as virulence factors. *Current Topics in Microbiology and Immunology* **150**, 65-85.

Murakami, K., Kimura, M., Owens, J.T., Meares, C.F. and Ishihama, A. (1997) The two alpha subunits of *Escherichia coli* RNA polymerase are asymmetrically arranged and contact different halves of the DNA upstream element. *Proceedings of the National Academy of Science U S A* **94**, 1709-14

Newlands, J.T., Ross, W., Gosink, K.K. and Gourse, R.L. (1991) Factor-independent activation of *Escherichia coli* rRNA transcription, characterization of complexes of *rrnB* P1 promoters containing or lacking the upstream activator region with *Escherichia coli* RNA polymerase. *Journal of Molecular Biology* **220**, 569-83.

Nguyen, C.C. and Saier, M.H. Jr. (1995) Phylogenetic, structural and functional analyses of the LacI-GalR family of bacterial transcription factors. *FEBS Letters* **377**, 98-102.

Nieto, J.M., Bailey, M.J., Hughes, C. and Koronakis, V. (1996) Suppression of transcription polarity in the *Escherichia coli* haemolysin operon by a short upstream element shared by polysaccharide and DNA transfer determinants. *Molecular Microbiology* **19**, 705-13.

Nieto, J.M., Mourino, M., Balsalobre, C.,Madrid, C., Prenafeta, A., Munoa, F.J. and Juarez, A. (1997) Construction of a double *hha hns* mutant of *Escherichia coli*: effect on DNA supercoiling and alpha-haemolysin production. *FEMS Microbiology Letters* **155**, 39-44.

Nieto, J.M., Madrid, C., Prenafeta, A., Miquelay, E., Balsalobre, C., Carrascal, M. and Juarez, A. (2000) Expression of the hemolysin operon in *Escherichia coli* is modulated by a nucleoid-protein complex that includes the proteins Hha and H-NS. *Molecular and General Genetics.* **263**, 349-58.

Nilsson, L., Vanet, A., Vijgenboom, E. and Bosch, L. (1990) The role of FIS in trans activation of stable RNA operons of *E. coli. EMBO J.* **9**, 727-34.

Noel, K. (1992) Rhizobial polysaccharides required for symbiosis with legumes. In *Molecular Signals in Plant-Microbe Interaction*, ed D. Verma, 341-357. Boca Raton. CRC Press.

Nye, M.B., Pfau, J.D., Skorupski, K., and Taylor, R.K. (2000) *Vibrio cholerae* H-NS silences virulence gene expression at multiple steps in the ToxR regulatory cascade. *Journal of Bacteriology* **182**, 4295-303.

Ophir, T. and Gutnick, D.L. (1994) A role for exopolysaccharides in the protection of microorganisms from desiccation. *Applied and Environmental Microbiology* **60**, 740-745.

Ørskov and Nyman (1974) Genetic mapping of the determinants of two polysaccharide K antigens, K10 and K54, in *Escherichia coli. Journal of Bacteriology* **120**, 43-51.

Ørskov, F., Sharma, V. and Ørskov, I. (1984) Influence of growth temperature on the development of *Escherichia coli* polysaccharide K antigens. *Journal of General Microbiology* **130**, 2681-2684.

Ørskov, F. and Ørskov, I. (1992) *Escherichia coli* serotyping and disease in man and animals. *Canadian Journal of Microbiology* **38**, 699-704.

Ørskov, I. Ørskov, F., Jann, B. and Jann, K. (1977) Serology, chemistry and genetics of O and K antigens of *Escherichia coli. Bacteriological Reviews* **41**, 667-710.

Osuna, R., Finkel, S.E. and Johnson, R.C. (1991) Identification of two functional regions in Fis: the N-terminus is required to promote Hin-mediated DNA inversion but not lambda excision. *EMBO J.* **10,** 1593-603.

Oyston, P.C., Dorrell, N., Williams, K., Li, S.R., Green, M., Titball, R.W. and Wren B.W. (2000) The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in *Yersinia pestis. Infection and Immunity* **68**, 3419-3425.

Ozoline, O.N., Fujita, N., Murakami, K. and Ishihama, A. (1998) Monitoring of RNA polymerase-DNA UP element interaction by a fluorescent probe conjugated to alpha subunit. *European Journal of Biochemistry* **253**, 371-81.

Pavelka, M., Wright, L. and Silver, R. (1991) Identification of two genes, *kpsM* and *kpsT*, in region 3 of the polysialic acid gene cluster of *Escherichia coli* K1. *Journal of Bacteriology* **173**, 4603-4610

Pazzani, C. (1992) Molecular and genetic analysis of the *Escherichia coli* K5 capsule gene cluster. Ph.D Thesis. University of Leicester. Leicester. UK.

Pazzani, C., Smith, A., Bronner, D., Jann, K. and Boulnois, G.J. (1993a) The export of capsular polysaccharides by *Escherichia coli.* In *Polysialic Acid: from Microbes to Man II* pp.125-136. Ed. J. Roth, U. Rutishauser and F. Troy. Basel Birkhauser Verlag

Pazzani, C., Rosenow, C., Boulnois, G.J., Bronner, D., Jann, K. and Roberts, I.S. (1993b) Molecular analysis of region 1 of the *Escherichia coli* K5 antigen gene cluster: a region encoding proteins involved in cell surface expression of capsular polysaccharide. *Journal of Bacteriology* **175**, 5978-5983.

Peacock, S., Weissbach, H. and Nash, H.A. (1984) *In vitro* regulation of phage lambda cII gene expression by *Escherichia coli* integration host factor. *Proceedings of the National Academy of Science U S A.* **81,** 6009-13.

Pearce, R. and Roberts, I.S. (1995) Cloning and analysis of gene clusters for production of the *Escherichia coli* K10 and K54 antigens: identification of a new group of *serA*-linked capsule gene clusters. *Journal of Bacteriology* **177**, 3992-3997.

Perez-Martin, J., Rojo, F. and Lorenzo, V. (1994) Promoters responsive to DNA bending: a common theme in prokaryotic gene expression. *Microbiological Reviews* **58**, 268-290.

Petit, C., Rigg, G.P., Pazzani, C., Smith, A., Sieberth, V., Stevens, M., Boulnois, G.J., Jann, K. and Roberts, I.S. (1995) Region 2 of the *Escherichia coli* K5 capsule gene cluster encoding proteins for the biosynthesis of the K5 polysaccharide. *Molecular Microbiology* **17**, 611-620.

Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustaffson, M. Magnusson, K.E. and Wolf-Watz, H. (1996) Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**, 1231-1233.

Plunkett, G. 3d, Burlan, V., Daniels, D.L., and Blattner, F.R. (1993) Analysis of the *Escherichia coli* genome. III. DNA sequence of the region from 87.2 to 89.2 minutes. *Nucleic Acids Research* **21**, 3391-8.

Porter, M. and Dorman, C. (1997) Positive regulation of *Shigella flexneri* virulence genes by integration host factor. *Journal of Bacteriology* **179**, 6537-50.

Porter, M. and Dorman, C. (1994) A role for H-NS in the thermo-osmotic regulation of virulence gene expression in *Shigella flexneri. Journal of Bacteriology* **176**, 4187-4191.

Pradel, E. and Schnaitman, C. (1991) Effect of *rfaH* (*sfrB*) and temperature on expression of *rfa* genes of *Escherichia coli* K-12. *Journal of Bacteriology* **173**, 6428-6431.

Prigent-Combaret, C., Vidal, O., Dorel, C. and Leieune, P. (1999) Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *Journal of Bacteriology* **181**, 5993-6002.

Qi, S.Y., Li, Y., Szyroki, A., Giles, I.G., Moir, A., and O'Connor, C.D. (1995) *Salmonella typhimurium* responses to a bactericidal protein from human neutrophils. *Molecular Microbiology* **17**, 523-31.

Rahn, A., Drummelsmith, J. and Whitfield, C. (1999) Conserved organisation in the *cps* gene clusters for expression of *Escherichia coli* group I K antigens: relationship to the colanic acid biosynthesis locus and the *cps* genes from *Klebsiella pneumoniae*. *Journal of Bacteriology* **181**, 2307-2313.

Rao, L., Ross, W., Appleman, J.A., Gaal, T., Leirmo, S., Schlax, P.J., record, M.T.Jr. and Gourse, R.L. (1994) Factor independent activation of *rrnB* P1. An "extended" promoter with an upstream element that dramatically increases promoter strength. *Journal of Molecular Biology* **235**, 1421-35

Rhodius, V.A. and Busby, S.J.W. (1998) Positive activation of gene expression. *Current Opinion in Microbiology* **1**, 152-159.

Rice, P.A., Yang, S., Mizuuchi, K., and Nash, H.A. (1996) Crystal structure of an IHF-DNA complex: a protein-induced DNA U-turn. *Cell* **87**, 1295-306.

Rice, P.A. (1997) Making DNA do a U-turn: IHF and related proteins. *Current Opinion in Structural Biology* **7**, 86-93.

Rigg, G.P., Barrett, B. and Roberts, I.S. (1998) The localization of KpsC, S and T, and KfiA, C and D proteins involved in the biosynthesis of the *Escherichia coli* K5 capsular polysaccharide: evidence for a membrane-bound complex. *Microbiology* **144**, 2905-2914.

Roberson, E. and Firestone, M. (1992) Relationship between desiccation and exopolysaccharide production in soil *Pseudomonas* sp. *Applied and Environmental Microbiology* **58**, 1284-1291.

Roberts, I., Mountford, R., High, N., Bitter-Suermann, D., Jann, K., Timmis, K. and Boulnois, G. (1986) Molecular cloning and analysis of genes for the production of the K5, K7, K12 and K92 capsular polysaccharides in *Escherichia coli*. *Journal of Bacteriology* **170**, 1228-1233.

Roberts, I., Mountford, R., Hodge, R., Jann, K. and Boulnois, G. (1988) Common organisation of gene clusters for production of different capsular polysaccharides (Kantigens) in *Escherichia coli*. *Journal of Bacteriology* **170**, 1305-1310.

Roberts, I.S. (1995) Bacterial polysaccharides in sickness and in health. *Microbiology* **141**, 2023-2031.

Roberts, I.S. (1996) The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual Review of Microbiology* **50**, 285-315.

Rosenow, C., Esumeh, F., Roberts, I.S. and Jann, K. (1995a) Characterization and localisation of the KpsE protein of *Escherichia coli* K5, which is involved in polysaccharide export. *Journal of Bacteriology* **177**, 1137-1143.

Rosenow, C., Roberts, I.S. and Jann, K. (1995b) Isolation from recombinant *Escherichia coli* and characterization of CMP-KDO synthetase, involved in the expression of the capsular K5 polysaccharide. *FEMS Microbiolgy Letters* **125**, 159-164.

Ross, W., Thompson, J.F., Newlands, J.T. and Gourse, R.L. (1990) *E.coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. *EMBO J.* **9**, 3733-42.

Ross, W., Gosink, K.K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K. and Gourse, R.L. (1993) A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science.* **262**, 1407-13.

Rowe, S., Hodson, N. Griffiths, G. and Roberts, I.S. (2000) Regulation of the *Escherichia coli* K5 capsule gene cluster: evidence for the roles of H-NS, BipA, and integration host factor in regulation of group 2 capsule gene clusters in pathogenic *E. coli*. *J Bacteriol* 2000 182, 2741-5

Rutberg, B. (1997) Control of transcription termination in prokaryotes. *Annual Review of Genetics* **30**, 35-57.

Salmond, G.P.C., Bycroft, B.W., Stewart, G.S.A.B. and Williams, P. (1995) The bacterial 'enigma': cracking the code of cell-cell communication. *Molecular Microbiology* **16**, 615-624.

Salmond, G.P and Reeves,P.J. (1993) Membrane traffic wardens and protein secretion in gram-negative bacteria. *Trends in Biochemical Sciences.* **18,** 7-12.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor

Schell, M.A. (1993) Molecular biology of the LysR family of transcriptional regulators. *Annual Review of Microbiology* **47**, 597-626.

Schmid, M.B. (1990) More than just "histone-like" proteins. *Cell* **63**, 451-3.

Schmidt, A. and Jann, K. (1982) Phospholipid substitution of capsular (K) polysaccharide antigens from *Escherichia coli* causing extraintestinal infections. *FEMS Microbiology Letters* **14**, 69-74.

Schneider, K. and Beck, C.F. (1986) Promoter-probe vectors for the analysis of divergently arranged promoters. *Gene* **42**, 37-48

Schneider, R., Travers, A. and Muskhelishvili, G. (1997) FIS modulates growth phase-dependent topological transitions of DNA in *Escherichia coli*. *Molecular Microbiology* **26,** 519-30.

Schurr, M.J., Yu, H., Martinez-Salazar, J.M., Boucher, J.C. and Deretic, V. (1996) Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *Journal of Bacteriology* **178**, 4997-5004.

Scott, S., Busby, S. and Beacham, I. (1995) Transcriptional coactivation of the *ansB* promoters: Involvement of the activating regions of CRP and FNR when bound in tandem. *Molecular Microbiology* **18**, 521-532.

Sieberth, V., Rigg, G.P., Roberts, I.S. and Jann, K. (1995) Expression and characterization of UDPGlc dehydrogenase (KfiD) which is encoded in the type-specifc region 2 of the *Escherichia coli* K5 capsule genes. *Journal of Bacteriology* **177**, 4562-4565.

Silhavy, T., Berman, M. and Enquist, L. (1984) Experiments with gene fusions. Cold Spring Harbor Laboratory Press. Cold Spring Harbor.

Silver, R., Finn, C., Vann, W., Aaronson, W., Schneerson, R., Kretschner, P. and Garon, C. (1981) Molecular cloning of the K1 capsular polysaccharide genes of *Escherichia coli*. *Nature* **289**, 696-698.

Simpson, D.A, Hammarton, T.C. and Roberts, I.S. (1996) Transcriptional organisation and regulation of expression of region 1 of the *Escherichia coli* K5 capsule gene cluster. *Journal of Bacteriology* **178**, 6466-6474.

Sledjeski, D. and Gottesman, S. (1995) A small RNA acts as an antisilencer of the H-NS-silenced *rcsA* gene of *Escherichia coli*. *Proceedings of the National Academy of Science U S A.* **92**, 2003-7.

Smith, A.N., Boulnois, G.J. and Roberts, I.S. (1990) Molecular analysis of the *Escherichia coli* K5 *kps* locus: Identification and characterization of an inner-membrane capsular polysaccharide transport system. *Molecular Microbiology* **4**, 1863-69.

Smyth, C.P., Lundback, T., Renzoni, D., Siligardi, G., Beavil, R., Layton, M., Sidebotham, J.M., Hinton, J.C., Driscoll, P.C., Higgins, C.F. and Ladbury, J.E. (2000) Oligomerization of the chromatin-structuring protein H-NS. *Molecular Microbiology* **36,** 962-72.

Spira, B. and Yagil, E. (1999) The integration host factor (IHF) affects the expression of the phosphate-binding protein and of alkaline phosphatase in *Escherichia coli*. *Current Microbiology* **38**, 80-5.

Spiro, S. and Guest, J.R. (1990) FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. *FEMS Microbiological Reviews* **6**, 399-428.

Spurio, R., Falconi, M., Brandi, A., Pon, C.L. and Gualerzi, C.O. (1997) The oligomeric structure of nucleoid protein H-NS is necessary for recognition of intrinsically curved DNA and for DNA bending. *EMBO J.* **16,** 1795-805.

Stevens, M.P., Hänfling, P., Jann, B., Jann, K. and Roberts, I.S. (1994) Regulation of *Escherichia coli* K5 capsular polysaccharide expression: evidence for involvement of RfaH in the expresion of group II capsules. *FEMS Microbiology Letters* **124**, 93-98.

Stevens, M.P. (1995) Regulation of *Escherichia coli* K5 capsular polysaccharide expression. Ph.D Thesis. (JR) University of Leicester, Leicester, UK.

Stevens, M.P., Clarke, B.R. and Roberts, I.S. (1997) Regulation of the *Escherichia coli* K5 capsule gene cluster by transcription antitermination. *Molecular Microbiology* **24**, 1001-1012.

Stevenson, G., Adrianoupolis, K., Hobbs, M. and Reeves, P.R. (1996) Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. *Journal of Bacteriology* **178,** 4885-93.

Straley, S.C and Perry, R.D. (1995) Environmental modulation of gene expression and pathogenesis in *Yersinia. Trends in Microbiology* **3**, 310-317.

Swartley, J.S., Ahn, J.H., Liu, L.J., Kahler, C.M. and Stephens, D.S. (1996) Expression of sialic acid and polysialic acid in serogroup B *Neisseria meningitidis*: divergent transcription of biosynthesis and transport operons through a common promoter region. *Journal of Bacteriology* **178,** 4052-9.

Tatnell, P.J., Russell, N.J. and Gacesa, P. (1994) GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies. *Microbiology* **140**, 1745-54.

Taylor, R.K., Manoil, C. and Mekalanos, J.J. (1989) Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. *Journal of Bacteriology* **171**, 1870-1878.

Tendeng, C., Badaut, C., Krin, E., Gounon, P., Ngo, S., Danchin, A., Rimsky, S. and Bertin, P. (2000) Isolation and characterization of *vicH*, encoding a new pleiotropic regulator in *Vibrio cholerae*. *Journal of Bacteriology* **182,** 2026-32.

Thompson, J.F., Moitoso de Vargas, L., Koch, C., Kahmann, R. and Landy, A. (1987) Cellular factors couple recombination with growth phase: characterization of a new component in the lambda site-specific recombination pathway. *Cell.* **50,** 901-8.

Tobe, T., Yoshikawa, M., Mizuno, T. and Sasakawa, C. (1993) Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by *virF* and repression by H-NS. *Journal of Bacteriology* **175**, 6142-6149.

Tobe, T., Schoolnik, G.K., Sohel, I., Bustamante, V.H. and Puente, J.L. (1996) Cloning and characterization of *bfpTVW*, genes required for the transcriptional activation of *bfpA* in enteropathogenic *Escherichia coli*. *Molecular Microbiology*. **21**, 963-75.

Ueguchi, C., Suzuki, T., Yoshida, T., Tanaka, K. and Mizuno, T. (1996) Systematic mutational analysis revealing the functional domain organization of *Escherichia coli* nucleoid protein H-NS. *Journal of Molecular Biology* **263**, 149-62.

Ueguchi, C., Kakeda, M. and Mizuno, T. (1993) Autoregulatory expression of the *Escherichia coli hns* gene encoding a nucleoid protein: H-NS functions as a repressor of its own transcription. *Molecular and General Genetics.* **236,** 171-8.

Uhl, M.A. and Miller, J.F. (1995) BvgAS is sufficient for activation of the *Bordetella pertussis ptx* locus in *Escherichia coli*. *Journal of Bacteriology* **177,** 6477-85.

Van der Woude, M.W., Kaltenbach, L.S. anf Low, D.A. (1995) Leucine-responsive regulatory protein plays dual roles as both an activator and a repressor of the *Escherichia coli pap* fimbrial operon. *Molecular Microbiology* **17**, 303-312.

Van Dijk, W.C., Verburgh, H.A., van der Tol, M.E., Peters, R. and Verhoef, J. (1979) Role of *Escherichia coli* K capsular antigens during complement activation, C3 fixation, and opsonization. *Infection and Immunity* **25,** 603-9.

Van Eldere, J., Brophy, L., Loynds, B., Celis, P., Hancock, I., Carman, S. Kroll, J.S. and Moxon, E.R. (1995) Region II of the *Haemophilus influenzae* type be capsulation locus is involved in serotype-specific polysaccharide synthesis. *Molecular Microbiology***15,** 107-18.

van Ullsen, P. Hillebrand, M., Zulianello, L., van de Putte, P. and Goosen, N. (1997a) The integration host factor-DNA complex upstream of the early promoter of bacteriophage Mu is functionally symmetric. *Journal of Bacteriology* **179,** 3073-5.

van Ullsen, P. Hillebrand, M., Kainz, M., Collard, R., Zulianello, L., van de Putte, P., Gourse, R.L. and Goosen, N. (1997b) Function of the C-terminal domain of the alpha subunit of *Escherichia coli* RNA polymerase in basal expression and integration host factor-mediated activation of the early promoter of bacteriophage Mu. *Journal of Bacteriology* **179,** 530-7.

Vann, W.F., Schmidt, M.A., Jann, B. and Jann, K. (1981) The stucture of the capsular polysaccharide (K5 antigen) of urinary tract infective *Escherichia coli* O10:K5:H4. A polymer similar to desulfo-heparin. *European Journal of Biochemistry* **116**, 359-364.

Vann, W., Zapata, G., Roberts, I., Boulnois, G. and Silver, R. (1993) Structure and function of enzymes in sialic acid metabolism in polysialic acid producing bacteria. In *Polysialic acid: from microbes to man*, pp. 125-136. Edited by J. Roth, U. Rutishauser and F. Troy II. Birkhauser Verlag. Basel.

Verbeek, H., Nilsson, L., Baliko, G. and Bosch, L. (1990) Potential binding sites of the trans-activator FIS are present upstream of all rRNA operons and of many but not all tRNA operons. *Biochimica et Biophysica Acta.* **1050**, 302-6.

Vimr, E., Aaronson, W. and Silver, R. (1989) Genetic analysis of chromosomal mutations in the polysialic gene cluster of *Escherichia coli* K1. *Journal of Bacteriology* **171**, 1106-1117.

Vimr, E. (1991) Map position and genome organisation of the *kps* cluster for polysialic acid biosynthesis in *Escherichia coli* K1. *Journal of Bacteriology* **173**, 1335-1338.

Wang, L., Curd, H., Qu, W., and Reeves, P.R. (1998) Sequencing of *Escherichia coli* O111 O-antigen gene cluster and identification of O111-specific genes. *Journal of Clinical Microbiology* **36**, 3182-3187.

White-Zieglier, C.A., Angus Hill, M.L., Braaten, B.A., van der Woude, M.W. and Low, D.A. (1998) Thermoregulation of *Escherichia coli pap* transcription: H-NS is a temperature-dependent DNA methylation blocking factor. *Molecular Microbiology* **28**, 1121-37.

Whitfield, C., Vimr, E., Costerton, J. and Troy, F. (1984) Protein synthesis is required for in *vivo* activation of polysialic acid synthesis in *E. coli* K1. *Journal of Bacteriology* **161**, 743-749.

Whitfield, C., Schoenhals, G. and Graham, L. (1989) Mutants of *Escherichia coli* O9:K30 with altered synthesis and expression of the capsular K antigen. *Journal of General Microbiology* **135**, 2589-2599.

Whitfield, C. and Valvano, M.A. (1993) Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. *Advances in Microbial Physiology* **35**, 135-246.

Whitfield, C. and Roberts, I.S. (1999) Structure, assembly and regulation of expression of capsules in *Escherichia coli. Molecular Microbiology* **31**, 1307-1319.

Weickert, M.J. and Adhya, S. (1992) A family of bacterial regulators homologous to Gal and Lac repressors. *Journal of Biologicial Chemistry.* **267,** 15869-74.

Williams, R.M., Rimsky, S. and Buc, H. (1996) Probing the structure, function, and interactions of the *Escherichia coli* H-NS and StpA proteins by using dominant negative derivatives. *Journal of Bacteriology* **178,** 4335-43.

Williams, S.M., Savery, N.J., Busby, S.J. and Wing, H.J. (1997) Transcription activation at class I FNR-dependent promoters: identification of the activating surface of FNR and the corresponding contact site in the C-terminal domain of the RNA polymerase alpha subunit. *Nucleic Acids Research* **25,** 4028-34.

Winkelstein, J.A. (1981) The role of complement in the host's defence against *Streptococcus pneumoniae. Reviews of Infectious Diseases* **3**, 289

Wold, S., Crooke, E. and Skarstad, K. (1996) The *Escherichia coli* Fis protein prevents initiation of DNA replication from oriC *in vitro*. *Nucleic Acids Research* **24,** 3527-32.

Woolwine, S.C. and Wozniak, D.J. (1999) Identification of an *Escherichia coli pepA* homolog and its involvement in suppression of the *algB* phenotype in mucoid *Pseudomonas aeruginosa*. *Journal of Bacteriology* **181,** 107-16.

Wozniak, D.J. (1994) Integration host factor and sequences downstream of the *Pseudomonas aeruginosa algD* transcription start site are required for expression. *Journal of Bacteriology* **176**, 5068-76.

Wozniak, D.J. and Ohman, D.E. (1994) Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. *Journal of Bacteriology* **176**, 6007-14.

Wren, B.W., Henderson, J. and Ketley, J.W. (1994) A PCR-based strategy for the rapid construction of defined bacterial deletion mutants. *Biotechniques* **16**, 994-996.

Wunder, D., Aaronson, W., Hayes, S., Bliss, J. and Silver, R. (1994) Nucleotide sequence and mutational analysis of the gene encoding KpsD, a periplasmic protein involved in the transport of polysialic acid in *Escherichia coli* K1. *Journal of Bacteriology.* **176**, 4025-4033.

Yanisch-Perron, C. Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

Yu, H., Mudd, M., Boucher, J.C., Schurr, M.J. and Deretic, V. (1997) Identification of the *algZ* gene upstream of the response regulator *algR* and its participation in control of alginate production in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **179**, 187-93.

Zhang, J.P. and Normark, S. (1996) Induction of gene expression in *Escherichia coli* after pilus-mediated adherence. *Science* **273,** 1234-1236.

Zierler, M.K. and Galan, J.E. (1995) Contact with cultured epithelial cells stimulates secretion of *Salmonella typhimurium* invasion protein InvJ. *Infection and Immunity* **63**, 4024-4028.