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Gene Regulation and the Leucine-responsive Regulatory Protein of *Salmonella enterica* serovar Typhimurium

by

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A dissertation presented for the degree of Doctor of Philosophy, in the Faculty of Science, University of Dublin, Trinity College

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DECLARATION

I, Kirsty A. McFarland, am the sole author of this thesis. The work presented herein represents my own work, except where duly acknowledged in the text, and has not been previously presented for a higher degree at this or any other University.

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SUMMARY

The leucine-responsive regulatory protein of *Escherichia coli* (*E. coli*) is a well characterised global regulator of transcription. Comparatively little is known about the Lrp regulon of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). In this study, the *lrp* gene was found to be regulated by nutrient availability, and negatively autoregulated, in a similar manner to that previously shown in *E. coli*. Autolysis of *S.* Typhimurium in MOPS minimal medium was examined, and conditions that reduced autolysis were determined, which allowed for further experimentation. The regulon of Lrp was examined in *S.* Typhimurium, during growth in rich and nutrient poor media, using DNA microarray analysis. This revealed that Lrp regulates many genes that have previously been identified as Lrp-regulated in *E. coli* as well as *Salmonella*-specific virulence determinants, type 1 fimbriae of the *fim* gene cluster, and the genes of SPI-2. This also revealed genes that are affected by leucine in *S.* Typhimurium.

DNA microarray analysis suggested the genes belonging to the type 1 fimbriae gene cluster, *fim*, were positively Lrp-regulated. The *lrp* mutant was found to be afimbriate, and had strongly reduced *fimA* and *fimZ* gene transcript levels. Lrp was found to interact with neither the *fimA* nor *fimW-U* promoter regions, but was found to interact with the *fimZ* promoter region. EMSA analysis and DNase I footprinting demonstrated the regions of Lrp binding, and putative binding sites were identified. It was found that the effect of Lrp on type 1 fimbriation was likely to be through its positive effect on *fimZ*, which is an essential positive regulator of the *fim* structural genes. Leucine was shown to increase the positive effect of Lrp on *fimZ* and *fimA* expression.

Lrp appeared to affect the expression of SPI-2 negatively: in the *lrp* mutant at logarithmic phase of growth in MOPS minimal medium, the levels of SPI-2 genes were upregulated. Lrp was found to be important in the response of *ompR* and *ssrA* to acidic pH, and it was also found to have a negative effect on *ssrA* expression at neutral pH. It was shown that Lrp directly regulates *ompR* expression in *S.* Typhimurium, and the region involved in Lrp binding on the *ompR* promoter was identified. In addition, acidic pH was found to repress *lrp* expression.

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Chapter 1

Introduction

1.1 *Salmonella*

Salmonella is a food-borne, Gram-negative pathogen belonging to the *Enterobacteriaceae* family. The *Salmonella* genus contains two species: *S. enterica* and *S. bongori*. *S. enterica* is divided into six subspecies: *arizonae, diarizonae, enterica, houtenae, indica,* and *salamae* (Popoff *et al.*, 2004). *Salmonella* species and subspecies are further divided into serovars by their flagellar, carbohydrate and lipopolysaccharide (LPS) structures (Coburn *et al.*, 2007). By 2004, over 2,541 *Salmonella* serovars had been described: 99 % of these belong to *S. enterica,* and *S. bongori* accounted for 22 serovars. Out of the *S. enterica* category, almost 60 % belong to subspecies *enterica,* which has over 1500 serovars (Popoff *et al.*, 2004).

The clinical manifestations of *Salmonella* infection, called salmonellosis, include typhoid fever and gastroenteritis. *S. enterica* subspecies *enterica* serovar Typhi and Paratyphi are exclusive to human and higher primate hosts, where they can cause typhoid fever, with a 10-15 % mortality rate without treatment (Ohl and Miller, 2001). They are endemic to parts of the world with poor sanitation and inadequate sewage treatment (Merrell and Falkow, 2004). Typhoid fever occurs when *Salmonella* establish a systemic infection, through their ability to survive and proliferate in mononuclear phagocytes (Ohl and Miller, 2001). *S. enterica* subspecies *enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) is a broad host range serovar, capable of infecting poultry, pigs, cattle and humans. *S.* Typhimurium is a non-typhoidal strain in humans, but can cause self-limiting gastroenteritis. Non-typhoidal salmonellosis is most frequently associated with *S.* Typhimurium (Olsen *et al.*, 2000), and it is estimated that annually between 200 million and 1.3 billion cases of non-typhoidal salmonellosis lead to 3 million deaths worldwide (Coburn *et al.*, 2007). In mice, *S.* Typhimurium is a natural pathogen, and causes a systemic, typhoid-like disease, which serves as an animal model of typhoid fever with intestinal and extraintestinal lesions closely resembling those observed in typhoid fever victims (Santos *et al.*, 2001; Zhang *et al.*, 2003). Natural and experimental infection of calves with *S.* Typhimurium is used to study pathogenesis of gastroenteritis, as the clinical and pathological manifestations parallel those found in humans (Santos *et al.*, 2001). *S.* Typhimurium has also been studied intensively as a model organism for genetic studies.

1.2 The infective process of *S. enterica*

Salmonella infections begin with the ingestion of organisms in contaminated food or water. During intestinal colonisation, *Salmonella* are exposed to a number of stresses: they must survive the acidic pH of the stomach, exposure to bile, anoxic conditions, the presence of metabolites produced by the normal gut microflora and exposure to cationic antimicrobial peptides present on the surface of epithelial cells (Rychlik and Barrow, 2005). *Salmonella* colonise the small intestine, where they are believed to adhere to the intestinal barrier using fimbriae and non-fimbrial adhesins (Gerlach *et al.*, 2007; Humphries *et al.*, 2001; Morgan *et al.*, 2007). Here, they target specialised M cells of the follicle-associated epithelium of Peyer's patches, which are collections of lymphoid follicles making up the gut-associated lymphoid tissue (Jones *et al.*, 1994). Using a type-three secretion system (TTSS) encoded by SPI-1, *Salmonella* secrete protein factors into these non-phagocytic cells that mediates the uptake of the *Salmonella* in a vacuole, by a process called bacterial-mediated endocytosis (Francis *et al.*, 1993). *Salmonella* can also be taken up by migratory and phagocytic dendritic cells, which open the tight junctions between epithelial cells and send dendrites outside the epithelium to directly sample bacteria (Rescigno *et al.*, 2001). From inside the epithelial cell, non-typhoidal *Salmonella* begin to enter both the apical and basolateral surfaces of enterocytes adjacent to infected M cells (Jones *et al.*, 1994), where they secrete effector proteins into the host cytosol, causing cytokine release, inflammation and fluid secretion, the symptoms of gastroenteritis (Galan, 1999). Typhoidal *Salmonella* cause systemic infection when they are transcytosed to the basolateral surface of the intestinal epithelium, where they are phagocytosed by submucosal macrophages (Alpuche-Aranda *et al.*, 1994). Within the macrophage, in a *Salmonella*-containing vacuole (SCV), *Salmonella* survives, replicates, and evades the host immune system. A second TTSS, encoded by SPI-2, is used to secrete effector proteins into the macrophage, to interfere with its antimicrobial properties, and is required for survival of *Salmonella* in macrophages (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Vazquez-Torres *et al.*, 1999).

1.2.1 Adhesion of *Salmonella* to the intestinal barrier

Adhesion is mediated by filamentous appendages on bacterial cell surfaces, called fimbriae, or pili. *S.* Typhimurium strain LT2 contains 13 fimbrial operons (McClelland *et al.*, 2001). Some studies suggest that these fimbriae each have a specific tropism for a certain cell type, or for cells from particular animal species (Baumler *et al.*, 1996b). It

has been shown that *S.* Typhi also contains 13 operons: one of the operons, *agf,* is well characterised, whereas the remaining 12 are putative (Townsend *et al.*, 2001). *S.* Typhi differs from other *Salmonella* serovars investigated in that it contains a unique combination of putative fimbrial operons (Townsend *et al.*, 2001). Typically, *S. enterica* serovars contain large numbers of putative fimbrial operons and it is thought that they may have a role in niche specialisation within *S. enterica* (Anjum *et al.*, 2005). Interestingly, it has been shown that the *fim, bcf* and *stb* operons appear to be conserved in *S. enterica* serovars (Townsend *et al.*, 2001). In bovine ligated ileal loops, expression of the major antigens of 9 fimbrial operons of *S.* Typhimurium has been demonstrated by flow cytometry, including *fim, bcf* and *stb* (Humphries *et al.*, 2003). There is evidence for a synergistic action of fimbrial operons *fim, agf, pef* and *lpf* during colonization of the mouse intestine and the development of murine typhoid fever (van der Velden *et al.*, 1998).

Non-fimbrial bacterial adhesion factors have also been identified, the most recent of these is SiiE of SPI-4, involved in adhesion to epithelial cells, which is discussed later (Gerlach *et al.*, 2007; Morgan *et al.*, 2007).

1.2.2 *Salmonella* pathogenicity islands (SPIs)

The pathogenesis of *Salmonella* is mainly attributable to horizontally acquired DNA from gene islands, prophages and plasmid sources. Perhaps the most important of these are the chromosomally-encoded gene islands, called pathogenicity islands (PAIs) (Schmidt and Hensel, 2004). Characteristically, PAIs are regions of DNA between 10-200 kb in size that are present in pathogenic strains but absent from non-pathogenic strains, their G+C content usually differs from the host chromosome, they are often flanked by short direct repeats, and they are frequently associated with tRNA genes (Hacker and Kaper, 2000). *Salmonella* pathogenicity islands, called SPIs, of which there are 5 in *S.* Typhimurium, are important contributors to the virulence associated with salmonellosis (Schmidt and Hensel, 2004). Type three secretion systems (TTSS) are often encoded by PAIs, as will be discussed in the following sections.

1.2.2.1 SPI-1

Salmonella pathogenicity island-1 (SPI-1) is located at centisome (cs) 63 on the *S.* Typhimurium chromosome. Galan and Curtiss identified *Salmonella* genes that were

essential for bacterial invasiveness in cell culture and complete oral virulence (Galan and Curtiss, 1989), which were later shown to be part of a 40 kb, *Salmonella*-specific PAI, that is not present in the *E. coli* chromosome (Mills *et al.*, 1995). SPI-1 is required by *Salmonella* for invasion of the non-phagocytic epithelial cells of the small intestine (Galan, 1999). SPI-1 contains at least 37 genes which encode the structural, effector, chaperone and regulatory proteins for the assembly and function of a type three secretion system (TTSS) (Lostroh and Lee, 2001a). The SPI-1 structural proteins form a needle complex that spans the inner and outer bacterial membranes (Kubori *et al.*, 1998), and when in close contact with the epithelial cells of the small intestine, delivers at least 19 effector proteins into the host cells (Galan, 1999). Both SPI-1-encoded, and non-SPI-1-encoded effector proteins are secreted through the needle complex, including the SPI-5-encoded SopB, and SopE1, which is encoded on the SopEΦ phage insertion at cs 60 (Darwin *et al.*, 2001; Figueroa-Bossi *et al.*, 2001). The effector proteins modify signal transduction pathways and remodel the actin cytoskeleton, which manifests itself as ruffling of the host cell membrane (Francis *et al.*, 1993). The membrane ruffles engulf the bacteria, causing them to be taken up in a vacuole where they survive, spread to adjacent epithelial cells and continue to secrete effector proteins through the membrane of the enclosing vesicle (Galan, 2001). This promotes inflammation, cytokine release and fluid secretion, which result in the symptoms of gastroenteritis. Gastroenteritis-causing *Salmonella* infection ends at this point, with bacteria limited to the lymphoid tissue of the intestinal tract (Altier, 2005).

SPI-1 expression is affected by a number of environmental signals, including pH, oxygen tension, osmolarity, bile, Mg^{2+} concentration, and short chain fatty acids (Altier, 2005). SPI-1 expression is regulated in a complex manner. The SPI-1-encoded *hilA* gene encodes an OmpR/ToxR-like protein that activates the transcription of *invF,* an AraC/XylS-like protein (Bajaj *et al.*, 1995). HilA and InvF both bind to the promoter region of the SPI-1 structural operon, activating transcription (Darwin and Miller, 1999; Lostroh and Lee, 2001b). The HilC and HilD proteins of SPI-1 positively regulate the expression of *hilA* by direct interaction with the *hilA* promoter (Olekhnovich and Kadner, 2002). SPI-1 expression is also known to be controlled by regulatory factors outside SPI-1: the nucleoid-associated proteins H-NS, HU, Fis and Hha; Lon protease; RtsA; HilE and Mlc all affect SPI-1 expression (Boddicker and Jones, 2004; Ellermeier and Slauch, 2003; Ellermeier *et al.*, 2005; Fahlen *et al.*, 2001; Lim *et al.*, 2007;

Schechter *et al.*, 2003). HilE is a repressor of *hilA* expression that was shown to interact via protein:protein interactions with HilD to inhibit transcriptional activation of *hilA*. The *hilE* gene is unique to *Salmonella* strains, and is resident on a part of the chromosome that strongly resembles a pathogenicity island (Baxter *et al.*, 2003). Recently, it was shown that Mlc, a sensor of sugar availability, causes activation of SPI-1 through repression of *hilE* expression (Lim *et al.*, 2007). Baxter and Jones have shown that *hilE* expression is activated by FimZ and FimY, two positive regulators of type 1 fimbrial gene expression: it is presumed that this leads to coordination of adhesion and invasion, presumably to delay expression of the SPI-1 TTSS, to first allow adhesion to epithelial cells to take place (Baxter and Jones, 2005).

1.2.2.2 SPI-2

SPI-2 is 40 kb in size and is located at cs 31 on the chromosome of *S.* Typhimurium. In mice, SPI-1 mutants are attenuated for virulence when infected by the peroral route, but are fully virulent infected by the intraperitoneal route (Galan and Curtiss, 1989). After the discovery of SPI-1, signature-tagged mutagenesis (STM) screening was used to isolate mutants attenuated for virulence in the systemic phase of infection in mice, when administered intraperitoneally (Hensel *et al.*, 1995). Sequencing and mapping of DNA flanking the mutation sites revealed that the genes were clustered on a pathogenicity island distinct from SPI-1 (Shea *et al.*, 1996), and this was also confirmed by an independent study (Ochman *et al.*, 1996). SPI-2 is inserted adjacent to the $tRNA^{ValV}$ gene, and has a lower G+C content than its flanking host chromosome, suggesting that it was acquired by horizontal transfer (Shea *et al.*, 1996). However, analysis of the genomic structure identified that SPI-2 is composed of at least two independently acquired genetic elements, of 25.3 kb and 14.5 kb (Hensel *et al.*, 1999). It is believed that the 14.5-kb region, which is present in the phylogenetically older *Salmonella* species *S. bongori,* as well as *S. enterica,* represents an earlier insertion than the 25.3-kb region, which is not present in *S. bongori* serotypes (Hensel *et al.*, 1999). In addition, the 25.3-kb insertion contains different classes of genes to that of the 14.5-kb insertion, which do not contribute significantly to systemic infection in the mouse model (Hensel *et al.*, 1999). The 14.5-kb region contains *ttr* genes, which encode anaerobic tetrathionate reductase, and a cluster of seven ORFs of unknown function (Hensel *et al.*, 1999). The 25.3-kb region of SPI-2 encodes a TTSS, secreted effector proteins, chaperones, and regulatory elements. The 31 genes are organised in four operons (Fig.

1.1): the structural I, structural II, regulatory, and effector/chaperone operons (Cirillo *et al.*, 1998; Hensel *et al.*, 1997; Shea *et al.*, 1996), although there appears to be more than one promoter for the structural II operon. Hensel *et al.* organise the genes *ssaK/U* into a single operon, and place *ssaJ* as the terminal gene of another transcriptional unit (Hensel *et al.*, 1997). A later report found a promoter upstream of *ssaH,* and proposed that all downstream genes were transcribed from this promoter (Cirillo *et al.*, 1998). In addition, the *ssrA* and *ssrB* genes of the regulatory operon do not appear to be co-transcribed, as there is evidence to suggest that their regulation is uncoupled (Feng *et al.*, 2003; Feng *et al.*, 2004). TTSS structural operon genes are designated *ssa,* for secretion system apparatus; *ssr*, for secretion system regulators; *sse*, for secretion system effectors; and *ssc* for secretion system chaperones (Hensel *et al.* , 1997). In retrospect, some of these genes have been incorrectly named, as SseA was later revealed to be a chaperone of translocon components SseB and SseD (Ruiz-Albert *et al.*, 2003; Zurawski and Stein, 2003). In addition, SsaB was revealed to be a secreted effector protein and renamed SpiC (Uchiya *et al.*, 1999).

SPI-2 is required for the survival and replication of intracellular *Salmonella* in SCVs within macrophages and epithelial cells (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). SPI-2, through the secretion of effector molecules, interferes with intracellular transport processes, host-cell actin cytoskeleton, and the host cell microtubule cytoskeleton (Abrahams and Hensel, 2006). The targeting of the SCV to the Golgi apparatus is important for replication of *Salmonella* in the SCV, and the positioning of the SCV is affected by SPI-2 TTSS effectors (Abrahams *et al.*, 2006; Deiwick *et al.*, 2006; Salcedo and Holden, 2003). Recently, it has been shown that effector proteins SseG and SseF affect this positioning by restricting the microtubule-dependent motility of the SCV (Ramsden *et al.*, 2007). The SPI-2-secreted effector proteins also deviate the SCV from the degradative endosomal pathway, and prevent the recruitment of NADPH oxidase to the SCV (Kuhle and Hensel, 2004). A total of 16 effector proteins, encoded both in SPI-2 and elsewhere on the chromosome, have been described as translocated by the SPI-2 TTSS, and have various functions (reviewed in Kuhle and Hensel, 2004; Abrahams and Hensel, 2006).

The environmental signals affecting SPI-2 gene expression are not clearly understood, as many conflicting reports have examined this subject. Acidic pH was identified as an

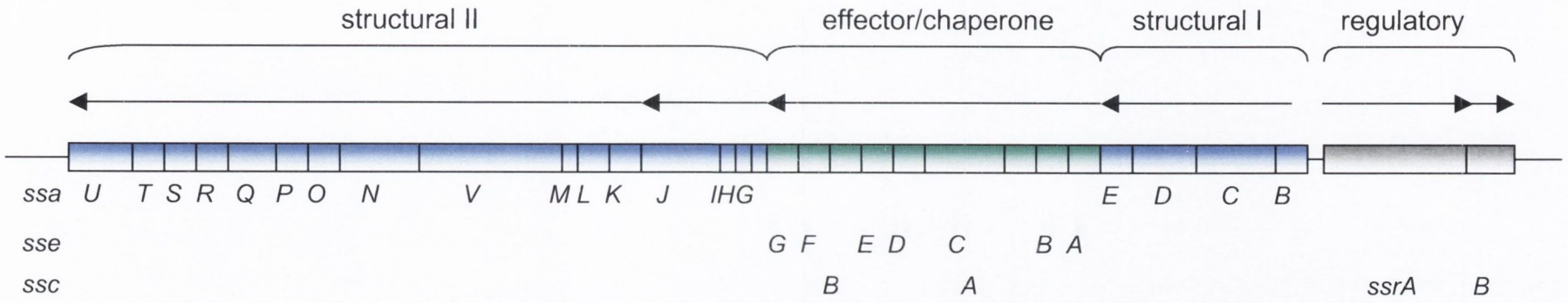


Fig. 1.1. Structural organization of the 25-kb virulence region of SPI-2. The structural genes of SPI-2, encoding a TTSS, are shown in blue, and the effector/chaperone operon is shown in green. The regulatory genes of SPI-2, *ssrAB,* are shown in grey. The arrows denote the direction of transcription. As described in the text, the promoter positions of the structural II operon are unclear, and it is thought that there may be more than one promoter controlling these genes. Hensel *et al.* organise *ssaK/U* as one operon, and *ssaJ* as the terminal gene of another operon (Hensel *et al.*, 1997). Figure adapted from Hensel *et al.,* 1998. The uncoupled transcription of *ssrA* and *ssrB* of the regulatory operon (Feng *et al.*, 2003), is shown by two arrows.

environmental signal by a number of studies. It has been shown that in murine macrophages, the SCVs undergo acidification to pH 4.0-5.0 between 20 to 30 min after formation, and that inhibition of acidification decreased *Salmonella* survival in macrophages (Rathman *et al.*, 1996). Eriksson *et al.* showed by microarray analysis that in J774-A.1 macrophage-like cells, *S.* Typhimurium strain SL1344 showed up-regulation of acid-inducible genes *cadB, cysB,* and *adiY,* but did not show significant transcript increases of genes involved in the acid tolerance response (ATR) of *Salmonella*, such as *ompR* (Eriksson *et al.*, 2003). They concluded that regulation may occur post-transcriptionally, or that the intravacuolar pH was not acidic enough, or sudden enough to induce an ATR. Other studies have shown that SPI-2 gene expression was greatly diminished in macrophages where acidification of vacuoles was blocked (Cirillo *et al.*, 1998). Miao *et al.* examined the induction of the SPI-2 effector *sspH2,* which is highly regulated by SsrA-B, and conclude that low pH was not required for SsrA-B expression, and that alkaline pH inhibited expression of SsrA-B (Miao *et al.*, 2002). Deiwick *et al.* also disagree that acidic pH is an inducing factor for SPI-2, as they did not see a change in expression that they considered significant (Deiwick *et al.*, 1999). Lee *et al.* showed that acidic pH induced the expression of SPI-2 genes *ssrA* and *ssaH* in wild type SL1344 and, using immunoblot analysis, detected the secretion of effector proteins under conditions of acidic pH in minimal medium, indicating that the SPI-2 machinery is likely to be expressed under these conditions (Lee *et al.*, 2000). Recently, it was shown that exposure of bacteria to minimal media with acidic pH of 6.2 or below resulted in a rapid induction of SPI-2 expression (Löber *et al.*, 2006). Rappl *et al.* demonstrated that acidic pH is required for the functional assembly of the SPI-2 TTSS (Rappl *et al.*, 2003), and Beuzón *et al.* showed that SseB is only secreted if the bacteria are grown at low pH or if the pH is shifted after growth from 7.0 to below pH 5.0. They suggest that low pH might be a physiological stimulus for SPI-2-mediated secretion *in vivo* (Beuzón *et al.*, 1999). *In vitro* analyses suggested that minimal media with low concentrations of Mg^{2+}, and phosphate starvation led to the induction of SPI-2 gene fusions (Deiwick *et al.*, 1999). However, Lee *et al.* describe induction of *ssrA* and *ssaB* in minimal media at pH 4.5 compared with pH 7.2 in the presence of both low (1 μM) and high concentrations (1 mM) of Mg^{2+}, failing to reproduce the effects of low Mg^{2+} (Lee *et al.*, 2000). In agreement, Miao *et al.* showed that limitation of Mg^{2+} or phosphate was not required for induction of the SPI-2 effector gene *sspH2* (Miao *et al.*, 2002). Garmendia *et al.* have shown that low osmolarity and absence of Ca^{2+} are major

signals that affect SPI-2 gene expression during growth in magnesium minimal medium (Garmendia *et al.*, 2003).

The SPI-2-encoded two-component signal transduction system SsrA-B controls SPI-2 TTSS gene expression, as well as the expression of SPI-2-secreted effector molecules located outside the SPI-2 pathogenicity island. SsrA-B responds to low osmolarity, the absence of Ca^{2+} and acidic pH (Garmendia *et al.*, 2003). Expression of *ssrAB* is also regulated by a second two-component signal transduction system, OmpR-EnvZ (Lee *et al.*, 2000). The sensor kinase, EnvZ, senses a signal, becomes autophosphorylated, and passes the phosphate to OmpR by phosphorelay. Phosphorylation of OmpR (OmpR-P) induces a change in conformation (Kenney *et al.*, 1995), which is thought to influence its DNA binding. Importantly, *ompR* of *Salmonella* appears to be repressed by osmolarity, and induced by acid stress, and is thought to transduce the acidic pH signal to *ssrA* (Bang *et al.*, 2000; Bang *et al.*, 2002; Garmendia *et al.*, 2003). It has been shown that the response of SPI-2 to acidic pH, low osmolarity and Ca^{2+} concentration is SsrA-B-dependent, but only partially dependent on OmpR-EnvZ (Garmendia *et al.*, 2003). OmpR-P directly regulates the expression of the sensor kinase, *ssrA,* by interaction with the *ssrA* promoter region (Feng *et al.*, 2003; Lee *et al.*, 2000). After entry into macrophages, *ssrA* transcription is most efficient in the presence of OmpR (Lee *et al.*, 2000) and the *S.* Typhimurium *ompR* mutant is highly attenuated for virulence (Dorman *et al.*, 1989). OmpR also regulates the expression of the response regulator, *ssrB,* directly by interaction with the *ssrB* promoter region (Feng *et al.*, 2003). SsrB expression is autoregulated positively, and SsrB directly affects the expression of *ssrA* and *ssaB* positively (Feng *et al.*, 2003).

SlyA is a transcriptional regulator that regulates genes that are expressed in the intracellular environment, and is important for systemic virulence in mice (Libby *et al.*, 1994). It has been shown to be important for survival in macrophages, due to its positive effect on SPI-2 genes (Linehan *et al.*, 2005). It had been suggested by a number of studies that SlyA might act positively on *ssrA* to promote SPI-2 function (Feng *et al.*, 2003; Feng *et al.*, 2004; Linehan *et al.*, 2005; Navarre *et al.*, 2005). More recently, Okada *et al.* showed a direct interaction of SlyA with the promoter region of *ssrA* (Okada *et al.*, 2007). Expression of *ssrA* is also induced by DNA relaxation: it was demonstrated that *Salmonella* DNA became more relaxed in the macrophage than in

epithelial cells, indicating that DNA supercoiling may have a role in niche discrimination, and Fis also influences this (Ó Cróinín *et al.*, 2006).

1.2.2.3 SPI-3

SPI-3 is a 17-kb region with a mosaic structure that encodes at least 10 genes, and is inserted at the *selC* tRNA locus at cs 82 (Blanc-Potard *et al.*, 1999). It harbours the cotranscribed *mgtCB* genes, which are *Salmonella*-specific and required for intramacrophage survival, virulence in mice, and growth in low Mg^{2+} media (Blanc-Potard and Groisman, 1997; Snavely *et al.*, 1991). The expression of *mgtCB* is controlled by PhoP-PhoQ, a major two-component system and virulence regulator in *Salmonella* (Garcia Vescovi *et al.*, 1996). The *mgtB* gene encodes an Mg^{2+} transporter, which is not required for virulence in BALB/c mice, but the function of MgtC is unknown (Blanc-Potard and Groisman, 1997; Schmidt and Hensel, 2004). A further putative virulence factor in SPI-3 is *misL,* which encodes a putative autotransporter with similarity to VirG of *Shigella flexneri* (Blanc-Potard *et al.*, 1999).

1.2.2.4 SPI-4

Until recently, little analysis had been performed on SPI-4. Wong *et al.* identified the *Salmonella*-specific 27-kb region at cs 92, identifying 18 putative operons encoding a type I protein secretion system (Wong *et al.*, 1998): it was later shown by genome sequencing that SPI-4 contains 6 ORFs, *siiABCDEF* (McClelland *et al.*, 2001; Morgan *et al.*, 2004). It was shown to be required as a colonisation factor, and to be required for long-term persistence in calves (Lawley *et al.*, 2006; Morgan *et al.*, 2004). Recent studies have shown that the SPI-4 gene *siiE* encodes a very large non-fimbrial adhesin of 600 kDa, with 53 repeats of Ig domains, which mediates binding to epithelial surfaces in a contact-dependent manner, and is the first of its kind to be described (Gerlach *et al.*, 2007; Morgan *et al.*, 2007). The *siiCDF* genes encode a type I protein secretion system, of which SiiE is the secreted substrate. The two remaining genes, *siiAB*, encode inner membrane proteins that are neither secreted nor required for SiiE secretion (Gerlach *et al.*, 2007). A previous study had shown that *siiE* is regulated by the transcriptional regulator SirA, in a HilA-dependent manner (Ahmer *et al.*, 1999), and HilA was also found to affect the transcription or secretion of SiiE (Morgan *et al.*, 2007). Very recently, DNA microarray analysis showed that HilA is a regulator of *siiA* (Thijs *et al.*, 2007), strongly linking SPI-1 and SPI-4 expression.

1.2.2.5 SPI-5

SPI-5 is a 7-kb region, adjacent to the *serT* tRNA gene at cs 25 (Schmidt and Hensel, 2004). SPI-5 encodes SopD, and PipB, which are effector proteins for SPI-1 and SPI-2, respectively (Knodler *et al.*, 2002; Wood *et al.*, 1998). SPI-5 is also found in several other *S. enterica* serovars, and it is involved in invasion of epithelial cells of the small intestine, and not systemic infection (Wood *et al.*, 1998).

1.2.2.6 Other *Salmonella* pathogenicity islands

S. Typhimurium contain only SPI-1 to SPI-5, whereas *S.* Typhi contains further pathogenicity islands, SPI-6 to SPI-10 (Schmidt and Hensel, 2004). The genome of *S.* Typhimurium strain DT104, a multidrug-resistant strain, contains the pathogenicity island *Salmonella* genomic island 1, or SGI-1 (Boyd *et al.*, 2000). SGI-1 is 43 kb in length, and carries a multidrug resistance region with genes that confer resistance to ampicillin, chloramphenicol, florfenicol, sulfonamides, streptomycin, and tetracycline (Quinn *et al.*, 2006). SGI-1 has also been found in *S.* Agona, which suggests that it may be transmissible to other serovars (Boyd *et al.*, 2001).

1.2.2.7 The large virulence plasmid, pSLT

S. Typhimurium contains a 90-kb virulence plasmid that was initially shown to increase the growth rate of *Salmonella* in mice, through the plasmid-encoded *spvRABCD* genes (Gulig and Doyle, 1993). The *spv* genes are essential for efficient systemic infection beyond the intestines in orally inoculated mice as a model for enteric fever (Guiney *et al.*, 1995; Libby *et al.*, 1997). The *spvR* gene encodes a LysR-like trancription factor, and it is an essential positive regulator of its own expression, and of the *spvABCD* operon (Guiney *et al.*, 1995; Libby *et al.*, 1997). RpoS is an alternative sigma factor that is induced upon entry into stationary phase and by stress (Chen *et al.*, 1995). Expression of *spv* genes is RpoS-dependent, which results in the *in vitro* activation of *spv* expression on the entry into stationary phase (Coynault *et al.*, 1992; Norel *et al.*, 1992). IHF (integration host factor) and Lrp (leucine-responsive regulatory protein) have also been shown to regulate *spv* gene expression (Marshall *et al.*, 1999). Lrp interacts directly with the *spvA* promoter region to negatively regulate transcription by occluding the SpvR binding site, while IHF binds upstream of *spvR* to stimulate its transcription and, thus, the transcription of the *spv* genes (Marshall *et al.*, 1999)

The pSLT plasmid also harbours the *pef* fimbrial operon, which is homologous to the *pap* system of *E. coli* (Friedrich *et al.*, 1993). The expression of *pef* fimbriae is induced by acidic pH, and as in the *pap* operon, is regulated at the transcriptional level by Lrp and Dam methylase (Nicholson and Low, 2000; van der Woude *et al.*, 1995). Pef fimbriae mediate adhesion to the mouse intestinal epithelium, and a *pefC* fimbrial usher mutant gave a two-fold increase in the LD_{50} oral dose (Baumler *et al.*, 1996a). The *pef* operon is also found in *Salmonella* strains associated with other animals and may facilitate colonisation (Baumler *et al.*, 1997).

1.3 The phages of *S. enterica*

Horizontal gene transfer is thought to be the main mechanism that drives the evolution of *Salmonella* pathogenicity (Figueroa-Bossi *et al.*, 2001). *S.* Typhimurium serovars contain a range of distinct prophages, some of which confer virulence traits. The virulent strain, SL1344, harbours the lambdoid phages *Gifsy*-1 and -2, as well as ST64B and SopEΦ. Strain ATCC 14028s contains *Gifsy*-1, -2 and -3 in its genome, with the latter absent from SL1344. ATCC 14028s does not contain SopEΦ. The strain LT2 contains the prophages *Gifsy*-1 and -2, and *Fels*-1 and -2.

The fully functional *Gifsy* prophages are known to contribute to virulence in mice: curing of the *Gifsy*-2 prophage resulted in >100-fold attenuation, whereas curing for the *Gifsy*-1 reduced virulence slightly, but only in the absence of *Gifsy*-2 (Figueroa-Bossi and Bossi, 1999). *Gifsy*-2, when released as a phage, is also capable of transferring its virulence-conferring traits to cured attenuated strains (Figueroa-Bossi and Bossi, 1999). The *Gifsy*-3 prophage of ATCC 14028s encodes *pagJ* and *sspH1* (Gunn *et al.*, 1998; Miao *et al.*, 1999). The *Gifsy* prophages are all capable of infecting and lysogenising strains from serovars other than Typhimurium (Figueroa-Bossi *et al.*, 2001). The *Fels* phages are specific to strain LT2 (Yamamoto, 1969). These phages are fully functional and can produce infectious particles upon induction (Affolter *et al.*, 1983). They are morphologically and serologically unrelated to P22, a natural phage of *Salmonella*, but are capable of recombination with P22 at low frequencies (Yamamoto, 1969). *Fels*-1 has been shown to contain *nanH*, a neuraminidase gene, and a new *sodC* gene, *sodCIII*, for superoxide dismutase (Figueroa-Bossi *et al.*, 2001).

The SopEΦ phage of SL1344 is closely related to the *Fels*-2 phage (Pelludat *et al.*, 2003). SopEΦ carries the virulence factor SopE. SopE is secreted into host cells as an effector protein by SPI-1 (Figueroa-Bossi *et al.*, 2001), where it modulates host cell signalling, membrane ruffling and host cell invasion (Hardt *et al.*, 1998). Lysogenic conversion of strain ATCC 14028s with SopEΦ, which it does not normally contain, enhances the invasiveness of ATCC 14028s in cultured cells, and its enteropathogenesis in a bovine infection model (Pelludat *et al.*, 2003; Zhang *et al.*, 2002). SL1344 also contains the ST64B phage. ST64B is a defective, lambdoid phage with a mosaic genetic structure, which can be induced by treatment with mitomycin C (Mmolawa *et al.*, 2003). ST64B forms tailless phage particles and is therefore defective, but can revert to form functional phage particles on occasions. Coculture of ST64B-containing strains with an isogenic strain lacking the prophage leads to active phage forms (Figueroa-Bossi and Bossi, 2004). Fragments of the virulence genes *sspH2, sopE* and *orgA* are found in the genome of ST64B. ST64B is also present in the genome of the *S.* Typhimurium epidemic strain DT64, where it was first identified (Mmolawa *et al.*, 2003). However, ST64B does not appear to contribute to virulence in the mouse model (Alonso *et al.*, 2005).

1.4 The nucleoid

The estimated length of the linearised chromosome of *E. coli* is 1.5 mm, whereas the cylindrical cell measures 1 μm by 0.5 μm of which the nucleoid occupies approximately half (Pettijohn, 1996). Molecular crowding and supercoiling contribute to the considerable compaction of DNA that is required (Luijsterburg *et al.*, 2006). Chromosome condensation is also achieved by nucleoid-associated proteins (NAPs), which act as architectural elements: NAPs wrap and package the DNA, but also introduce bending or coiling (Drlica and Rouviere-Yaniv, 1987; Luijsterburg *et al.*, 2006). This conformation still allows DNA transactions such as replication, recombination, repair, and transcription to occur. The genomic DNA of *Escherichia coli* is associated with a core set of 10-20 DNA-binding proteins, which form the nucleoid (Pettijohn, 1996).

1.4.1 Nucleoid-associated proteins

The nucleoid-associated proteins (NAPs) are functionally similar, but do not show homology (Azam and Ishihama, 1999). These proteins also function in DNA replication,

transcriptional regulation and recombination (McLeod and Johnson, 2001). The best-characterised NAPs of *S.* Typhimurium include Fis, H-NS, HU and IHF. IHF and Fis bind to regions of DNA that contain a consensus binding site sequence. IHF binds with a high degree of specificity, whereas Fis binds to a more degenerate consensus sequence (Finkel and Johnson, 1992; Goosen and van de Putte, 1995). Fis mediates the formation and stabilisation of planar DNA loops, which can bend DNA 160° (Travers and Muskhelishvili, 2005). H-NS is believed to be dimeric, but can form larger multimers in solution (Smyth *et al.*, 2000). Scanning force microscopy has shown that has shown that H-NS-DNA complexes allow bridging between adjacent DNA duplexes (Dame *et al.*, 2000; Dame *et al.*, 2005). H-NS has recently been shown to bind to and silence DNA regions containing a high A+T content, and thus a high degree of intrinsic curvature (Lucchini *et al.*, 2006; Navarre *et al.*, 2006). HU also binds DNA without sequence specificity (Azam and Ishihama, 1999). In *E. coli,* HU has been shown to contribute to DNA looping (Becker *et al.*, 2007). HU produces many flexible bends in DNA, of approximately 180°, by random, non-specific DNA binding (Sagi *et al.*, 2004). It is also capable of reducing DNA length by 50 % (van Noort *et al.*, 2004).

The NAPs of *S.* Typhimurium have also been shown to regulate *Salmonella* virulence determinants: Fis has been shown to affect the expression of SPI-1 and SPI-2 genes (Kelly *et al.*, 2004; Lim *et al.*, 2006; Ó Cróinín *et al.*, 2006; Schechter *et al.*, 2003; Wilson *et al.*, 2001). H-NS is a regulator of *spv*, *csg* fimbriae and SPI-1 genes (Gerstel and Römling, 2003; O'Byrne and Dorman, 1994; Schechter *et al.*, 2003), HU has been shown to affect the control of SPI-1 expression, and IHF is a regulator of *spvR* of pSLT (Marshall *et al.*, 1999; Schechter *et al.*, 2003).

The leucine-responsive regulatory protein (Lrp) is a small, basic DNA-binding and bending regulatory protein that is present in the cell in large numbers (3000 dimers/cell), thus making it possible that Lrp is a DNA-organising protein, like the nucleoid-associated proteins Fis, H-NS, IHF and HU (Newman, 1996). A recent study has shown that Lrp is capable of binding to non-specific DNA sequences, which supports this idea, as nucleoid-associated proteins tend to have low DNA-binding sequence specificity (Peterson *et al.*, 2007). This study suggests that Lrp may have evolved from having a role in the condensation of the bacterial nucleoid, to a global and local gene regulatory role.

1.5 Lrp

The Lrp protein of *S.* Typhimurium is a transcriptional regulator and a member of the Lrp/AsnC family of regulatory proteins. The archetypal and best-characterised member of the Lrp/AsnC family of regulatory proteins is Lrp of *E. coli.* Extensive studies on this family member have examined many complex aspects of Lrp structure and regulation. Comparatively few analyses of the *lrp* gene and Lrp protein of *S.* Typhimurium have been performed: these are listed in the relevant sections that follow. However, henceforth, in this chapter, Lrp refers to *E. coli* Lrp, unless otherwise stated.

1.5.1 The structure of Lrp

1.5.1.1 The functional domains of the Lrp monomer

The Lrp monomer of *E. coli* has been divided into three domains based on analysis of mutations in Lrp and their ability to affect expression of the *ilvIH* promoter (Platko and Calvo, 1993). Mutations in a putative helix-turn-helix (HTH) domain in the N-terminal third affected the ability of Lrp to bind DNA, which suggested that the N-terminus contains the DNA binding region. The flexible linker region third was shown to have residues important for the activation of the *ilvIH* promoter by Lrp. Mutations in the C-terminal third of the monomer produced leucine response mutants: Lrp no longer showed leucine-mediated repression of the *ilvIH* operon (Platko and Calvo, 1993). In support of this, the recently solved crystal structure of Lrp of *E. coli* confirmed the domains of the monomer fold (de los Rios and Perona, 2007).

The N-terminal HTH consists of three α-helices with a tight turn between two of the three helices (de los Rios and Perona, 2007) (Fig. 1.2 (A)). The third recognition helix binds in the major groove of DNA. The C-terminal domain contains the so-called 'regulation of amino acid metabolism' (RAM) domain. RAM domains are structural features common to amino acid metabolic enzymes and transcriptional regulators, the structural confirmation of which is affected by amino acid binding (Ettema *et al.*, 2002). In Lrp, this domain binds the effector molecule, leucine. The C-terminus, and hence RAM domains, are composed of a βαββαβ fold forming a four-stranded antiparallel β-sheet that is flanked by two α-helices (de los Rios and Perona, 2007). The position of leucine binding is believed to be contained within the β3-β4 loop (Fig. 1.2 (A)). Monomer-monomer interactions occur at high affinity, having an estimated dissociation

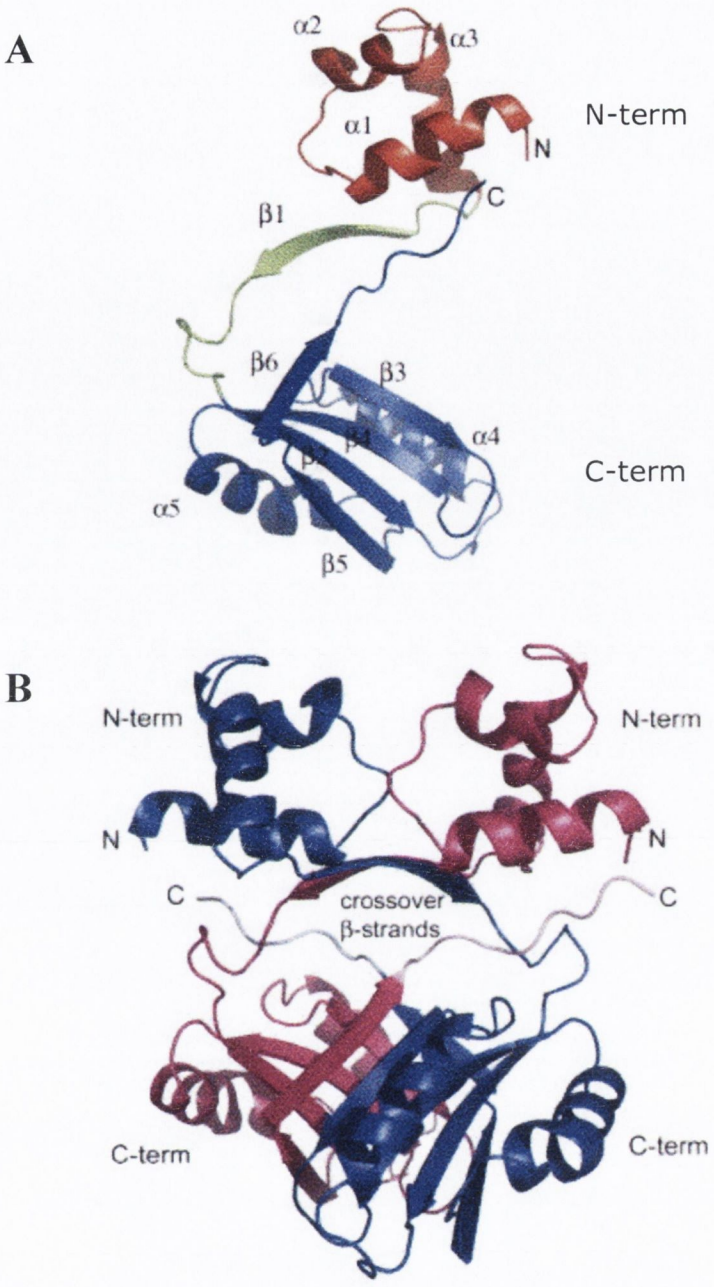


Fig. 1.2. Crystal structure of Lrp. The crystal structure of Lrp of *E. coli* has been solved. The monomer (A) shows an N-terminal HTH motif of three α-helices with a tight turn between two of the three helices. The C-terminus is composed of a βαββαβ fold forming a four-stranded antiparallel β-sheet that is flanked by two α-helices. The dimer (B) forms in a head-to-head orientation, with the flexible β-strand linker domains of each monomer crossing over each other. A second crossover of the C-terminal β-strand, β6, joins the β-sheet in the opposite subunit. Figures taken from de los Rios and Perona, 2007.

constant of less than 100 fM (Chen *et al.*, 2005), and the smallest unit of DNA binding is the Lrp homodimer (Cui *et al.*, 1996). The Lrp monomer dimerises in a head-to-head orientation, with the flexible β-strand linker domains of each monomer crossing over each other. A second crossover of the C-terminal β-strand, β6, joins the β-sheet in the opposite subunit. This results in the N-terminus of one monomer placed over the C-terminus of the other monomer (de los Rios and Perona, 2007)(Fig. 1.2 (B)).

The structure of Lrp of *S.* Typhimurium LT2 is expected to be similar to that of *E. coli* K-12, as the amino acid sequences are 99 % identical (Friedberg *et al.*, 1995). Friedberg *et al.* report two amino acid substitutions, however, sequence comparisons show only one: the threonine at position 95 in *E. coli* is substituted for an alanine in *S.* Typhimurium, which is considered a conservative substitution in terms of charge and side chain structure. The position of this substitution is not thought to significantly affect the structure of Lrp: the *S.* Typhimurium *lrp* gene was able to complement the *E. coli* *lrp-35*::*Tn*10 allele of strain CV1008 (Friedberg *et al.*, 1995).

1.5.1.2 Multimerisation and the leucine response of Lrp

Lrp is capable of self-association to form higher-order oligomers of octamers and hexadecamers. This requires the C-terminus of Lrp, which has been shown to be involved in dimer and multimer formation (Chen *et al.*, 2001b). The concentration of Lrp influences its multimerisation: at concentrations of 50 nM, Lrp dimers are the predominant species. However, at micromolar concentrations of Lrp in the absence of L-leucine, the association state of Lrp is hexadecamer and octamer *in vitro*, with hexadecamers predominating (Chen *et al.*, 2001b). Multimerisation of Lrp is also influenced by the effector molecule, L-leucine. Lrp has two leucine-binding sites, each with a different affinity for L-leucine: the high affinity site binds L-leucine with micromolar dissociation constants, while the low affinity site binds it with millimolar dissociation constants (Chen and Calvo, 2002). The binding of L-leucine to the low affinity site on Lrp affects the association state of Lrp: the presence of millimolar concentrations of L-leucine leads to leucine-bound octamer predominance (Chen and Calvo, 2002).

The first crystal structure of a member of the Lrp/AsnC family to be solved was that of LrpA of *Pyrococcus furiosus* (Leonard *et al.*, 2001). LrpA, like Lrp of *E. coli*, forms a

homodimer mainly through interactions between the antiparallel β-sheets of its C-terminal domain, and further interactions lead to octamer formation. X-ray crystallisation revealed a symmetrical octamer structure, composed of a tetramer of LrpA homodimers in ring formation, around a central hole (Fig. 1.3 (A)). The more recent crystal structures of two other Lrp homologues, AsnC of *E. coli*, and LrpC of *Bacillus subtilis*, have revealed similar symmetrical octameric assemblies (Thaw *et al.*, 2006), with 90° angles between the dimer 2-fold axes. However, the recently solved crystal structure of Lrp of *E. coli* reveals a novel octameric assembly. One of the four dimer-dimer interfaces, which are closed to form a ring structure in the crystal structures of the Lrp homologues, are disrupted in Lrp of *E. coli,* forming an asymmetrical unit (Fig. 1.3 (B)). This results in a 110° angle between the disrupted dimer 2-fold axes, an 80° angle at the opposite side, and 85° angles between the two other intradimer axes (de los Rios and Perona, 2007). Importantly, Lrp of *E. coli* was the only protein in the family to be crystallised in the presence of DNA, which may have led to the change in protein conformation. The authors suggest verification of the structure in the absence of DNA (de los Rios and Perona, 2007).

1.5.2 DNA binding by Lrp

1.5.2.1 The Lrp consensus binding site sequence

Comparison of the DNA sequences of twelve sites to which Lrp was shown to bind by methidiumpropyl-EDTA (MPE) footprinting, led to the elucidation of a preliminary 13-bp consensus binding site sequence (Wang and Calvo, 1993a; Wang *et al.*, 1993). However, this consensus sequence was not discernible in other known Lrp-regulated gene promoter regions. The consensus sequence was verified and extended by analysis of 63 sequences using SELEX (systematic evolution of ligands by exponential enrichment). This gave rise to the degenerate consensus binding site sequence YAGHAWATTWTDCTR (Cui *et al.*, 1995), a 15 bp, part-palindromic sequence, where Y= C/T, H= A/T/C, W= A/T, D=A/T/G and R= A/G. It has been demonstrated that a double-stranded 15-bp fragment closely matching this consensus sequence does not bind Lrp, as Lrp requires at least 3 bp of DNA flanking the recognition sequence to allow binding (Cui *et al.*, 1996). The consensus sequence has been used to successfully identify Lrp binding sites in promoters (Lahooti *et al.*, 2005; Rhee *et al.*, 1996), but does not always correctly predict Lrp binding sites: it has been shown recently that DNA

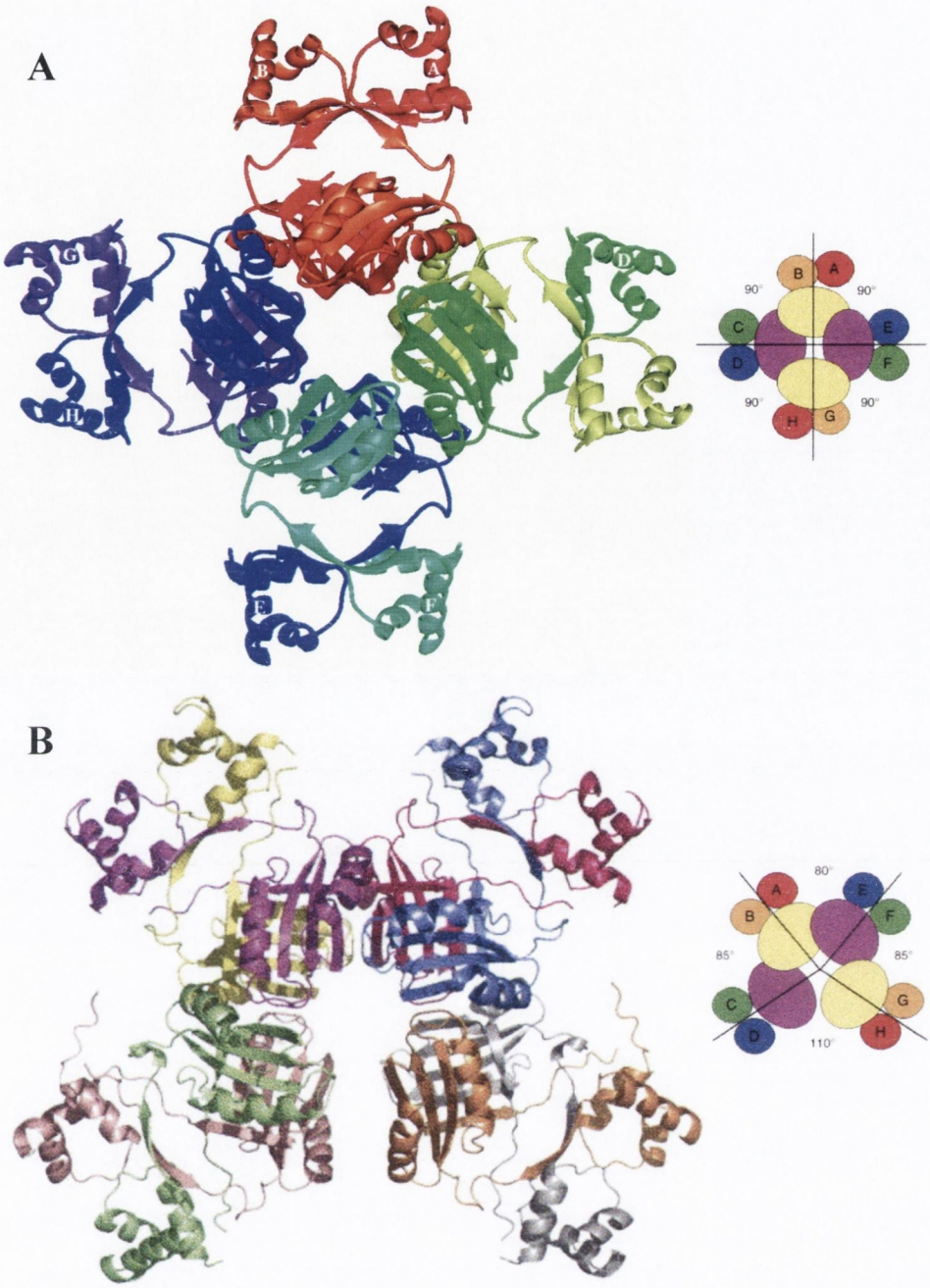


Fig. 1.3. Comparison of the crystal structures of LrpA of *P. furiosus,* and Lrp of *E. coli*. LrpA (A) was shown to have a symmetrical octameric assembly, with 90° angles between the dimer 2-fold axes (Leonard *et. al,* 2001; de los Rios and Perona, 2007). However, the structure of Lrp of *E. coli* (B) reveals a disruption between one dimer-dimer interface, forming an asymmetrical unit, with 110° angle between the disrupted dimer 2-fold axes, an 80° angle at the opposite side, and 85° angles between the two other intradimer axes (de los Rios and Perona, 2007). Figures adapted from Leonard *et. al*, 2001 and de los Rios and Perona, 2007.

secondary structure may also influence the ability of Lrp to recognise certain DNA sequences (Pul *et al.*, 2007).

1.5.2.2 Binding and bending of DNA by Lrp

Lrp often binds to multiple sites at promoters over which it exerts regulatory control. Circular permutation assays have estimated that DNA bends approximately 52° upon binding of an Lrp dimer to a single binding site, and at least 135° in a DNA molecule containing two adjacent sites (Wang and Calvo, 1993b). A model of *pap* operon DNA binding generated using the crystal structure of Lrp of *E. coli* showed bending angles in agreement with the previous study (de los Rios and Perona, 2007). In addition, this model also suggested a bending angle of 180° or more over three Lrp binding sites (Fig. 1.4). The hypothesis of DNA-wrapping around an Lrp protein core, which has been suggested by DNase I footprinting analyses in many studies, is supported by this model. Multiple Lrp binding sites are often spaced by approximately 30 bp, which is approximately equal to 3 helical turns: examples of this are found many Lrp-regulated genes, including the *pap, dadAX,* and *ilvIH* operons (de los Rios and Perona, 2007; Jafri *et al.*, 2002; Zhi *et al.*, 1999). This is also supported by the positions of the binding sites in relation to the spacing of dimers, shown in Figure 1.4 (de los Rios and Perona, 2007).

The binding of multiple sites, and subsequent bending and wrapping of DNA by Lrp leads to the protection of large regions, often over 100 bp, from DNase I-mediated cleavage (Jafri *et al.*, 2002; Mathew *et al.*, 1996; Wiese *et al.*, 1997), which is also supported by the model shown in Figure 1.4 (de los Rios and Perona, 2007). DNase I footprinting of DNA-binding and bending proteins often results in the appearance of hypersensitive sites. Bending of a DNA segment leads to the narrowing of the minor groove in the inside of the bend, and a widening of the minor groove on the outside of the bend (Drew and Travers, 1985). Hypersensitivity occurs when DNA cleavage by DNase I is facilitated by the widening of the minor groove (Drew and Travers, 1984). Phased hypersensitivity is a feature that is seen at many Lrp-regulated promoters (Gazeau *et al.*, 1994; Nou *et al.*, 1995; Stauffer and Stauffer, 1994; Wang and Calvo, 1993b; Wiese *et al.*, 1997). The phased hypersensitive sites are often located on one face of the double helix, which together with analysis of regions of protection, can enable elucidation of the direction of DNA bending by Lrp (Wiese *et al.*, 1997).

1.5.2.3 Cooperative binding of Lrp to DNA

Lrp binds DNA cooperatively, where binding of Lrp to one site strongly influences the binding of Lrp to additional adjacent sites. This was first described in studies on the well-characterised *ilvIH* operon (Wang and Calvo, 1993a). The *ilvIH* operon, of which Lrp is a positive regulator, contains six Lrp binding sites within a 200-bp region, two within an upstream region (sites 1 and 2, centred at –231), and four within a downstream region (sites 3, 4, 5, and 6, centred on –95). Lrp was shown to bind cooperatively to the two upstream sites 1 and 2, as mutations in site 2 reduced binding to both sites 1 and 2 by 10 fold (Wang and Calvo, 1993a). Binding to sites 3, 4, 5 and 6 was also found to be highly cooperative. DNA addition mutations that changed both the phasing and the distance between upstream and downstream binding sites substantially reduced promoter expression, indicative of cooperative binding between upstream and downstream sites (Jafri *et al.*, 2002). Cooperative Lrp-binding to adjacent DNA sites has been identified in many gene promoter regions, including the *pap* system (Kaltenbach *et al.*, 1995) and the *ilvGMEDA* operon (Rhee *et al.*, 1996). In the *gltBDF* operon, Lrp binds to three sites to allow maximal expression. Lrp appears to bind a central palindromic site, allowing additional dimers to bind flanking sites to form a nucleoprotein complex (Wiese *et al.*, 1997). Very recently, it has been suggested that cooperativity of DNA-binding favours non-specific DNA interactions at sites adjacent to specific interaction sites (Peterson *et al.*, 2007).

1.5.3 Regulation of *lrp* gene expression

1.5.3.1 The *lrp* gene

The transcriptional start site of the *lrp* gene was identified by primer extension analysis and is located 267 bp upstream of the translational start codon: the function of the long untranslated region is unknown (Wang *et al.*, 1994). The predicted promoter contains a perfect –35 element, TTGACA, and a –10 element, GAAAAC, with three mismatches to the perfect *E. coli* σ^{70} promoter sequence TATAAT (Wang *et al.*, 1994).

The nucleotide sequence of the *lrp* ORF of *S.* Typhimurium is 88 % identical to that of *E. coli* (Friedberg *et al.*, 1995). However, the transcriptional start site of the *S.* Typhimurium *lrp* gene is unknown. A putative promoter sequence, assigned by genome annotation, places a –35 and a –10 element centred at approximately 71 bp and 44 bp,

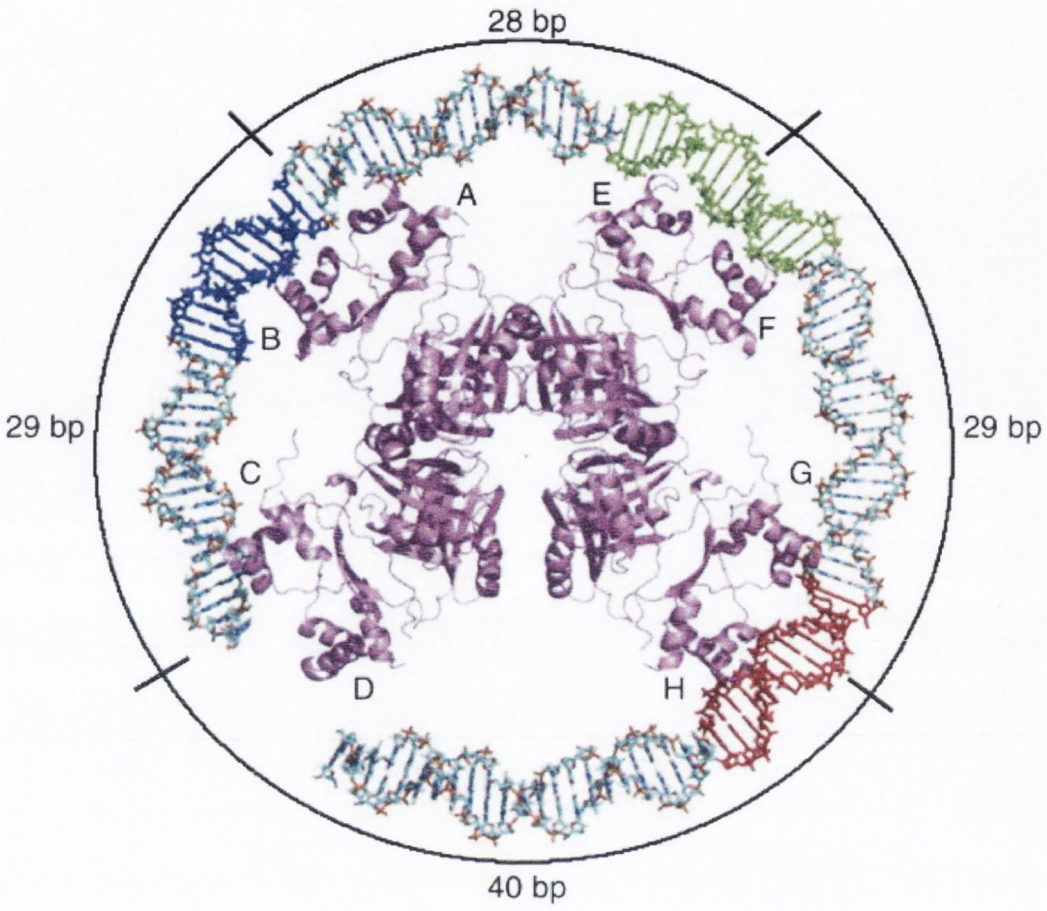


Fig. 1.4. Model of the Lrp-*pap* DNA complex. The crystal structure of Lrp of *E. coli* was solved in the presence of *pap* DNA. The known Lrp binding sites are shown in this model in blue, green and red on the DNA structure. This model suggested a bending angle of 180° or more over three Lrp binding sites. The hypothesis of DNA-wrapping around an Lrp protein core, suggested by DNase I footprinting analyses in many studies, is supported by this model. Multiple Lrp binding sites are often spaced by approximately 30 bp, this is supported by the spacing of the dimer-DNA interfaces, shown in this model. This figure is taken from de los Rios and Perona, 2007.

respectively, upstream of the translation initiation codon of *lrp* (McClelland *et al.*, 2001). If this putative promoter of *S.* Typhimurium is functional, a much shorter 5' untranslated region would be found in the *lrp* transcript of *S.* Typhimurium than in *E. coli*.

1.5.3.2 Regulation of *lrp* expression

Expression of *lrp* is regulated by a number of factors. Firstly, *lrp* expression is regulated by nutrient availability. During growth in minimal medium, *lrp* expression levels are high. Two independent analyses estimate the concentration of Lrp, in *E. coli* strains CSH26 and BE3479, during growth in MOPS minimal medium supplemented with glucose to be 5 μM, or approximately 3,000 dimers per cell (Borst *et al.*, 1996; Willins *et al.*, 1991). In rich medium, *lrp* promoter activity and Lrp protein levels are repressed by 3-4 fold (Landgraf *et al.*, 1996). The positive effects on *lrp* transcription in minimal media are mediated by the stringent response nucleotide, guanosine 3',5'-bispyrophosphate or ppGpp (Landgraf *et al.*, 1996). ppGpp levels are increased in the cell in response to amino acid or energy source limitations, and are inversely related to growth rate (Cashel, 1996). ppGpp directly interacts with RNA polymerase to influence transcription of promoters (Chatterji *et al.*, 1998; Reddy *et al.*, 1995; Toulokhonov *et al.*, 2001). *lrp* expression levels are also inversely related to growth rate, and it is believed that growth rate may affect Lrp levels through ppGpp (Landgraf *et al.*, 1996).

Analysis of *lrp-lacZ* reporter fusions identified that the *lrp* gene is negatively autoregulated (Wang *et al.*, 1994). Lrp is believed to exert this effect by direct interaction with two sites upstream of the *lrp* promoter. Wang *et al.* describe two sequences in the *lrp* promoter that match the Lrp consensus binding sequence at –67 to –53 and at +14 to +28. Mutational analysis of the DNA sequence from –60 to –53 abolished Lrp-binding and reduced Lrp-mediated repression of *lrp* expression (Wang *et al.*, 1994). This has been the only Lrp binding site to be verified by mutational analysis in the *lrp* promoter.

1.5.4 Factors that affect gene expression by Lrp

1.5.4.1 The sensitivity of Lrp-regulated genes to Lrp protein concentration

Lrp-regulated genes vary in their sensitivities to Lrp concentrations. *In vivo* titration studies of the Lrp binding of different promoter regions have been used to determine the relative sensitivities of Lrp-regulated genes. Borst *et al.* examined the effect of varying Lrp levels on the expression of *gltBDF* in the presence and absence of 30 mM leucine (Borst *et al.*, 1996). Their method involved the determination of apparent dissociation constants of the Lrp-DNA complex that activates expression of *gltB::lacZ*. This gave a dissociation constant of 2.0 nM in the absence of leucine, and 6.6 nM in the presence of 30 mM leucine. The dissociation constant of the Lrp-*ilvIH* complex was determined to be 6.9 nM in the absence of leucine, and 14.1 nM in the presence of 30 mM leucine, indicating that the Lrp-*ilvIH* complex has a lower affinity for Lrp than the Lrp-*gltB* complex (Borst *et al.*, 1996). Wang *et al.* determined the dissociation constant of an Lrp-*lrp* DNA complex interaction by estimation of the fraction of total DNA that formed a complex, against Lrp protein concentration. Under these conditions, the dissociation constant for Lrp-*lrp* was 35 nM, approximately 4 times higher than their estimated Lrp-*ilvIH* dissociation constant of 8 nM (Wang *et al.*, 1994), indicating a lower affinity of Lrp for the promoter region of *lrp* than *ilvIH*. Chen and Newman analysed half-maximal induction of *serA* and *gltD* by controlling *lrp* expression from a plasmid with an arabinose-inducible promoter. They conclude that the affinity of Lrp for *serA* was 4-5-times greater than for *gltD* (Chen and Newman, 1998). In addition, the affinity of Lrp for the *gcv* and *gltD* promoter regions has been reported as being higher than that of the *sdaC* gene (Newman, 1996). It is believed that the varying sensitivities of Lrp-regulated genes may reflect the varying levels of Lrp in response to nutrient availability (Ernsting *et al.*, 1993).

1.5.4.2 The effect of leucine on the expression of Lrp-regulated genes

The effect of leucine on Lrp is highly complex. Many studies have been performed and these findings are summarised in this section. As discussed in section 1.5.1.2, leucine can bind to two sites on Lrp to influence its multimerisation. The effect of leucine is believed to be relevant to the regulatory patterns exhibited by Lrp (Chen *et al.*, 2001b).

Six different patterns of regulation by Lrp have been observed, which depend on whether Lrp has a positive or a negative effect on gene expression, and whether this regulation is affected by leucine. Where Lrp acts as a positive regulator of expression, leucine may antagonise the effect, be required for the effect, or may have no effect.

Where Lrp acts as a negative regulator of expression, the same cases apply: leucine may antagonise or potentiate the negative effect, or may have no effect. Given the effects that leucine has on Lrp multimerisation, a hypothesis to account for the diverse regulatory patterns described was suggested by Chen and Calvo. They propose that some Lrp-regulated genes and operons must preferentially respond to Lrp that does not contain leucine, some respond to Lrp as a leucine-bound octamer, and others respond to both (Chen and Calvo, 2002). Genes that are activated or repressed by hexadecameric or octameric states would be expected to show reduced activation or repression in the presence of leucine, as leucine converts hexadecamer and octamer to leucine-bound octamer. Similarly, genes activated or repressed by leucine-bound octamer are expected to require leucine for the effect of Lrp. Leucine is expected to have no effect on genes that are repressed or activated by hexadecameric, octameric, or leucine-bound octameric forms of Lrp (Chen and Calvo, 2002).

The effect of leucine on the distribution of Lrp in the cytoplasm (free Lrp) and the nucleoid (DNA-bound Lrp) was examined using minicells. The addition of leucine to MOPS medium caused a 2-3-fold reduction in the amount of free Lrp (Chen *et al.*, 2001a). Addition of nutrients into the MOPS medium, to make rich MOPS medium, reduced the total level of Lrp by three fold, consistent with previous studies (Landgraf *et al.*, 1996), and reduced the level of free Lrp by four fold, compared with nutrient-poor MOPS medium (Chen *et al.*, 2001a). Chen and Calvo also showed that the intracellular concentrations of hexadecamer, free octamer and leucine-bound octamer are strongly affected by growth in these media (Chen and Calvo, 2002). They found that in minimal medium, Lrp exists predominantly as a hexadecamer, with smaller amounts of octamer and leucine-bound octamer in the ratio 70:18:12, respectively. Addition of leucine to MOPS medium decreased the amount of hexadecamer and octamer by 10 fold and 3 fold, respectively, but doubled the amount of leucine-bound octamer, changing the ratio in the presence of leucine to 18:15:67 (Chen and Calvo, 2002). To summarise, this indicated that the hexadecameric form (predominant in minimal medium) has a weaker affinity for DNA than the octameric form of Lrp (predominant in MOPS medium with leucine), as the amount of free Lrp was reduced 3-4 fold by addition of leucine to MOPS medium.

Much analysis of the effect of leucine on Lrp has been performed in conjunction with the *ilvIH* operon. The addition of leucine to minimal medium leads to an Lrp-mediated 5-10-fold decrease in *ilvIH* expression (Squires *et al.*, 1981). Chen *et al.* hypothesised that low levels of *ilvIH* expression would be associated with leucine-bound octamer, and that as a corollary, leucine-bound octamer is a poor activator and hexadecamer is a good activator of *ilvIH* (Chen *et al.*, 2005). To evaluate the effectiveness of octamer and hexadecamer as activators, they used a mutated Lrp, Lrp-1, which forms octamers but no significant amounts of hexadecamer, and does not respond to leucine: leucine does not affect the expression of *ilvIH* in an *lrp-1* strain. They found that Lrp-1 could interact with, and activate *ilvIH* expression as well as Lrp, which suggested that *ilvIH* expression does not require a hexadecamer (Chen *et al.*, 2005). This also suggested that Lrp interactions over the octamer level are unimportant for *ilvIH* expression. The cooperative Lrp dimer-dimer interactions that lead to long-range interactions between distant sites on DNA are known to be important for *ilvIH* expression (Jafri *et al.*, 2002). Chen *et al.* conclude that leucine must affect *ilvIH* expression by enhancing dimer-dimer interactions that lead to octamer formation, but reduce dimer-dimer interactions that lead to long-range interactions. Therefore, the mutation in Lrp-1 is expected to cause dimer-dimer interactions that primarily lead to strong long-range interactions, rather than weaker octamer formation (Chen *et al.*, 2005). Finally, they suggest that leucine may increase the cooperative binding of Lrp to adjacent sites on the *ilvIH* promoter. How leucine might do this has not been experimentally examined.

1.5.5 Lrp-regulated genes in *E. coli*

Lrp has a well-characterised role as a regulator of metabolism, but it has also been shown to regulate diverse gene types, such as fimbrial genes and plasmid conjugation genes. High-throughput DNA microarrays estimate that approximately 10 % of *E. coli* genes are affected by Lrp in MOPS minimal medium (Hung *et al.*, 2002).

1.5.5.1 Genes involved in metabolism

Lrp has been described as a stimulator of anabolic pathways, and a repressor of catabolic pathways. Landgraf *et al.* suggest that in rich media where there are available precursors, growth rates are high and the level of ppGpp is low, which leads to low Lrp levels. With low Lrp levels, the tendency is towards catabolic pathway induction, with repression of anabolic reactions. Conversely, in minimal media, ppGpp levels are high,

thus Lrp levels are high, which results in upregulation of anabolic operons, and repression of catabolism (Landgraf *et al.*, 1996). Lrp is best characterised for its role in the regulation of genes involved in amino acid transport and metabolism. Some of these genes are discussed below.

The best-characterised amino acid biosynthetic operon regulated by Lrp is the *ilvIH* operon, which encodes acetohydroxy acid synthase III (AHAS III), one of three isoenzymes present in *E. coli* K-12, which is the first enzyme in the biosynthesis of branched-chain amino acids. As described in section 1.5.2.3, the *ilvIH* operon is positively regulated by Lrp, and this positive regulation is reduced in the presence of leucine. The effect of leucine on Lrp has been extensively studied in this system (see section 1.5.4.2). Lrp also positively regulates the *leuABCD* operon, for leucine biosynthesis, but this is not thought to be direct (Landgraf *et al.*, 1999).

Lrp is an essential positive regulator of the *gcvTHP* operon, which is a relatively leucine-insensitive operon (Stauffer and Stauffer, 1994). The *E. coli lrp* mutant is unable to utilise glycine as the sole nitrogen source, because of severely down-regulated *gcvTHP* expression (Lin *et al.*, 1992).The *gcvTHP* operon is involved in the catabolism of glycine, in the reaction shown below (Calvo and Matthews, 1994):

$$\text{glycine} + \text{NADP}^+ + \text{tetrahydrofolate} \rightarrow \text{CO}_2 + \text{NH}_3 + \text{NADPH} + \text{methylenetetrahydrofolate}$$

Lrp interacts directly at multiple sites in the *gcvTHP* promoter region and induces curvature in the DNA upon bending, allowing the interaction of another positive regulator, GcvA, with RNA polymerase (Stauffer and Stauffer, 1998). The structural role for Lrp was shown to be important for *gcv* regulation by replacement of the Lrp binding sites with the I1A IHF binding site from phage λ, which also induces DNA curvature (Stauffer and Stauffer, 1999).

Lrp is also involved in the regulation of the catabolism of threonine to acetyl CoA and glycine, by oxidation to 2-amino-3-ketobutyrate, followed by cleavage. The two enzymes that perform this are 2-amino-3-ketobutyrate coenzyme A lyase and threonine dehydrogenase, products of the *kbl-tdh* operon, respectively (Rex *et al.*, 1991). Lrp represses the expression of these genes, and leucine partly relieves this repression (Lin *et al.*, 1990; Rex *et al.*, 1991).

Lrp negatively affects the expression of the leucine transport operons, *livJ* and *livKHMGF*. The *livJ* product, LIV-I, binds to isoleucine, leucine and valine, while the *livK* product, LS, is a leucine-specific periplasmic binding protein (Anderson and Oxender, 1977). The membrane components, encoded by *livHMGF*, are shared by the two transport systems (Landick and Oxender, 1985). The two operons are turned off only in the presence of high leucine concentrations in an Lrp-dependent manner (Ernsting *et al.*, 1992; Landick and Oxender, 1985). Strains that lack Lrp were shown to have a high constitutive level of *livJ* and *livK* expression, and in an *lrp-1* mutant strain, which encodes an Lrp mutant that is insensitive to leucine, *livJ* and *livK* expression were no longer leucine-repressed (Haney *et al.*, 1992).

1.5.5.2 Fimbrial genes

Lrp is also a well-characterised regulator of fimbrial gene expression. Phase variation of the *fim* operon in *E. coli* is controlled by DNA inversion (Abraham *et al.*, 1985). The promoter for *fim* transcription is carried on an invertible DNA element, *fimS*, and the expression of the *fim* structural genes depends on the orientation of *fimS*. The FimB and FimE recombinases catalyse the inversion of *fimS* (McClain *et al.*, 1991), which also requires Lrp. Lrp binds to two sites in *fimS*, where it positively influences DNA inversion (Blomfield *et al.*, 1993; Gally *et al.*, 1994), and leucine is known to enhance this effect (Roesch and Blomfield, 1998). Recently, Lrp has also been shown to interact with DNA supercoiling to influence switching of *fimS* (Kelly *et al.*, 2006).

Lrp regulates the expression of the P-regulatory *pap* operon in conjunction with Dam methylase. The *papBA* operon contains two sets of Lrp-binding sites called [4, 5, 6] and [1, 2, 3], which are located distal and proximal to the *papBA* promoter, respectively (Hernday *et al.*, 2003). Lrp has a higher affinity for [1, 2, 3] and binding of this site blocks *papBA* transcription as [1, 2, 3] overlaps the *papBA* promoter, resulting in a phase OFF phenotype (Braaten *et al.*, 1994; Weyand and Low, 2000). Binding of Lrp to [4, 5, 6] is mediated by PapI, a *pap* regulatory protein, which is essential for *papBA* transcription, and leads to a phase ON phenotype (Kaltenbach *et al.*, 1995). Furthermore, methylation of GATC sequences within [4, 5, 6], called GATC I, and [1, 2, 3], called GATC II, modulates phase ON-OFF switching. Addition of a methyl group to GATC I reduces the affinity of Lrp, locking transcription in phase OFF, until DNA replication

takes place: similarly, binding of Lrp to GATC I blocks Dam methylation, allowing a phase ON phenotype (Nou *et al.*, 1995; van der Woude *et al.*, 1998). The P-regulatory family of fimbriae, including *sfa, daa, foo, clp* and *fae* fimbriae, are characterised by their phase variable expression, regulation by Lrp and a PapI homolog, the spacing of the two GATC sites by 102-103 bp, and the influence of differential methylation status of these GATC sites (Harel and Martin, 1999).

1.5.5.3 Genes involved in anaerobic metabolism

In *E. coli*, comparative DNA microarray analyses of FNR and ArcA (Salmon *et al.*, 2003), which respond to oxygen availability, and Lrp (Hung *et al.*, 2002) have revealed that these regulators have overlapping regulons. A number of previously unidentified genes that are regulated by ArcA are also regulated by Lrp under aerobic conditions, and by FNR under anaerobic conditions (Salmon *et al.*, 2003).

1.5.6 Lrp-regulated genes in *Salmonella*

Comparatively little is known about Lrp of *Salmonella*, and few Lrp-regulated genes in *S. enterica* have been identified. The majority of these are *Salmonella*-specific genes, present on the large virulence plasmid, pSLT.

Camacho and Casadesús have shown that Lrp is required for the expression of *traJ* of pSLT (Camacho and Casadesús, 2002). *traJ* encodes a transcriptional activator of the remaining transfer operon. Two Lrp binding sites, LRP-1 and LRP-2, located upstream of the *traJ* promoter contain GATC sequences, which become methylated by Dam (Camacho and Casadesús, 2005). Methylation of the GATC sequence in LRP-2 hinders the binding affinity of Lrp. Non-methylated DNA allows the occupation of Lrp at both LRP-1 and LRP-2, which is essential for *traJ* transcription. In addition, hemi-methylated DNA produces strand specific results: methylation of the coding strand inhibits the binding of Lrp to LRP-2, whereas methylation of the non-coding strand allows the Lrp-*traJ* complex to form, promoting transcription, and suggesting that Lrp will be able to bind to the *traJ* promoter in only one daughter plasmid (Camacho and Casadesús, 2005).

A role for Lrp in regulation of the *spvABCD* genes of pSLT has also been identified (Marshall *et al.*, 1999). As described in section 1.2.2.7, Lrp represses *spvA* and its repression is relieved by the presence of leucine. It is believed that Lrp occludes the

binding site of the essential positive *spv* regulator, SpvR, which prevents *spv* transcription. High leucine levels reduce the occupation of this site, which allows SpvR binding, and thus, *spvABCD* expression (Marshall *et al.*, 1999). The effect of Lrp on *spv* expression was more severe during growth in intracellular salts medium, which mimics some of the conditions encountered by bacteria in the intracellular niche, than in LB, suggesting that Lrp may have a more significant role in *spv* expression *in vivo* than *in vitro* (Marshall *et al.*, 1999). This was also the first indication that Lrp is a regulator of genes that are important in the intracellular environment, and hints that Lrp may be a regulator of virulence genes in *Salmonella.*

The phase variable *pef* (plasmid-encoded fimbriae) system of *S.* Typhimurium pSLT plasmid is analogous to the *pap* system of *E. coli* (Nicholson and Low, 2000), and is a member of the P-regulatory family of fimbriae. Lrp is required for the expression of *pef* genes, which is regulated by methylation of GATC sequences within Lrp binding sites. However, unlike the *pap* system of *E. coli*, the *pef* system has three GATC sequences, GATC I, GATC II, and GATC X: methylation of GATC II is important for *pef* expression, whereas methylation of both GATC I and GATC X inhibit transcription (Nicholson and Low, 2000). This is similar to the *pap* system, whereby methylation of GATC I leads to repression whereas methylation of GATC II is essential for transcription. PefI acts negatively, in contrast to its homolog PapI, by allowing binding of Lrp to GATC II, as opposed to GATC I, thereby preventing *papBA* transcription (Nicholson and Low, 2000).

1.5.7 Regulatory proteins that are associated with Lrp

Lrp generally interacts with other regulatory proteins at Lrp-regulated promoters. It is expected that this leads to the formation of transcription complexes that represent the regulatory requirements of certain promoters, allowing them to be expressed appropriately (Newman, 1996). The P-regulatory fimbriae, which are discussed above, are characterised by their phase variable expression, the spacing of two GATC sites by 102-103 bp, and the influence of differential methylation of the GATC sites (Harel and Martin, 1999). In addition, these fimbrial operons encode a PapI-homolog. The PapI homolog interacts with Lrp to determine fimbrial phase variation (Kaltenbach *et al.*, 1995).

Transcriptional regulators, such as the nucleoid-associated proteins IHF and H-NS are often found at Lrp-regulated promoters. IHF has been shown to interact with Lrp at gene promoters. At the *osmY* promoter region, Lrp binds in the presence of IHF, while the presence of CRP leads to its exclusion from this promoter sequence (Colland *et al.*, 2000). IHF and Lrp also interact at the *gltB* promoter, and are required for activation of *gltB*. The effect of IHF and Lrp is antagonised by CRP (Paul *et al.*, 2007). At the *serA* promoter, Lrp and cAMP/CRP are rival activators. Lrp activates the P1 promoter, and represses the P2 promoter of *serA*, whereas CRP activates transcription from the P2 promoter. The effect of CRP is inhibited by Lrp (Yang *et al.*, 2002).

H-NS has been shown to affect Lrp activity at the *ilvIH, kbl-tdh* and *gcv* promoters (Landgraf *et al.*, 1994; Levinthal *et al.*, 1994). It has been shown that Fis, H-NS and Lrp are all capable of binding to the upstream regions of the seven rRNA P1 promoters of *E. coli*, *rrnA, rrnB, rrnC, rrnD, rrnE, rrnG and rrnH* (Hillebrand *et al.*, 2005; Pul *et al.*, 2005). More recently, it has been shown that H-NS facilitates the formation of an Lrp-DNA complex at the rRNA *rrnB* promoter, where Lrp acts as a transcriptional repressor. It is believed that H-NS makes transient contacts with the DNA target which allow higher order Lrp-DNA complex formation (Pul *et al.*, 2007).

1.5.8 Aims and objectives

The Lrp regulon in *S.* Typhimurium is unknown: however, previous analyses of Lrp-regulated gene expression in *S.* Typhimurium have shown that Lrp is associated with virulence genes (Marshall *et al.*, 1999; Nicholson and Low, 2000). The aim of this study was to construct an *lrp* mutant in *S.* Typhimurium SL1344, which was then to be used to elucidate the Lrp regulon in *S.* Typhimurium, using DNA microarray technology. Genes that were identified by DNA microarrays were then examined in depth to gain further understanding of gene regulation by Lrp in *S.* Typhimurium.

Chapter 2

Materials and Methods

2.1 Chemicals and growth media

2.1.1 Chemicals, reagents and radionucleotides

The suppliers for each chemical/reagent used are indicated in parentheses after the product. DNA restriction and modifying enzymes were obtained from New England Biolabs, or Roche Molecular Biochemicals. Radionucleotides were supplied by Amersham GE Biotech. Commercial sequencing was performed by GATC Biotech, Konstanz, Germany. Several molecular biology kits were utilised in this study: the basic principles of which are outlined in the relevant sections.

2.1.2 Growth media

Ingredients for preparation of rich media were obtained from Bacto, Difco, or Oxoid. All rich media were sterilised by autoclaving at 120 °C for 20 min prior to use. Ingredients for preparation of minimal media were obtained from Sigma, or BDH. Minimal media constituents, and other solutions not suitable for autoclaving, e.g. antibiotic solutions, were sterilised by filtration through sterile 0.2 μm Acrodisc Syringe Filters (Pall). Quantities for preparation of media listed below are for 1 litre, in sterile MillQ deionised distilled water (ddH_2O). Note that minimal media were prepared with sterile AnalaR grade ddH_2O, commercially available from BDH. Media were supplemented with the appropriate antibiotics as required. Media were allowed to cool to 50 °C before the addition of heat-labile solutions, e.g. antibiotic solutions.

LB (lysogeny broth) media were routinely used for culture of bacteria in nutrient-rich media (Miller, 1992) (Bertani, 1951). Lennox medium, a low salt variation of LB medium (Lennox, 1955), was also used in this study.

Lennox LB medium (L medium)

Lennox media were routinely used for revival of bacterial strains from glycerol stocks, general culturing of strains, and selection of transformants and transductants.

L broth:	10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl.
L agar:	10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl, 15 g agar.

LB medium

LB media were used for microarray analysis of SL1344 in rich media, and for subsequent analyses resulting from these data.

LB broth: 10 g Bacto-tryptone, 5 g Bacto-yeast, 10 g NaCl.

LB agar: 10 g Bacto-tryptone, 5 g Bacto-yeast, 10 g NaCl, 15 g agar.

MOPS minimal medium broth

MOPS minimal medium is a chemically defined medium used for the analysis of the Lrp regulon in nutrient-poor conditions. As SL1344 is a histidine auxotroph, all minimal media without a source of amino acids must be supplemented with 0.5 mM L-histidine. For analysis of the effect of leucine on the Lrp regulon, MOPS minimal media were supplemented with 10 mM L-leucine.

10X MOPS minimal broth:

400 ml 1.0 M potassium morpholinopropane sulfonate (MOPS), freshly prepared
40 ml 1.0 M N-Tris(hydroxymethyl)-methyl glycine (Tricine), freshly prepared
Adjust to pH 7.4 with KOH

Add in order:
10 ml 0.01 M $FeSO_4$, freshly prepared
50 ml 1.9 M NH_4Cl
10 ml 0.276 M K_2SO_4
0.25 ml 0.02 M $CaC1_2 \bullet 2H_2O$
2.1 ml 2.5 M $MgCl_2$
100 ml 5.0 M NaCl
0.2 ml micronutrient solution
(Add to 40 ml ddH_2O: 9 mg $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$; 62 mg H_3BO_3; 18 mg $CoCl_2$, 6 mg $CuSO_4$, 40 mg $MnCl_2$, 7 mg $ZnSO_4$. Bring total volume to 50 ml.)
387 ml AnalaR ddH_2O

The 10X MOPS minimal solution is filter sterilised and stored in aliquots at –20 °C.

MOPS minimal medium:

100 ml 10X MOPS minimal medium
10 ml 132 mM K_2HPO_4
10 ml 40 % (w/v) glucose
2.5 ml 200 mM L-histidine
877.5 ml AnalaR ddH_2O

This is adjusted to pH 7.2 and sterilised by filtration.

MOPS minimal medium agar

MOPS minimal agar:

875 ml AnalaR ddH_2O and 15 g agar are mixed and autoclaved. The solution is cooled to 50 °C, and the following solutions, and antibiotics if required, are added.

100 ml 10X MOPS minimal medium
10 ml 132 mM K_2HPO_4
10 ml 40 % (w/v) glucose
2.5 ml 200 mM L-histidine

M9 minimal medium

M9 minimal medium is a nutrient-poor medium. It is prepared from a sterilised 10X concentrate of M9 salts.

M9 broth:

60 g Na_2HPO_4-$7H_2O$, 15 g K_2HPO_4, 2.5 g NaCl, 5 g NH_4Cl. The pH is adjusted to 7.4.

After sterilisation, 100 ml M9 concentrate, 2 ml 1 M $MgSO_4$, 100 µl 1 M $CaCl_2$, 10 ml 40 % (w/v) glucose are added to 981 ml sterile dH_2O, plus antibiotics where required.

SOC medium

SOC medium is a rich recovery medium used following electroporation of *S.* Typhimurium to increase efficiency of transformation.

SOC medium: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl. After autoclaving, 1 ml 1 M $MgCl_2$, 1 ml 1M $MgSO_4$, and 2 ml 1M glucose are added.

Motility agar

Motility agar was used to prepare swarm plates to determine the motility of SL1344. The swarm plates are inoculated centrally, and incubated at 37 °C for 8 to 12 h. The rate of spreading of bacteria was examined by measurement of the diameter of the swarm.

Motility agar: 3 g agar, 10 g Bacto-tryptone, 5 g NaCl.

Green agar

Green agar plates were routinely used following bacteriophage P22-mediated generalised transduction, to obtain isolates of *S.* Typhimurium free of phage. On Green plates, phage-free colonies appear light green, whereas pseudolysogens appear dark green/blue. Excess glucose that is added to the agar is fermented by the strains, producing more acid than usually produced. When cells containing pseudolysogens lyse, the pH indicator incorporated into the agar causes the colonies to turn a dark green/blue colour.

Green agar: 8 g tryptone, 1 g yeast extract, 5 g NaCl, 15 g agar.
After autoclaving, 21 ml 40 % (w/v) glucose, 25 ml 2.5 % (w/v) Alizarin yellow (freshly prepared) and 3.3 ml 2 % (w/v) Aniline blue were added.

2.1.3 Antibiotics, X-Gal and IPTG

All antibiotic stock solutions were stored at –20 °C. Solutions prepared in dH_2O were sterilised by filtration through 0.2 μm Acrodisc filters (Pall). Carbenicillin, kanamycin, and spectinomycin were prepared as 50 mg ml^{-1} solutions in ddH_2O, and used to supplement media at a concentration of 50 μg ml^{-1}. Chloramphenicol was prepared as a 25 mg ml^{-1} stock solution in 95 % ethanol, and was used at a final concentration of 25 μg ml^{-1}. Tetracycline was prepared in 95 % ethanol as a stock solution of 12.5 mg ml^{-1}, and was used at a final concentration of 12.5 μg ml^{-1}.

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), a chromogenic substrate for β-galactosidase, was prepared as a 20 mg ml^{-1} stock solution in *N, N*-dimethyl formamide and stored in darkness at –20 °C. X-gal was used in agar plates at a final concentration of 40 μg ml^{-1}.

IPTG (isopropyl β-D-1-thiogalactopyranoside), a molecular mimic of allolactose, was prepared as a 1 M stock solution in dH_2O, and stored at –20 °C. It was used at a final concentration of 1 mM.

2.2 Bacterial strains and culture conditions

2.2.1 Bacterial strains

All bacterial stains used in this study were either derivatives of *Salmonella enterica* serovar Typhimurium SL1344, LT2, or *Escherichia coli* and are listed in Table 2.1. Bacterial strains were maintained as permanent stocks in 15 % (v/v) glycerol in L media and stored at –70 °C.

2.2.2 Bacterial culture conditions

Cultures to be grown in LB broth were first cultured overnight from single colonies on L agar plates in 3 ml L broth, with antibiotic supplemented where necessary. The optical densities of the overnight cultures were measured by spectrophotometry, equalised to the same OD and 250 μl inoculated into 25 ml LB broth.

MOPS minimal broth-grown bacteria were first grown overnight in 3 ml L broth, by inoculating from single colonies on L agar plates. Two-ml volumes of each culture were harvested and washed by centrifugation at 10,000 x *g* for 5 min, removal of supernatant using suction, followed by thorough resuspension of the cell pellet in 2 ml warm MOPS minimal broth. This step was repeated to ensure removal of traces of L broth and thus residual nutrients. The cell pellets were then resuspended in 2 ml warm MOPS minimal medium, and the OD_{600} measured. The concentrations were equalised to OD_{600} of 2.5, and 500-μl volumes used to inoculate 25-ml volumes of warm MOPS minimal broth.

Bacteria were routinely cultured aerobically in liquid media at 37 °C with shaking at 250 rpm, unless where otherwise stated. Cultures were inoculated into 25-ml volumes of

Table 2.1. *S.* Typhimurium and *E. coli* strains used in this study.

Strain	Relevant genotype	Reference/Source
S. Typhimurium		
SL1344	*rpsL hisG*	(Hoiseth and Stocker, 1981)
LT2	*rpoS*	Lab stocks
CJD3129	LT2 *lrp*::*kan*	This study
CJD3130	SL1344 *lrp*::*kan*	This study
CJD3131	CJD3130 pKMC102	This study
CJD3132	SL1344 pZEP*lrp*	This study
CJD3133	CJD3130 pZEP*lrp*	This study
CJD3134	SL1344 pBAD24	This study
CJD3135	SL1344 pBAD*fimZ*	This study
CJD3136	CJD3130 pBAD*fimZ*	This study
CJD3137	SL1344 pZEP*ompR*	This study
CJD3138	CJD3130 pZEP*ompR*	This study
CJD3139	SL1344 pZEP*ssrA*	This study
CJD3140	CJD3130 pZEP*ssrA*	This study
CJD3141	SL1344 *lrp*::*kan ompR*::*Tn*10	This study
CJD3142	CJD3141 pZEP*ssrA*	This study
CJD3143	CJD365 pZEP*ssrA*	This study
CJD365	SL1344 *ompR*::*Tn*10	Lab stocks
CJD2089	SL1344 *fis*::*cat*	(Keane and Dorman, 2003)
CJD2090	SL1344 *rpoS*::*kan*	(Keane, 2002)
CJD2112	SL1344 *hns*::*kan*	(Keane, 2002)
E. coli		
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*qZΔM15 *Tn10*]	Stratagene
BL21 λDE3 pLysS	F^- *dcm ompT hsdS gal* Cml^R	(Studier and Moffatt, 1986)
VL386 *lrp*::*Tn10*	*lrp*::Tn10 ϕ (*fimA-lacZ*) λ pL(209) *fimE*::*IS*1	(Kelly *et al.*, 2006)

liquid media in 250 ml conical flasks. Overnight cultures were grown in 3 ml L broth in culture tubes. Bacteria were cultured on agar plates by incubation at 37 °C for 12 to 16 h.

2.2.3 Growth of SL1344 in media of acidic pH

Bacteria were cultured as described previously (Lee *et al.*, 2000). Briefly, strains were grown statically overnight in 3 ml L broth at 37 °C. Bacteria were harvested and washed with PBS, before inoculation into 3 ml MOPS minimal medium at pH 7.2 or adjusted to pH 4.5. Bacteria were incubated without shaking at 37 °C in a jet waterbath for the appropriate amount of time, before sampling for flow cytometry.

2.3 Plasmids, bacteriophage and oligonucleotides

Plasmids used in this study are listed in Table 2.2 with relevant details and source. Necessary details of plasmid construction will be detailed in the appropriate chapters. Generalised transduction was carried out using the bacteriophage P22HT105/1 *int*-201. Phage lysates were stored at 4 °C in 5 ml volumes, over chloroform. The sequences and features of all oligonucleotides used in this study are listed in Table 2.3. Oligonucleotides were purchased from MWG-Biotech, Germany.

2.4 Transformation of bacterial cells with plasmid DNA

Plasmid DNA was transformed by two distinct mechanisms. Recipient cells were either made competent by treatment with calcium chloride, coupled with a heat-shock triggered uptake of plasmid DNA, or made competent for transformation by electroporation, a high-voltage electroshock treatment.

2.4.1 Preparation of calcium chloride-competent cells

Bacterial cells to be made competent were grown by overnight culture, and used to inoculate 100 ml of Lennox broth. The cells were grown to an OD_{600} of 0.5 to 0.6. The cells were incubated on ice for 10 min, and then harvested by centrifugation at 10,000 x *g*, at 4 °C for 10 min. The bacterial pellet is resuspended in 50 ml ice-cold $CaCl_2$ solution (0.1 M $CaCl_2$) and incubated on ice for 30 min. Cells were harvested again as above, resuspended in 2 ml ice-cold 0.1 M $CaCl_2$, 10% glycerol (v/v) solution, and then incubated on ice for at least 1 h. Cells were distributed into 100 µl aliquots and stored at –70 °C.

2.4.2 Transformation of calcium chloride-competent cells

0.1-1 μg DNA, in a volume not exceeding 10 μl, is added to 100 μl competent cells, and incubated on ice for 20-40 min. This allows the DNA to come into contact with the bacterial surface. The samples are heat-shocked by incubation for 1.5 min at 42 °C, followed by incubation on ice for 30 s. The heat-shock treatment allows uptake of the plasmid DNA through the $CaCl_2$-induced competent cell membrane, by an unknown mechanism (Mandel and Higa, 1970). Five hundred μl L broth warmed to 37 °C is added to the culture, and the culture is incubated for 1 h at 37 °C with shaking at 200 rpm to allow for phenotypic expression of the plasmid-borne resistance cassette. Samples are then plated onto appropriate selection plates. Following overnight incubation at 37 °C, single-colony transformants were purified on fresh selective agar plates.

2.4.3 Preparation of electro-competent cells

Bacterial cells to be made competent were grown by overnight culture, and used to inoculate 1 l of Lennox broth. The cells were grown to an OD_{600} of 0.5 to 0.6. The cells were harvested by centrifugation at 5,000 x *g* for 15 min. The bacterial pellet is resuspended in 1 l sterile ddH_2O at 4 °C, incubated on ice for 30 min, pelleted by centrifugation as above, and resuspended in 500 ml sterile ddH_2O at 4 °C. Cells were then harvested and resuspended in 20 ml ddH_2O, 10 % (v/v) glycerol at 4 °C. Cells were again harvested as above, resuspended in 2 ml ddH_2O, 10 % (v/v) glycerol at 4 °C, distributed into 40 μl aliquots and stored at –70 °C.

2.4.4 Transformation of electro-competent cells

0.1-1 μg DNA, in a volume not exceeding 4 μl, was added to 40 μl electro-competent cells, and incubated on ice for 20 to 40 min to allow the DNA to come into contact with the bacterial surface. The samples were transferred to a 2 mm electroporation cuvette (Cell Projects) and placed in the electroporation chamber set to 2500 V, with resistance at 200 Ω. The cells were pulsed and 500 μl SOC broth warmed to 37 °C was added. The mixture was transferred to 1.5 ml tubes and incubated for 1 h at 37 °C to allow for phenotypic expression of the plasmid-borne resistance cassette. Samples were then plated onto appropriate selection plates. Following overnight incubation at 37 °C, single colony transformants were purified on fresh selective agar plates.

Table 2.2. Plasmids used in this study.

Plasmid	Relevant characteristics	Reference / Source
pCL1921	pSC101 replicon, Spc^R, Str^R	(Lerner and Inouye, 1990)
pHP45Ω*kan*	Contains Ω element-flanked kanamycin resistance cassette	(Blondelet-Rouault *et al.*, 1997)
pKMC101	1.3 kb *lrp* gene and flanking regions cloned into pCL1921	This study
pKMC101Ω*kan*	Kanamycin-disrupted *lrp* gene in pKMC101	This study
pZC320	F replicon, Amp^R	(Shi and Biek, 1995)
pKMC102	*lrp* gene cloned into pZC320	This study
pZEP08	Promoterless *gfp*, Cml^R, Amp^R	(Hautefort *et al.*, 2003)
pZEP*lrp*	612 bp *lrp* promoter region upstream of promoterless *gfp* of pZEP08	This study
pZEP*ompR*	363 bp *ompR* promoter region upstream of promoterless *gfp* of pZEP08	This study
pZEP*ssrA*	*ssrA* promoter upstream of promoterless *gfp* of pZEP08	(Ó Cróinín *et al.*, 2006)
pKOBEGA	Arabinose-inducible λ Red system, Amp^R, T_s origin of replication	(Chaveroche *et al.*, 2000)
pBAD24	pMB1 replicon, Amp^R	(Guzman *et al.*, 1995)
pBAD*fimZ*	*fimZ* ORF downstream of the arabinose-inducible promoter of pBAD24	This study
pET22b	Protein expression vector, Amp^R	Novagen
pKMC301	*lrp*-6xHis under control of T7*lac* promoter of pET22b	This study
pBluescript II SK (–)	Cloning vector, Amp^R	Stratagene
pBSK*fimZ*	317 bp *fimZ* promoter in pBluescript	This study
pBSK*lrp*	418 bp *lrp* promoter in pBluescript	This study
pBSK*ompR*	290 bp *ompR* promoter in pBluescript	This study
pPHI-1	*Eco*RV phage DNA fragment in pBluescript	This study
pPHI-2	*Eco*RV phage DNA fragment in pBluescript	This study

pPHI-4	*Eco*RV phage DNA fragment in pBluescript	This study
pPHI-8	*Eco*RV phage DNA fragment in pBluescript	This study
pPHI-10	*Eco*RV phage DNA fragment in pBluescript	This study
pPHI-14	*Eco*RV phage DNA fragment in pBluescript	This study
pPHI-12	*Eco*RV phage DNA fragment in pBluescript	This study
pPHI-16	*Eco*RV phage DNA fragment in pBluescript	This study
pPHI-17	*Eco*RV phage DNA fragment in pBluescript	This study
pPHI-18	*Eco*RV phage DNA fragment in pBluescript	This study

Table 2.3. Oligonucleotides used throughout this study.

Name	Sequence (5'-3')
Rev1.3	GGG CAA TAA GTA TCA ACA ACG CTT CCA AAA G
Fwd1.3	GTT GTT GGC AGA CAA TGA GCA GAA TTG TAG G
Rev1.3-*Pst*I	ATA CTG CAG GGG CAA TAA GTA TCA ACA ACG CTT CCA AAA G
Fwd1.3-*Eco*RI	ATA GAA TTC GTT GTT GGC AGA CAA TGA GCA GAA TTG TAG G
DIG-*lrp*-F	CAA CGT CGA GCT TTC TAA ACG AGT AGG
DIG-*lrp*-R	GCG TGT CTT AAT AAC CAG ACG ATT AC
lrp-pET22b-F	TAC CAT ATG GTA GAT AGC AAG AAG CGC
lrp-pET22b-R	GGC TGC TCG AGG CGT GTC TTA ATA ACC AG
fimZ-F.*Bam*HI	ATA GGA TCC GAT TAT ACC TGG TCT GAT TTC
fimZ-R.*Eco*RI	ATA GAA TTC TAT TTA CAA CTC GTC CTG GTA AG
fimZ-BAD-F	AAA CCT GCA TCT GTT ATC ATT ATG GAC G
fimZ-BAD-R.*Hin*dIII	ATA AAG CTT AAC GCG GAT GCG ACC TTC
lrp-F.*Bam*HI	ATC GGA TCC GTT GTT GGC AGA CAA TGA G
lrp-R.*Eco*RI	GCG GAA TTC GCC TGG TCT ATG GTG AGA AG
ompR-F.*Bam*HI	GTG GGA TCC GGG CGC TCT TCA CG
ompR-R.*Eco*RI	CAA GAA TTC GCA ACA AAT GTA AGT GTG TAT TC
PE-*lrp*	GAA TGT TAC GAT CGA TAC GGT CGA GAT CTT TG
pBSK-F	GCA AGG CGA TTA AGT TGG GTA AC
pBSK-R	GTG TGG AAT TGT GAG CGG ATA AC
*fimA*1/3-R	ATT AAC GAC GAG GGA TTA CGG
*fimA*2/3-F	AAC AAT TCA TCA CCC TGG C
*fimA*2/3-R	TCC CCT GAG TAT CAG ACG CAG
*fimA*3/3-F	GTG ACG AAA TGT CAT ATT CGC
fimA-F	CCT GAA CTT TTT GAG CAA CCT C
fimA-R.Bio	GGA GTA GGA TCA GCC GCA AC
fimW-F.Bio	TGA CTG TTT TGT TCC TTA ATA GC
fimW-R	GCG CCT TGT GAA GTG AAG AC
fimZ-F.Bio	ATA ACA GAT GCA GGT TTC ATT G
fimZ-F.Bio-2	GAG CTA TTT TCT TTA GAG TTG AC
fimZ-F.3	AAT AAC AGA AAG ATG TTG ACG C
fimZ-F.4	GTG GAG CTA TTT TCT TTA GAG TTG

fimZ-F.5	GAT TAT ACC TGG TCT GAT TTC TC
fimZ-R	ACT ACT CAA TGT CAA CTC TAA AG
fimZ-R.2	TAT TTA CAA CTC GTC CTG GTA AG
fimZ-R.3	CAC AGG CAA AGC TTT CTT C
lrp-EMSA-F	GTT GTT GGC AGA CAA TGA GC
lrp-EMSA-F3	TGT AAA TAC CAT GTT TAC CGG
lrp-EMSA-F4	ATC GAT GTT TTG CTT TGA C
lrp-EMSA-F5	GAC AGC GAC GTT ATC ATC AC
lrp-R.Bio	GCC TGG TCT ATG GTG AGA AG
lrp-EMSA-R3	GTT CAT CGG TTC ATG CTA TTA C
lrp-EMSA-R4	CAG TTA TTC ATT AGA GAG GGC G
lrp-EMSA-R5/Bio	TAC ATG CAT GAT TAT GCA GC
StyLP-10-F	TGA CAG TTT ACC GCC CTC TC
EcLP-10-F	TGA TGA ATA AAC GCC CCT G
LP-10-R.Bio	CTC GAA TGT TTT CGC AAA AC
ompR-EMSA-F.1	CTT CAC GCC AGA GAT AAT TG
ompR-EMSA-F.2	AGC TGC TGT TAA ATA TGC TTT G
ompR-EMSA-F.3	TGT CGA TAT TGC GCA CAC GGG G
ompR-R.Bio.1	CGT CAT CAA CCA CCA GAA TC
ompR-R.Bio.2	TGA ATT TCA GGC TAA ATT GTT AC
ssrA-EMSA-F	ATA CCT TGT CAC AGG CGA TTC T
ssrA-EMSA-F.2	GCA AGA CAA GGC TTA GGT AAG C
ssrA-EMSA-R-Bio	GAT GTT TGC AGC GTA TTC TTG A
ompR-gfp-F	ATA TCT AGA CGC ATA TCG TCA TCA ACC
ompR-gfp-R	ATA GCG GCC GCT CTT CAC GCC AGA G
lrp-gfp-F	ATA CCC GGG GTT GTT GGC AGA CAA TG
lrp-gfp-R	CGC TCT AGA TAA GAA TGT TAC GAT CG
dadA-RT-F	GGG CTT TAA TAC CGA CTT AC
dadA-RT-R	CCG ACA AGA TAT CGC TTA AC
fimA-RT-F	GTT GCG GCT GAT CCT AC
fimA-RT-R	GTC CGC AGA GGA GAC AG
fimF-RT-F	TTC ATC GCT ATC GGT TGT
fimF-RT-R	CAG CAA GCG CCA GTA AT
fimH-RT-F	CGC GCT CTT TTT CAC C

fimH-RT-R	CGC CCA GAA GGT AGT CA
fimW-RT-F	AAC AGT CAC TTT GAG CAT GG
fimW-RT-R	ATT TTC CGG GTA ATT TCT TC
fimY-RT-F	TGA CAA CTA CCT CGG CTA TTC
fimY-RT-R	GCC ATA CGG ATA AAC TGT G
fimZ-RT-F	ATA ACA GAT GCA GGT TTC ATT G
fimZ-RT-R	ACT ACT CAA TGT CAA CTC TAA AG
hilE-RT-F	GTT CCG TCC TTG TCA GC
hilE-RT-R	AGA GGC CTG GTG TAG CA
iadA-RT-F	GGA GTT TAC CTT ATT AC
iadA-RT-R	CCG ACG ACC GTT GTT AT
leuA-RT-F	AAC AGC AAG AAG AAC CAG AG
leuA-RT-R	GCG TTC AGG TCG TAT TTA AC
livJ-RT-F	GGA TGT ATC GCC CTG TC
livJ-RT-R	CAG CTA CCG CCT GTT TC
livK-RT-F	GAA TAA AGC GAT TGT CGA AG
livK-RT-R	TTA AGA TCG CCT TTT TCA TC
osmY-RT-F	CGA AAC CAA TCA GAA AGT TG
osmY-RT-R	TTC CTG AGT TTC AAC GGT AC
sdaC-RT-F	AAG GCT TTA ACG GGA TGG TG
sdaC-RT-R	GTA CTT TCT GGA TGG CAT AC
serA-RT-F	TCG CAG GAA AAC TGA TTA AG
serA-RT-R	AAT TAC CAC ATA CCC CAT TC
ssaB-RT-F	AAG GCA TTC CAT TAA TTC AG
ssaB-RT-R	GCT GTA TAG CAT AAT CAT GGA C
ssaG-RT-F	TGC ACA ATT AGT GGA TAT GC
ssaG-RT-R	TTA GCA ATG ATT CCA CTA AGC
ssaI-RT-F	GTA AAG TCC TCT GCA GAA CC
ssaI-RT-R	CAT GTT ATC CAG CTT ATT AAG
sseA-RT-F	GGC TGC GTT TAG TGA ATA TC
sseA-RT-R	GGG CTT GAG CAT TAA GTT G
ssrA-RT-F	GAT TTA TCC GTT CTA TCC GAG
ssrA-RT-R	CAG AGG ACT GCA GAA AGA G
STM2800-RT-F	GTC AAC ATT TTG ATT GCA TG

STM2800-RT-R	AGC TGC TCA CTT TAA ACA GG
STM3038-RT-F	GTA TTG CGG TGC TGT TAT G
STM3038-RT-R	CTG TTT TAT TGC TGG TTT TG
STM4510-RT-F	ACA TCC CGC TCA TTG TC
STM4510-RT-R	ATA TCC CCC AGG GTA GC
yhhK-RT-F	TCA TTA AGC GTA GAT GAA ACG
yhhK-RT-R	ACC TAA CGC CTG CAT AAA C
yjiE-RT-F	GTG CCG CAA TTT CTC TC
yjiE-RT-R	GCG GCA ATC TTG ATT TT
yjiG-RT-F	CGA CCA ACC TGT TAC CC
yjiG-RT-R	GGC CGA CAT TCT GTA CC
yjiH-RT-F	CGC GTT ATA CCG TCC AC
yjiH-RT-R	CCC TTT ATC CCG ACC TT
P22-F	GCT TCA TGA TGT GCT GAG AC
P22-R	GTT TGC CAT TGA ACA CGC C
sb21-F	ATA GGA TCC TGC CGG TTA TTG CTG ATG
sb21-R	AGA GAA TTC CGG CAA AAT ATG GTC ACG

2.5 Allelic replacement

2.5.1 Preparation of electro-competent cells for allelic replacement using pKOBEGA-containing cells.

The bacteriophage λ Red recombinase system consists of the following genes: *exo,* producing lambda exonuclease, which processively digests the 5'-ended strand of a dsDNA end; *bet*, encoding beta protein, which binds to ssDNA and promotes strand annealing; and *gam,* producing gamma protein, which binds to the host RecBCD enzyme and inhibits its activities (Poteete, 2001). The plasmid pKOBEGA contains the λ Red genes *exo*, *bet,* and *gam*, the expression of which are under the control of an arabinose-inducible promoter. When expressed, these genes produce the λ Red recombinase machinery, which allows recombination of homologous DNA sequences at a high frequency. pKOBEGA is a low copy-number, temperature-sensitive (T_s) plasmid, which is maintained at 30 °C, but lost at 37 °C (Datsenko and Wanner, 2000).

Bacterial cells were transformed with pKOBEGA as described in section 2.4.4. pKOBEGA-containing cells were grown by overnight culture at 30 °C, and used to inoculate 1 l of L broth with antibiotics. The cells were grown at 30 °C to an OD_{600} of 0.4. The expression of the *exo, bet,* and *gam* genes was induced by the addition of 0.2 % arabinose. Growth is continued for approx. 2 h at 30 °C (OD_{600} of 0.6 to 0.7). The bacteria were harvested and made electro-competent as described in section 2.4.3.

2.5.2 Allelic replacement using pKOBEGA-containing cells

Pfu polymerase was used to PCR amplify the disrupted *lrp* gene and flanking regions from pKMC101ΩKm using primer pair Fwd1.3-plain and Rev1.3-plain. The PCR products were pooled and subsequently digested with *Dpn*I for 2 h. *Dpn*I digests only methylated DNA, so digests the plasmid template but not the PCR product. The desired product of 3504 bp was purified from the PCR reactions by agarose gel electrophoresis, and ethanol precipitated. The DNA was resuspended to 0.1 μg μl^{-1}, and 4 μl was transformed into the pKOBEGA-containing strain as described in section 2.4.4. The cells were recovered for 2 h at 37 °C to encourage loss of the T_s pKOBEGA, and plated on L agar supplemented with kanamycin. Plates were grown at 37 °C overnight. Colonies were replica-plated on kanamycin and kanamycin/ampicillin to check for

plasmid curing. DNA from Kan^R Amp^S colonies was transduced into fresh background strains by P22 transduction.

2.6 Transduction with bacteriophage P22

Bacteriophage P22 is a specific phage of *Salmonella*. Its receptor is the O-antigen of lipopolysaccharide on the outer membrane of the cell. Phage P22 binds and injects double-stranded linear DNA into the bacterial host. The DNA circularises and replicates by O replication, and then by rolling circle replication to generate concatemers of double-stranded P22 DNA. The concatemers are resolved by cleavage by a phage-encoded nuclease that cuts the DNA at Pac sites, which are 44 kb apart. After 50-100 phage particles have been produced, the DNA is packaged into new phage particles that are released by the host by lysis.

The P22 derivative used in this study for generalised transduction was P22 HT105/1 *int*-201 (Schmieger, 1972). This high transducing (HT) phage contains a nuclease with lower specificity for Pac sites. This increases the likelihood of packaging of host genomic DNA, whereby approximately 50 % of phage heads carry random fragments of host DNA. This phage also carries a mutation in the *int* gene, which is required for phage integration into host DNA, thus preventing phage lysogeny.

2.6.1 Preparation of P22 phage lysate

Overnight starter cultures of the lysate strain were used to inoculate 5 ml of L broth by 1:1,000 dilution, and incubated at 37 °C with shaking at 200 rpm. At OD_{600} 0.15, 10 µl of P22 phage stock is added (approximately 10^8 p.f.u ml^{-1}). Growth is continued for 4 h, and then 500 µl of chloroform is added to the culture and mixed thoroughly by vortex. This is incubated at 4 °C for 1 h, and then cleared by centrifugation in bench-top centrifuge (MSE Mistral 2000) at 6,000 rpm for 20 min. The supernatant is transferred to a clean tube and a few drops of chloroform added. This is stored at 4 °C.

2.6.2 P22 phage transduction

The recipient strain is grown by overnight culture, and 100 µl is transferred to a 1.5 ml tube containing 20 µl of the donor strain P22 lysate. This mixture is incubated without shaking at 37 °C for 1 h, and then plated on L agar plates containing the relevant antibiotic. Plates are incubated at 37 °C overnight. Transduced, phage-cured bacteria are

distinguished from pseudolysogens by single colony purification two to three times on Green agar plates supplemented with the relevant antibiotic. On Green agar (see section 2.1.2), transduced, phage-free cells appear light green, while pseudolysogens appear dark blue/green. The light green colonies are then used to make glycerol stocks. True lysogens never arise due to the *int* mutation in P22 HT105/1 *int*-201.

2.7 Spectrophotometric assays

Samples were analysed on a ThermoSpectronic Genesys 10 UV spectrophotometer. Where necessary, samples were diluted to be within the spectrophotometric range of 0.1 to 1, and the values obtained corrected by multiplying by the dilution factor.

2.7.1 Monitoring bacterial growth

Bacterial growth was monitored by measurement of the optical density at 600 nm (OD_{600}). For routine measurement, 1 ml of the culture to be tested was transferred to a disposable plastic cuvette (Greiner). The OD_{600} measurement was performed using sterile medium as a blank.

2.7.2 Determination of nucleic acid concentration

Nucleic acid concentration was determined by dilution in DEPC-treated dH_2O, transfer to a quartz cuvette, and measurement of absorbance at 260 nm (A_{260}). The A_{260} measurement was performed using only DEPC-dH_2O as a blank. An OD_{260} value of 1.0 is equal to 50 μg ml^{-1} dsDNA or 40 μg ml^{-1} ssDNA or RNA. The purity of nucleic acid is determined by measuring the A_{280} value. For pure DNA or RNA uncontaminated by proteins the ratio of A_{260} to A_{280} is 1.8 and 2, respectively.

2.7.3 Determination of protein concentration by the Bradford assay

Lrp-6xHis protein concentration was determined by the Bradford assay (Bradford, 1976). The dialysis buffer components used (section 2.13) do not interfere with this assay. Known concentrations of BSA from 0 to 20 mg ml^{-1} PBS are used to produce a standard curve for protein concentration. One-ml volumes of Coomassie Brilliant Blue solution were added to 100 μl volumes of the protein standards, and to 10 μl, 50 μl, and 100μl of the rLrp in a total volume 100 μl of PBS. The samples were mixed by vortex, transferred into a 1 ml disposable cuvette, and the absorbance at 595 nm (A_{595}) read by spectrophotometry.

Coomassie Brilliant Blue solution

10.0 mg Coomassie Brilliant Blue G-250

4.75 ml absolute ethanol

10.0 ml 85 % phosphoric acid

Add dH_2O to a final volume of 100 ml. Store at 4 °C.

2.8 Assays based on flow cytometry

Flow cytometric analysis was used to monitor gene expression using a green fluorescent protein (GFP)-based assay, in which the *gfp* gene is placed under the regulatory control of the promoter of interest. The GFP protein from *Aequoria victoria* is fluorescent under UV light (Chalfie *et al.*, 1994).

2.8.1 Assay of *gfp* reporter gene expression

Samples to be analysed were harvested and fixed immediately by dilution to approximately 10^6 cells ml^{-1} into 500 µl PBS containing 3 % (v/v) formaldehyde. PBS-formaldehyde solution was freshly prepared and stored at 4 °C. Flow cytometry was performed on an EPICS-XL flow cytometer (Beckman Coulter). Ten thousand bacterial cells are analysed per sample, and the relative GFP fluorescence expressed as the mean fluorescence of the population after analysis with EXPO-32 analysis software (Beckman Coulter). Assays performed were the average of triplicate values.

2.9 Preparation of plasmid, chromosomal DNA and RNA

2.9.1 Small-scale isolation of high purity plasmid DNA

The Qiaprep Spin Miniprep kit (Qiagen) was routinely used to extract plasmid DNA from 5-10 ml cultures, according to the guidelines provided. This procedure is based on a modified alkaline lysis method. After harvesting, bacteria are resuspended in NaOH-SDS buffer in the presence of RNase. SDS leads to lysis, NaOH denatures chromosomal and plasmid DNA, and bacterial RNA is degraded by RNase. The alkaline lysate is then neutralised by the addition of acidic potassium acetate: the high salt concentration causes protein and chromosomal DNA precipitation. Plasmid DNA renatures and remains in solution. The cell debris is pelleted by centrifugation. Plasmid DNA remains

in the supernatant, which is washed and desalted through a mini-column, and eluted in 100 µl ddH_2O.

2.9.2 Large-scale isolation of high purity plasmid DNA

The QIAGEN Plasmid Midi kit was used to extract up to 100 µg plasmid DNA from 25 to 100 ml overnight cultures, depending on plasmid copy number. Purification is based on an alkaline lysis method similar to that described in section 2.8.1, followed by binding of plasmid DNA to a column-based anion-exchange resin under low salt and pH conditions. The column is washed with a medium-salt buffer to remove remaining contaminants such as RNA and proteins, and the plasmid DNA is eluted using a high-salt buffer. The plasmid DNA is desalted and concentrated by isopropanol precipitation, and resuspended with 50 to 100 µl ddH_2O.

2.9.3 Purification of chromosomal DNA

Purification of chromosomal DNA was performed using the AGTC Bacterial Genomic DNA Purification kit (Edge Biosystems). A 5-ml sample of an overnight culture was used for DNA extraction according to the guidelines provided. This procedure involves the conversion of bacteria to sphaeroplasts by incubation in a Tris-buffered solution containing lysozyme (to cleave peptidoglycan), sucrose (to prevent osmotic stress), and EDTA (to chelate divalent metal ions, cofactors for protease and DNase activity). Lysis is achieved by heating to 65 °C in the presence of SDS, to denature protein, and NaCl, to produce osmotic shock, supplemented with RNase. Latex beads are added: these bind and aggregate denatured protein and cellular debris. The mixture is cleared by centrifugation, and the supernatant precipitated with isopropanol. Chromosomal DNA is desalted and resuspended in a final volume of 100 µl ddH_2O.

2.9.4 Isolation of RNA

Briefly, bacterial cultures to be examined are mixed with 0.2 volumes of 5 % (v/v) phenol: 95 % (v/v) ethanol and incubated on ice for at least 30 min. The phenol:ethanol solution halts bacterial growth, and helps to stabilise RNA and prevent degradation (Tedin and Blasi, 1996). Samples equal to 2.0 OD_{600} units (eg. 2 ml of OD_{600} 1.0, or 4 ml of OD_{600} 0.5) were pelleted by centrifugation, and stored at –20 °C until required. The RNA is extracted from the cell pellets using the SV Total RNA Isolation kit (Promega) according to the manufacturer's instructions. The basis of this procedure is as follows:

bacterial cells are lysed by resuspension in lysozyme solution, followed by incubation in a solution containing B-mercaptoethanol and guanidine thiocyanate. This serves to inactivate the ribonucleases present in cell extracts. Guanidine thiocyanate precipitates cellular proteins, while the RNA remains in solution. After the sample is cleared of cellular debris and precipitated proteins by centrifugation, the supernatant is applied to a glass fibre spin-column. RNase-free DNase I is applied to the column to digest contaminating genomic DNA, and the column is then washed with 60 mM potassium acetate, 60 % (v/v) ethanol solution. The RNA is then eluted from the column by addition of nuclease-free dH_2O.

DNA contamination of RNA preparations was removed where necessary by using the DNA-*free* kit (Ambion). This is performed by incubation of the RNA samples with a solution of RNase-free rDNase I, to allow digestion of the contaminating DNA, followed by inactivation of the DNase I and chelation of divalent cations.

2.10 Manipulation of DNA *in vitro*

2.10.1 Restriction endonuclease cleavage of DNA

Typically, 0.5-2 μg of DNA was digested with 10 U of restriction enzyme in an appropriate volume containing the reaction buffer supplied with the enzyme of choice. For double digests, the buffer in which both enzymes have maximal activity was chosen. Alternatively, double digests were performed sequentially in the appropriate buffer, with purification between steps using the Wizard *Plus* SV Minipreps DNA Purification System kit (Promega). Reactions were incubated at 37 °C for 2-3 h, unless otherwise stated.

2.10.2 Phosphatase treatment of restriction endonuclease-cleaved DNA

Cleavage of DNA by restriction enzymes leaves a 5'-protruding and 3'-recessive, or blunt end phosphoryl groups on the DNA. These 5'-phosphoryl termini are required for formation of phosphodiester bonds between adjacent ends of DNA. Consequently, removal of the 5'-phosphoryl group of vector DNA molecules prior to use prevents self-ligation and decreases vector background in cloning strategies. Shrimp alkaline phosphatase was used to dephosphorylate phosphoryl groups when a blunt ended cloning was performed, or if cloning into a single restriction site. After restriction

digestion of 50 ng vector DNA, 1 U shrimp alkaline phosphatase and an appropriate volume of 10X dephosphorylation buffer (Roche Molecular Biochemicals) were added directly to the reaction tube, and incubated at 37 °C for 1 h. Alkaline phosphatase was inactivated by heating to 65 °C for 15 min.

2.10.3 Purification of linear DNA

Linear DNA fragments were either purified directly using the Wizard *Plus* SV Minipreps DNA Purification System kit (Promega), or purified from agarose gel slices using the High Pure PCR Purification Kit (Roche). DNA is subjected to gel electrophoresis in 1X TAE agarose containing 1 μg ml^{-1} ethidium bromide. The DNA fragment of interest is excised from the agarose gel using a surgical blade, and subsequently purified as described in the manufacturer's instructions. In both procedures, the DNA is mixed with a buffer that selectively binds DNA with high affinity to a silica-based resin. The resin is then trapped in a minicolumn, and washed with 80 % isopropanol. The resin is then dried, and the DNA eluted with ddH_2O.

2.10.4 Ligation of DNA molecules

T4 DNA ligase was used to catalyse the ATP-dependent formation of phosphodiester bonds between adjacent 5'-phosphate and 3'-hydroxyl ends in double-stranded DNA. Phage-derived T4 DNA ligase (Roche Molecular Biochemicals), supplied with ligation buffer, was used to ligate insert DNA into appropriately digested vectors according to the manufacturer's instructions. Reactions were performed by incubating molar ratios of purified vector and insert DNA (<1 μg total DNA) in a 30 μl reaction, with 10X ligase buffer and 1-5 U T4 DNA ligase. For cohesive ends, the molar ratio of vector to insert DNA was 1:2; for blunt end ligation, the molar ratio was 1:5. Reactions were incubated at 4 °C for 16 h for ligation of cohesive ends, and 20 °C for 16 h for blunt ends.

2.10.5 Ethanol precipitation of DNA and RNA

Ethanol precipitation was used to concentrate nucleic acid solutions. For DNA preparations, 0.1 volumes 3 M sodium acetate (pH 5.2) and 2 volumes of ice-cold absolute ethanol were added to the DNA sample and mixed thoroughly. The samples were then incubated at –20 °C for at least 1 h, before centrifugation at 16,000 x *g* at 4 °C for 30 min to 1 h. The pellet was washed with 500 μl ice-cold 70 % ethanol, air-dried, and resuspended in an appropriate volume of dH_2O.

For ethanol precipitation of RNA, 3 M sodium acetate (pH 5.2) was treated with RNAsecure reagent (Ambion) before use. RNAsecure reagent is supplied as a 25X concentrate, which was added to the sodium acetate solution, mixed and heated to 60 °C for 10 min. 0.1 volumes of RNAsecure-treated 3 M sodium acetate (pH 5.2) were added to the RNA solution, followed by 2.5 volumes of ice-cold ethanol. This was stored at –80 °C for at least 1 h, followed by centrifugation at 16,000 x *g* at 4 °C, for 1 h. The supernatant was carefully removed, and 500 µl 70 % (v/v) RNase-free ethanol, made with RNase-free dH_2O (Sigma) was added and decanted immediately. The samples were air-dried and resuspended in a suitable volume of RNase-free dH_2O.

2.10.6 Phenol: chloroform purification of DNA

For DNA extractions, an equal volume of phenol:chloroform (mixed in a 4:1 ratio) was added to the DNA sample, mixed by vortex, and centrifuged in a minifuge (Eppendorf 5415R) for 2 min at 16,000 x *g*. The aqueous upper layer was carefully removed and purified by ethanol precipitation. Two volumes of ice-cold absolute ethanol were added and mixed by vortex. The sample was stored at –20 °C overnight, before centrifugation at 16,000 x *g* for at least 30 min at 4 °C.

2.11 Polymerase Chain Reaction (PCR)

2.11.1 Amplification of DNA

PCR reactions were carried out by mixing 10X *Pfu* buffer, 1 mM dNTP mix (0.25 mM of each deoxynucleotide triphosphate), 25 pmol of each oligonucleotide, 1.5 U of *Pfu* polymerase, 5-50 ng template DNA, and sterile dH_2O to a final volume of 50 µl in a 500 µl thin-walled PCR tube (Sarstedt). PCR reactions using *Taq* polymerase were performed as above, except using 10X *Taq* buffer, and 1.5 U *Taq* polymerase. Reactions were assembled on ice, and placed into a PTC-200 Peltier Thermal Cycler (MJ Research). Reaction cycles were routinely set up as follows:

1. 94 °C, 1 min
2. 94 °C, 30 s
3. Appropriate annealing temperature, 30 s
4. 72 °C, appropriate extension time

5. Repeat steps 2-4, 30 cycles
6. 72 °C, 10 min

The annealing temperature was set at 2 °C below the theoretical melting temperature (T_m) of the relevant oligonucleotides. T_m is calculated using the following formula:

$$T_m = 2\ °C\ (No.\ A + T) + 4\ °C\ (No.\ G + C)$$

Extension time was dependent on the amplimer size: for amplification using *Pfu* polymerase, 2 min per kilobase was allowed. For *Taq* polymerase, 1 min per kilobase was allowed. PCR amplification was carried out with purified chromosomal or plasmid DNA templates.

2.11.2 Reverse Transcriptase-PCR (RT-PCR)

Reverse transcriptase-PCR was used to examine relative mRNA levels of genes of interest. RNA was isolated (see section 2.9.4), and its concentration equalised to 0.6 μg ul^{-1}. Oligonucleotides were designed to hybridise to and amplify a region towards the 5' end of the cDNA molecule, to have a T_m of 54-56 °C, and to generate an amplicon length of between 200 to 300 bp.

RT-PCR was carried out using the OneStep RT-PCR kit (Qiagen), which provides an enzyme solution containing reverse transcriptase and *Taq* polymerase, RNase-free 5X RT-PCR buffer and RNase-free dNTPs. Instructions were carried out as described by the manufacturer. Briefly, the reactions are set up on ice and the thermal cycler is pre-heated to 50 °C. An initial cycle for 30 min at 50 °C allows the reverse transcriptase to convert RNA to cDNA. This is followed by incubation at 95 °C for 15 min to inactivate the reverse transcriptase, activate the hot-start *Taq* polymerase, and to denature the newly synthesised cDNA template. This is followed by the usual PCR cycle settings (see section 2.10.1) with a variable number of cycles, between 24-27, depending on abundance of the mRNA transcript of the gene of interest. RT-PCR products were analysed by 1 % agarose gel electrophoresis.

2.12 Gel electrophoresis

2.12.1 Agarose gel electrophoresis

DNA samples were analysed by agarose gel electrophoresis. The appropriate percentage of agarose, depending on the size of the DNA molecules being analysed, was added to TAE buffer (40 mM Tris, 1 mM EDTA, 0.114 % (v/v) glacial acetic acid), and heated to 100 °C. This was cooled to 50 °C, and ethidium bromide was added to a final concentration of 1 $\mu g\ ml^{-1}$. Ethidium bromide intercalates into DNA and fluoresces in long-wavelength UV light allowing DNA to be visualised on a UV imager. Samples were prepared by adding 6X loading dye (Promega) to the samples, followed by electrophoresis through the gel at 80 to 120 V in TAE buffer. The appropriate molecular weight markers were analysed alongside DNA samples as a reference (NEB, Promega).

2.12.2 SDS-PAGE

Proteins were separated by discontinuous polyacrylamide gel electrophoresis as described (Laemmli, 1970; Maniatis *et al.*, 1982). The discontinuous buffer system uses buffers of different pH and composition in the stacking and separating gels. Proteins migrate rapidly through the large pores in the stacking gel (5 % acrylamide) and become concentrated into a narrow band. Proteins then migrate according to size through the higher percent-acrylamide separating gel. Sodium dodecyl sulphate, present in both stacking and separating gels and running buffer, binds to most proteins in a constant weight ratio, so that the distance of migration through the gel can be assumed to be directly related to the size, and not charge, of the protein.

2.12.2.1 Staining of proteins

Gels were washed in ddH_2O prior to staining with Coomassie Protein Staining solution overnight. Coomassie Protein Staining solution is prepared by mixing 2.5 g Coomassie Brilliant Blue G-250, 100 ml glacial acetic acid, 450 ml methanol, and 450 ml dH_2O. The gels were decolourised by repeated washing in destaining solution: 100 ml glacial acetic acid, 450 ml methanol, and 450 ml dH_2O. Once used, the destain solution was filtered through activated charcoal to decolourise for re-use.

2.13 Southern blotting

Southern blotting (Southern, 1992) involves separation of DNA by agarose gel electrophoresis, which is denatured and transferred to a nylon membrane. Immobilised DNA is hybridised with a digoxigenin (DIG)-labelled probe that can be detected by a chemiluminescent immunoassay. This was used to confirm the genetic manipulation of mutant bacterial strains.

2.13.1 DIG (Digoxigenin) probe synthesis

A DIG-labelled DNA probe was synthesised by PCR using the PCR DIG labelling Mix (Roche Molecular Biochemicals). This mix contains 2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.9 mM dTTP and 0.1 mM DIG-11-dUTP. Of this, 5 µl was added to a 50 µl PCR reaction volume in place of standard dNTP mix. The PCR products synthesised have DIG-labelled nucleotides incorporated. These products were purified by agarose gel electrophoresis and eluted with dH_2O. The concentration of the probe was diluted to 30 ng μl^{-1}.

2.13.2 Preparation, electrophoresis, and capillary transfer of DNA to nylon membrane

Genomic DNA for examination by Southern blotting was isolated from SL1344 and SL1344 *lrp* and the concentration of the gDNA was quantified. Ten micrograms of gDNA of each strain was digested with 100 U *Sac*II in a reaction volume of 100 µl at 37 °C for 16-18 h. Equal volumes were loaded onto a 1 % TAE-agarose gel, along with DIG-labelled DNA Molecular Weight Marker III (Roche Molecular Biochemicals), and subjected to electrophoresis at 200 V for 1.5 to 2 h. After electrophoresis, the DNA was depurinated *in situ* by submerging and incubating the gel in 0.25 M HCl for 8 min, followed by washing in dH_2O. The DNA was then denatured by incubation in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 45 min at room temperature, followed by rinsing in dH_2O. The DNA was then submerged in neutralisation solution (1 M Tris pH 8.0, 1.5 M NaCl) for 45 min with shaking at room temperature. The DNA was transferred to Biodyne B 0.45 µm (PALL) nylon membrane by overnight capillary transfer, as described previously (Maniatis *et al.*, 1982), and crosslinked in a UV chamber (GS GeneLinker, Bio-Rad) at 254 nm UV.

2.13.3 Hybridisation and detection of DIG probe

Hybridisation of DIG-labelled probe was carried out using the DIG Easy Hyb kit (Roche Molecular Biochemicals) as described in the manufacturer's instructions. The pre-hybridisation and hybridisation temperature used was 44 °C, and 2 µl of probe mix per ml of hybridisation buffer was used. The probe was denatured at 95 °C for 10 min, then cooled on ice for 10 min prior to use. Fab fragments from an anti-digoxigenin antibody conjugated to alkaline phosphatase (Anti-DIG-AP Fab fragments), and CDP-*Star* chemiluminescent substrate for alkaline phosphatase were used to detect DIG labelled probe as described in the manufacturer's instructions (Roche Molecular Biochemicals).

2.14 Purification of 6xHis-rLrp

pKMC301-containing BL21 λDE3 pLysS were cultured overnight in 3 ml L broth. This was diluted 1:1,000 into 1 litre L broth, and monitored for growth by optical density at 600 nm (OD_{600}). At an OD_{600} of 0.5, IPTG was added to a final concentration of 1 mM to induce expression from the T7*lac* promoter of pKMC301. The bacterial cells were allowed to grow for another 2.5 h, before harvesting by centrifugation at 12,000 x *g* at 4 °C for 20 min.

6xHis-tagged Lrp was purified from cell pellets using the His•Bind Quick Buffer Kit and His•Bind Quick Columns, as described by the manufacturer (Novagen). The cell pellets were resuspended thoroughly in 40 ml binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl pH 7.9) with 100 µg ml^{-1} lyzoyme. An EDTA-free protease inhibitor cocktail tablet was added (Roche). The cell suspension was incubated on ice for 30 min to 1 h. The cells were lysed by sonication with Soniprep 150 (Sanyo), on setting 5 for 30 s pulses, with incubation on ice between pulses. The sonicated suspension was cleared by centrifugation at 4 °C at 14,000 x *g* for 20 min. The supernatant was removed and passed through a 0.45 µm syringe filter. At 4 °C, the supernatant was applied to a His•Bind Quick Nickel column (Novagen) and allowed to pass through. The column was washed with 10 ml binding buffer, followed by 10 ml wash buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl pH 7.9). The bound protein was eluted from the column in 500-µl fractions by addition of 5 ml elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9). The fractions were analysed for presence of rLrp by 10 % (v/v) acrylamide SDS-PAGE. Fractions containing Lrp were pooled and dialysed against 100 mM Na_2HPO_4 pH 8.0, 1 mM EDTA, 10 % (v/v) glycerol, 300 mM NaCl, 0.1 mM

phenylmethanesulphonylfluoride (PMSF), 1 mM DTT. To prevent aggregation, the Lrp solution was diluted with dialysis buffer and, as it was found to be unstable at –20 °C, was stored at 4 °C. The concentration of Lrp was estimated by comparison to known concentrations of lysozyme and BSA by SDS-PAGE (section 2.12.2), and by Bradford assay (section 2.7.3).

2.15 Electrophoretic mobility shift assay of protein: DNA interactions

The interaction of Lrp with the promoters of genes was examined by electrophoretic mobility shift assay. Biotinylated primers were used to generate labelled probes for EMSA analysis. DNA probes were amplified by PCR with *Pfu* polymerase, using *S.* Typhimurium SL1344 chromosomal DNA as template. Amplified probes were gel purified as detailed in section 2.10.3.

2.15.1 Formation and separation of protein: DNA complexes

DNA binding reactions were prepared to obtain a final volume of 20 µl. Approximately 50 pg of labelled DNA was incubated with increasing concentrations of His-tag purified Lrp (section 2.14) for 25-30 min at room temperature. Each reaction contained 20 mM Tris-HCl pH 8.0, 75 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 12.5 % (v/v) glycerol, 0.1 mg ml^{-1} BSA and 25 µg ml^{-1} poly-[d(I-C)•(dI-dC)] as previously described (Camacho and Casadesús, 2002). Samples included 15 mM L-leucine, or 15 mM glycine, where applicable. After incubation, 10 µl of the EMSA reaction, plus loading dye, were subjected to electrophoresis at 100 V on Novex 6% DNA Retardation non-denaturing gels (Invitrogen) in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3), for an appropriate amount of time depending on probe length.

2.15.2 Transfer of DNA to nylon membrane

Following electrophoresis, gels were electroblotted onto Biodyne B 0.45 µm nylon membrane (PALL) using the Novex XCell II blot module (Invitrogen) filled with 0.5X TBE at 30 V for 1 h. Membranes were crosslinked by exposure to UV light (254 nm) for 150 s using the GS Genelinker (Bio-Rad), and developed using the Chemiluminescent Nucleic Acid Detection Module (Pierce). This procedure involves blocking of the membrane, followed by incubation with streptavidin-horseradish peroxidase (HRP) conjugate. The membrane was washed, incubated with a chemiluminescent substrate, and exposed to autoradiography film (section 2.16).

2.16 Autoradiography

Autoradiography was used to visualise and quantitate photon emissions on X-ray film from procedures using radionucleotides [α-^{35}S]dATP or [γ-^{32}P]dATP (sequencing reactions, primer extension products, DNase I footprinting), or chemiluminescent emissions derived from horseradish peroxidase cleavage of chromogenic substrates (Southern blotting, or EMSA analyses). Hyperfilm MP (Amersham Biosciences) film was used in all cases. When photon emissions strike a silver halide crystal, the crystal adsorbs energy and releases an electron. This electron is attracted to a positively charged silver ion forming an atom of metallic silver. After an appropriate exposure time, the film was placed into a tray containing Kodak LX-24 X-ray Developer for 3 min, a chemical solution that amplifies the signal by reducing exposed silver halide crystals to metallic silver. The film was washed briefly in water and fixed in Kodak Industrex liquid fixer for further 3 min. The fixer serves to convert any silver halide not reduced by into soluble silver thiosulphate. Developed films were rinsed in a large volume of water.

2.17 Mapping of Lrp binding sites

Deoxyribonuclease I (DNase I) footprinting was introduced by Galas and Schmitz to identify the DNA sequences that constitute binding sites for site-specific DNA-binding proteins. The basis of footprinting techniques is that DNA-bound proteins protect the phosphodiester backbone of the DNA from cleavage by DNase I (Galas and Schmitz, 1978). The DNase I reactions are inactivated, the DNA denatured, cleavage products separated by sequencing gel electrophoresis (section 2.17.5) and visualised by autoradiography.

2.17.1 DNA sequencing

The DNA sequence to be analysed for Lrp binding was amplified using primers with incorporated restriction endonuclease sites. The resulting amplimer was cloned into pBluescript II SK (–) and verified by commercial sequencing. DNA was then sequenced in the laboratory by the Sanger method (Sanger *et al.*, 1977) using the T7 Sequencing Kit (Amersham GE Biotech) according to the manufacturer's instructions.

Briefly, T7 polymerase is used to amplify the product of interest. Depending on the DNA strand to be sequenced, the relevant primer used for amplimer cloning was used to

produce single-stranded DNA, which is ^{35}S-dATP-labelled. Deoxynucleotides (dNTPs) and dideoxynucleotides (ddNTPs) are incorporated to allow chain elongation, and chain termination, respectively. The 3' carbon of ddNTPs contains a hydrogen group instead of a hydroxyl group. This prevents the formation of a phosphodiester bond between the ddNTP and the next incoming nucleotide, and thus the DNA chain is terminated. Four different reaction mixes are produced, corresponding to each ddNTP: ddATP, ddCTP, ddGTP, and ddTTP. Each separate reaction mix produces all possible amplimers, which terminate in their corresponding ddNTP, and are radiolabelled.

2.17.2 5'-end labelling of DNA using γ-^{32}P-ATP

For generation of DNA probes for DNase I footprinting, PCR was used to amplify the DNA of interest (section 2.11.1). The region of interest was amplified from the vector used for DNA sequencing. Two primers were used: firstly, the relevant primer used for amplimer cloning, depending on the DNA strand sequenced; secondly, a primer located outside the region of interest, separated by a restriction endonuclease site (Figure 2.1). This DNA fragment was purified by agarose gel electrophoresis, ethanol precipitated in the absence of sodium acetate, and resuspended to 0.3 μg μl^{-1}. For the labelling reaction, detailed below, T4 polynucleotide kinase (T4 PNK; New England Biolabs) catalyses the phosphorylation the 5'-ends of the double-stranded probe with [γ-^{32}P]dATP (Amersham GE), resulting in a 5'-radiolabelled fragment.

PNK labelling reaction:

20 μl DNA fragment
4 μl T4 PNK buffer
2 μl T4 PNK
2 μl [γ-^{32}P]dATP (10 mCi/ml, 6000 Ci/mmol)
12 μl ddH_2O

This mixture is incubated at 37 °C for at least 2 h.

For labelling of oligonucleotide PE-*lrp* for primer extension analysis, 100 pmol of PE-*lrp* were incubated with 1 μl 10X kinase buffer, 25 μCi of [γ-^{32}P]dATP (10 mCi/ml, 6,000 Ci/mmol) and 15 U T4 PNK in a 1.5 ml screw-cap tube. The reaction was

incubated for 30 min at 37 °C. The reaction was diluted with RNase-free dH_2O (Sigma) to 1 pmol μl^{-1}, and stored at –20 °C.

2.17.3 Generation of 5'-end labelled DNA probes for DNase I footprinting

The ^{32}P-labelled DNA fragment is purified using the High Pure PCR kit (Roche) as described in section 2.10.3, and eluted with 80 µl dH_2O. To remove one of the labelled ends, the fragment was digested using the incorporated restriction endonuclease site (see Figure 2.1). The probe was purified by non-denaturing polyacrylamide gel electrophoresis (section 2.15.1), extracted from the gel and placed in 3 ml elution buffer at 56 °C with shaking at 200 rpm, for 24 h.

Elution buffer:

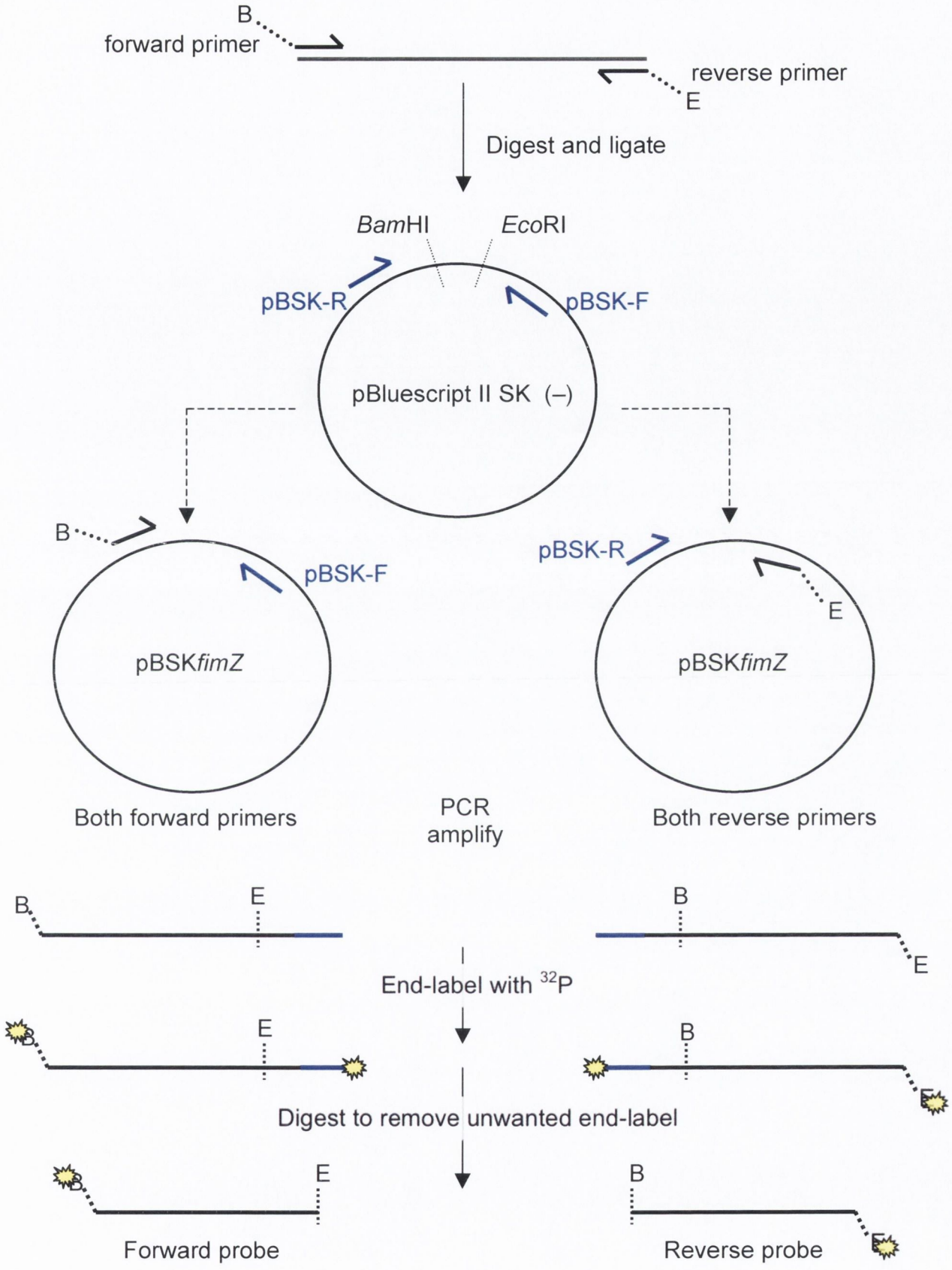
10 mM Tris-HCl pH 8.8
300 mM $NaCO_2CH_3$ pH 5.2
1 mM EDTA
0.2 % (w/v) SDS

The labelled probe was purified from the elution buffer by phenol extraction.

2.17.4 DNase I footprinting

Four µl of labelled DNA probe was incubated with increasing concentrations of rLrp-6xHis in each footprinting reaction. Protein-DNA complexes were formed in 50-µl reaction volumes at 37 °C for 30 min. Fifty-µl volumes of 10 mM $MgCl_2$, 5 mM $CaCl_2$ solution were added, and incubation continued for 10 min. 1 mU DNase I (Roche Molecular Biochemicals) was added, and digestion was allowed to proceed for 1 min. This reaction was stopped by addition of 90 µl stop solution (200 mM NaCl, 30 mM EDTA pH 8.0, 1 % (w/v) SDS, and 100 µg ml^{-1} yeast tRNA; Sigma). Samples were then extracted once with an equal volume of phenol:chloroform, purified by ethanol precipitation, and then resuspended in 6 µl loading dye (95 % (v/v) formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue, 0.05 % (w/v) xylene cyanol FF). The products of DNase I digestion were then denatured by incubation at 90 °C for 3 min, and subjected to electrophoresis on a 7 % urea-polyacrylamide gel (section 2.16.5) alongside the appropriate sequencing reactions.

Fig. 2.1. Production of end-labelled probes for DNase I footprinting analyses. The appropriate oligonucleotides containing restriction sites for *Eco*RI and *Bam*HI, denoted by E and B, respectively, were used to amplify the region of interest, which was then ligated into the *Eco*RI and *Bam*HI restriction enzyme sites in pBluescript II SK (–). Oligonucleotides located external to the MCS of pBluescript II SK (–), pBSK-F and pBSK-R, were used in conjunction with the cloning primers to produce probes. The probes were end-labelled with ^{32}P, and digested with the appropriate enzyme to remove one end label. The desired probes were purified by polyacrylamide gel electrophoresis, and eluted as described in the text.


B
forward primer
reverse primer
E
Digest and ligate
BamHI
EcoRI
pBSK-R
pBSK-F
pBluescript II SK (–)
B
pBSK-F
pBSKfimZ
pBSK-R
E
pBSKfimZ
Both forward primers
PCR
amplify
Both reverse primers
B
E
B
E
End-label with 32P
B
E
B
E
Digest to remove unwanted end-label
B
E
B
E
Forward probe
Reverse probe

2.17.5 Denaturing polyacrylamide gel electrophoresis

The products of sequencing, primer extension analysis and DNase I footprinting were separated by electrophoresis on 7.5 M urea, 6 % polyacrylamide gels in 1X TBE buffer. The gels were prepared by combining 24 ml SequaGel concentrate, 10 ml SequaGel buffer (National Diagnostics), and 66 ml 7.5 M urea (BDH). To this, 300 µl 10 % (w/v) ammonium persulphate and 50 µl *N, N, N', N'*-tetramethylethylenediamine (TEMED; Sigma) were added. The polymerised gel was pre-run in 1X TBE at 80 W until the temperature of the gel had reached at least 50 °C. The heating of the gel to this temperature combined with the presence of urea help to maintain the sequencing reactions, primer extension products and DNase I digestion products in a denatured state. Prior to loading, samples are heated (see individual sections). The gel was then run at 80 W for 90 to 120 min, dried under vacuum, and exposed to autoradiography film.

2.18 Primer extension analysis

Primer extension analysis involves the isolation of total cellular RNA and reverse transcription of mRNA to cDNA using a ^{32}P-labelled oligonucleotide primer. The radiolabelled oligonucleotide primer hybridises to the complementary region of the RNA transcript of interest. This primer is then used by reverse transcriptase to synthesise cDNA using the mRNA as the template. The length of the cDNA reflects the number of bases between the labelled nucleotide of the primer and 5'-end of the mRNA. The resulting cDNA is subsequently analysed on a denaturing polyacrylamide gel. In addition, the quantity of cDNA product is proportional to the amount of target mRNA.

Primer extension analysis was used to map the transcriptional start site of the *lrp* promoter in *S.* Typhimurium SL1344. SL1344 were grown to OD_{600} of 0.5 in MOPS minimal medium, and OD_{600} of 3.5 in LB broth. Volumes equal to 1 ml OD_{600} of 2.0 were harvested and total RNA isolated. The oligonucleotide PE-*lrp* was designed to be complementary to *lrp* RNA, 58 nt into the ORF, and was labelled as described in section 2.17.2.

The following components were assembled on ice: 2 pmol radiolabelled PE-*lrp*, 5 µg total RNA, and 1 µl RNase-free 10 mM dNTP mix (Qiagen OneStep RT-PCR kit) in a total volume of 13 µl RNase-free dH_2O. This mixture was heated to 65 °C for 5 min, and cooled on ice for 5 min. To this mixture, 1 µl (200 U) SuperScript III Reverse

Transcriptase (Invitrogen), 4 μl 5X First-Strand Buffer, 1 μl 0.1 M DTT (supplied with SuperScript III) and 1 μl RNase Inhibitor (Roche Molecular Biochemicals) were added, mixed gently by pipetting, and incubated at 55 °C for 1 h. The reaction was inactivated by incubation at 70 °C for 15 min. The samples were purified by ethanol precipitation.

The cDNA transcripts were resuspended in 6 μl loading dye (95 % (v/v) formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue, 0.05 % (w/v) xylene cyanol FF) and heated to 95 °C for 10 min. The samples were analysed by denaturing 6 % polyacrylamide-urea gel electrophoresis (section 2.17.5) alongside sequencing reactions that were generated using the same unlabelled oligonucleotide, and pKMC101 as template, as described (section 2.17.1).

2.19 DNA microarray analysis

DNA microarray analyses were carried out to examine the Lrp regulon of *Salmonella enterica* sv. Typhimurium during growth in LB, and MOPS minimal broth. This procedure was carried out as described previously (Kelly *et al.*, 2004). A PCR product *Salmonella* Typhimurium SL1344 and LT2 genome array was used in collaboration with the Hinton laboratory in the Institute for Food Research in Norwich, UK. The procedure involves isolation of RNA, reverse transcription of RNA to cDNA incorporating labelled nucleotide dCTP-Cy5, hybridisation of the cDNA and dCTP-Cy3-labelled gDNA to the array, and detection in an array scanner. Results are normalised and imported into the GeneSpring 7.0 software program for analysis. Arrays were hybridised in triplicate.

2.19.1 cDNA synthesis and Cy5-dye labelling

For each array to be hybridised, the following reaction was assembled. In a sterile microfuge tube, 16 μg total RNA was added to 5 μg random primers in a total volume of 15.1 μl. This was incubated at 70 °C for 10 min, and then on ice for 10 min to allow primer annealing. On ice, 3 μl of 10X RT buffer (Stratagene), 0.6 μl 50X dNTP mix (25 mM dATP, dGTP, dTTP, 10 mM dCTP), 0.3 μl 1 M DTT, 2 μl 1 mM Cy5-dCTP, and 100 U Stratascript were added. This mix was incubated overnight at 37 °C.

Following overnight incubation, 15 μl 0.1 M NaOH was added, and the mixture incubated at 70 °C for 15 min to hydolyse the RNA. To neutralise the reaction mix, 15

µl 0.1 M HCl was added, and the resulting cDNA was purified using the Wizard PCR Prep DNA purification system (Promega). The samples were dried in a SpeedVac.

2.19.2 Cy3-labelling of genomic DNA

Type II microarray analysis was used in this study. Type II arrays use genomic DNA as an internal positive control and reference. Genomic DNA is labelled with Cy3-dCTP, while cDNA derived from sample RNA is labelled with Cy5-dCTP. Both Cy-labelled products are included in the array hybridisations. The binding of labelled gDNA to each spot on the array acts as a reference, to which binding of cDNA is compared.

gDNA was isolated from SL1344 using the Genomic Tip kit (Qiagen), as described in the manufacturer's instructions. The gDNA was eluted with 295 µl dH_2O, and digested with 200 U *Eco*RI in a 450 µl reaction volume at 37 °C overnight. After digestion, the gDNA was purified by ethanol precipitation, quantified by A_{260} and resuspended in ddH_2O to 2 µg in 21 µl.

gDNA labelling was carried out using the Bioprime DNA Labelling System (Gibco). The protocol detailed below provides enough labelled gDNA for 5 hybridisations. To 21 µl digested gDNA (2 µg), 20 µl 2.5X random primer/reaction buffer mix was added and this mix heated to 100 °C for 5 min, and subsequently incubated on ice for 5 min. On ice, the following solutions were added: 5 µl 10X dNTP mix (1.2 mM dATP, dTTP, dGTP, 0.6 mM dCTP), 3 µl mM Cy3-dCTP (Amersham), 10 mM Tris (pH 8.0), 1 mM EDTA, and 1 µl Klenow polymerase from the Bioprime kit. This mix was incubated overnight at 37 °C. The reaction was then purified using the Wizard PCR Prep DNA purification system. The samples were dried in a Speed Vac, and resuspended to 41 ng μl^{-1}.

2.19.3 DCE blocking of microarray slides

DNA microarrays are printed on Corning CMT-GAPS coated glass slides. Each slide contains 2 arrays, each consisting of 16 blocks of printed PCR products. The corners of each array were marked with a diamond-tipped pencil before blocking. Slides were blocked with 1,2-dichloroethane (DCE) as follows:

Microarray slides were crosslinked twice with UV light to immobilise the DNA. Blocking solution was made up using 15 g succinic anhydride dissolved in 300 ml anhydrous DCE, to which 3.75 ml N-methyl-imidazol was added. Slides were incubated in this solution for 1 h with gentle agitation. After blocking, slides were washed with 300 ml fresh DCE for 2 to 3 min. The slides were transferred to boiling dH_2O for 2 min to denature the DNA, and then immediately transferred to 96 % ethanol for 1 min. The slides were then thoroughly dried by centrifugation (Jouan MR23i) at 1,200 rpm for 5 min.

2.19.4 Hybridisations

For each sample hybridisation, the following procedure was performed. Each cDNA sample was resuspended with 9.75 µl of Cy3-labelled gDNA solution. To this reaction, 1.125 µl yeast tRNA (10 µg μl^{-1}), 2.25 µl 20X SSC, 0.375 µl 1M HEPES (pH 7.0) were added and mixed, followed by 0.338 µl 10 % SDS and 1.5 µl 50X Denhardts Solution. This was denatured by heating to 100 °C for 2 min, and then allowed to cool for 5 to 10 min at room temperature. The samples were subjected to centrifugation for 10 min at maximum speed in a microfuge, transferred to a clean microfuge tube, and the centrifugation step repeated. The array slides were placed in a hybridisation chamber. The samples (15 µl) were then added to the edge of the array and a glass coverslip, cleaned with 96 % ethanol, was applied to the edge and carefully lowered to allow for even distribution of the solution across the array, and exclusion of bubbles. Four 5-µl drops of 3X SSC were placed along each side of the glass slide to maintain the correct humidity in the chamber. The chambers were fastened securely and placed in a waterbath overnight at 63 °C. The chambers were opened and the slides washed as follows. The first wash was performed twice in 1 litre volumes of 2X SSC, 0.1 % SDS at 68 °C for 5 min, with a stir bar to remove coverslips. The following wash in 250 ml 1X SSC was performed twice at room temperature with vigorous shaking. The final wash in 0.2X SSC was performed in 250-ml volumes at room temperature with shaking. The slides were dried by centrifugation for 5 min at 600 rpm.

2.19.5 Scanning of microarray slides and data handling

After hybridisation, the slides were scanned using a GenePix 4000 A scanner (Axon Instruments). Fluorescent spot intensities and local background were quantified using the GenePix 3.0 software for LB broth arrays, but BlueFuse software for the MOPS

minimal media arrays. Data were passed through quality control procedures previously described (Eriksson *et al.*, 2003), and the subsequent data were saved in .gpr file format, converted to .txt text files, and imported into Microsoft Excel. Using a custom designed macro program (S. Lucchini and A. Thompson), the cDNA data were normalised against the genomic DNA data. The resulting Excel file was converted into a .txt file and imported into the microarray analysis program GeneSpring 7.0 (Silicon Genetics/ Agilent Technologies). All subsequent array analysis was carried out using GeneSpring 7.0.

2.20 Mannose-sensitive haemagglutination assays

Guinea pig or horse erythrocytes are used for haemagglutination assays with *S. enterica* (Ewen *et al.*, 1997; Tinker and Clegg, 2000). The erythrocytes were washed in PBS three times by centrifugation for 2 min at 1,000 x *g*. A 9% erythrocyte solution was made by dilution in PBS. Bacterial cultures were induced for fimbriation by growth in 10 ml L broth in a 250 ml conical flask for 48 h to 72 h at 37 °C without shaking. Bacterial cells were equalised by OD_{600}, and 100 µl-volumes in either 24-well cell culture plates, or on glass microscope slides, mixed with washed erythrocytes to a final concentration of 3 % (v/v). Where used, mannose was added to a final concentration of 3 % (w/v).

2.21 Bacteriophage particle purification and DNA isolation

2.21.1 Phage particle purification

Phage particles were purified using a modified version of an established protocol for purification of bacteriophage λ particles, as described previously (Maniatis *et al.*, 1982). Briefly, the lysed cultures were treated with DNase I and RNase to digest cellular nucleic acid which otherwise traps large amounts of phage particles. NaCl is added to dissociate the phage particles from bacterial debris, and the solution is cleared by centrifugation. PEG 8000 is added to the supernatant, mixed, and cooled on ice to allow the phage particles to form a precipitate. The precipitated phage are recovered by centrifugation, and resuspended in TM buffer (10 mM Tris-Cl pH 8.0, 10 mM $MgCl_2$). The PEG 8000 and cellular debris are then extracted from the phage suspension by addition of chloroform, followed by vigorous mixing by vortex, and centrifugation to separate the organic and aqueous phases, the latter containing the phage particles. The

phage sample was purified by glycerol step gradient, by layering of the phage suspension over a 10 %: 40 % (v/v) glycerol step in TM buffer. Only phage particles should be able to penetrate the 40 % (v/v) glycerol cushion, forming a pellet. This was then subjected to ultracentrifugation at 25,000 rpm for 2 h in an Optima™ L-100 XP ultracentrifuge (Beckman Coulter). Upon recovery of the tubes, white debris at the gradient interface and a glassy pellet were observed. The supernatant was carefully removed, and the pellet resuspended in 500 µl TM buffer.

2.21.2 Phage DNA isolation

The protocol used for phage DNA isolation detailed below is combined from two established protocols (Maniatis *et al.*, 1982; Weiss *et al.*, 1994). Purified phage particle solution (200 µl) was removed from the stock solution. To remove contaminating host nucleic acid present in the phage particle preparation, a final concentration of 5 µg ml^{-1} of both DNase I and RNase were added, followed by incubation at 37 °C. After 30 min, the reaction was stopped by addition of 10 µl 0.5 M EDTA solution, to chelate divalent cations necessary for nuclease activity, and to aid in disintegration of phage particles. An equal volume of 4 M guanidine hydrochloride was added and briefly vortexed, to denature phage proteins. The DNA present in the sample was purified by ethanol precipitation, as described in section 2.9.5.

DNA was resuspended in 200 µl TE-SDS buffer (25 mM Tris pH 8.0, 0.6 % (w/v) SDS, 25 µM EDTA) and incubated for no longer than 10 min at 65 °C. Proteinase K is a serine protease which inactivates nucleases in DNA and RNA preparations. It is not inhibited by the presence of EDTA. Fresh proteinase K (200 µg) was added to the solubilised DNA, and the suspension incubated at 50 °C for 2 h. DNA was precipitated again, as above, resuspended in TE-$CaCl_2$-proteinase K buffer (TE pH 7.4, 10 mM $CaCl_2$, 2 mg ml^{-1} proteinase K), and incubated at 50 °C for 2 h. To inactivate proteinase K, the samples were incubated at 70 °C for 1 h. The DNA was precipitated again, and resuspended in 100 µl TE buffer and a further 100 µl dH_2O added.

2.21.3 Pulsed-field gel electrophoresis of phage DNA

Pulsed-field gel electrophoresis was used to separate phage DNA alongside 0.5 µg of Lambda Mono Cut Mix DNA ladder (New England Biolabs). DNA was analysed in a

1% agarose gel (w/v) in 0.5X TBE. The gel was run at 6 V/cm at 15 °C for 20 hours. Switch times were ramped from 0.5-1.5 seconds.

2.22 Software for data analysis

2.22.1 Bioinformatic analysis

DNA alignments were performed using the T-COFFEE web-based programme (Notredame *et al.*, 2000). DNA homology searches were analysed using BLAST (www.ncbi.nlm.nih.gov/BLAST). The LT2 genome was searched for Lrp consensus sequences using either the web-based pattern search (colibase.bham.ac.uk), or GeneSpring 7.0 (Agilent Technologies). The intrinsic curvature of DNA was examined using the bend.it server (Vlahovicek *et al.*, 2003).

2.22.2 Data presentation software

Unless otherwise stated, numerical data was analysed using Microsoft Excel. Plasmid maps were generated using Enhance Map Draw 4.0 (Sci Ed Software).

Chapter 3

Characterisation of the Lrp protein of *Salmonella enterica* serovar Typhimurium

3.1 Introduction

The leucine-responsive regulatory protein (Lrp) of *Escherichia coli* is the best-studied member of the Lrp family, with most of the data on *lrp* gene regulation obtained in studies in this organism. Other than sequence analysis of the ORF, there has been no characterisation of the *lrp* gene and its regulation in *Salmonella* species. The *lrp* gene sequences of *E. coli* and *Salmonella* show only 88 % identity, whereas the amino acid sequences of the Lrp proteins are 99 % identical (Friedberg *et al.*, 1995).

The *E. coli lrp* mutant MEW26 has a doubling time of 80 min, compared to 58 min for the wild type in glucose minimal media, with growth of the *lrp* mutant in leucine reducing the doubling time to 65 min (Lin *et al.*, 1990). Lrp is strongly regulated by the nutrient content of the medium in which the bacterium is cultured. Nutrient-poor media lead to high levels of *lrp* expression whereas rich media, e.g. LB, lead to reduced levels of *lrp* expression in *E. coli*. In log phase, Lrp levels are 3- to 4-fold higher in cells growing in minimal medium, compared to rich medium (Landgraf *et al.*, 1996). Lrp expression *in vivo* is positively regulated at the transcriptional level by guanosine tetraphosphate (ppGpp), the stringent response alarmone nucleotide. Levels of ppGpp increase in response to amino acid and energy source limitation. The expression of *lrp* is also affected by growth rate: it appears that the growth rate and Lrp levels are inversely related, as are ppGpp levels (Landgraf *et al.*, 1996).

The predicted promoter of the *E. coli lrp* gene contains a perfect –35 element (TTGACA), and a –10 element (GAAAAC) with a mismatch of three to a perfect σ^{70} promoter (Wang *et al.*, 1994). The transcriptional start site of *lrp* of *E. coli* was shown by primer extension analysis to be 267 bp upstream of the translational start codon (Wang *et al.*, 1994). The purpose of the long untranslated region is unknown at present. The *lrp* gene of *E. coli* is known to be directly negatively autoregulated, with Lrp binding to a site upstream of the transcription start site. Lrp protects a region of 85 bp (from –105 to –20) at the *lrp* regulatory region, overlapping the promoter: it is thought to affect RNA polymerase by reducing its affinity for the promoter, or its ability to initiate transcription (Wang *et al.*, 1994). Within the *lrp* regulatory region of *E coli*, Wang *et al.* describe two sequences that match the consensus sequence, at –67 to –53, and at +14 to +28. Mutational analysis of the site located at –60 to –53 prevented binding of Lrp *in vitro*, and reduced Lrp-mediated repression of the *lrp* gene (Wang *et*

al., 1994), leading to the identification of the only characterised Lrp binding site in the *lrp* promoter of *E. coli*. In *S.* Typhimurium LT2, a putative binding site for Lrp centred at +49, has been identified by whole genome sequencing (McClelland *et al.*, 2001).

L-leucine is capable of strongly modulating the effect of Lrp on gene regulation (examples in Chapters 1 and 4). It has been shown that leucine can have a number of effects on this regulation: leucine can enhance the effect of Lrp, leucine is capable of antagonising the effect of Lrp, and leucine may have no effect on regulation by Lrp. The negative regulation of the *lrp* gene by Lrp has been described as leucine-insensitive: β-galactosidase activity from an *E. coli* strain containing an *lrp-lacZ* fusion did not show a significant change between leucine-deplete and leucine-replete media (Lin *et al.*, 1992). In addition, the presence of leucine did not modulate Lrp-mediated DNase I-protection of the *lrp* promoter (Wang *et al.*, 1994). However, another study has described slightly greater intracellular Lrp levels in the presence (6.42 μM) than the absence of leucine (4.95 μM) (Borst *et al.*, 1996).

Lrp has been well characterised in *E. coli* and, thus far, it is not known whether Lrp is regulated similarly in *Salmonella*. The aim of this study was to examine the regulation of Lrp of *S.* Typhimurium, and to enable a comparison with that of *E. coli*.

3.2 Results

3.2.1 Construction of SL1344 *lrp*

In order to examine the effect of Lrp on gene expression in SL1344, a mutation to prevent expression of the *lrp* gene was required. The *lrp* gene was disrupted in the following manner, depicted in Figure 3.1. A 1,274-bp fragment containing the *lrp* gene plus 533 bp upstream and 227 bp downstream DNA was PCR amplified from the SL1344 genome using primer pair Fwd1.3-*Eco*RI and Rev1.3-*Pst*I. This fragment was digested with *Eco*RI and *Pst*I, yielding a 1,256-bp fragment, purified by agarose gel electrophoresis, and ligated into the same restriction sites in the MCS of the low-copy number vector pCL1921, creating plasmid pKMC101. Cells containing the *lrp* gene in a high copy-number vector were not viable, presumably because overproduction of Lrp inhibits growth and can be lethal to the cell (Borst *et al.*, 1996; Chen and Newman, 1998). The sequence integrity of pKMC101 was verified commercially. pKMC101 was then digested with *Bgl*II, which digests this plasmid at a unique site located 27 bp into the *lrp* ORF.

The purified plasmid pHP45Ω*kan* was digested using the restriction enzyme *Bam*HI. This liberated a fragment of 2,248 bp, containing the kanamycin resistance gene *kan*, flanked by Ω elements which contain transcriptional and translational stop signals in six reading frames. The Ω elements prevent read-through originating from the *kan* gene. The *Bam*HI-digested Ω*kan* element was ligated into the compatible cohesive ends of the *Bgl*II-digested pKMC101, and cloned into *E. coli* XL-1 Blue cells. The structure of this plasmid, pKMC101Ω*kan*, was also verified by commercial sequencing. A 3,504-bp fragment containing the Ω*kan*-disrupted *lrp* gene was PCR amplified using pKMC101-Ω*kan* as template, in combination with primer pair Fwd1.3 and Rev1.3. The product was treated with *Dpn*I to remove plasmid template, and then purified and transformed by electroporation into pKOBEGA-containing SL1344. The *lrp* lesion was transduced from SL1344 *lrp* into fresh SL1344 and LT2 strains using P22-mediated transduction.

3.2.2 Genetic confirmation of the *lrp* mutation

Southern blotting was employed to confirm the *lrp* mutation. A DIG-labelled DNA fragment complementary to the intact 3'-region of the *lrp* gene was used as a probe. The probe was PCR amplified using primer pair DIG-*lrp*-F and DIG-*lrp*-R. Southern blotting

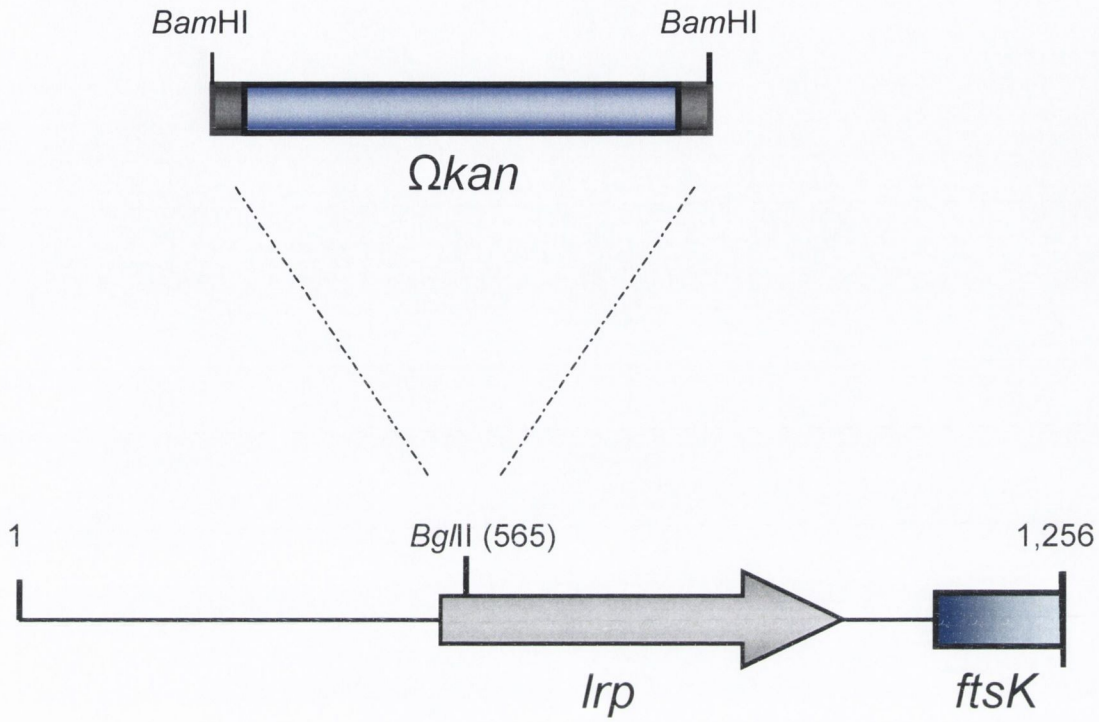


Fig. 3.1. Disruption of the *lrp* open reading frame of SL1344. A 1,256 bp fragment containing the *lrp* gene was cloned into pCL1921, producing pKM101. This plasmid was restriction digested with *Bgl*II, and ligated, via compatible overhangs, to the Ω*kan* cassette liberated from pHP45Ω*kan* by *Bam*HI digestion.

was carried out as described in section 2.13. SL1344 and SL1344 *lrp* genomic DNA was harvested and digested with *Sac*II. In the wild type, the *lrp* gene migrated as a 2.88 kb *Sac*II fragment. In the *lrp* mutant however, the *lrp* gene contained a Ω*kan*-mediated disruption, so it migrated on a 5.13 kb *Sac*II fragment (Fig. 3.2).

3.2.3 Glycine utilisation by the *lrp* mutant

In *E. coli,* Lrp is an essential positive regulator of the *gcv* operon for glycine cleavage. As a result of the low expression of *gcv*, *E. coli lrp* mutants are unable to utilise glycine as their sole nitrogen source (Lin *et al.*, 1992). To confirm the *lrp* mutation in *S.* Typhimurium, the ability of the *lrp* mutant to grow in minimal A glycine medium was examined. LT2 has been shown to be unable to utilise histidine as a nitrogen source, although rare mutants may be generated which are capable of doing so (Meiss *et al.*, 1969). The strain of interest SL1344 is an auxotroph for histidine, which results in the need to supplement all minimal media with 0.5 mM L-histidine. As a result, it was decided to analyse the ability of the LT2 *lrp* mutant to grow in minimal A glycine medium, as this would not require supplementation with histidine. Wild type LT2 grew poorly in minimal A glycine, reaching an average OD_{600} of 0.5 after 24 h. The LT2 *lrp* mutant did not grow in minimal A glycine, with an average OD_{600} of 0.07 after 24 h. This showed that the ability of the *lrp* mutant to utilise glycine as the sole nitrogen source was strongly repressed.

3.2.4 Growth of *S.* Typhimurium in LB broth

SL1344 and SL1344 *lrp* were examined for their ability to grow in LB broth, as described in section 2.2.2. Briefly, the optical densities of overnight cultures of each strain were equalised at OD_{600} and used to inoculate 25-ml volumes of LB broth with equalised numbers of cells. Cultures were incubated at 37 °C, with shaking at 250 rpm. Samples were taken at timed intervals, and a growth curve produced by the plotting of OD_{600} against time. From these growth curves, shown in Figure 3.3, doubling times for both strains were calculated. The mean doubling time of SL1344 was 28 min, and 30 min for SL1344 *lrp*, indicating a slight growth defect in the *lrp* mutant.

3.2.5 Growth of *S.* Typhimurium in MOPS minimal medium in the presence and absence of leucine

In *E. coli,* Lrp is expressed most highly during growth in minimal medium compared to rich medium, and is an important regulator during starvation conditions. In addition, it is possible to alter the concentration of the Lrp effector molecule, L-leucine, in a chemically-defined medium such as MOPS minimal medium. To analyse the effect of the *lrp* mutation on growth of SL1344 in minimal medium, SL1344 and SL1344 *lrp* were cultured in MOPS minimal medium in both the absence and presence of 10 mM L-leucine, as described in section 2.2.2.

During growth in MOPS minimal medium in the absence of L-leucine, the *lrp* mutant displayed a clear growth defect, with an average doubling time of 76 min, compared to 58 min for the wild type SL1344 (Fig. 3.4 (A)). The growth of the *lrp* mutant was characterised by a long lag phase, followed by exponential growth, and a terminal OD_{600} relatively lower than that of the wild type. Figure 3.4 (B) displays the growth curve of SL1344 and SL1344 *lrp* in MOPS minimal medium supplemented with 10 mM L-leucine. Here, the growth defect of SL1344 *lrp* compared to SL1344 was reduced. On further inspection, it was observed that the presence of 10 mM L-leucine strongly affected the growth of wild type SL1344. SL1344 had a longer lag phase duration, and a slower mean doubling rate (67 min) in the presence of leucine compared to its absence. In addition, SL1344 *lrp* had a similar mean doubling rate in the presence (80 min) and absence (76 min) of 10 mM L-leucine, but a shorter lag phase in the presence of leucine: in the presence of leucine, SL1344 *lrp* reached an OD_{600} of 0.2 30 min before SL1344 *lrp* in the absence of leucine. However, SL1344 *lrp* reached stationary phase after an approximately equal growth period (630 min) in both the absence and presence of 10 mM L-leucine.

3.2.6 Construction of an *lrp* expression vector

Analyses to be undertaken using Lrp required the expression and purification of recombinant Lrp protein. An Lrp expression vector was constructed as follows. The *lrp* ORF was PCR amplified using the primers *lrp*-pET22b-F and *lrp*-pET22b-R. This led to the amplification of a 509-bp fragment, flanked with *Nde*I and *Xho*I restriction sites, which was digested and purified. The IPTG-inducible protein expression plasmid pET22b, which contains a 6xHis-tag at the C-terminal end of the MCS, was digested

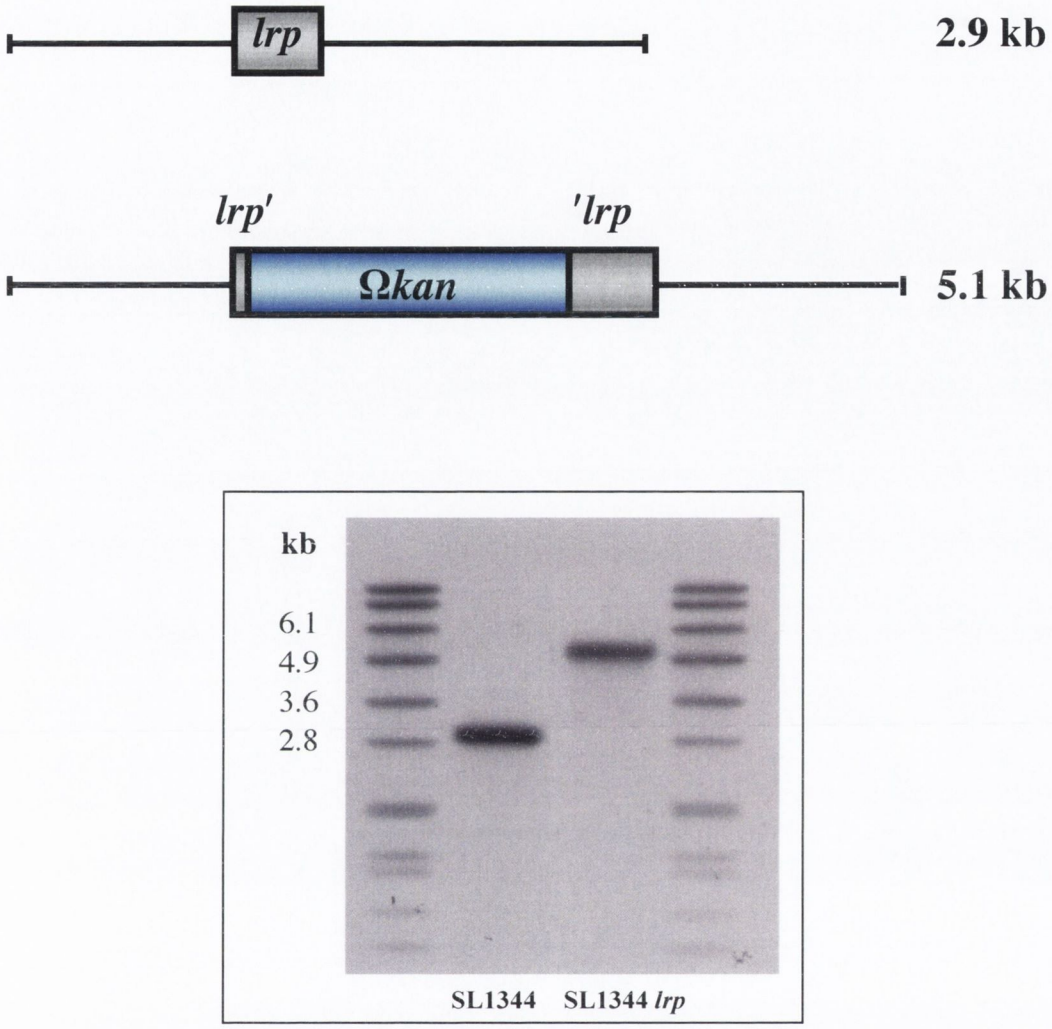


Fig. 3.2. Genetic analysis of the *lrp* mutant. Southern hybridisation analysis was employed to examine the genetic structure of the *lrp* mutant. Genomic DNA from SL1344 and SL1344 *lrp* was treated with *Sac*II as described. The wild type *lrp* gene (top) migrates on ~2.9 kb *Sac*II fragment, whereas the *lrp* mutant gene migrates on ~5.1 kb *Sac*II fragment.

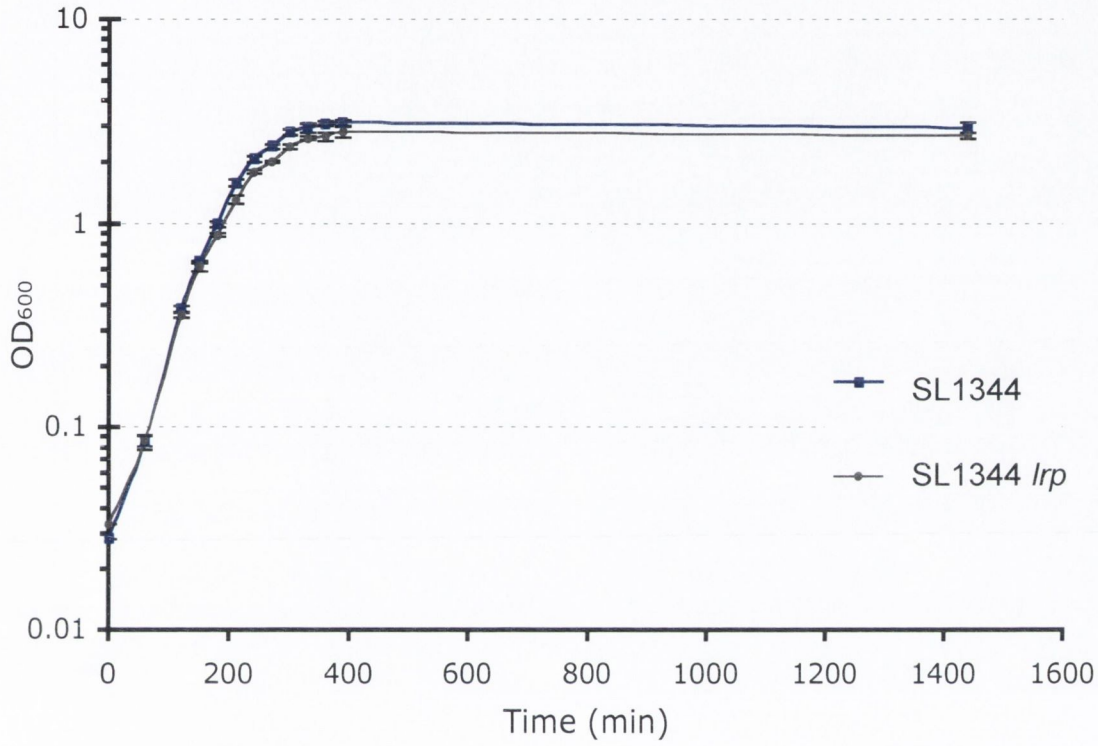
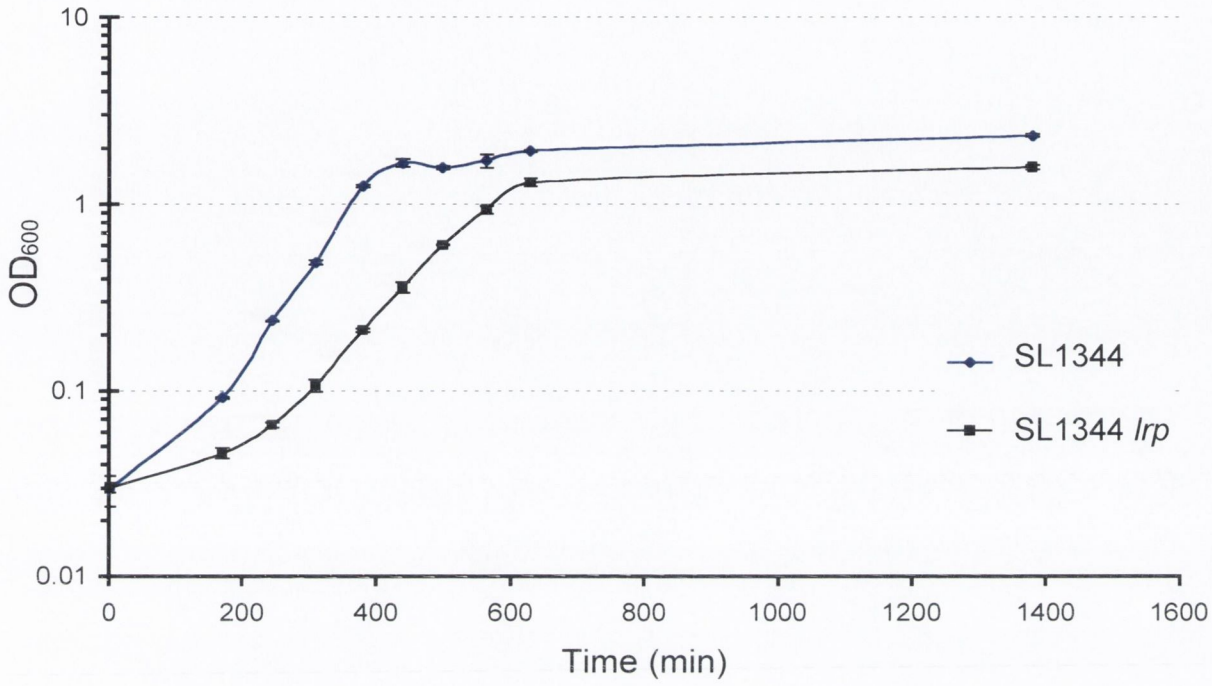


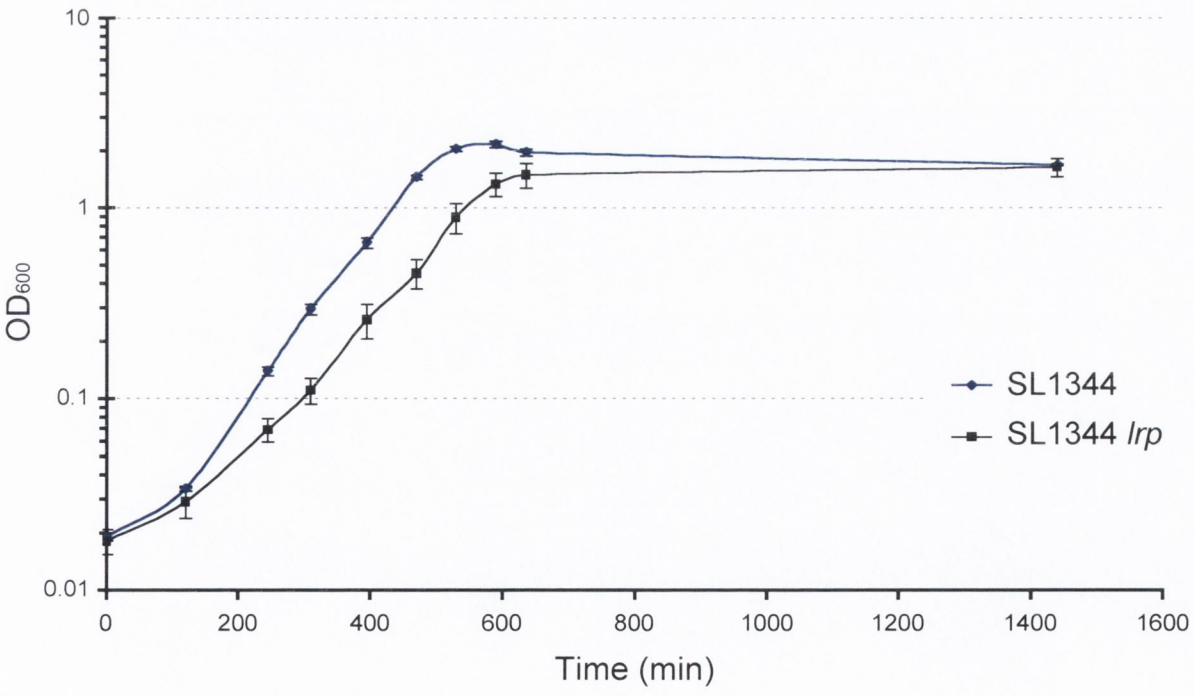
Fig. 3.3. Growth of SL1344 and SL1344 *lrp* in LB broth. Overnight cultures of SL1344 and SL1344 *lrp* were inoculated 1:100 into 25 ml LB broth at 37 °C with shaking. Optical density of the cell culture at 600 nm was monitored and plotted against time to produce a semi-logarithmic growth curve. SL1344 had a doubling time of 28 min, while SL1344 *lrp* had a slower doubling time of 30 min. Experiments were performed at least three times in triplicate with similar results. Error bars denote ± SEM.

Fig 3.4. Growth of SL1344 and SL1344 *lrp* in MOPS minimal medium. *S.* Typhimurium SL1344 and SL1344 *lrp* were cultured overnight in 3 ml L broth. Two-ml volumes of each culture were washed twice in pre-warmed MOPS minimal medium, before equalisation of optical density and inoculation into 25 ml pre-warmed MOPS minimal medium (A) the absence of L-leucine, and (B) supplemented with 10 mM L-leucine. The optical density at 600 nm (OD_{600}) was monitored at various timepoints throughout growth, and plotted as a semi-logarithmic growth curve. The *lrp* mutant shows a slower doubling time compared to the wild type in both media. This experiment was performed at least three times in triplicate, with similar results. Error bars denote ±SEM.

A


10
1
0.1
0.01
OD600
0
200
400
600
800
1000
1200
1400
1600
Time (min)
SL1344
SL1344 lrp

B


10
1
0.1
0.01
OD600
0
200
400
600
800
1000
1200
1400
1600
Time (min)
SL1344
SL1344 lrp

with the same restriction enzymes and ligated to the digested *lrp* ORF (494 bp) to produce pKMC301 (Fig. 3.5 (A)), which was capable of producing rLrp (Fig. 3.5 (C)).

3.2.7 Functionality of rLrp-6xHis protein

Plasmid pKMC301 was engineered to express C-terminal 6xHis-tagged rLrp protein. The C-terminus is known to be important in the oligomerisation of Lrp, a feature essential for its ability to interact with DNA, as the smallest unit of DNA-binding is the Lrp dimer (Cui *et al.*, 1996). In addition, the labelling of proteins with affinity tags may lead to structural changes or may affect the biological activity of the protein (Waugh, 2005). In order to test whether the C-terminal 6xHis-tag interfered with its ability to multimerise or its structural integrity, the functionality of rLrp *in vivo* was examined using a *lacZ* reporter fusion to a known Lrp-regulated target gene. When pET22b-derivative plasmids are transformed into λDE3 lysogen protein-expression strains, stringent inhibition of expression from the T7 *lac* promoter prevents uncontrolled protein expression. When these plasmids are transformed into wild type cells, expression is less controlled, leading to small amounts of transcription. pKMC301, and the parental plasmid pET22b, were transformed into the *E. coli* strain VL386 *lrp*::*Tn*10 ϕ(*fimA-lacZ*) *fimE*::IS*I*. VL386 *lrp* is unable to invert the *fimS* element of the *E. coli fim* switch as a result of the *Tn*10 insertion in the *lrp* gene (Dove and Dorman, 1996). If Lrp is restored, switching occurs and *fimA* will become active, resulting in expression of the reporter gene *lacZ*. MacConkey-lactose plates were used to monitor the Lac phenotype of the cells. VL386 *lrp* with the protein expression plasmid pKMC301 showed a Lac^+ phenotype, whereas VL386 *lrp* containing pET22b gave a Lac^- phenotype (Fig. 3.5 (B)).

3.2.8 Sequence analysis of the *lrp* promoter

The *lrp* promoter region of *E. coli* has been well characterised, and is known to contain Lrp binding sites, as *lrp* gene expression is negatively autoregulated. Friedberg *et al.* noted that the DNA sequences of the *E.coli* and *S. enterica* Lrp ORFs are 88 % identical, yet the amino acid sequences of the gene products are 99 % identical (Friedberg *et al.*, 1995). In this study, bioinformatics were used to examine the homology of the *lrp* promoter regions between *E. coli* and *S.* Typhimurium, with particular interest in the positions of the characterised Lrp binding site of *E. coli*. A DNA alignment of the 550 bp *lrp* upstream region, shown in Fig. 3.6, showed 83.3 % identity between *S.* Typhimurium LT2 and *E. coli* K-12 MG1655. The areas with least identity (49 %)

between the strains lie between –168 and –100, with respect to the transcriptional start site of *E. coli lrp*. The putative promoter of *S.* Typhimurium *lrp* has been assigned by bioinformatic analyses of the genome sequence of LT2, as have putative Lrp binding sites (McClelland *et al.*, 2001). The putative promoter has both a highly degenerate –35 (ATGGGCAGC) and –10 element (AGTAGAGTC). The promoter and putative promoter of both *E. coli* and *S.* Typhimurium *lrp* are highlighted in the respective sequences. The putative Lrp binding sites of *S.* Typhimurium as identified by McClelland *et al.* are highlighted in blue text, while the putative, and characterised Lrp binding sites of *E. coli* are highlighted in blue, and boxed, respectively (Wang *et al.*, 1994). Interestingly, the characterised Lrp binding site in *E. coli* is conserved in *S.* Typhimurium.

3.2.9 The transcriptional start site of the *lrp* transcript

The transcriptional start site of the *lrp* transcript of *S. enterica* is unknown. Annotation of whole genome sequencing has placed a putative –35 element at 67 to 75 bp upstream, and –10 element from 39 to 48 bp upstream of the translation initiation codon ATG of the *S.* Typhimurium *lrp* gene (McClelland *et al.*, 2001). In *E. coli*, *lrp* transcription initiates with a G, mapped to 267 bp upstream of the ATG initiation codon (Wang *et al.*, 1994). In *S.* Typhimurium, the corresponding base is 268 bp upstream, due to gaps in the sequence alignment.

To identify the position of the *lrp* promoter in *Salmonella*, primer extension analysis was undertaken as described in section 2.18. This was performed on RNA samples taken from SL1344 after 5.5 h growth in LB, at an OD_{600} corresponding to approximately 3.5. Primer extension analysis was also performed on RNA taken from mid-logarithmic phase SL1344 in MOPS minimal medium, at an OD_{600} of 0.5. Equal concentrations of total RNA were used for each assay. The oligonucleotide used for promoter mapping, PE-*lrp*, has reverse complementarity to 32 bp in the 5' region of the *lrp* gene. DNA sequencing reactions were performed as described in section 2.17.1, also using oligonucleotide PE-*lrp*, and plasmid pKMC101 as template. The results of primer extension analysis, shown in Figure 3.7, showed one product band from both LB and MOPS minimal media RNA sample analyses. This band was positioned adjacent to one of six adenines from between 258 and 263 bp upstream of the *lrp* promoter. This showed that the *lrp* transcription start site of SL1344 was located further downstream than that

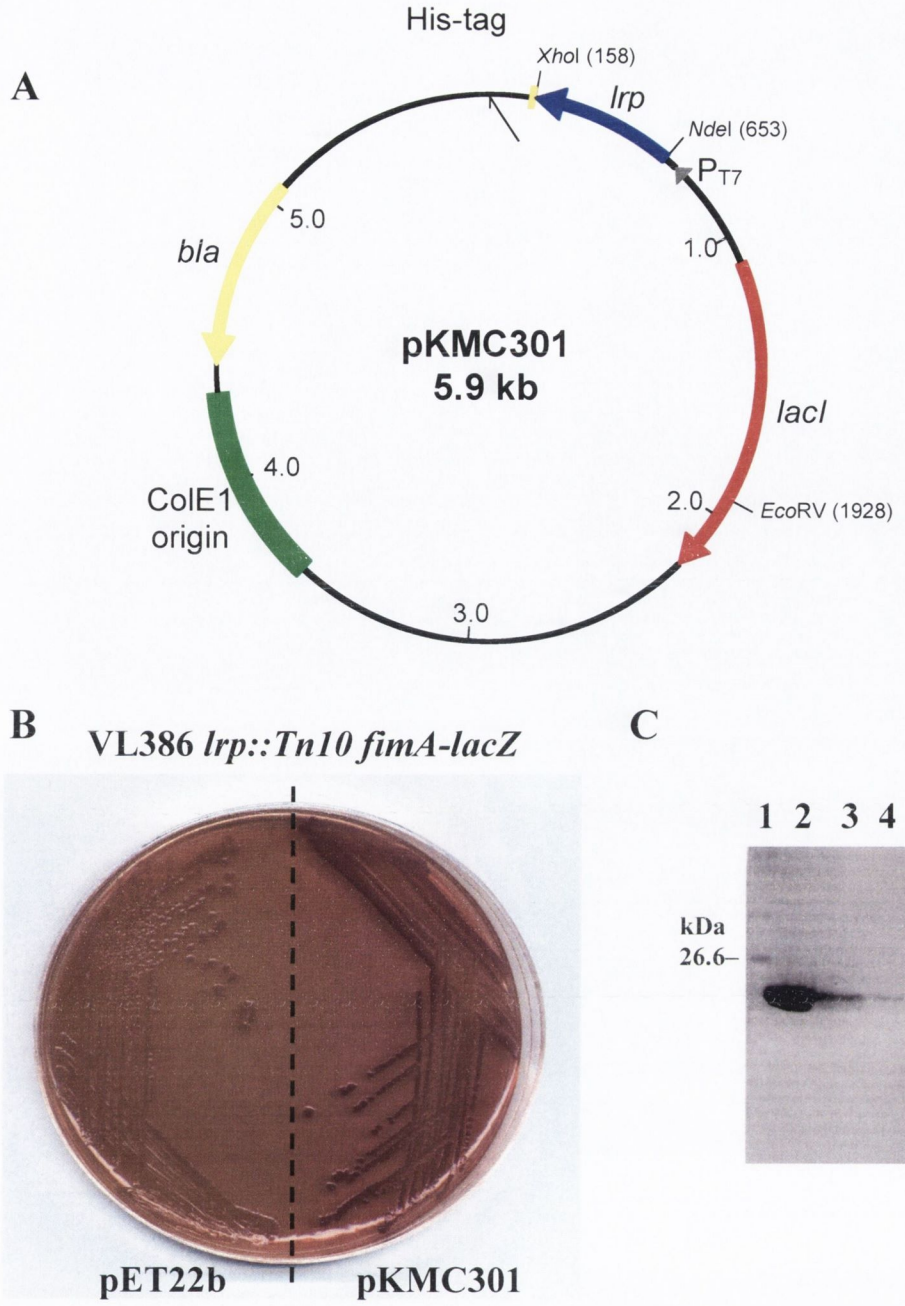
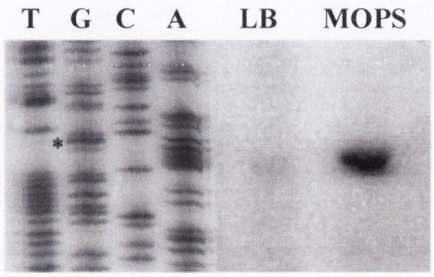


Fig. 3.5. The 6xHis-Lrp expression plasmid, pKMC301. The *lrp* ORF was placed under the control of the IPTG-inducible T7 *lac* promoter in pET22b, creating plasmid pKMC301 (A), which expresses Lrp with a C-terminal His-tag. The fusion protein expressed was tested for functionality by transformation of pKMC301 into the *E. coli* strain VL386 *lrp*::Tn10, which also contains a *lacZ* reporter fusion to *fimA*. Expression of *lacZ*, indicating expression of functional Lrp, was monitored by growth on MacConkey lactose agar (B). Lrp expressed from this plasmid was examined by SDS-PAGE: 5, 1 and 0.5 μl-volumes of purified Lrp (18.8 kDa) were analysed (C: lanes 2-4), in comparison with lysozyme standards of 5, 2 and 1 μg (lanes 5-7). Molecular mass protein marker is shown in lane 1. The Lrp solution, estimated to be approximately 5 μg μl^{-1}, was diluted 3 fold with dialysis buffer, and analysed by Bradford assay.

Fig. 3.6. *lrp* gene regulatory sequence comparison between *E. coli* and *S.* Typhimurium. The 550-bp region upstream of the translation initiation site of *lrp* was examined for homology between *S.* Typhimurium (top) and *E. coli* (bottom). The identical nucleotides between the two sequences are denoted by an asterisk, and gaps in the sequences are shown by a hyphen. The *lrp* ORFs are shown boxed with capitalised, bold text. The putative –35 and –10 regions of both *E. coli* and *S.* Typhimurium are highlighted in green in the respective DNA sequences. The predicted *S.* Typhimurium promoter, located between 39 to 75 bp from the *lrp* ATG, shows little homology to the consensus σ^{70} –35 and –10 promoter elements. Blue text indicates putative Lrp binding sites, while the characterised Lrp binding site is boxed in the *E. coli* DNA sequence. The base pair positions indicated refer to the *E. coli lrp* transcriptional start site (Wang *et al.*, 1994), which is shaded in capitalised, bold text.


-270 -250 -230
-tgagatccccatagttgttggcagacaatgagcagaattgtagggaatttacagacgta
atgagatccccatagttgttggcagacaatgggcaggattgtagggaatttacagacgta
-210 -190 -170
aaaaaagagcataacgattttgttaacaatatgtgtaatagcatgaaccgatgaacggcc
aaaaaagagtatgacgattttgttaacaatttgtgcaatcggcagcatcgataagcaggt
-150 -130 -110
gcgacagcgacgttatcatcacaaactttaattaaaatcggtaacttataaggtgacgaa
caaattctcccgtcattatcacctctgctacttaaatttcccgctttataagccgattaa
-90 -70 -50
atgacagtttaccgccctctctaatgaataactggcatgttgtactaaaaatcgatgttt
atgatgaataaacgcccctgttaatgaatatctggcatgttgtactaaaaatcgatgttt
-35 -10 +1 +20
tgctttgacaatcacctgctgttttgcgaaaacattcgaggaagaaaaaactgtgttatg
tgcttttgacaatcccctggtgttttgcgaaaacattcgaGgaagaaaaaaaacag-tatt
--tatgtgctgcataatcatgcatgtaaataccatgtttaccgggctagtgaaatctacg
cttatatgc-gcataaccatgcatgtaaataccatgtttaccgtgctagtgaaatctacg
catggcgtggacagacgccattcgtgatgtcgatagctgccgcgaggcaacggtcttctc
tatggcgtggacagacgccattcgtgatgtcgatagctgccacaaggcaacggtcttctc
accatagacc-aggcattgcgcgccgttaatccctctgggtttcggtctatcgtgatggg
accgtagacccaggcattgcgcgccgtgaat-cttcatgatttcggtctatcgtgacggg
cagcgactctgaacagtgatgtgagtagagtcaggcaggagtagggaaggaatacagaga
tagcgactctgaacagtgatgt-ttcagggtcagacaggagtagggaaggaatacagaga
gacaataataATGG...
gacaataataATGG...



*
TTTGACAATCACCTGCTGTTTTGCGAAAACATTCGAGGAAGAAAAAACTG
–35 –10

Fig. 3.7. Primer extension analysis of the *lrp* promoter of *S.* Typhimurium. Primer extension analysis showed that the *lrp* transcriptional start site is positioned within a group of six As, between 258 and 263 bp upstream of the translation initiation codon ATG. The DNA sequence of the corresponding region is alongside: the lanes were re-labelled to allow reading of the complementary coding strand. An asterisk is placed at the base corresponding to the transcriptional start site of *E. coli* (Wang *et al.,* 1994). This is a composite figure, with the DNA sequence from one exposure with the primer extension analysis bands from a different exposure.

of *E. coli*. In addition, densitometric analyses showed that the transcript signal was 4.2-fold higher in MOPS minimal medium compared to LB medium. This suggested that the level of *lrp* RNA, and thus transcription, was relatively higher in MOPS minimal medium than LB medium at the OD_{600} values examined. For consistency, and because an exact transcriptional start site was not identified in this study, positions of binding sites etc. are referred to in terms of the *E. coli lrp* transcriptional start site for the remainder of this study.

3.2.10 Construction of an *lrp* transcriptional fusion

In order to monitor the activity of the *lrp* gene, a transcriptional reporter fusion to the *lrp* promoter of SL1344 was constructed. The vector used was pZEP08, which contains a promoterless *gfp* gene. When an active promoter is placed 5' to the *gfp* gene, green fluorescent protein is transcribed and translated. Flow cytometry is used to monitor the average level of fluorescence, which represents the level of promoter activity.

PCR was used to amplify a 612-bp fragment, containing 61 bp of the *lrp* ORF, and 533 bp upstream DNA containing the *lrp* promoter region, from the SL1344 genome using primer pair *lrp*-gfp-F and *lrp*-gfp-R, incorporating *Sma*I and *Xba*I restriction sites, respectively. The fragment produced was digested with restriction enzymes, purified, and subsequently ligated to pZEP08 digested with the same restriction enzymes. The resulting plasmid, pZEP*lrp*, was transformed into XL1-Blue, purified and verified by sequencing.

3.2.11 Construction of an *lrp* complementation plasmid

To confirm that phenotypes shown by SL1344 *lrp* were attributable to the mutation in *lrp*, it was necessary to complement the *lrp* mutation. To do this, an *lrp* complementation plasmid was constructed. A 1,256 bp *lrp* gene and flanking regions were PCR amplified from the SL1344 genome using primer pair Fwd1.3 and Rev1.3, and then digested with *Aat*II. The single copy vector pZC320 was digested with *Pml*I and *Aat*II restriction enzymes, purified and mixed with the digested *lrp* gene fragment in a ligation reaction, producing pKMC102 (Fig. 3.8 (A)).

pKMC102 was tested for its ability to complement an *lrp* mutation in the *E. coli* strain VL386 *lrp*. This strain is unable to invert the *fimS* element of the *E. coli fim* switch due

to the *Tn*10 insertion in the *lrp* gene (Dove and Dorman, 1996). If switching does occur, *fimA* becomes active, resulting in expression of the reporter gene *lacZ*. MacConkey-lactose plates were used to monitor the Lac phenotype of the cells. VL386 *lrp* with the complementation plasmid pKMC102 showed a Lac^+ phenotype, whereas VL386 *lrp* containing pZC320 gave a Lac^- phenotype (Fig. 3.8 (B)).

3.2.12 Activity of the *lrp* promoter in LB medium

To assess the activity of the *lrp* promoter in rich media, SL1344 and SL1344 *lrp* were transformed with the *lrp-gfp* reporter fusion plasmid pZEP*lrp*. Strains were cultured as described in section 2.2.2, and samples taken at specific timepoints. These samples were analysed by spectrophotometry at OD_{600}, and tested for *gfp* expression by flow cytometry. The growth curve data showed that the carriage of pZEP*lrp* imparted a minor growth deficit on these cells and the growth rate difference between SL1344 and SL1344 *lrp* was slightly increased (Fig. 3.9 (A)). The *gfp* expression data showed that the activity of the *lrp* promoter initially increased slightly at lag to early exponential phase, and then decreased to its lowest level at mid-exponential phase, approximately 3 h after inoculation (Fig. 3.9 (B)). The activity increased again, coinciding with a slowing of growth in late-exponential to stationary phase, between 5 and 6 h after inoculation. Thereafter, levels of activity remained relatively stable through to late stationary phase. The profile of *lrp* promoter activity was similar between SL1344 and SL1344 *lrp*, but interestingly, the level of *lrp* expression was higher in the *lrp* mutant, suggesting that Lrp negatively controls its own expression in *S.* Typhimurium, as well as in *E. coli*. This experiment was performed at least three times with similar trends. Error bars denote ±SEM.

3.2.13 Activity of the *lrp* promoter in MOPS minimal medium in the absence and presence of L-leucine

The activity of the *lrp* promoter in nutrient-poor medium was analysed by the growth of SL1344 and SL1344 *lrp* transformed with pZEP*lrp* in MOPS minimal medium in the absence of L-leucine, and SL1344 in the presence of 10 mM L-leucine. Samples were harvested at specific timepoints, their OD_{600} measured by spectrophotometry, and the expression levels of *gfp* monitored by flow cytometry. The growth curve data obtained (Fig. 3.10 (A)) showed that the growth rate of all strains was strongly affected by the carriage of pZEP*lrp*. However, the growth patterns of all three strains followed the same

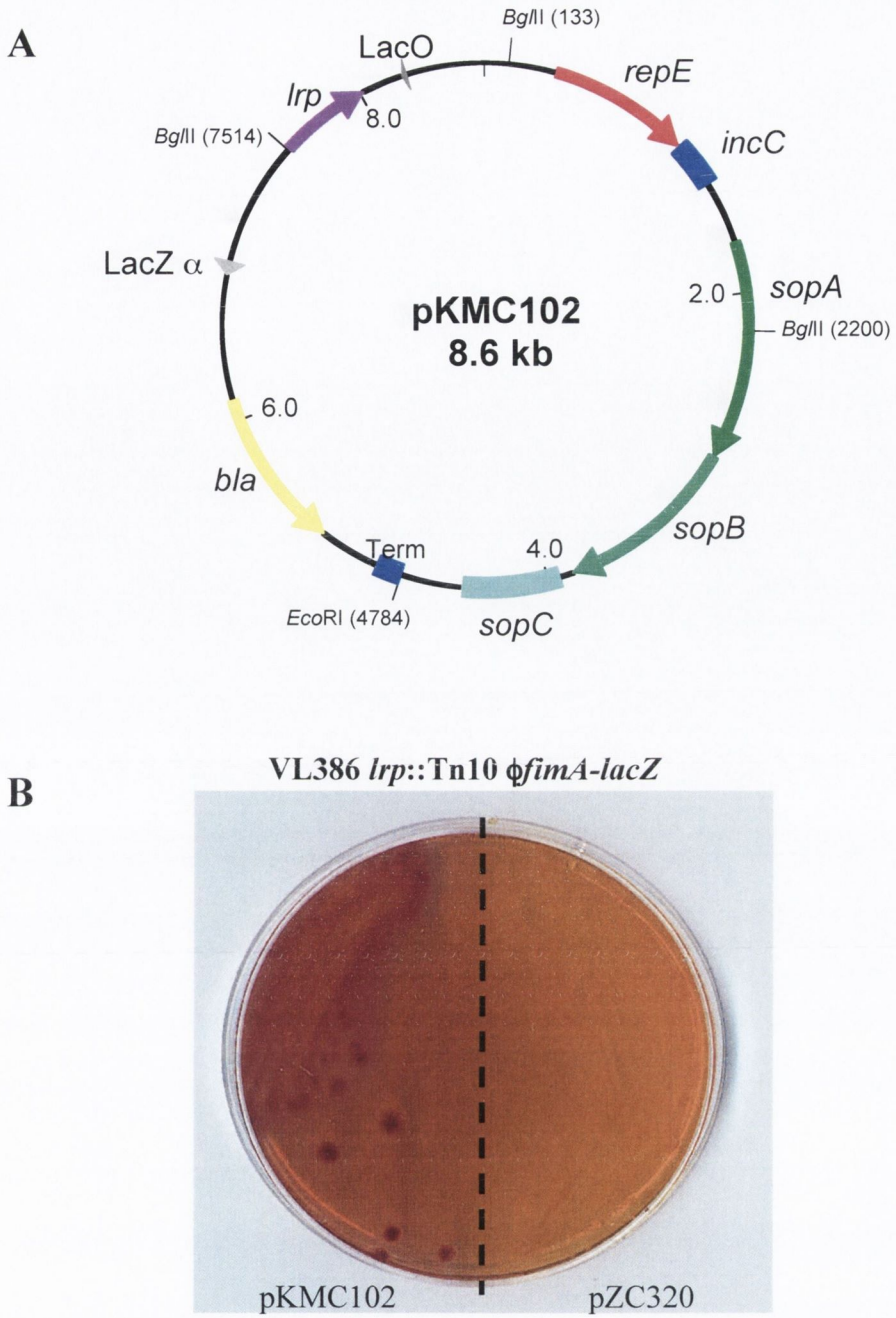


Fig. 3.8. Construction and verification of functionality of pKMC102, the *lrp* complementation plasmid. The *lrp* gene and flanking regions were PCR amplified and ligated into the MCS of pZC320 (**A**). The ability of this construct to complement the *lrp* mutation was tested by transformation into the *E. coli* strain VL386 *lrp*::Tn10, which also contains a *lacZ* reporter fusion to *fimA*. Expression of *lacZ*, indicating a restoration of *lrp* activity, was monitored by growth on MacConkey lactose agar (**B**).

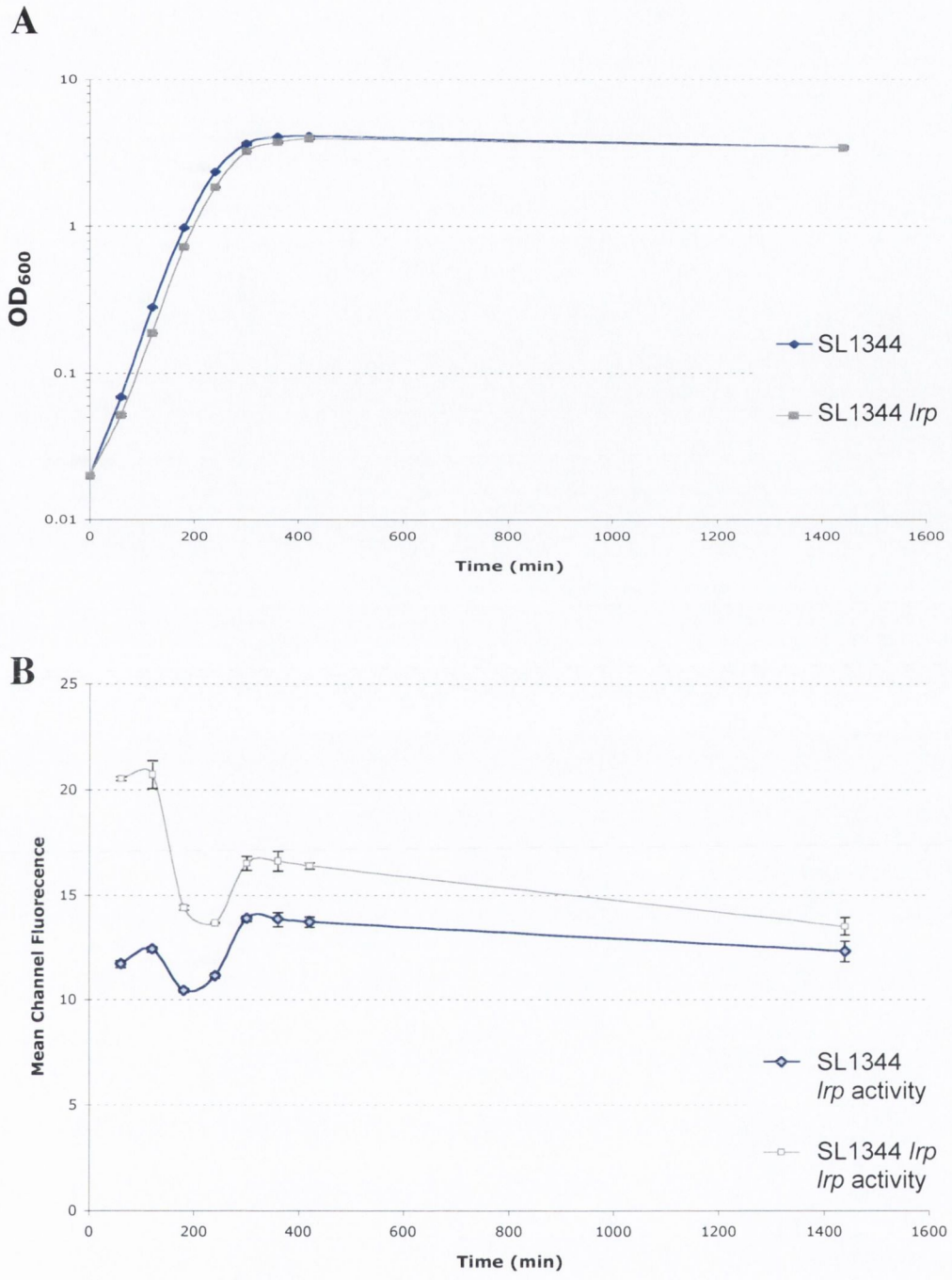


Fig. 3.9. The activity of the *lrp* promoter during growth in LB broth. SL1344 and SL1344 *lrp* containing pZEP*lrp*, the *lrp-gfp* reporter fusion vector, were assessed at specific time points for growth (**A**) by measurement of optical density at 600 nm (OD_{600}), and subsequently analysed for *gfp* expression by flow cytometry (**B**). The experiment shown was performed in triplicate and is representative of similar experiments. Error bars: ± SEM.

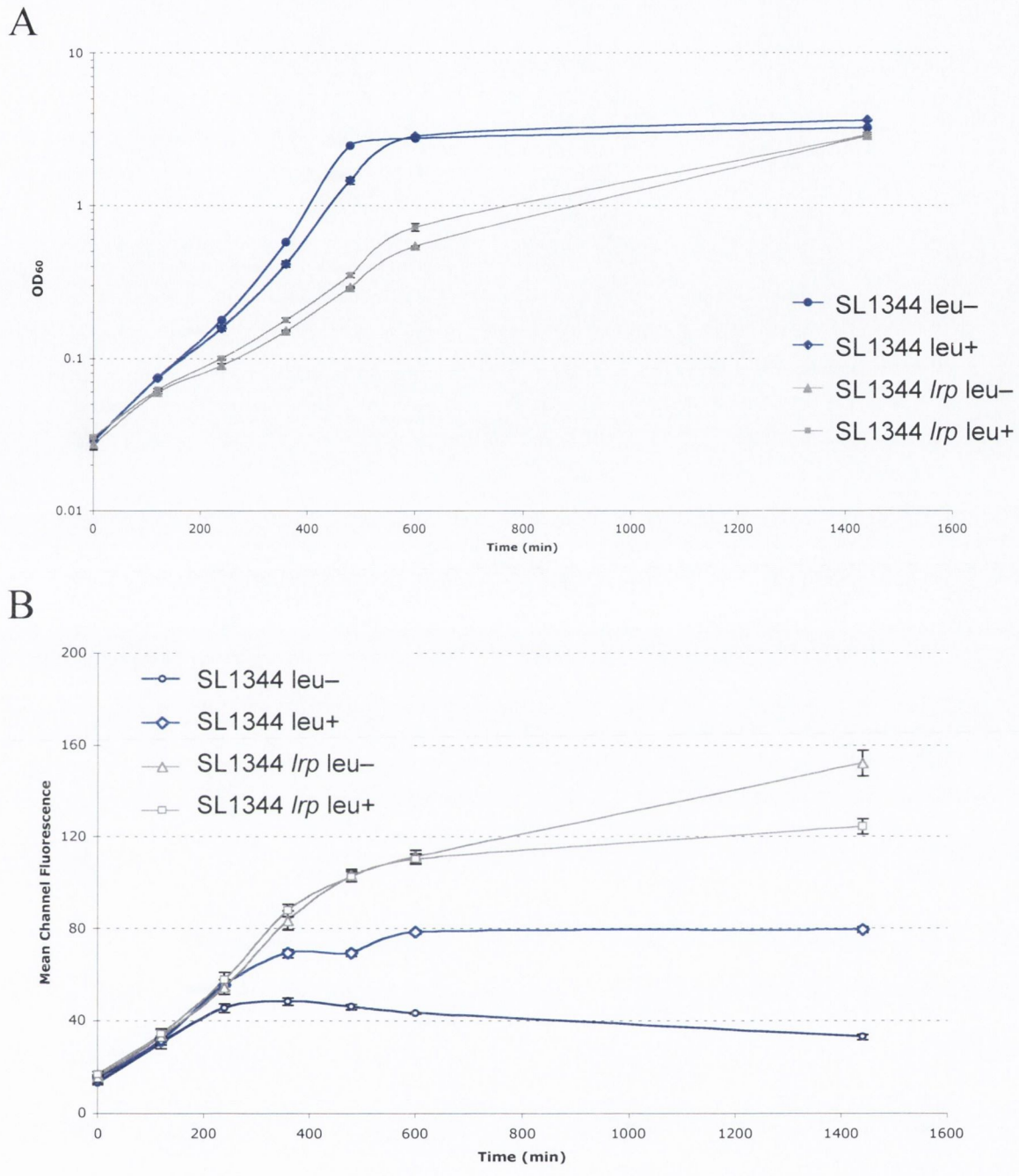


Fig. 3.10. The activity of the *lrp* promoter during growth in MOPS minimal broth. SL1344, in the absence and presence of leucine, and SL1344 *lrp* containing pZEP*lrp*, the *lrp-gfp* reporter fusion vector, were assessed at specific time points for growth by measurement of optical density at 600 nm (**A**), and subsequently analysed for *gfp* expression by flow cytometry (**B**). The data shown was performed in triplicate and is representative of similar experiments. Error bars denote ± SEM.

trends as previously shown: the addition of leucine reduced the doubling time of the wild type, and SL1344 *lrp* experienced a considerable growth defect in comparison to SL1344. The expression data (Fig. 3.10 (B)) showed that from inoculation to an OD_{600} of approximately 0.5, *lrp* expression in SL1344 increased. Upon entry into stationary phase, the levels of *lrp* expression appeared to decrease slightly, possibly a result of negative feedback by Lrp. By stationary phase, expression began to plateau into late-stationary phase. SL1344 supplemented with leucine showed a similar expression profile, but levels of *lrp* expression were generally higher in the presence of leucine. The expression level appeared similar between the two strains until an approximate OD_{600} of 0.2: thereafter *lrp* expression increased by 1.5 to 2 fold in the presence of leucine. Interestingly, the change in expression patterns seemed to coincide with the change in growth profile between the two growth conditions. Compared to SL1344, SL1344 *lrp* also showed an increased level of *lrp* expression along the growth curve, suggesting that Lrp represses its own transcription. The levels of *lrp* expression in the *lrp* mutant in the absence and presence of leucine were strongly similar, and ranged from 2- to 5-fold higher than that of the wild type in the absence of leucine. *lrp* expression was increased slightly at late stationary phase in the absence of leucine, compared with its presence.

3.2.14 Direct interaction of Lrp with the *lrp* promoter

Analyses of *lrp* promoter activity between the SL1344 wild type and *lrp* mutant strongly suggested that Lrp represses its own transcription. In *E. coli*, Lrp has been shown to directly auto-repress its transcription (Wang *et al.*, 1994), however, as shown in section 3.2.8, the sequence of the 550 bp *trxB-lrp* intergenic regions of *E. coli* and *S.* Typhimurium have only 83.3 % identity. In addition, two putative sites for Lrp binding, one of which correlates with a characterised *E. coli* Lrp site, have been identified in the genome annotation of *S.* Typhimurium LT2 (McClelland *et al.*, 2001), centred at –61 and +50, with respect to the corresponding transcription initiation codon of *E. coli lrp* (Figure 3.11 (A)). To examine whether Lrp was capable of binding directly to its own regulatory region, EMSA analyses were employed. Increasing concentrations of Lrp were incubated with biotinylated DNA probes, and analysed by polyacrylamide gel electrophoresis.

EMSA analyses were undertaken using a biotinylated probe of 418 bp, amplified using primer pair *lrp*-EMSA-F and *lrp*-R.Bio, and encompassing 266 bp upstream and 152 bp

downstream of the *lrp* transcriptional start site (Figure 3.11 (A)). This was incubated with 0, 13.3, 26.6, 53.2 and 133 nM Lrp in the absence and presence of 15 mM L-leucine, and additionally in the presence of 15 mM glycine. Like leucine, glycine is a non-polar, neutral amino acid that, unlike leucine, is not an effector molecule for Lrp. Glycine was added to act as a control for non-specific ionic strength effects that L-leucine may have on the interaction of Lrp with its target sites (Wiese *et al.*, 1997). This experiment showed that Lrp interacted with the 418 bp probe to form two complexes (Figure 3.11 (B)). As expected, glycine did not have a significant effect on the binding of Lrp (lanes 9-12), whereas the addition of leucine appeared to favour the production of complex 2 over complex 1 at higher concentrations of Lrp (lane 10). In addition, the presence of leucine seemed to enhance complex formation, compared to the absence of leucine (lanes 5-6 and lanes 1-2, respectively).

3.2.15 Nested deletion analysis of the *lrp* promoter

Two putative Lrp-binding sites had previously been identified in the *lrp* promoter region by whole genome sequencing (McClelland *et al.*, 2001). The current study demonstrated that a 418-bp fragment encompassing this area formed two protein-DNA complexes upon incubation with Lrp. In order to analyse the position of these two sites, and whether there were other, uncharacterised Lrp-binding sites located in the *lrp* regulatory region, nested deletion analysis was undertaken. This involved the production of a series of biotinylated DNA probes. The probes, shown in Fig. 3.12 (A), increased in length by increments of approximately 100 bp: the F probes were PCR amplified using primer *lrp*-EMSA-F with primer *lrp*-EMSA-R3 (F102); with primer *lrp*-EMSA-R4 to produce F200, and primer *lrp*-EMSA-R5 to produce F311. The R probes were PCR amplified using primer *lrp*-R.Bio in conjunction with primer *lrp*-EMSA-F3 (R111); primer *lrp*-EMSA-F4 (R202) and primer *lrp*-EMSA-F5 (R310).

Upon incubation of the probes F102, F200, and R111 with Lrp, no interactions were observed, indicating that the region important for Lrp binding was located between –66 and +43, with respect to the transcriptional start site (Fig. 3.12 (B)). Probe R202 (–49/+153) showed a small amount of complex formation at the highest concentrations of Lrp (160, 213 and 266 nM). Probes F311 and R310 showed the most significant Lrp interaction, with three and two apparent complexes, respectively, indicating that the region overlapping F311 and R310 (–157 to +46) was important for Lrp binding (see

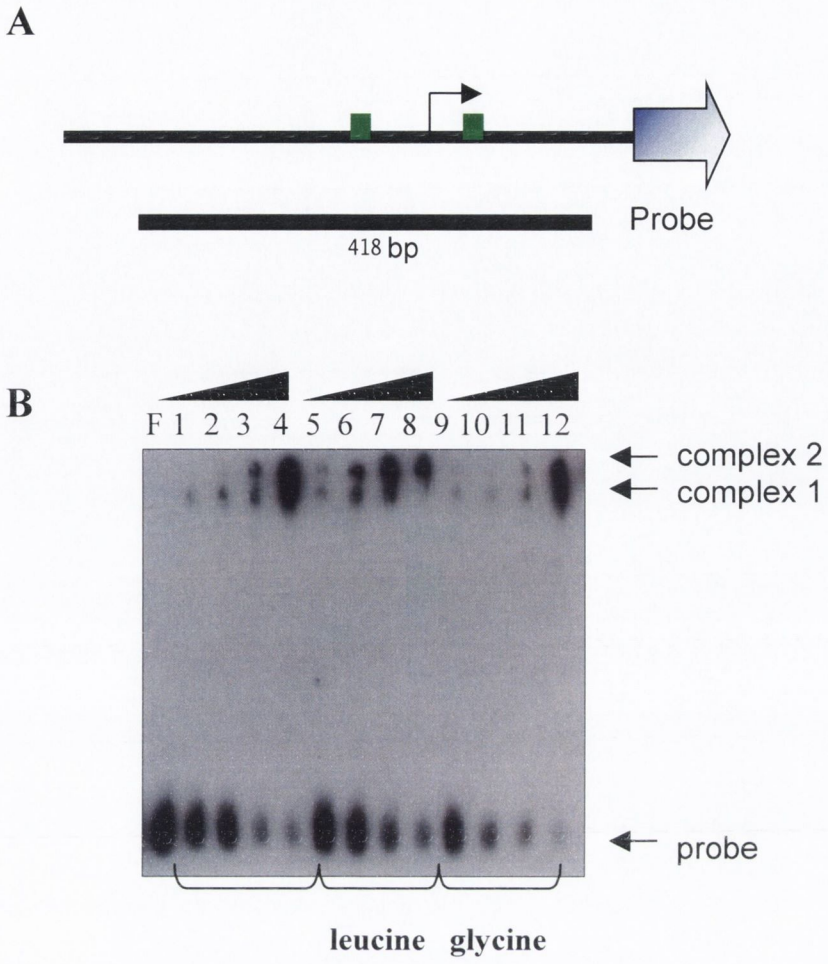


Fig. 3.11. EMSA analyses of the *lrp* promoter with Lrp. The *lrp* promoter region was examined for Lrp binding using EMSA analysis. The position of this probe in relation to the *lrp* promoter is shown in A, where the green blocks denote putative Lrp binding sites, and the arrow denotes the transcriptional start site. A 418-bp probe was incubated with increasing concentrations of Lrp (13.3, 26.6, 53.2, 133 nM), in the absence of amino acid supplements (lanes 1-4), presence of 15 mM leucine (lanes 5-8) or 15 mM glycine (lanes 9-12). Free probe, without added protein or amino acids, is denoted by F.

Fig. 3.12. Further EMSA analyses of the *lrp* promoter with Lrp. The *lrp* promoter region was examined for Lrp binding using nested EMSA analysis with an array of biotinylated probes, designated F probes and R probes. The position of these probes in relation to the *lrp* promoter is shown in A, where the green blocks denote putative Lrp binding sites identified by whole genome sequencing, and the arrow denotes the approximate position of the transcriptional start site. A probe of 418 bp that had been shown to form two complexes in the presence of Lrp is shown for comparison. This series of probes was amplified to examine more closely the ability of Lrp to interact with the *lrp* promoter. EMSA analyses of the F102, R111, F200 and R202 probes did not show any significant interaction with increasing concentrations of Lrp (0, 26.6, 53.2, 106.4, 160, 212.8, 266 nM), whereas both F311 and R310 showed the formation of three and two complexes respectively, denoted by arrows, when incubated with increasing concentrations of Lrp (0, 13.3, 26.6, 53.2, 133, 266 nM).

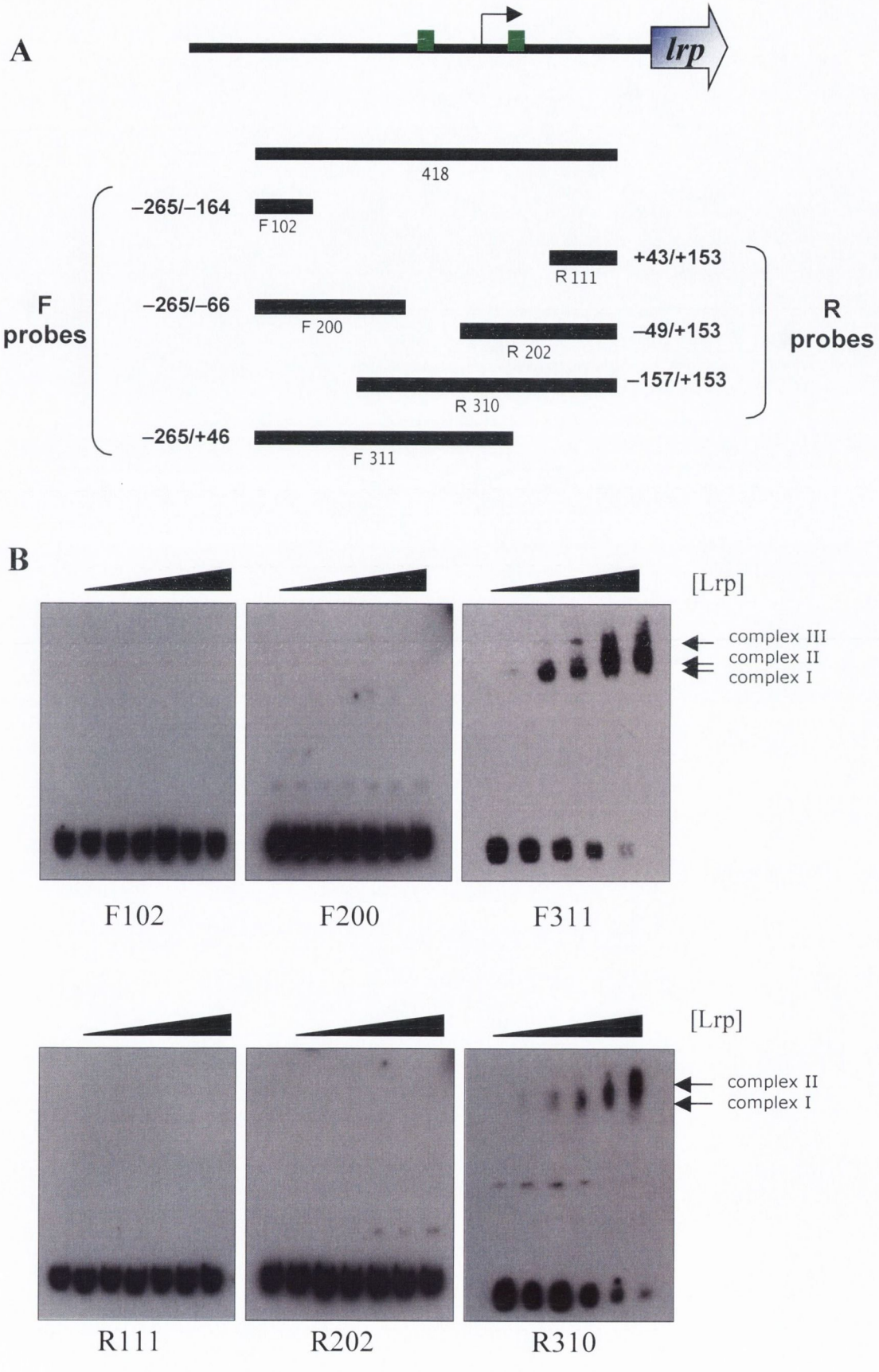

A
lrp
418
–265/–164
F 102
+43/+153
R 111
F
probes
–265/–66
F 200
–49/+153
R 202
R
probes
–157/+153
R 310
–265/+46
F 311
B
[Lrp]
complex III
complex II
complex I
F102
F200
F311
[Lrp]
complex II
complex I
R111
R202
R310

Fig. 3.12). Of particular interest was the region from –66 to +43, which was present in all probes with significant Lrp interaction.

In *E. coli*, it has been shown that Lrp can interact with a region named LP-10 (Wang *et al.*, 1994). LP-10 is positioned between –98 and +1, with respect to the transcriptional start site of *lrp* of *E. coli.* In order to examine the ability of Lrp to interact with the corresponding region in SL1344, primers EcLP-10-F and StyLP-10-F were used to PCR amplify the region from both *E. coli* MC4100 and SL1344, respectively, in conjunction with biotinylated primer LP-10-R.Bio. Both probes were incubated with 0, 26.6, 53.2, 106.4, 212.8 and 266 nM Lrp, and both showed formation of two complexes (Figure 3.13 (A)). In previous studies, the Calvo group showed that Lrp formed one complex with LP-10 of *E. coli* at 65.5 nM Lrp (Wang *et al.*, 1994): my assay was in agreement, but additionally shows that higher concentrations of Lrp lead to the appearance of a second complex (lanes 4 and 5). LP-10 was capable of forming two complexes within a region that overlapped the position of one of the putative Lrp sites, centred at –61, but not containing the second of the putative sites (centred at +50) that had been identified by whole genome sequencing (McClelland *et al.*, 2001). In addition, another putative Lrp site in the *E. coli* sequence centred at +21 (Wang *et al.*, 1994) was also not contained within this probe.

To examine further the interaction of Lrp with its regulatory region, a 203-bp probe, consisting of the overlapping regions of F311 and R310 (–157 to +46), was PCR-amplified using primer pair *lrp*-EMSA-F5 and *lrp*-EMSA-R5/Bio. This was incubated with increasing concentrations of Lrp, in the absence and presence of leucine or glycine, which showed that Lrp was capable of forming three discrete complexes with this region (Fig. 3.13 (B)). In addition, there appeared to be a putative fourth complex (lane 4 in all cases). In this analysis, leucine had no clear effect on the ability of Lrp to interact with the *lrp* promoter. This probe contains regions of DNA that have been shown by EMSA analysis with F200 to be unimportant for Lrp interaction, i.e. between –157 to –66. This probe also extends LP-10 downstream by 45 bp, which suggests that there may be more Lrp-binding sites between +1 and +46, to account for increased complex formation. Taken together, the EMSA analyses suggested that Lrp is capable of binding to three sites in the *lrp* promoter region.

3.2.16 Mapping the binding sites of Lrp on the *lrp* promoter

DNase I footprinting was used to map the binding sites of Lrp on the *lrp* promoter. It had been established that Lrp is most likely to interact with a region between 66 bp downstream and 46 bp upstream of the transcription initiation codon of *lrp*. A 436-bp fragment containing this region was amplified using primer pair *lrp*-F.*Bam*HI and *lrp*-R.*Eco*RI. This fragment was ligated to pBluescript II SK (–), producing pBSK*lrp*. Using pBSK-F and *lrp*-F.*Bam*HI primer pair, a 569-bp forward probe was amplified, and labelled with ^{32}P as described in section 2.17.2. This was digested with *Eco*RI to remove one end-label and purified, leaving a probe of 418 bp. The probe was incubated with increasing concentrations of Lrp, as described in section 2.17.4. The data, shown in Figure 3.14 (A), show that Lrp protects two regions from DNase I cleavage. Hypersensitive sites, indicative of DNA bending, are shown throughout the protected regions. These have been divided into two groups: dominant hypersensitive bases, and less prominent hypersensitive bases. In the first protected region, from –122 to –12, the most dominant bases occur at –107, –75, and –34, while less sensitive bases are located at –117, –98, –54 and –42. In the second region of protection, from +15 to +50, the hypersensitive base at +22 is most dominant, while the base at +41 appears less hypersensitive. To examine the reverse, non-coding strand of *lrp*, a 583-bp reverse probe was amplified, using the pBSK-R and *lrp*-R.*Eco*RI primer pair, and labelled with ^{32}P. This was digested with *Bam*HI to remove one end-label, and purified, resulting in a probe of 418 bp. The data, shown in Figure 3.14 (B), show that Lrp protects a region from +4 to –100 from DNase I cleavage. Many hypersensitive sites occur throughout the protected region, the most dominant at –1, –12, –36, –68, –78, –88 and –98. Less dominant bands appear at –46/–47 and at –57. The data obtained from DNase I footprints is collated in Figure 3.15, this figure also highlights the position of fragments LP-10 and the 203-bp fragment.

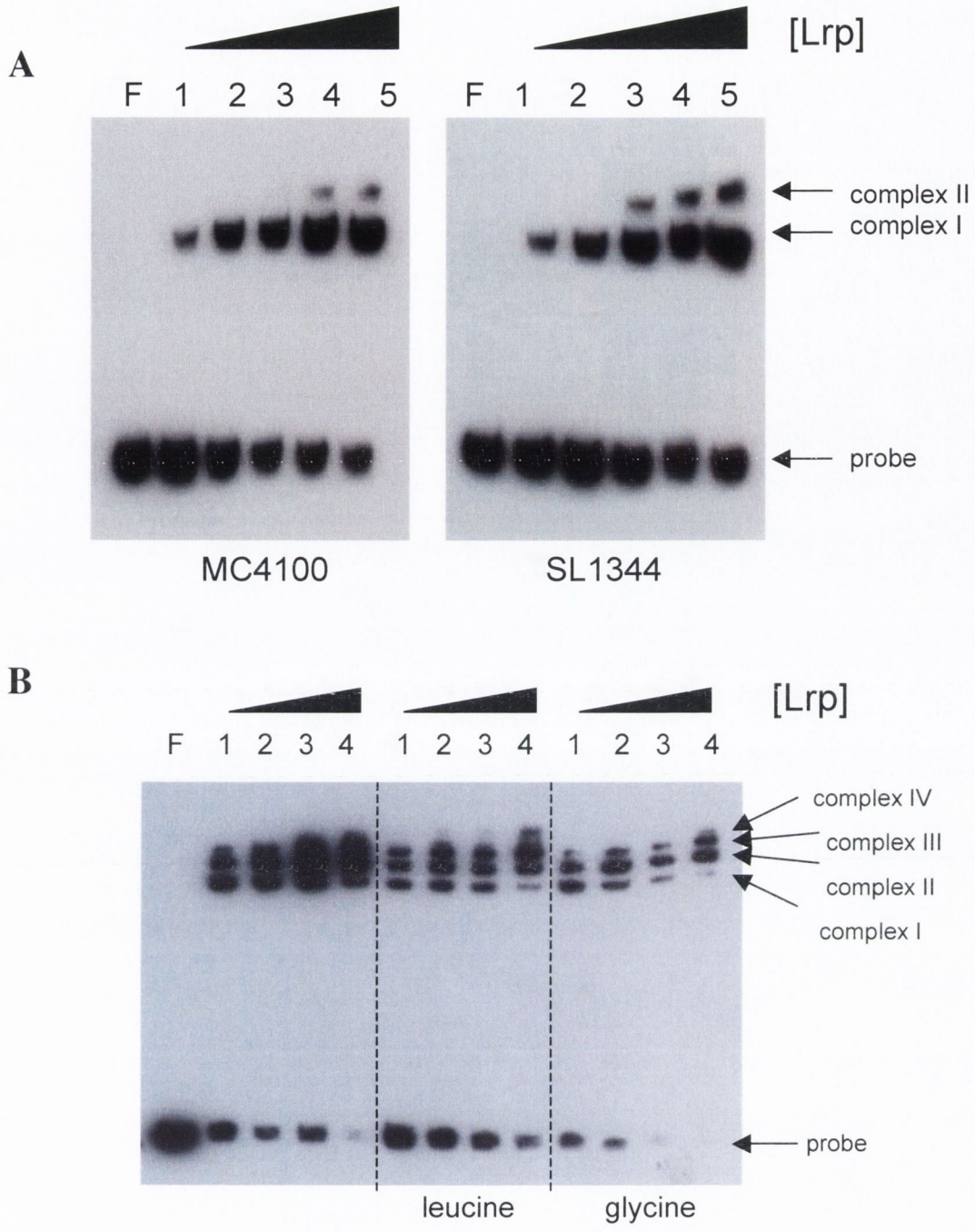
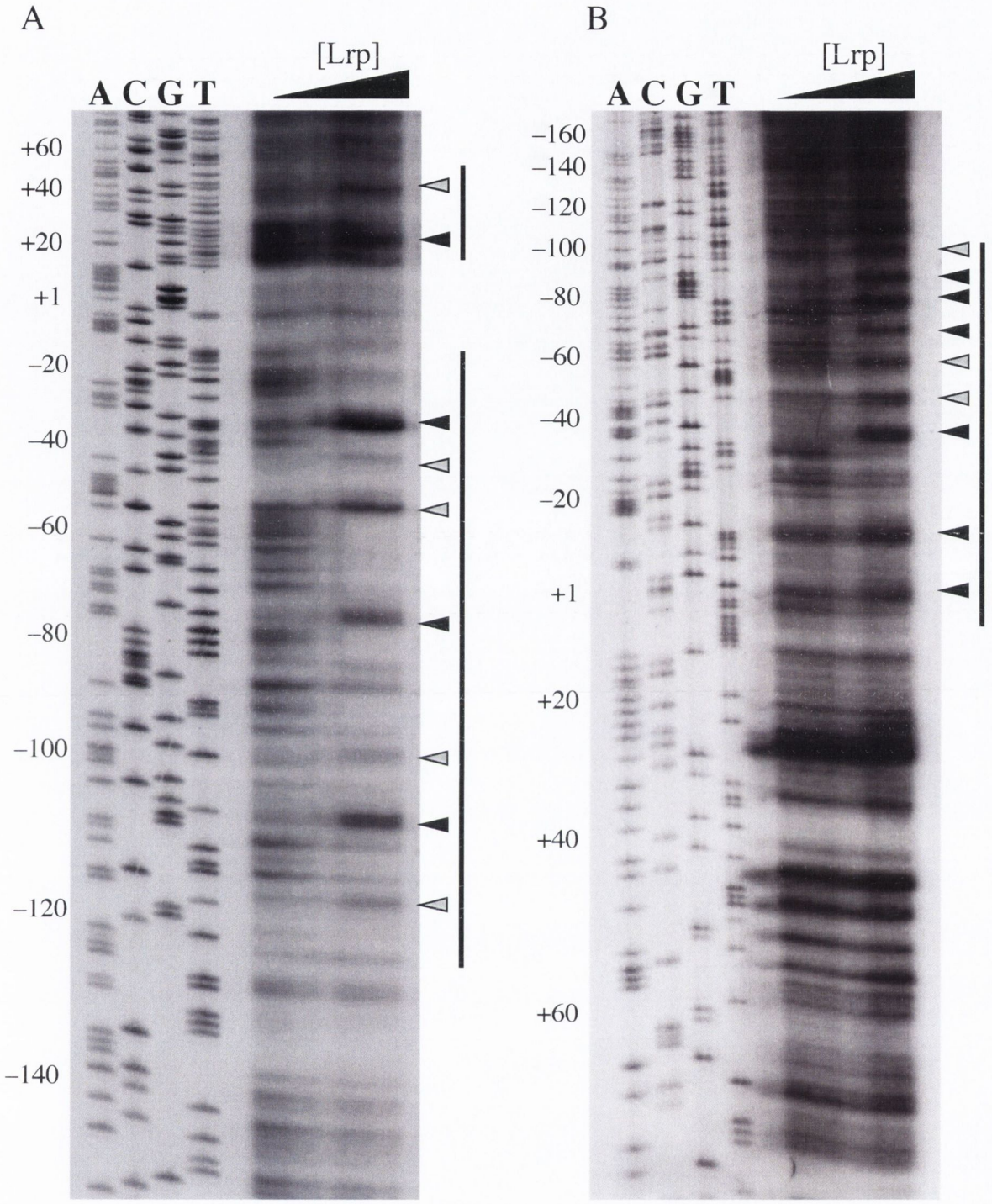


Fig. 3.13. EMSA analysis of the Lrp binding site-containing regions. (A) LP-10, the 99-bp fragment of the *lrp* promoter region from –98 to +1, of *E. coli* and the corresponding region in *S.* Typhimurium were analysed for Lrp interaction by incubation with increasing concentrations of Lrp (26.6, 53.2, 106.4, 212.8 and 266 nM: lanes 1-5). In addition, a 203-bp probe from –157 to +46 was also examined for its interaction with Lrp (B). This probe was incubated with 13.3, 26.6, 53.2, 133.38 nM Lrp (lanes 1-4). Incubations were performed in the absence, or in the presence of 15 mM leucine or glycine. F denotes free probe.

Fig. 3.14. DNase I footprinting of the Lrp-protected regions within the *lrp* promoter. The *lrp* promoter probes were incubated with 0, 25, 50, 100 and 150 nM Lrp as described in Chapter 2, and analysed by denaturing polyacrylamide gel elctrophoresis, alongside the relevant DNA sequencing products. Both coding strand (A) and non-coding strand (B) of the *lrp* promoter were analysed. Regions of protection by Lrp are shown by a black line, while hypersensitive bases are denoted by arrowheads. Hypersensitive bases are in two groups: the more dominant hypersensitive bases are shown by filled arrowheads, while the less prominent hypersensitive bases are depicted by shaded arrowheads. Footprints of both strands were performed twice, with similar results.


A
[Lrp]
A C G T
+60
+40
+20
+1
–20
–40
–60
–80
–100
–120
–140
B
[Lrp]
A C G T
–160
–140
–120
–100
–80
–60
–40
–20
+1
+20
+40
+60

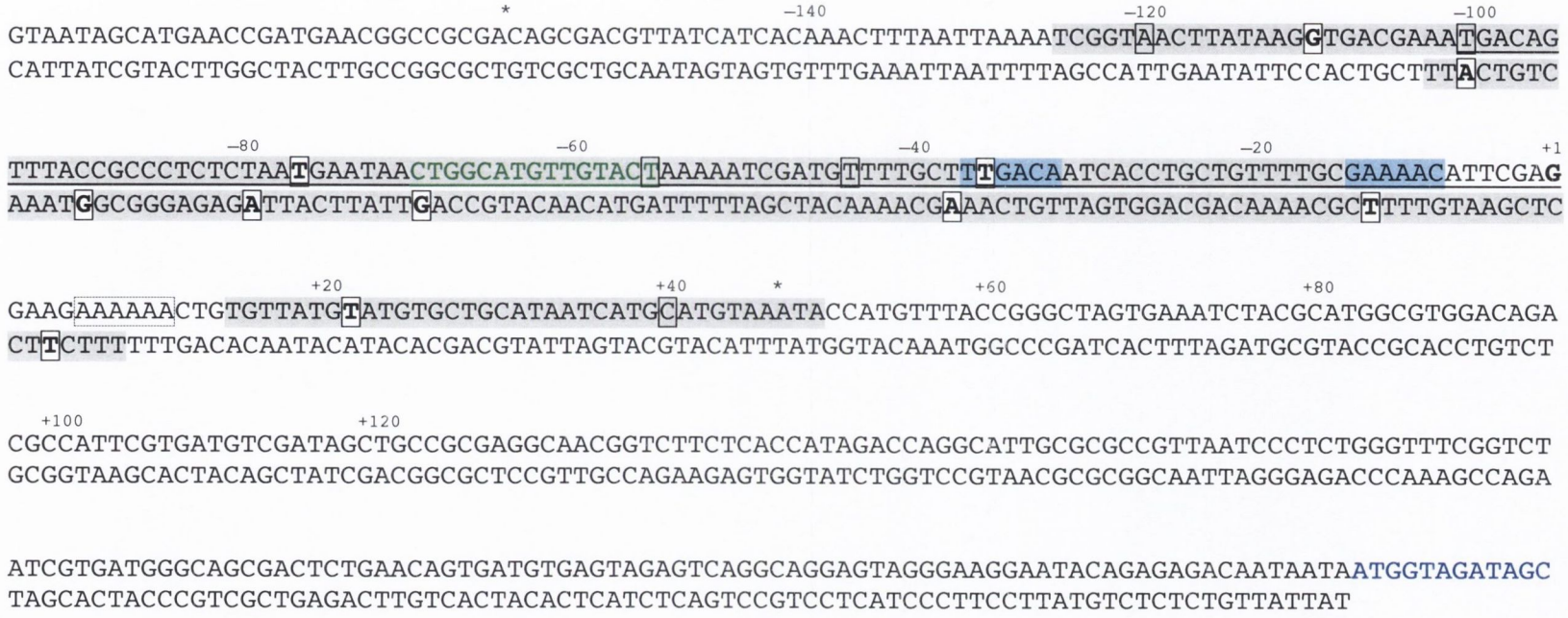


Fig. 3.15. Protection of the *lrp* promoter by Lrp. DNase I footprinting of both strands of the *lrp* promoter revealed areas of protection by Lrp (shaded), and regions of hypersensitivity (boxed nucleotides) strongly suggesting protein-induced bending of DNA. Hypersensitive bases that were the most sensitive to DNase I cleavage are additionally in bold, in a white box. Putative Lrp binding sites are denoted by green text. The transcriptional start site of the *lrp* gene in *E. coli* (circled, in bold text) is located 267 bp upstream of the ATG initiation codon (Wang *et. al.,* 1994) and numbers denote positions with respect to this start site. Primer extension analyses performed in this study places the transcriptional start site of *S.* Typhimurium *lrp* (dashed box) at between 258 to 263 bp upstream of the translational start codon (blue letters). The DNA sequences encompassed by LP-10 and the 203-bp probe are underlined, and denoted by two asterisks, respectively.

3.3 Discussion

In this chapter, the *lrp* promoter of *S.* Typhimurium was examined. The aims of this work were two-fold: to enable comparisons with the *E. coli lrp* promoter, the best-studied member of the *lrp* gene family, and to increase understanding of both SL1344 *lrp* and the *lrp* promoter to facilitate further study.

In previous studies, the *E. coli lrp* insertion mutant has been described as growing only slightly more slowly than its parent strain, with a doubling time of 80 min in glucose minimal medium, compared to 58 min for the wild type (Lin *et al.*, 1990). With mean doubling times of 58 min and 76 min in the absence of leucine supplementation, the growth rates of SL1344 and SL1344 *lrp* are comparable to that of *E. coli* (Fig. 3.4 (A)). Much of the physiological analysis of the *lrp* mutant was carried out in an *ilvA* background conferring isoleucine auxotrophy (Ambartsoumian *et al.*, 1994; Lin *et al.*, 1990). This is because *E. coli* strains show sensitivity to L-serine and L-leucine, the presence of which cause partial isoleucine-valine starvation, which can be relieved by the presence of exogenous isoleucine and valine in the growth medium (Bouloc *et al.*, 1992). As a result, the growth analysis of the *E. coli lrp* mutant in minimal medium was carried out in the presence of isoleucine and valine. Under these conditions, the growth rate of the *lrp* mutant (*ilvA lrp*) in glucose minimal medium was increased by the addition of leucine, with no effect on the growth of the parental (*ilvA* lrp^+) strain, whereas in an $ilvA^+$ background, the *lrp* mutant was shown to be hypersensitive to leucine (Ambartsoumian *et al.*, 1994). In the present study, the addition of leucine to SL1344 caused a slight growth defect, increasing the average doubling time to 67 min (Fig. 3.4 (B)). This could perhaps be explained by a negative effect of leucine on the Lrp-mediated regulation of the *ilvIH* biosynthetic operon, causing isoleucine-valine starvation upon addition of leucine, as was described in *E. coli.* Importantly, the *lrp* mutant of *S.* Typhimurium was not sensitive to leucine during growth in glucose minimal media, and did not require amino acid supplementation (section 3.2.5; Fig. 3.4 (B)). Currently, there is no explanation for the discrepancy between the effect of leucine on *E. coli lrp* and *S.* Typhimurium *lrp* mutant growth. If the addition of leucine causes a change in gene expression, e.g. at the *ilvIH* operon, through its effect on Lrp in SL1344, then the insensitivity of SL1344 *lrp* growth to leucine could be because Lrp is absent, and would therefore have no effect on Lrp/leucine-regulated genes. A number of

unknown factors and differences in cell metabolism between *E. coli* and *S.* Typhimurium presumably account for these observations.

The activity of the *lrp* promoter was examined during growth in rich and minimal media. During growth in LB broth, the *lrp* promoter activity initially increased slightly and then decreased, with its lowest expression level between 180 and 200 min after inoculation (Fig. 3.9). The expression level then peaked at between 300 and 360 min, staying at a similar level until late stationary phase. Expression studies on *lrp* in *E. coli* have shown similar trends in rich medium, with the lowest activity at approximately 2 h into growth and a peak of expression after 6 h growth (Landgraf *et al.*, 1996). In the *E. coli* studies, cell growth was monitored by μg total protein ml^{-1} culture, so it was not possible to compare growth by optical density, but comparison of the growth curves against time was possible. During growth in MOPS minimal medium, the activity of the *lrp* promoter increased from the point of inoculation, until approximately 240 to 360 min, coinciding with an OD_{600} of around 0.15 to 0.5 (Fig. 3.10 (A)). Thereafter, the activity stayed approximately level, with a slight decrease by late-stationary phase. In *E. coli,* promoter activity in MOPS minimal medium initially decreased slightly until 3 h after inoculation, after which a similar trend to *S.* Typhimurium was seen, with an increase of activity up to between 4 to 6 h, which stayed approximately level to late stationary phase (Landgraf *et al.*, 1996). In both LB and MOPS minimal medium, *lrp* promoter activity was higher in SL1344 *lrp* than in SL1344, which suggested that Lrp represses its own transcription. The *lrp* expression level was essentially the same in SL1344 *lrp* in the absence and presence of leucine.

The Lrp-mediated negative regulation of the *lrp* promoter has been described as leucine-insensitive (Lin *et al.*, 1992; Wang *et al.*, 1994) and leucine-sensitive (Borst *et al.*, 1996). Analyses in this study showed that SL1344 consistently displayed between 1.5 to 2 fold higher levels of *lrp* promoter activity during growth in MOPS minimal medium supplemented with 10 mM L-leucine (Fig. 3.10 (B)), suggesting that leucine positively affects Lrp expression. Importantly, the increased *lrp* expression in SL1344 *lrp* was of a similar level in both the absence and presence of leucine. This implies that the increased level of *lrp* expression in SL1344 in the presence of leucine, compared to its absence, was Lrp-dependent. There could be another explanation for the apparent increase in *lrp* expression: it was shown that the presence of leucine in MOPS minimal media reduced

the growth rate of SL1344 (Fig. 3.4). During the course of *lrp* expression analyses in MOPS minimal medium, it was observed that an increase in *lrp* expression in SL1344 in the presence of leucine correlates with a decrease in growth rate. As discussed above, *E. coli* strains exhibit varying sensitivities to leucine, which leads to isoleucine-valine starvation. This starvation is thought to lead to increased levels of ppGpp (Bouloc *et al.*, 1992). Landgraf *et. al.* showed that the expression level of Lrp is inversely related to growth rate and is positively regulated by ppGpp levels (Landgraf *et al.*, 1996). Perhaps the effect of leucine on the *lrp* promoter is not a direct one, but as leucine slows the growth of SL1344, the expression of *lrp* increases because of increased ppGpp levels. EMSA analyses showed that the effect of leucine on the interaction of Lrp with its own promoter was relatively minor. When a 418-bp region of the *lrp* promoter was incubated with increasing concentrations of Lrp in the absence and presence of leucine and glycine, leucine appeared to affect the production of complexes (Fig. 3.11 (B)), but glycine had no effect, suggesting that the effect of leucine is specific. A 203-bp probe was also examined for an Lrp interaction in the presence and absence of leucine (Fig. 3.13 (B)). Leucine did not appear to affect significantly Lrp-binding under these conditions. DNA microarray analysis comparing SL1344 grown in the presence and absence of leucine suggested that there was no change in the level of transcription of *lrp* grown in the presence of leucine (see Chapter 4). Taken together, it appears that in *S.* Typhimurium, leucine affects *lrp* regulation in an Lrp-dependent manner, but it is not known how this may occur.

Expression of *lrp* in *E. coli* is affected by nutrient availability and it has been shown that in log phase, Lrp levels are 3- to 4-fold higher in minimal than rich medium (Landgraf *et al.*, 1996). In the present study, comparison of *lrp-gfp* expression levels showed that the *lrp* promoter is between 3- to 5-fold more active in MOPS minimal than in LB broth culture. Primer extension analysis was undertaken to examine the transcriptional start site of *lrp* in *S.* Typhimurium. RNA was isolated from MOPS minimal broth-grown SL1344, at an OD_{600} of 0.5, and from LB broth-grown SL1344 after 5.5 h growth, corresponding to an OD_{600} of approximately 3.5. Upon inspection of the *lrp* expression data, it can be seen that these OD_{600} values represent high levels of *lrp* promoter activity in the respective media. The primer extension analysis was undertaken using equal amounts of total RNA and radiolabelled primer, which allows a quantitative comparison of the relative densities of the bands. Comparison of the densities of the bands

representing the transcriptional start site of *lrp*, showed that promoter activity in minimal medium was approximately 4.2-fold higher than that of rich medium.

Primer extension analysis revealed the position of the *lrp* transcriptional start site as between 258 and 263 bp upstream of the *lrp* translation initiation codon ATG, placing the start site further downstream of the *lrp* transcriptional start site of *E. coli* (Fig. 3.7). This places the promoter over 200 bp further upstream than predicted by bioinformatic analysis of the genome sequence of LT2 (McClelland *et al.*, 2001). It is striking that the *lrp* transcripts of both *E. coli* and *S.* Typhimurium have a long 5' untranslated region (UTR). The translation of the transcriptional regulator Fis is controlled by BipA, a ribosome-binding translation factor that binds to the 5' UTR of *fis* to enhance its translation by the ribosome (Owens *et al.*, 2004). The 5' UTR region of *lrp* may be important for post-transcriptional regulation, and may influence the stability of the *lrp* mRNA molecule. It has been shown that transcription and translation of *lrp* follow a similar pattern in *E. coli* (Landgraf *et al.*, 1996).

Studies with *E. coli* identified two putative sites for Lrp-binding on the *lrp* promoter, one of which has been confirmed and is considered a characterised site. Wang *et al.* demonstrate that incubation of LP-10 with 65.5 nM Lrp produces one complex (Wang *et al.*, 1994). However, the present study shows that at higher concentrations of Lrp, two complexes in the 99-bp region of both *S.* Typhimurium and *E. coli* are observed (Fig. 3.13 (A)). The fragment LP-10 is described in the original work as spanning –88 to –12, making it a 77-bp fragment (Wang *et al.*, 1994). Furthermore, the oligonucleotides described do not perfectly match the sequence of *E. coli* K-12 MG1655, but have three and four mismatches. The current study used oligonucleotide sequences with no mismatches to the same regions, to amplify the corresponding LP-10 fragments of *S.* Typhimurium and *E. coli* K-12, generating 99-bp fragments spanning –98 to +1, with respect to the transcriptional start site of *E. coli lrp*. From this study, two protein-DNA complexes formed with LP-10 suggest two putative binding sites for Lrp between –98 and +1. Further EMSA analysis using a 203-bp probe extending from –157 to +46 clearly shows three complexes, which may suggest a further Lrp-binding site in the region from +1 to +46. The LP-10 fragment of 99 bp showed production of two complexes, as did the 418-bp probe. The 203-bp probe, however, showed three complexes and a putative fourth complex (Fig. 3.13 (B)). Given that the 203-bp probe is

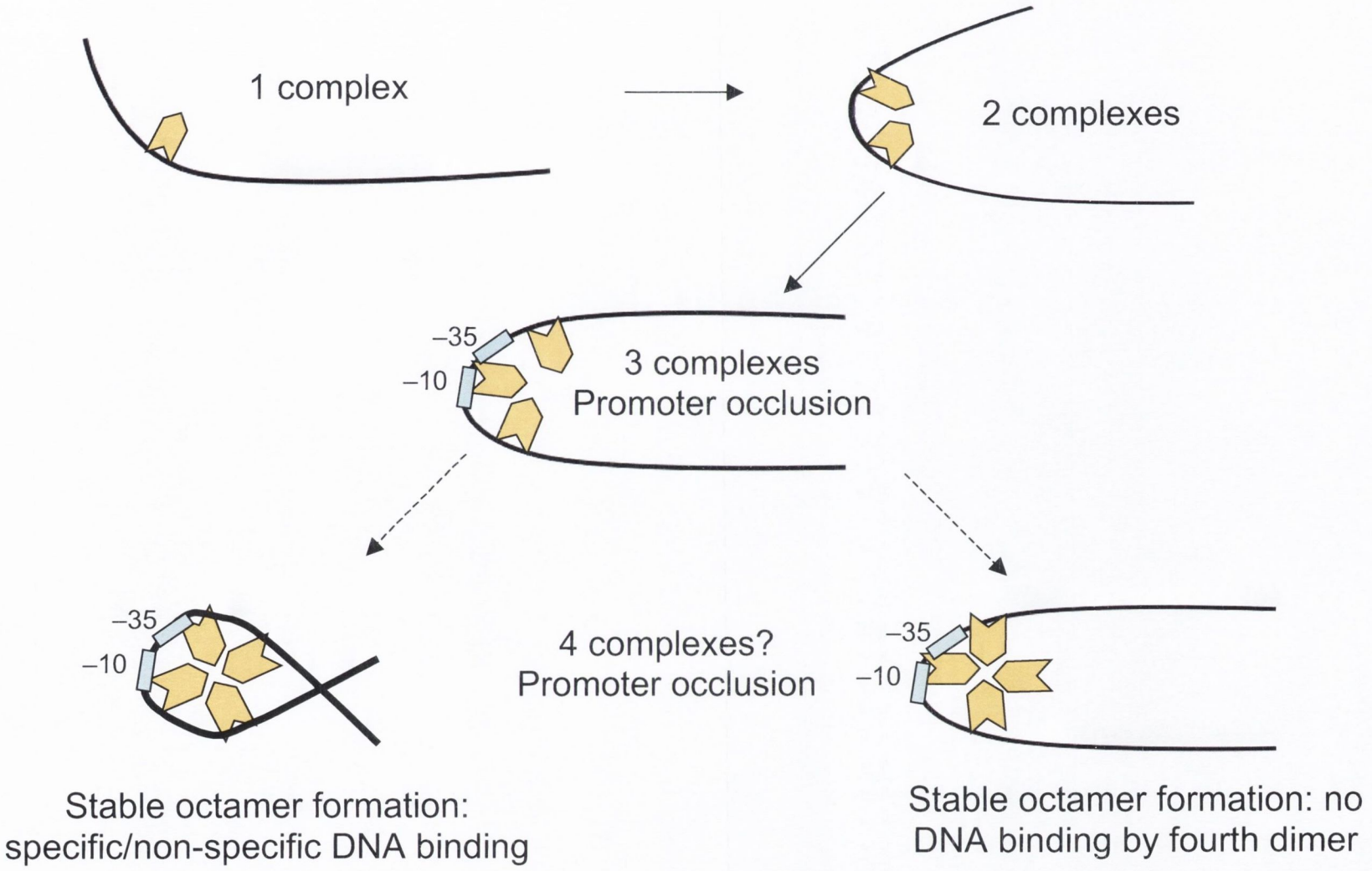
a subset of the 418-bp probe, it is difficult to understand how more complexes have formed on a smaller DNA probe. One explanation could be that there are three Lrp sites on the 203-bp probe, and two on the LP-10 fragment, which implies that the third site is located in the +1 to +46 region. If this is the case, then there are three sites on the 418-bp probe, but this probe only showed production of two complexes. Perhaps this anomaly represents a structural difference between the two probes: the extra 215 bp of DNA on the 418-bp probe could have an effect on DNA-bending by Lrp, possibly hindering the ability of Lrp to bind to additional sites.

DNase I footprinting revealed that Lrp protects two regions, from –122 to –12 and +15 to +50, on the *lrp* coding strand, and from –100 to +4 on the non-coding strand (Fig. 3.14). The positions of Lrp protection suggest that the –10 and –35 promoter regions would be bound by Lrp. It is also noteworthy that a strongly hypersensitive base identified by DNase I footprinting is located at –34 and –36, on the coding and non-coding strands respectively, which are of importance as they suggest wrapping in the –35 promoter element region. Presumably, the role of Lrp in negative regulation of *lrp* expression is promoter occlusion. Hypersensitive bases are located throughout the protected region on both strands, with most hypersensitive bases found on the non-coding strand. DNase I footprinting analyses were also undertaken during studies of Lrp autoregulation in *E. coli.* Interestingly, the region of protection by Lrp is described as having few regions clearly protected by Lrp, but obvious hypersensitive cleavage particularly in the non-coding strand (Wang *et al.*, 1994), which correlates well with the non-coding strand analysis of the *lrp* promoter in the present study (Fig. 3.14 (B)). Hypersensitivity due to the binding and bending of DNA by Lrp is a general phenomenon that has been observed at many Lrp-regulated promoters including *ilvIH* (Wang and Calvo, 1993), *pap* (Nou *et al.*, 1995), *lysU* (Gazeau *et al.*, 1994) and *gltBDF* (Wiese *et al.*, 1997).

A model of regulation of the *lrp* promoter is proposed in Figure 3.16. Three Lrp binding sites are proposed: two of these are likely to occur between –98 and +1, and are proposed to be positioned in regions conserved between *S.* Typhimurium and *E. coli,* as two complexes occur in the 99-bp fragment LP-10 generated from both strains. The characterised Lrp-binding site of the *E. coli lrp* promoter centred at –61 is fully conserved between the two strains, making it highly likely that this is also an Lrp-

binding site in *S.* Typhimurium. A third site is hypothesised to occur between +1 and +46, identified by EMSA analysis. Further evidence to support this is demonstrated by DNase I footprinting, which identified that a region from +15 to +50 is protected from DNase I cleavage by Lrp. A putative Lrp binding site at +21 was previously identified in *E. coli*, but this site is not well conserved in *S.* Typhimurium (see Figure 3.6): in addition, there is no evidence that this site binds Lrp in *E. coli*. EMSA analyses suggested that the region encompassed by probe R111 (spanning +43 to +153) does not contain any Lrp binding sites (Fig. 3.12). An additional fourth Lrp complex was identified by EMSA analysis with the 203-bp probe: this could represent an additional Lrp binding site in this region, or non-specific Lrp-DNA interactions (Fig. 3.13). Alternatively, the fourth complex could result from the addition of an Lrp dimer to the three existing Lrp dimers, resulting in formation of an Lrp octamer, which would increase the mass of the Lrp-DNA complex. An Lrp dimer bound to one Lrp binding site induces a bend of 52°: when two dimers are bound to adjacent sites, a bend of at least 135° is induced (Wang and Calvo, 1993). These data were supported by a model generated with the crystal structure of *E. coli* Lrp, which suggested that three dimers produce a bend of at least 180° over three binding sites, with DNA wrapping around an Lrp octamer core (Fig. 1.4; de los Rios and Perona, 2007). In the model of regulation proposed in Figure 3.16, these possibilities are considered. In all cases, after the first complex is formed, is difficult to predict whether subsequent complexes represent the binding of dimers to binding sites in either a specific or non-specific manner, or multimerisation of DNA-bound dimers with DNA-free dimers.

Fig. 3.16. Model of *lrp* regulation by Lrp. Lrp (depicted by yellow chevrons) was shown to regulate negatively its own expression, and was shown by EMSA analyses to form at least three complexes with its promoter. DNase I footprinting showed that Lrp protected a region from –122 to –12 and a further region from +15 to +50 on the coding strand, and from –100 to +4 on the non-coding strand of the *lrp* promoter. This strongly suggested that the –35 and –10 elements of the *lrp* promoter would be bound by Lrp, possibly resulting in promoter occlusion, and preventing access of RNA polymerase. Strongly hypersensitive bases were observed at the –34 and –36 positions, which suggested DNA bending in the –35 element. A putative fourth complex was observed. This may represent an additional Lrp binding site, with specific or non-specific DNA binding by an additional Lrp dimer, perhaps forming an Lrp octamer, or alternatively, the formation of an Lrp octamer, with no DNA binding activity of the fourth dimer.


1 complex
2 complexes
–35
–10
3 complexes
Promoter occlusion
–35
–10
4 complexes?
Promoter occlusion
–35
–10
Stable octamer formation:
specific/non-specific DNA binding
Stable octamer formation: no
DNA binding by fourth dimer

Chapter 4

Microarray analysis of the Lrp regulon of *Salmonella enterica* serovar Typhimurium

4.1 Introduction

As described in Chapter 1, gene regulation by Lrp has been well characterised in *E. coli*, and Lrp is best known as a regulator of genes involved in amino acid metabolism and transport, and fimbrial genes (Calvo and Matthews, 1994). Relatively little is known about the role of Lrp in gene regulation in *S. enterica*. In *S. enterica,* Lrp has been identified as a regulator of *pef* fimbrial genes (Nicholson and Low, 2000), the *spv* genes (Marshall *et al.*, 1999), and the *traJ* gene (Camacho and Casadesús, 2002) of the large virulence plasmid of *S. enterica,* pSLT.

DNA microarray analysis can be used to investigate the effect of a transcriptional regulator on gene expression. In previous studies, DNA microarray analysis was performed on *E. coli lrp* mutants, in glucose minimal MOPS medium in both the absence, and presence of L-leucine, the Lrp effector molecule. L-leucine modulates gene regulation by Lrp, and there are six known ways that L-leucine may affect Lrp-mediated regulation. Positive effects of Lrp on a given gene may be negated by, enhanced by, or unaffected by leucine. A negative regulatory effect of Lrp may also be affected by L-leucine in the same manner. Hung *et al.* performed microarray analysis on the *lrp* mutant in *E. coli* strain NO3434 during growth to an optical density (OD_{600}) of 0.5 to 0.6 in MOPS minimal broth (Hung *et al.*, 2002). The medium was supplemented with 0.4 % (w/v) glucose, and was undertaken in the absence of L-leucine. This study identified genes that were subsequently placed into functional categories. The categories of small molecule biosynthesis and small molecule transport together contained the majority of Lrp-regulated genes identified in this study: these consist of known Lrp-regulated genes such as genes of the *ilv, leu, liv* and *opp* operons, and some newly identified genes such as those of the *art* and *pot* operons, which will be discussed later. Regulatory proteins of stress responses constituted another functional category. The alternative sigma factors *rpoS* and *rpoE* were identified as upregulated 2 to 3 fold in the *lrp* mutant. In addition, several RpoH (σ^{32})-regulated genes were affected by Lrp, but the *rpoH* gene itself was unaffected. Genes involved in cell structure were also affected by Lrp. The *fimA* gene of *E. coli* was downregulated by > 4 fold in the *lrp* mutant strain, which had previously been reported to be Lrp-regulated.

Tani *et al.* also performed microarray analysis on an *E. coli* K-12 strain, W3110 and its isogenic *lrp* mutant derivative, BE1 (Tani *et al.*, 2002). These analyses were also

undertaken in glucose minimal MOPS medium, which was supplemented with isoleucine and valine and performed in logarithmic phase, in the presence and the absence of 10 mM L-leucine. This study revealed that more than 400 genes were significantly Lrp-responsive under the conditions examined, and that Lrp is a major regulator of stationary phase-induced genes. In *E. coli,* Tani *et al.* found that the majority of leucine-insensitive Lrp-regulated genes identified by the study were Lrp-repressed genes, and that many of these genes were annotated as being either functionally associated with, or induced in stationary phase (Tani *et al.*, 2002).

In this chapter, DNA microarray analysis was used to examine the Lrp regulon of *S.* Typhimurium SL1344 under conditions of high and low nutrient availability, reflecting the different expression levels of Lrp in these media (see Chapter 3).

4.2 Results

4.2.1 Microarray analysis of Lrp in rich media

The Lrp regulon of *S.* Typhimurium was examined in rich broth. This was performed by growth of SL1344 and SL1344 *lrp* under conditions described in section 2.2.2, followed by microarray analysis. The growth profile of SL1344 and SL1344 *lrp* in LB broth had been determined, and mean doubling times of 28 min and 30 min respectively, calculated from the growth curves (see Chapter 3; section 3.2.4). Microarray analysis was performed at timepoints corresponding to lag phase, mid-logarithmic phase, transition to stationary phase and late stationary phase in both SL1344 and SL1344 *lrp*. Due to the similar growth pattern, these timepoints occurred 1 h, 4 h, 5.5 h and 24 h after inoculation of both strains. Table 4.1 shows the results of statistical analysis (FDR [false discovery rate] 0.05) of the LB broth array data. The genes affected by the *lrp* mutation by ≥ 2 fold in LB broth are listed in Table 4.2. As Lrp levels are lowest in rich media, it was expected that the genes affected are those in the Lrp regulon with the highest affinity for Lrp. Genes of interest will be discussed in later sections. Microsoft Excel files created from the FDR 0.05 lists of lag phase, logarithmic phase, transition phase and stationary phase data in Table 4.1, are presented as supplementary data tables S4.1 to S4.4, respectively, in a CD accompanying this manuscript.

4.2.2 Microarray analysis of Lrp in minimal media in the absence and presence of leucine

The Lrp regulon of *S.* Typhimurium was examined in MOPS minimal broth. This was performed by growth of SL1344 and SL1344 *lrp* and microarray analysis as described in section 2.2.2. The growth profile of SL1344 and SL1344 *lrp* in MOPS minimal broth had been determined in both the presence and absence of 10 mM L-leucine. Average doubling times for SL1344 and SL1344 *lrp* of 58 min and 76 min respectively, were calculated from growth curves in MOPS minimal medium in the absence of L-leucine, whereas in the presence of 10 mM L-leucine, the mean doubling times were 67 and 80 min, respectively (see Chapter 3; section 3.2.5). Microarray analysis was performed at timepoints corresponding to logarithmic phase and late-stationary phase in both SL1344 and SL1344 *lrp*. Due to the growth defect of SL1344 *lrp*, bacteria were harvested at equal optical densities (OD_{600} 0.5) for logarithmic phase, and after 24 h growth, for stationary phase. Samples of SL1344 and SL1344 *lrp* were taken at the timepoints

above, during growth in MOPS minimal broth with and without 10 mM L-leucine. The arrays used were Type II arrays, which have labelled genomic DNA as a reference signal. This meant that each sample could be compared to any other sample using the genomic DNA as a common reference signal. The experiment was designed to allow for comparison of SL1344 and SL1344 *lrp* in MOPS minimal broth; SL1344 and SL1344 *lrp* in MOPS minimal broth in the presence of 10 mM L-leucine; SL1344 grown in the presence and absence of 10 mM L-leucine; and SL1344 *lrp* grown in the presence and absence of 10 mM L-leucine, at both logarithmic phase, and stationary phase. Hereafter, growth in MOPS minimal broth. without addition of leucine at logarithmic phase will be referred to as MOPS leu– log; at stationary phase will be referred to as MOPS leu– stat; MOPS supplemented with 10 mM L-leucine will be referred to as MOPS leu+ log and MOPS leu+ stat.

Table 4.3 shows the results of statistical analysis (FDR 0.05) of the MOPS minimal medium array data. Of particular interest was the analysis of gene expression between SL1344 and SL1344 *lrp*, however, statistical filtering of logarithmic phase data yielded few results. This appeared to be because of three reasons: firstly, the *lrp* mutant data displayed high variability between biological replicates. Secondly, a set of approximately 500 genes, which were positioned in the same region on each array, consistently gave spurious results in the data examined, adding to the variability and thus allowing fewer genes through the statistical filter. Thirdly, few changes were seen between SL1344 and SL1344 *lrp*, which meant that relatively few genes passed the statistical filter. As a result, the SL1344 *lrp* logarithmic minimal medium array comparisons yielded little to no data.

A number of steps were taken to obtain data from the array results. Firstly, biological replicates with the most variable results could be removed from the statistical analysis. Secondly, because of the high variability observed in the data, the significance threshold could be increased to FDR 0.1, giving a less stringent cut-off and thus allowing more genes to pass the statistical filter, but meaning that 10 % of the genes that passed the filter are likely to have arisen by chance.

Growth phase	FDR 0.05	≥ 2-fold up	≥ 2-fold down
Lag phase	183	11	17
Log phase	210	21	19
Transition phase	73	12	8
Stationary phase	306	13	8

Table 4.1. Numbers of genes affected by Lrp in LB broth. This table lists the numbers of genes that passed the statistical filter FDR 0.05 (5 %), and the number of those genes that were affected by 2 fold, in SL1344 *lrp* compared to SL1344 during growth in LB broth at the phases of growth listed.

priC	1.12	0.62	**0.48**	1.06	primosomal replication protein N
prlC	**2.04**				oligopeptidase A
prsA			**0.44**		phosphoribosylpyrophosphate synthetase
pSLT054	0.69		**0.50**	0.98	pSLT virulence plasmid: mutagenesis by UV; *umuDC*-like
recO		**0.20**	1.07	0.86	gap repair gene
rfbB	1.62	0.94	**0.49**	0.97	dTDP-glucose 4,6 dehydratase
rimM		0.67	1.22	**2.14**	16S rRNA processing protein
rpmH	0.92	1.44	1.21	**2.22**	50S ribosomal subunit protein L34
rpoE	**0.50**	**0.23**	1.05	1.31	sigma E (sigma 24) factor of RNA polymerase
rpsL	**4.14**			**3.01**	30S ribosomal subunit protein S12
sthB	1.04	1.32	**2.65**	**0.47**	putative fimbrial usher protein
STM0345	0.81	**2.04**	1.07	0.72	putative inner membrane protein
STM0809	1.16	**2.05**	1.08	0.66	putative inner membrane protein
STM0898	**0.39**		1.20		Fels-1 prophage; predicted transcriptional regulator
STM0910	1.34	**0.36**	0.64	0.77	Fels-1 prophage
STM0920	**0.30**	**0.40**	**0.20**	**0.41**	Fels-1 prophage; ail and ompX Homolog
STM0927	1.71	**2.23**	0.88	0.95	Fels-1 prophage; putative tail assembly protein
STM1600	1.10	0.76	0.65	**0.44**	putative inner membrane protein
STM1967	1.49	**2.22**	1.06	0.82	putative 50S ribosomal protein
STM2779	1.76	1.11	0.78	**0.41**	putative inner membrane protein
STM2800	**2.91**	**0.18**	**0.45**	**0.43**	putative inner membrane protein
STM3036	1.04	**2.22**	1.52	0.67	putative inner membrane protein
STM3052	0.77	**0.41**	1.97	0.75	putative outer membrane protein
STM3154	0.57	**0.29**	**0.32**	0.89	putative ATP-dependent RNA helicase-like protein
STM3566	1.54	**3.26**	**2.79**	1.91	putative cytoplasmic protein
STM3604	0.66	**0.16**	**0.37**	0.71	putative inner membrane protein
STM3605	0.75	0.93	**2.24**	0.57	putative phage endolysin
STM4157	1.29	**2.11**	1.73	0.83	putative cytoplasmic protein
STM4158	1.22	**2.18**	**2.06**	0.84	putative cytoplasmic protein
STM4255	1.34	1.30	**2.58**		putative cytoplasmic protein
STM4509	0.92	0.51	**0.45**	0.51	putative cytoplasmic protein
STM4510	0.93	**0.14**	1.21	0.83	putative aspartate racemase
STM4519	0.83	0.99	0.62	**2.02**	putative NAD-dependent aldehyde dehydrogenase
thiM	1.08	1.44	**2.65**	1.54	hydoxyethylthiazole kinase (THZ kinase)
trmD	0.59		1.07	**0.50**	tRNA (guanine-7-)-methyltransferase
trpB		1.13	**3.58**	1.86	tryptophan synthase, beta protein
uhpT	**0.21**		**0.44**	1.45	MFS family, hexose phosphate transport protein
ybaY	1.33	1.33	0.70	**2.11**	glycoprotein/polysaccharide metabolism
yeaJ	1.46	1.21	0.90	**0.50**	putative methyl-accepting chemotaxis protein
yfbQ	**0.23**	0.67	**0.49**	0.59	putative aminotransferase (ortho), paral putative regulator
ygiK	**0.37**		1.45	0.70	putative integral membrane protein, possible transporter
yhbH	0.95	0.87	0.53	**2.38**	putative sigma N modulation factor
yjcC	1.15	1.13	1.39	**0.50**	putative diguanylate cyclase/phosphodiesterase
yjiE	1.10	**0.22**	1.29	0.79	putative transcriptional regulator, LysR family
yjiG	0.96	**0.20**	0.86	0.88	putative permease
yjiH	0.57	**0.18**	1.01	0.79	putative inner membrane protein
ylaC	1.68	1.22	0.67	**2.06**	putative inner membrane protein
ynfC	1.59	**0.50**	**0.48**	0.91	putative inner membrane lipoprotein

Table 4.2. Lrp-regulated genes in *S.* Typhimurium during growth in LB broth. Genes upregulated by ≥ 2 fold are shown in red text, whereas downregulated genes are shown in blue text. Unavailable data is denoted by a space. Gene products are described.

Gene	1 h	4 h	5.5 h	24 h	Product
asrB	**0.17**	1.18	0.91	**3.23**	anaerobic sulfide reductase
basR	**0.30**	**0.29**	**2.54**	1.98	response regulator with BasS (OmpR family)
btuR	**0.38**	1.66	1.03	0.85	cob(I)alamin and cobinamide adenolsyltransferase
caiF	0.60	**0.50**	1.14	0.79	transcriptional regulator of cai and fix operon
carA	**0.22**		0.50	0.70	carbamoyl-phosphate synthetase,
cboQ	**2.44**	1.11	**2.04**	0.62	synthesis of vitamin B12 adenosyl cobalamide precursor
cheW	1.47	1.24	1.63	**0.49**	purine-binding chemotaxis protein; regulation
cobU	**0.34**	1.68	**3.65**	**2.69**	cobinamide kinase & phosphate guanylyltransferase
cydB	**2.22**	0.96	1.40	0.84	cytochrome d terminal oxidase polypeptide subunit II
dcoC	**0.49**	1.33	1.76	0.75	oxalacetate decarboxylase: gamma chain
eco	**3.22**	**2.13**	1.53	**4.12**	ecotin, a serine protease inhibitor
entD		1.31	**3.99**	1.21	enterochelin synthetase, component D
era	**0.28**		0.62	0.90	GTPase involved in coordination of cell cycle
eutM	0.66	**0.23**	1.37	1.34	putative detox protein in ethanolamine utilization
fimA	**0.20**	**0.09**	**0.06**	0.84	major type 1 subunit fimbrin (pilin)
fimD	**0.46**	**0.48**	**0.50**	0.69	outer membrane usher protein
fliD	0.59	1.15	**5.89**	1.46	flagellar biosynthesis; filament capping protein
fruR	1.89	1.91	1.71	**2.77**	transcriptional repressor of fru operon (GlaR/LacI family)
gcvP	0.79	0.51	0.66	**2.28**	glycine cleavage complex protein P
gcvT	0.74	0.62	0.61	**3.13**	glycine cleavage complex protein T
gltB	**0.49**	1.22	0.95	0.67	glutamate synthase, large subunit
glyA	**0.21**		**2.07**	1.23	serine hydroxymethyltransferase
hisF	0.71	**0.13**	0.84	1.60	imidazole glycerol phosphate synthase, subunit with HisH
hisM			**3.45**	**2.05**	histidine and lysine/arginine/ornithine transport protein
hybB	0.90	**0.44**	1.10	0.66	putative cytochrome Ni/Fe component of hydrogenase-2
hybE	0.94	**0.46**	1.00	0.90	putative hydrogenase
iadA	1.13	**0.16**	1.46	0.83	isoaspartyl dipeptidase
ilvC	**4.28**	**2.30**	1.01	0.97	ketol-acid reductoisomerase
ilvD	**2.73**	1.28	0.88	1.01	dihydroxyacid dehydratase
ilvE	**2.97**	1.48	0.69	1.13	branched-chain amino-acid aminotransferase
ilvH	**2.13**		1.28	1.67	acetolactate synthase III, small subunit
ilvM	**3.13**	1.19	0.80	1.45	acetolactate synthase II, small subunit
livH	1.23		**2.33**		branched-chain amino acid transporter, high-affinity
livJ	**9.18**	**27.68**	**17.85**	**18.05**	branched-chain amino acid transporter, high-affinity
livK	**4.08**	**2.90**	**2.46**	1.07	branched-chain amino acid transporter, high-affinity
lpfD			**2.50**		long polar fimbrial operon protein
lrp	**0.05**	**0.11**	**0.06**	**0.15**	**leucine-responsive regulatory protein: control gene**
napB	0.85	**5.40**	1.04	0.68	periplasmic nitrate reductase, cytochrome C550
napC	0.97	**5.81**	0.78	0.82	periplasmic nitrate reductase, cytochrome c-type protein
napD	0.81	**6.29**	**2.50**	0.64	periplasmic nitrate reductase
napG	0.64	**5.97**	1.23	**0.47**	ferredoxin-type protein: electron transfer
napH	0.81	**5.58**	**2.08**	0.78	ferredoxin-type protein: electron transfer
nirD	0.55	**3.08**	1.78	0.73	nitrite reductase, small subunit
nrfA	0.57	**4.82**	1.04	0.74	nitrite reductase periplasmic cytochrome c(552)
nrfC	**0.41**	**3.32**	1.56	0.70	putative nitrite reductase; formate-dependent, Fe-S centers
nrfD	**0.50**	**3.02**	1.54	0.61	putative nitrate reductase, formate dependent
nrfE	0.54	**2.43**	**2.16**	0.63	nitrite reductase formate-dependent
oat	0.88	0.58	**0.04**	**0.07**	putative acetylornithine aminotransferase
orgA	1.60	**2.09**	1.12	0.74	putative flagellar biosynthesis/TTS pathway protein
parE	**0.17**			**2.65**	DNA topoisomerase IV, subunit B
pduB	0.96	**0.17**	**0.49**	1.07	Propanediol utilization: polyhedral bodies
pduF	1.02	**0.33**	0.95	0.72	Propanediol utilization: propanediol diffusion facilitator
pduS	0.58	**0.38**	0.61	0.65	Propanediol utilization: polyhedral bodies

	SL1344 vs SL1344 lrp (leu –)	SL1344 vs SL1344 lrp (leu +)	SL1344 leu+ vs SL1344 leu-	SL1344 lrp leu+ vs SL1344 lrp leu-
Log phase	0	8	99	1
≥ 2 fold	↑0	↑2	↑35	↑1
	↓0	↓6	↓30	↓0
Stationary phase	2922	595	968	2092
≥ 2 fold	↑1125	↑238	↑248	↑703
	↓799	↓171	↓163	↓683

Table 4.3. Numbers of genes that passed statistical filtering of FDR 0.05 (5 %), from MOPS minimal broth microarray analysis. Arrows denote number of genes ≥ 2-fold upregulated, or downregulated.

4.2.2.1 Analysis of SL1344 and SL1344 *lrp* in MOPS minimal medium at logarithmic phase

To obtain data from the analysis of SL1344 and SL1344 *lrp* during growth in MOPS leu– log, statistical filtering was performed with an FDR of 0.1 (10 %). This gave one result, *dadA*, which is a gene known to be repressed by Lrp in *E. coli*, which was upregulated by 17 fold in SL1344 *lrp*. To increase the number of results, the most variable biological replicate was removed and statistical filtering (FDR 0.1) performed. This gave a list of 77 genes (Table 4.4), which, when examined in the most variable *lrp* replicate, mostly follow the same profile in all *lrp* mutant biological replicates. Thirty-six of the 77 genes had ≥ 2-fold higher levels in the *lrp* mutant (labelled in red text in Table 4.4), and 21 genes had ≥ 2-fold lower levels in the *lrp* mutant (blue text in Table 4.4). Interestingly, a large number of the genes identified were previously identified Lrp-regulated genes in *E. coli*: e.g. *malT, kbl* and *opp, pot, ilv, glt,* and *gln* genes. Out of the 57 genes which were affected ≥ 2 fold in the *lrp* mutant, 27 had been previously associated with Lrp in *E. coli*. The genes that were linked with Lrp regulation are identified and referenced in Table 4.4. This list contained additional genes, seven of which had been identified in the LB broth microarray (identified by an asterisk), and others that had not previously been shown to be Lrp-regulated. Among the latter group were a large percentage of *Salmonella*-specific SPI-2-related genes, including *sifB, ssaI* and *ssaJ*.

Analysis of SL1344 and SL1344 *lrp* in MOPS leu+ log also yielded few results (see Table 4.3). Increasing the significance threshold to FDR 0.1 led to 26 genes passing the filter (Table S4.5 in supplementary data CD). These genes consist mainly of genes that are strongly repressed or activated by leucine/Lrp, and thus show strong changes in expression in the *lrp* mutant.

4.2.2.1.1 Genes regulated by addition of leucine

The gene expression profiles of SL1344 during growth in MOPS leu– log and MOPS leu+ log were compared. This enabled the identification of leucine-regulated genes. Of the 99 differentially regulated genes, 35 were ≥ 2-fold upregulated in the presence of leucine, and 30 were downregulated ≥ 2 fold in the presence of leucine. The differentially regulated FDR 0.05 genes are listed in Table S4.6, while the ≥2-fold affected genes are listed in Table 4.5. Included in the up-regulated list are genes of the

art operon, involved in arginine transport; the *dadAX* genes, which encode D-amino acid dehydrogenase; and genes of the *frd* operon, for anaerobically-expressed fumarate reductase. In addition, genes of the *opp* operon, for oligopeptide transport; the *kbl-tdh* genes, enzymes that convert threonine to glycine; and *sdaBC*, for serine deaminase, all appeared to be positively regulated by the presence of leucine. Downregulated by leucine in SL1344 are, amongst others, the *glnHPQ* genes, for glutamine permease; the *ilvIH* operon, for isoleucine-valine biosynthesis; genes of the *leu* operon, for leucine biosynthesis; and various *liv* genes, for branched-chain amino acid uptake. In addition, the *potFGH* genes for putrecine transport also appeared downregulated by growth in 10 mM L-leucine.

The ≥ 2-fold differentially regulated genes were compared with the 77 genes identified as regulated by Lrp (section 4.2.2.1) with an FDR 0.1. This identified 30 genes present in both lists, which were strongly expected to be Lrp and leucine-regulated genes. These genes were analysed in GeneSpring 7.0 for the effect of leucine and the *lrp* mutation (Fig. 4.1). This was performed by normalising the expression data of the 30 genes to SL1344 in the absence of leucine, thereby using the gene level in SL1344 as a reference level and comparing the relative levels in SL1344 *lrp* and SL1344 in the presence of leucine. Examination of Figure 4.1 showed that the addition of leucine appears to mainly mirror the absence of Lrp, i.e. leucine antagonised the effect of Lrp. For example, *dadA* was repressed by Lrp (> 10-fold higher in the *lrp* mutant), and the addition of leucine increased *dadA* expression > 10 fold. Conversely, *glnK* was activated by Lrp (> 10-fold down in the *lrp* mutant), and repressed in the presence of leucine. Exceptions to this, where leucine potentiated the effect of Lrp, were also shown in Fig. 4.1: *fimA*, encoding the major fimbrial subunit of type-1 fimbriae, appeared repressed by > 5 fold in the *lrp* mutant, suggesting it requires Lrp for its expression. The addition of leucine, however, increased the *fimA* transcript level by approximately 4 fold. Another example visible in Fig. 4.1 is the gene *yhhK*. This gene encodes a putative acetyltransferase, and is located between *livK* and *livJ*, which are known Lrp-regulated genes, although *yhhK* itself has not previously been identified as Lrp regulated. The gene *yhhK* was repressed by Lrp (> 2 fold upregulated in SL1344 *lrp*), and repressed > 5 fold by the presence of leucine. The gene list created is in Table S4.7, in the supplementary data CD.

STM0717	2.04	putative inner membrane protein
STM0721	1.32	putative glycosyl transferase
STM1410	9.87	putative cytoplasmic protein
STM1493	1.55	putative periplasmic component, ABC transport system
STM1560	1.99	putative alpha amylase
STM1833	0.71	putative inner membrane protein
STM2800*	4.84	putative inner membrane protein
STM3129	1.33	putative NAD-dependent aldehyde dehydrogenase
STM4519*	7.57	putative NAD-dependent aldehyde dehydrogenase
tsr	0.48	methyl-accepting chemotaxis protein I, serine sensor receptor
yaeP	2.89	putative cytoplasmic protein
yaiC	1.29	putative diguanylate cyclase/phosphodiesterase domain 1
yaoF	3.67	putative hemolysin
ybaY[*, 4]	3.68	glycoprotein/polysaccharide metabolism
ybgS[4]	1.59	putative homeobox protein
ybiJ	2.36	putative periplasmic protein
ybjO	0.46	putative inner membrane protein
ydhE	3.20	putative MATE family transport protein
yeaB	1.69	putative NTP pyrophosphohydrolase
*yfbQ**	0.15	putative aminotransferase (ortho), paral putative regulator
yfhJ	0.77	believed to be involved in assembly of Fe-S clusters
ygaE	1.72	putative transcriptional repressor (GntR familiy)
yhhK	2.73	putative acetyltransferase
yhjE	0.09	putative MFS family transport protein
yjbA	2.40	putative inner membrane protein
yjgB[4]	1.53	putative alcohol dehydrogenase
ynfM	2.49	putative MFS family transport protein

Table 4.4: Lrp-regulated genes in logarithmic phase MOPS minimal broth-grown SL1344. Gene lists were filtered as described in the text (FDR 0.1). Gene names, the fold change observed in SL1344 *lrp*, and the product of the relevant gene as listed in KEGG (Kyoto Encyclopedia of Genes and Genomics) are listed in the table. Fold change text is coloured red for genes upregulated by ≥ 2 fold, and blue text denotes downregulation in the *lrp* mutant by ≥ 2 fold. Genes that were identified in previous studies are denoted with superscript: [1] Microarray analysis of Hung *et al.*, 2002; [2] Reviewed in Calvo and Matthews, 1994; [3] Microarray analysis of Tani *et al.*, 2002; [4] Tchetina and Newman, 1995; [5] Examined in Chapter 5. Asterisks denote genes identified in LB microarray analysis in this study.

Gene	Fold change	Product
argT	0.14	lysine/arginine/ornithine transport protein
aroG	0.37	2-dehydro-3-deoxyphosphoheptonate aldolase (phenylalanine repressible)
artM[1]	4.05	arginine third transport system
artQ[1]	4.76	arginine third transport system
avtA	0.18	valine-pyruvate aminotransferase
cbiD	154.97	synthesis of vitamin B12 adenosyl cobalamide precursor
cysC	0.71	adenosine 5'-phosphosulfate kinase
dacB	1.21	D-alanyl-D-alanine carboxypeptidase, penicillin-binding protein 4
dadA[2]	16.78	D-amino acid dehydrogenase subunit
fimA[*,5]	0.12	major type 1 subunit fimbrin (pilin)
fimZ[5]	0.45	fimbrial transcriptional regulator
fruA	2.02	Sugar Specific PTS system, fructose-specific transport protein
ftsK	0.28	required for cell division and chromosome partitioning
galP[1]	2.12	MFS family, galactose:proton symporter
glnK	0.03	nitrogen assimilation by glutamine synthetase, regulates GlnL and GlnE
glnQ[1]	0.27	ABC superfamily (atp_bind), glutamine high-affinity transporter
glpF	0.65	MIP channel, glycerol diffusion
gltB[*,1,2]	0.05	glutamate synthase, large subunit
gltP[2]	4.49	DAACS family, glutamate:aspartate symport protein
ilvB	3.53	acetolactate synthase I, large subunit, valine sensitive
ilvI[1,2]	0.05	acetolactate synthase III, valine sensitive, large subunit
kbl[2]	4.15	2-amino-3-ketobutyrate CoA ligase (glycine acetyltransferase)
leuC[1,2]	0.28	3-isopropylmalate isomerase (dehydratase), subunit with LeuD
leuD[1,2]	0.26	3-isopropylmalate isomerase (dehydratase), subunit with LeuC
lipA	1.72	lipoate synthase, an iron-sulfur enzyme
lrp[*]	**0.06**	**Leucine-responsive regulatory protein: control gene**
malT[3]	0.69	transcriptional activator of the mal genes
narW	1.95	nitrate reductase 2, delta subunit, assembly function
oppA[1,2]	4.93	oligopeptide transport protein with chaperone properties
oppD[1,2]	5.74	oligopeptide transport protein
oppF[1,2]	5.43	oligopeptide transport protein
osmY[2]	6.24	hyperosmotically inducible periplasmic protein, RpoS-dependent
otsA[4]	1.45	trehalose-6-phosphate synthase
pntB	0.36	pyridine nucleotide transhydrogenase (proton pump), beta subunit
potG[1]	0.19	ABC superfamily (atp_bind), putrescine transporter
potH[1]	0.19	ABC superfamily (membrane), putrescine transporter
pta	0.70	phosphotransacetylase
rpsF	0.78	30S ribosomal subunit protein S6
sdaC[2]	5.22	putative HAAAP family, serine transport protein
serA[1,2]	0.17	D-3-phosphoglycerate dehydrogenase
sifB	6.48	Salmonella translocated effector: translocated by SPI-2
sprB	2.52	transcriptional regulator
ssaI	16.69	Secretion system apparatus
ssaJ	13.15	Secretion system apparatus
ssaM	4.80	Secretion system apparatus
ssaR	4.65	Secretion system apparatus
ssaT	2.17	Secretion system apparatus
ssaV	2.98	Secretion system apparatus
sseD	4.08	Secretion system effector
STM0561	1.37	Sensor protein

STM3022	2.68	putative transport protein
STM4219	2.30	putative cytoplasmic protein
STM4518	4.32	putative inner membrane protein
STM4519	4.18	putative NAD-dependent aldehyde dehydrogenase
tdh	3.68	threonine 3-dehydrogenase
yaoF	2.85	putative hemolysin
ybaY	2.12	glycoprotein/polysaccharide metabolism
ydhE	2.85	putative MATE family transport protein
ygdI	3.03	putative lipoprotein
yhhK	0.16	putative acetyltransferase
yhiQ	0.36	putative SAM-dependent methyltransferase
yhjE	0.07	putative MFS family transport protein
yifK	3.16	putative APC family, amino-acid transport protein, permease
yihR	0.26	putative aldose-1-epimerase

Table 4.5. Genes affected by the presence of leucine during logarithmic growth in SL1344. Fold change in SL1344 grown in the presence of leucine, compared to the absence of leucine, is shown.

Gene	Fold Change	Product
argT	0.27	Lysine/arginine/ornithine transport protein
artI	4.09	arginine transport system
artM	3.54	arginine transport system
artP	4.07	arginine transport system
artQ	3.90	arginine transport system
avtA	0.13	valine-pyruvate aminotransferase
dadA	18.41	D-amino acid dehydrogenase subunit
dadX	6.36	D-alanine racemase
fimA	3.94	major type 1 subunit, fimbrin
fimC	2.60	periplasmic chaperone, required for type 1 fimbriae
frdB	3.42	fumarate reductase, anaerobic, Fe-S protein subunit
frdC	3.63	fumarate reductase, anaerobic, membrane anchor polypeptide
frdD	2.62	fumarate reductase, anaerobic, membrane anchor polypeptide
glnH	0.41	glutamine high-affinity transporter
glnK	0.06	nitrogen assimilation by glutamine synthetase
glnP	0.45	glutamine high-affinity transporter
glnQ	0.45	glutamine high-affinity transporter
gltB	0.17	glutamate synthase, large subunit
gltP	8.27	DAACS family, glutamate:aspartate symport protein
ilvH	0.18	acetolactate synthase III, small subunit
ilvI	0.10	acetolactate synthase III, valine sensitive, large subunit
kbl	4.06	2-amino-3-ketobutyrate CoA ligase
leuB	0.17	3-isopropylmalate dehydrogenase
leuC	0.14	3-isopropylmalate isomerase (dehydratase) subunit
leuD	0.11	3-isopropylmalate isomerase (dehydratase), subunit
livF	0.11	branched-chain amino acid transporter, high-affinity
livG	0.14	branched-chain amino acid transporter, high-affinity
livH	0.06	branched-chain amino acid transporter, high-affinity
livK	0.05	branched-chain amino acid transporter, high-affinity
lysC	2.13	aspartokinase III, lysine sensitive
oppA	2.87	oligopeptide transport protein with chaperone properties
oppD	2.60	oligopeptide transport protein
oppF	2.37	oligopeptide transport protein
osmY	3.33	hyperosmotically inducible protein, RpoS-dependent
pagC	0.39	PhoP regulated: reduced macrophage survival
pckA	8.42	phosphoenolpyruvate carboxykinase
pntB	0.44	pyridine nucleotide transhydrogenase, beta subunit
potF	0.21	putrescine transporter
potG	0.35	putrescine transporter
potH	0.31	putrescine transporter
sdaB	3.97	L-serine dehydratase (L-threonine deaminase 2)
sdaC	9.95	putative HAAAP family, serine transport protein
serA	0.31	D-3-phosphoglycerate dehydrogenase
STM0932	2.96	putative nucleoside-diphosphate-sugar epimerase
STM1494	2.32	ABC-type transport systems, permease components
STM1633	0.20	putative periplasmic binding protein
STM1635	0.37	putative ABC-type polar amino acid transport system
STM1795	9.35	putative homolog of glutamic dehyrogenase
STM2800	10.83	putative inner membrane protein

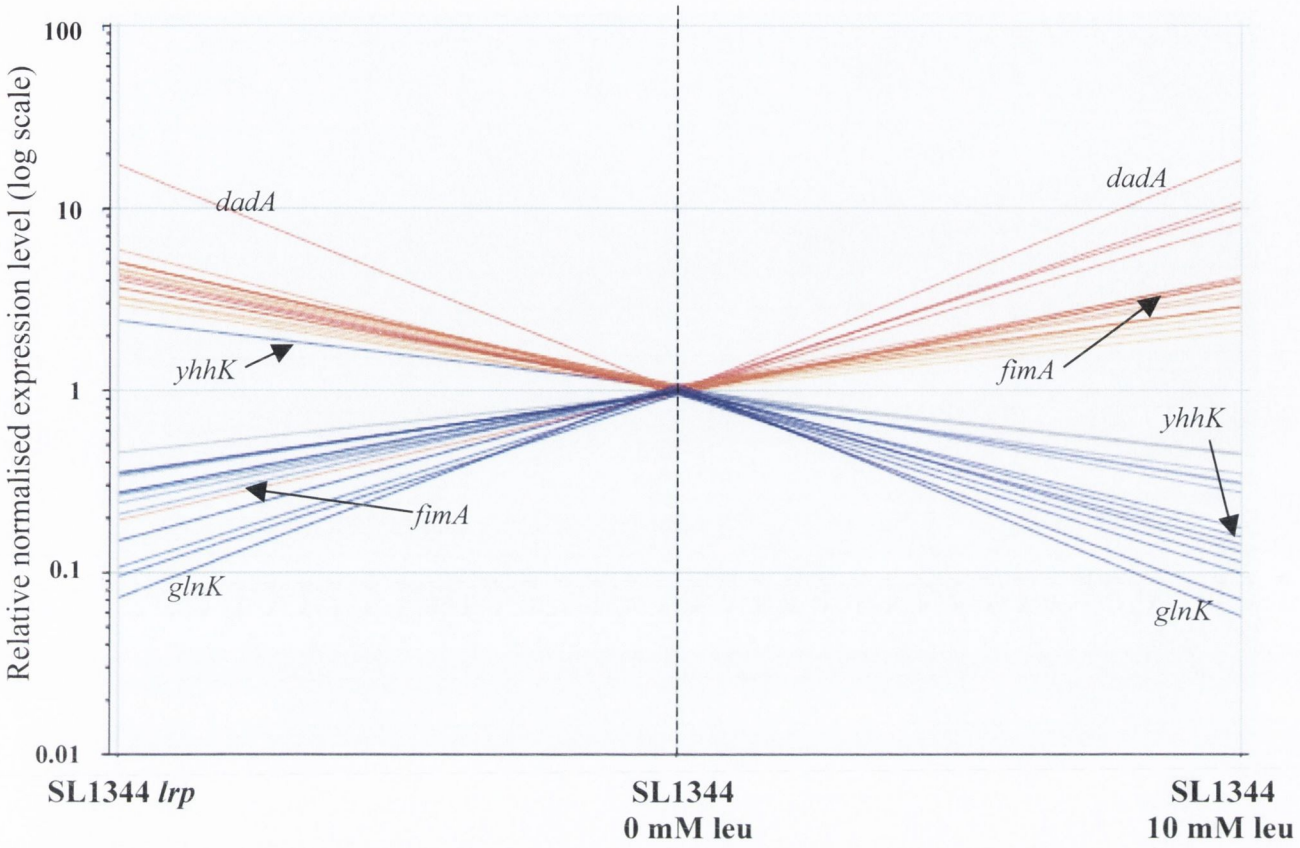


Fig. 4.1. Expression profile of Lrp/leucine regulated genes. Lrp/leucine-regulated genes in SL1344 *lrp* and SL1344 during exponential growth in MOPS minimal broth, and SL1344 during exponential growth in MOPS minimal broth supplemented with 10 mM L-leucine. Gene expression values were normalized to SL1344, 0 mM leucine. Blue denotes down-regulation, while red denotes up-regulation, according to the position in SL1344, 10 mM leucine. Leucine antagonizes the effect of Lrp in most cases: *dadA* is upregulated in both SL1344 *lrp* and SL1344 in the presence of leucine, compared to the SL1344 in the absence of leucine, whereas *glnK* is downregulated in both SL1344 *lrp* and SL1344 with leucine. The putative Lrp-mediated regulation of gene expression of *fimA* and *yhhK* appeared to be enhanced by leucine.

4.2.2.1.2 RT-PCR analysis of Lrp/leucine-regulated genes

Analyses of Lrp-regulated genes in logarithmic phase MOPS minimal media were performed with an FDR of 0.1, and with only 2 out of 3 biological replicates, due to high variability in the logarithmic phase SL1344 *lrp* data (section 4.2.2.1). To validate the effect of Lrp and leucine identified in section 4.2.2.1.1, RT-PCR analyses were carried out on a selection of genes. The ten genes selected were analysed in SL1344, SL1344 *lrp*, and the complemented *lrp* mutant (SL1344 *lrp* containing pKMC102), during growth in MOPS minimal media in the absence and presence of 10 mM L-leucine, from samples taken at logarithmic phase of growth (OD_{600} 0.5). The genes selected were a combination of known Lrp-regulated genes in *E. coli* and genes that were not previously identified as Lrp or leucine regulated, and an unchanging control gene STM3038. The RT-PCR analyses, shown in Figure 4.2 (A), suggested that the expression profiles of most genes tested were in overall agreement with the microarray results (Fig. 4.2 (B)). In addition, the complemented *lrp* mutant gave a highly similar profile to SL1344 in both the absence and presence of 10 mM L-leucine. Two genes did not agree with the microarray data: *yhhK* was expected to have reduced expression in the presence of leucine in SL1344, compared to its absence, but instead showed increased expression in the presence of leucine on three separate occasions. In addition, *serA* was expected to be reduced in the *lrp* mutant, and reduced in the presence of leucine, but instead showed little change between all samples. Additional expression data for SL1344 *lrp* in the presence of 10 mM L-leucine was also obtained, which had not been identified by the microarray due to low yield of data: in particular, *ssaI* was shown to decrease in SL1344 *lrp* in the presence of leucine compared to its absence. The control gene STM3038 was unaffected in all samples, as expected.

4.2.2.2 Analysis of SL1344 and SL1344 *lrp* in MOPS minimal medium at stationary phase

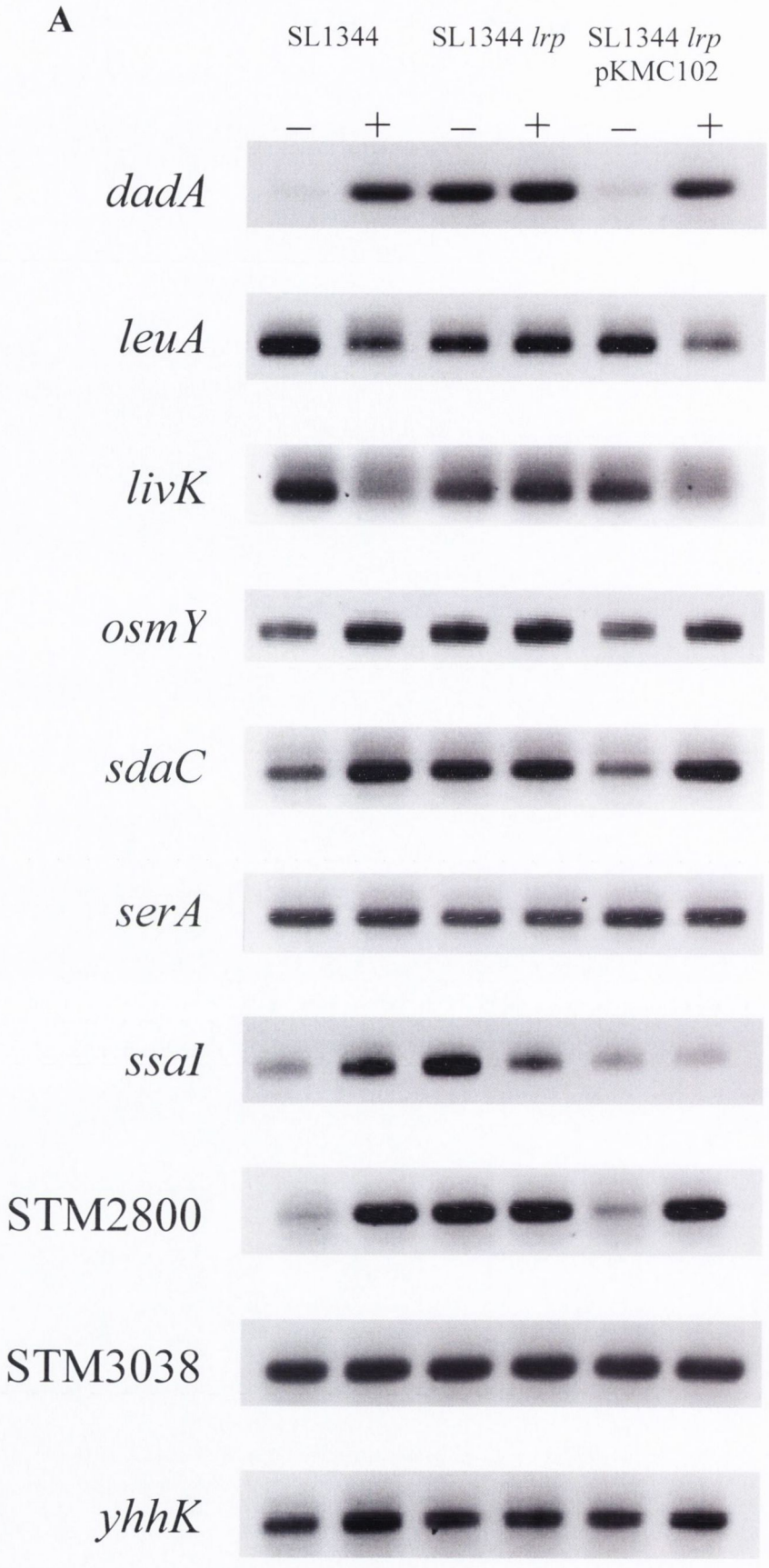
Stationary phase analysis of SL1344 and SL1344 *lrp* yielded more results than logarithmic phase analysis. As shown in Table 4.3, statistical filtering of MOPS leu– stat gave 2922 genes with an FDR of 0.05 between SL1344 and SL1344 *lrp*. Comparison of SL1344 *lrp* in the absence and presence of 10 mM L-leucine led to 2092 genes with a significant change, while between the two growth conditions in SL1344, only 968 genes were significantly affected. This suggested that the addition of leucine led to the greatest number of changes in the *lrp* mutant, which was interesting as Lrp is absent from the *lrp*

mutant. Table 4.3 shows the number of genes that were affected by a fold change ≥ 2 in each category. All MOPS minimal array data FDR 0.05 lists are presented in Microsoft Excel files in Tables S4.8 to S4.11.

The genes affected by ≥ 2 fold by the *lrp* mutation in SL1344, and those affected in SL1344 under the two growth conditions, were placed into functional categories (Fig. 4.3), based on the Kyoto Encyclopaedia of Genes and Genomics (KEGG). The analysis of functional categories showed that SPI-1 and SPI-2 genes were reciprocally regulated, with over 40 % of SPI-2 genes downregulated, and almost 80 % of SPI-1 genes upregulated in SL1344 *lrp* compared to SL1344. In addition, genes involved in propanediol utilisation, fimbriation and genes of the large virulence plasmid, pSLT, were upregulated in SL1344 *lrp*. Genes involved in peptidoglycan biosynthesis and translation, and genes of regulators were predominantly downregulated in SL1344 *lrp*. Interestingly, the gene expression patterns from SL1344 in the presence of leucine, compared with the absence of leucine, gave a similar profile to that of the *lrp* mutant.

4.2.3 Lrp and amino acid transport and metabolism

A large number of genes involved in amino acid transport and metabolism were identified as Lrp-regulated in this study. This was unsurprising, as Lrp is best known for its role in regulation of amino acid metabolism. These genes were identified by LB broth microarray analysis and MOPS minimal microarray analysis in logarithmic phase. Amino acid metabolism and transport was also affected by the *lrp* mutation and by the presence of leucine in MOPS minimal medium in stationary phase. Approximately 25 % of genes in this category (115 out of 450) were downregulated in the *lrp* mutant, and less than 20 % of genes were upregulated (72 out of 450). The majority of upregulated genes appeared to be involved in amino acid transport and biosynthesis, while the downregulated genes seemed to be mainly involved in amino acid transport and degradation. As expected, amino acid transport and metabolism was also affected by the presence of leucine, which affected approximately 5 % of those genes both positively and negatively. A number of newly identified, putatively Lrp-regulated genes involved in amino acid transport and metabolism were also identified, some of which are discussed in later sections.


A
SL1344
SL1344 lrp
SL1344 lrp pKMC102
– + – + – +
dadA
leuA
livK
osmY
sdaC
serA
ssaI
STM2800
STM3038
yhhK

B

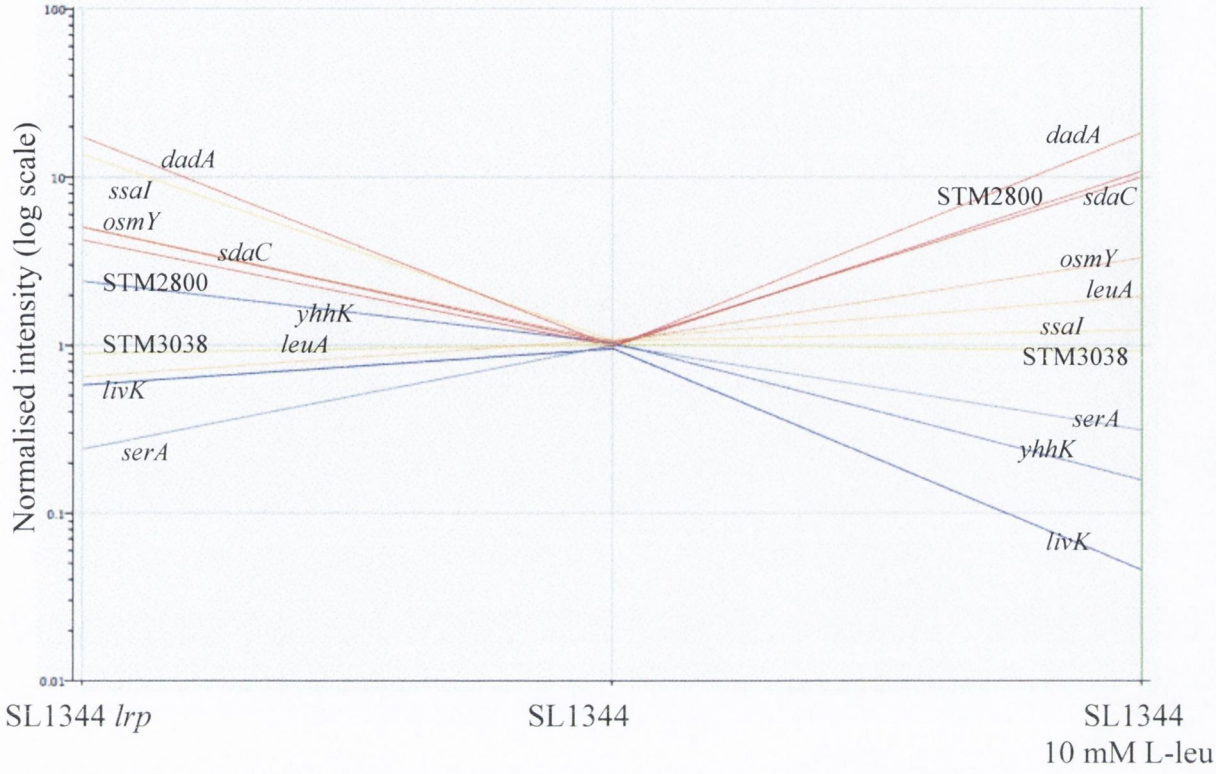


Fig. 4.2. RT-PCR analysis of Lrp-regulated genes. Genes identified by statistical analysis (FDR 0.1) of logarithmic phase data, using two out of three biological replicates were examined by RT-PCR analysis to verify the gene expression profile (A). RNA was extracted from SL1344, SL1344 *lrp* and SL1344 *lrp* containing the complementation plasmid pKMC102, grown to logarithmic phase in MOPS minimal broth in the absence and presence of 10 mM L-leucine. Absence and presence of 10 mM L-leucine is denoted by minus (–) and plus (+) signs, respectively. RT-PCRs were performed at least twice, with similar trends. Most genes follow the expected profile, as shown in part B, and the control gene STM3038, which was shown to be unaffected by Lrp or leucine, gave the same level of transcript in all samples.

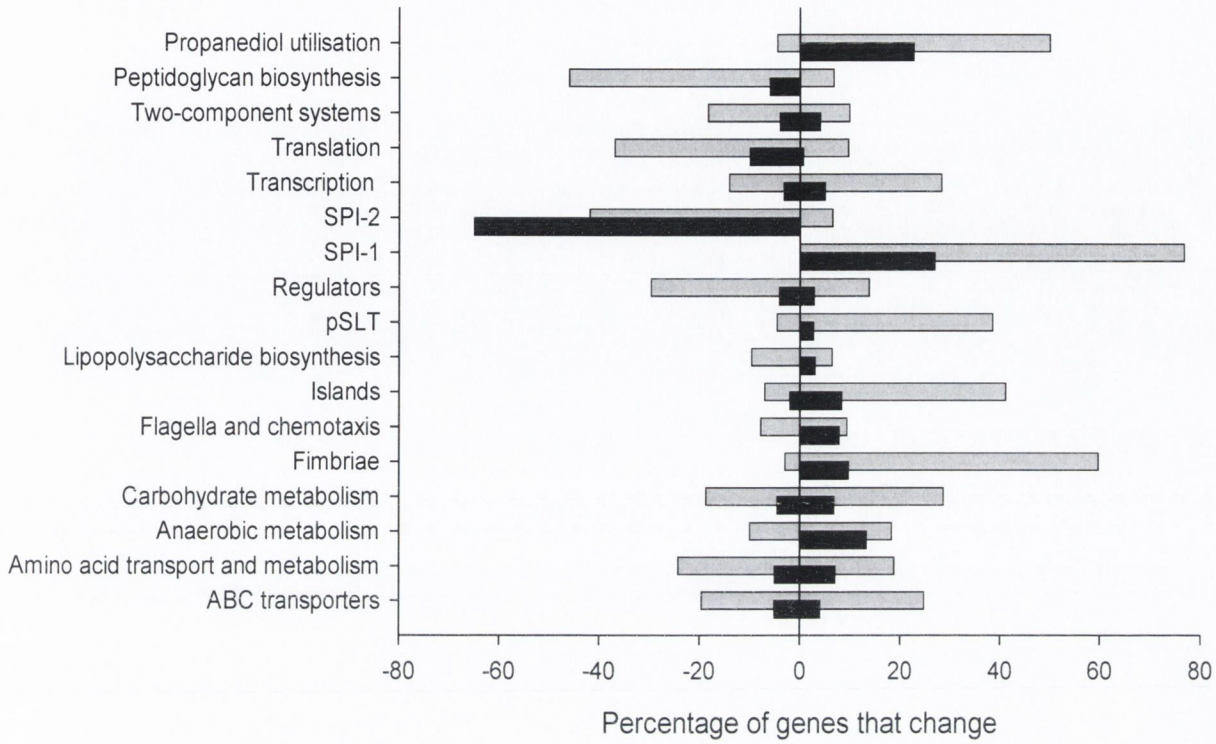


Fig. 4.3. Functional categories of genes affected by SL1344 *lrp* and 10 mM L-leucine, in stationary phase. In comparison with SL1344 in the absence of leucine, genes affected by SL1344 *lrp* are shown by shaded bars, and SL1344 in the presence of 10 mM L-leucine are shown by filled bars. Percentages denote the percentage of genes in a given category that are affected negatively (negative values) or positively (positive values) by the genetic characteristic, or growth condition. Genes were assigned functional categories using the Kyoto Encyclopaedia of Genes and Genomics (KEGG).

4.2.4 Regulation of nitrogen assimilation by Lrp

Lrp is required for the Ntr (nitrogen regulated) response in *E. coli* as it activates *gltBD* (Ernsting *et al.*, 1992), which codes for the two subunits of glutamate synthase. Lrp also controls the metabolism of alanine, serine and glycine, which are feedback inhibitors of glutamine synthetase. Ammonia assimilation by glutamate synthase and glutamine synthetase provides a nitrogen source, as glutamate and glutamine are major intracellular nitrogen donors (Reitzer, 2003). Nitrogen may also be assimilated from nitrogen-containing compounds such as polyamines, including ornithine and putrescine, oligopeptides, and amino acids that can provide nitrogen for glutamate or glutamine synthesis. Most Ntr genes specify transport proteins that scavenge nitrogen-containing compounds. Scavenging of nitrogen-containing compounds negates the need for ammonia assimilation (Reitzer, 2003).

Lrp and leucine have been shown in this study, and in previous studies, to regulate a number of members of the Ntr response. The effect of the *lrp* mutation on Ntr response genes was seen during logarithmic growth in MOPS minimal medium. In addition, leucine also appeared to have a strong effect on these genes under the conditions examined. The genes involved in nitrogen starvation that were affected are listed in Table 4.6. As expected, the *gltB* gene, which encodes the large subunit of glutamate synthase, was downregulated by 20 fold in SL1344 *lrp* and by > 5 fold in SL1344 in the presence of leucine. *S. enterica* has at least two arginine transport systems: the *argT-hisJMPQ* operon, and the *artPIQM-artJ* operon (Reitzer, 2003). In SL1344 *lrp,* and in SL1344 in the presence of leucine, the expression of *argT* was affected negatively, suggesting a positive role for Lrp and a negative role for leucine in regulation of *argT*. The *art* arginine transport system was positively affected in SL1344 *lrp* and in SL1344 in the presence of leucine, suggesting negative regulation by Lrp and reversal of negative regulation by leucine. Thus, according to these data, both the *art* and *argT* arginine transport systems are affected in a reciprocal manner by Lrp and leucine. The *glnK* gene, which encodes a signal transduction protein that regulates ammonia influx into the cell, was also strongly affected by both Lrp and leucine. In *E. coli, glnK* becomes strongly activated when cells stop growing due to ammonia starvation (Atkinson *et al.*, 2002). Lrp appeared to act as a positive regulator of *glnK* that was negatively affected by leucine. In *E. coli*, Lrp has been identified as a regulator of the *opp* and *pot* genes, which are responsible for oligopeptide and putrescine uptake,

respectively (Hung *et al.*, 2002). Oligopeptides and putrescine are nitrogen-containing compounds that are scavenged during periods of nitrogen starvation. Interestingly, *opp* gene expression appeared to be negatively affected by Lrp (> 4.9-fold upregulated in the *lrp* mutant) and positively regulated by leucine, as previously described (Calvo and Matthews, 1994; Hung *et al.*, 2002; Salmon *et al.*, 2003). The *pot* genes, however, appeared to be positively regulated by Lrp (downregulated by > 5 fold in SL1344 *lrp*), also in agreement with previous studies (Hung *et al.*, 2002). In addition, examination of the effect of leucine revealed that leucine downregulated *potF* and *potH* expression by 4.7 and 3.2 fold, respectively. A number of other genes involved in nitrogen assimilation were also affected by Lrp and leucine (Table 4.6).

4.2.5 Lrp/leucine and anaerobic gene expression

The regulatory network of Lrp is known to overlap the FNR regulon of *E. coli* (Salmon *et al.*, 2003). FNR is a sensor of oxygen availability in *E. coli*. The role of Lrp in anaerobic metabolism is not understood. Examination of the Lrp regulon of *S.* Typhimurium identified a number of genes involved in anaerobic metabolism under the control of Lrp/leucine. In LB broth, 11 out of 38 genes that were regulated by ≥ 2 fold in SL1344 *lrp* at the 4 h timepoint were genes associated with anaerobic metabolism. These genes, which were strongly induced between 1 h to 4 h growth, are shown in Fig. 4.4. The *nap* and *nrf* genes encode anaerobically-induced nitrate and nitrite reductases, respectively (Wei and Miller, 1999), and *nirD* encodes part of an FNR-dependent, anaerobically-induced nitrite reductase (Harborne *et al.*, 1992). The *asrB* gene encodes an anaerobic sulfide reductase (Huang and Barrett, 1991). Comparison of SL1344 during logarithmic growth in MOPS minimal medium in the absence and presence of 10 mM L-leucine showed that the *frdBCD* genes, which encode fumarate reductase, were upregulated by > 2.6 fold upon the addition of leucine. Fumarate can be used as a terminal electron acceptor for anaerobic oxidative phosphorylation, and fumarate reductase is induced by anaerobiosis and repressed by the presence of nitrate. Anaerobic metabolism was also affected in stationary phase in MOPS minimal broth. Less than 20 % of genes involved in anaerobic metabolism were upregulated, while approximately 10 % were downregulated in SL1344 *lrp*: among the upregulated genes were putative dimethyl sulfoxide reductase subunits; downregulated genes included the *glpABC* operon, which encodes an anaerobic glycerol phosphate dehydrogenase. Growth in 10 mM L-leucine increased the expression of >10 % of anaerobic genes at this timepoint.

Gene	SL1344 *lrp*	SL1344 + leucine	Product
argT	0.14	0.27	Lys/arg/ornithine transport
artI	–	4.09	Arginine third transport system
artP	–	4.07	Arginine third transport system
artQ	4.76	3.90	Arginine third transport system
artM	4.05	–	Arginine third transport system
glnK	0.03	0.06	Nitrogen starvation regulator
glnH	–	0.41	Glutamine high-affinity transport
glnP	–	0.45	Glutamine high-affinity transport
glnQ	0.27	0.45	Glutamine high-affinity transport
gltB	0.05	0.17	Glutamate synthase subunit
gltP	4.49	8.27	Glutamate:aspartate symport
oppA	4.93	2.87	Oligopeptide transport
oppD	5.74	2.60	Oligopeptide transport
oppF	5.43	2.37	Oligopeptide transport
pntB	0.36	–	Pyridine nucleotide transhydrogenase
potF	–	0.21	Putrescine transporter
potG	0.19	–	Putrescine transporter
potH	0.19	0.31	Putrescine transporter

Table 4.6. Genes involved in nitrogen starvation, and their regulation by Lrp and leucine. Fold changes of genes in SL1344 *lrp,* or SL1344 in the presence of leucine, with respect to SL1344 during growth in the absence of leucine, are listed along with a description of the gene product. A dash denotes unavailable data.

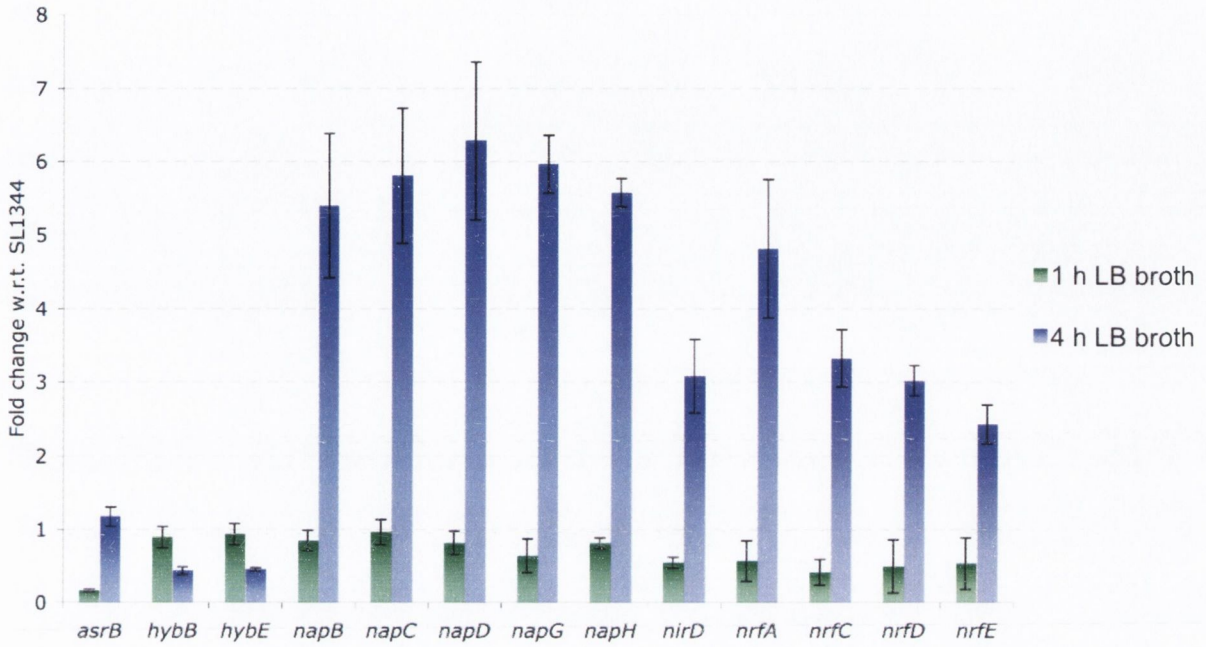


Fig. 4.4. Induction of genes involved in anaerobic metabolism in LB-grown SL1344 *lrp*. Fold change of gene expression is expressed with respect to SL1344. Error bars denote standard error of the mean. Genes were strongly induced from lag phase (1 h) to logarithmic phase (4 h) during growth in LB (see Fig. 3.3).

4.2.6 Fimbrial gene expression

Lrp and leucine are known to affect the expression of a number of fimbrial genes in *E. coli* and *Salmonella*. In *S.* Typhimurium LT2, whole genome sequencing identified 13 putative fimbrial operons (McClelland *et al.*, 2001), but *fimA* of the type 1 fimbrial *fim* operon is the only the major fimbrial subunit to be expressed on the bacterial surface after growth in LB broth (Humphries *et al.*, 2005). Microarray analysis of SL1344 and SL1344 *lrp* grown in LB broth in the present study showed that *fimA* and *fimD* were downregulated in the *lrp* mutant in lag and logarithmic phases, and at the transition phase (from logarithmic to stationary), suggesting that Lrp is a positive regulator of *fim* gene expression (see Table 4.2). In MOPS minimal medium arrays, *fimA* and *fimZ* were also identified as downregulated in SL1344 *lrp* in logarithmic phase, by > 8 fold and > 2 fold, respectively. In addition, inspection of gene expression of SL1344 in the presence of 10 mM L-leucine suggested that leucine had a positive effect on *fim* gene expression of approximately 2 to 4 fold (see Table 4.5). As a result, the *fimA* gene was present in the list of 30 genes that were Lrp/leucine-regulated (see Fig. 4.1, and supplementary data Table S4.7). Although a known regulator of fimbrial genes, Lrp had not previously been identified as a regulator of *fim* gene expression in *S. enterica*. The effect of Lrp on *fim* type 1 fimbriae of *S. enterica* is analysed in further detail in Chapter 5.

Lrp also appeared to affect the expression of other fimbrial genes. In LB microarray analyses, the *lpfD* gene, encoding a long polar fimbrial operon protein, was upregulated at transition phase by 2.5 fold in SL1344 *lrp*. No data were available for this gene at other timepoints. The *sthB* gene, encoding a putative fimbrial usher protein, was also upregulated at this timepoint in SL1344 *lrp*, but downregulated by > 2 fold by stationary phase. Analysis of gene expression after 24 h growth in MOPS minimal medium showed that approximately 60 % of fimbrial genes were ≥ 2-fold upregulated in SL1344 *lrp*, while approximately 10 % of fimbrial genes were upregulated by ≥ 2 fold in the presence of leucine. Less than 5 % of fimbrial genes were downregulated in SL1344 *lrp* after 24 h growth in MOPS minimal medium.

4.2.7 Virulence gene expression

A number of virulence genes were identified as affected by Lrp. Fimbrial genes of the *fim* operon were strongly affected by the *lrp* mutation (see section 4.2.6). Five pathogenicity islands (SPI-1 to SPI-5) have been characterised in *S.* Typhimurium,

mutations in which can lead to attenuation of virulence (Blanc-Potard *et al.*, 1999; Galan and Curtiss, 1989; Morgan *et al.*, 2004; Shea *et al.*, 1996; Wood *et al.*, 1998). The SPI-2 pathogenicity island encodes a TTSS that is required for systemic infection of mice, and survival and replication in macrophages and epithelial cells (Cirillo *et al.*, 1998; Hensel *et al.*, 1995; Hensel *et al.*, 1998). SPI-2 secretes effector proteins encoded in SPI-2, and other chromosomal locations, into the host cell from the SCV (Abrahams and Hensel, 2006). These effector proteins interfere with the degradative pathway of the SCV, and aid replication within the SCV, through interference with host intracellular transport processes, the host microtuble cytoskeleton and the actin cytoskeleton (Kuhle and Hensel, 2004; Ramsden *et al.*, 2007).

MOPS minimal medium analyses of SL1344 *lrp* in logarithmic phase growth identified a number of SPI-2 genes that were upregulated in the *lrp* mutant. Six SPI-2 genes, which occur in the large structural II region encoding the secretion system apparatus (Hensel *et al.*, 1997), the *sifB* gene, encoding a SPI-2 translocated effector protein, and the *sseD* translocon gene were all upregulated by 2-16 fold in the *lrp* mutant. Importantly, *sifB* is located outside SPI-2, but its expression is controlled by the SPI-2-encoded two-component regulatory system SsrA-B, and its promoter is responsive to a range of stimuli that affect SPI-2 gene expression (Miao and Miller, 2000). In addition, the gene STM1410, which is located immediately downstream of *ssaJ* and upstream of *ssaK,* was also upregulated by > 9.8 fold in the *lrp* mutant. The gene *ssaI*, which was upregulated by 16 fold in SL1344 *lrp*, was examined by RT-PCR analysis (shown in section 4.2.2.1.2, Fig. 4.1) and was in agreement with the microarray data. These data suggested that Lrp may have a role in the negative regulation of SPI-2 expression.

Analysis of gene expression after 24 h growth in MOPS minimal broth showed that a number of virulence factors were affected by both Lrp and leucine. Genes of SPI-2, which were upregulated in the *lrp* mutant at logarithmic phase, were downregulated in the *lrp* mutant by stationary phase, shown in Figure 4.5. This suggested that Lrp could have a positive role in SPI-2 gene expression, an opposite effect to that seen at logarithmic phase. In addition, leucine dramatically affected SPI-2 genes in SL1344 (Fig. 4.3), with > 60 % of SPI-2 genes downregulated in its presence, compared to its absence. SPI-1 genes were identified as affected by the *lrp* mutation in MOPS minimal medium at stationary phase. Interestingly, over 70 % of SPI-1 genes were upregulated in

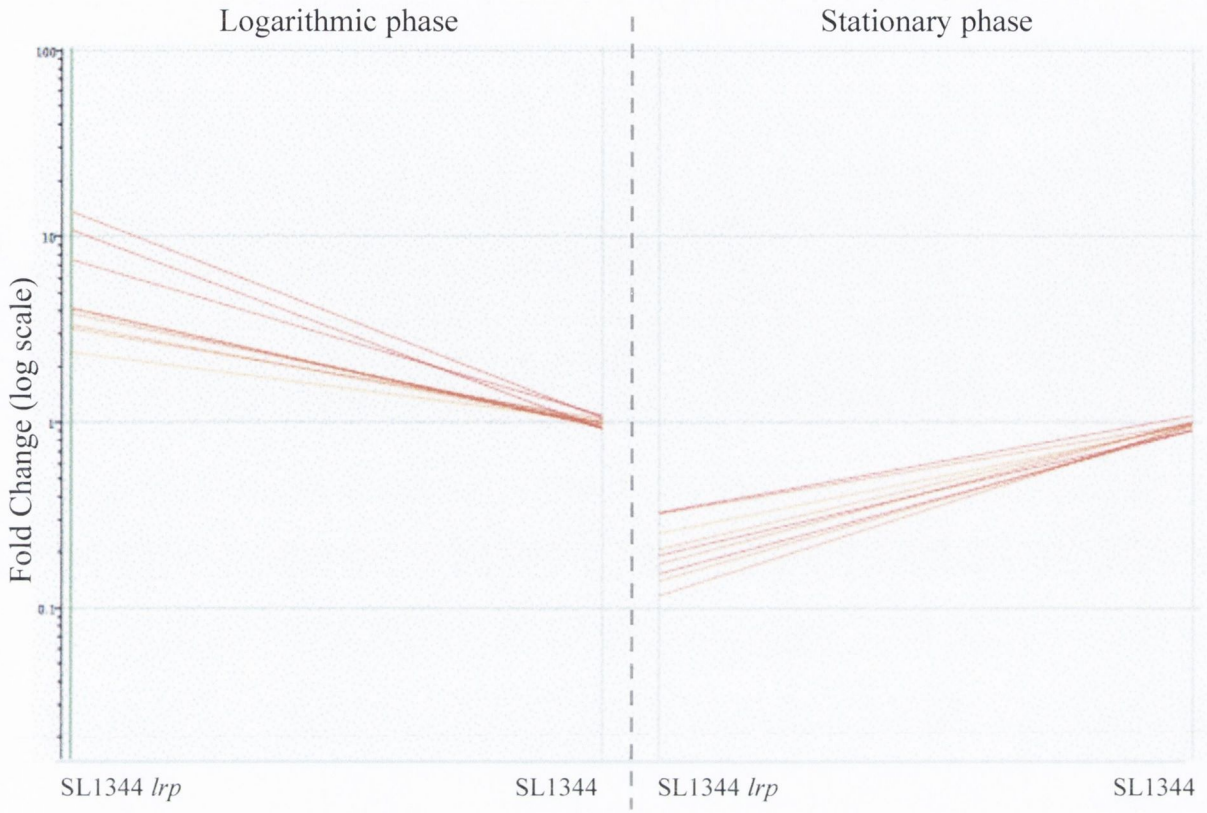


Fig. 4.5. Expression of SPI-2 genes in logarithmic and stationary phase. Nine SPI-2 genes that were downregulated in SL1344 *lrp*, compared to SL1344 at stationary phase in MOPS minimal medium were chosen. The profiles of gene expression at logarithmic phase and stationary phase are shown. At logarithmic phase, all nine genes showed upregulation in SL1344 *lrp* (denoted by the red colour), whereas the same nine genes showed downregulation in SL1344 *lrp* at stationary phase.

SL1344 *lrp*, and over 20 % of SPI-1 genes were upregulated by the presence of leucine, with no downregulated SPI-1 genes identified during stationary phase in MOPS minimal broth.

4.2.8 Putative newly-identified Lrp/leucine-regulated genes

In addition to the newly identified Lrp-regulated genes in the preceding sections, Lrp also appeared to regulate a number of uncharacterised genes and operons in SL1344, which have not been revealed by studies prior to this. Some of these genes are discussed in this section.

In LB, a putative operon consisting of *yjiHG-iadA-yjiE* was downregulated by 4 to 5 fold in SL1344 *lrp* in logarithmic phase (Fig. 4.6 (A)). Putative functions have been assigned to these genes: *yjiH* encodes a putative inner membrane protein, *yjiG* encodes a putative permease, *iadA* produces an isoaspartyl dipeptidase, while *yjiE* encodes a LysR-like transcriptional regulator. In addition to this, STM4510, whose 3' end converges with the 3' end of *yjiE*, is downregulated by > 7 fold at the same timepoint in SL1344 *lrp*. STM4510 encodes a putative apartate racemase. Upstream of STM4510, STM4509 was 1.96-fold downregulated at 4 h and 24 h, and > 2-fold downregulated at 5.5 h. This gene encodes the virulence regulator, HilE. Taken together, this gene cluster, which appears to partially require Lrp for its expression, may contribute to aspartate transport/metabolism. To confirm the effect of Lrp on this gene cluster, RT-PCRs were performed on these genes (Fig. 4.6 (B)), in SL1344 and SL1344 *lrp* from RNA isolated during growth in LB broth. The *livJ* gene, which is located elsewhere on the chromosome, and is strongly upregulated in the *lrp* mutant in LB broth and thus regulated oppositely to the newly identified operon, was used as a control gene.

The gene STM2800, which encodes a putative inner membrane protein, appeared to be strongly regulated by Lrp. In LB broth, this gene was 2.9-fold upregulated at lag phase, and then downregulated by > 5 fold in logarithmic phase, and > 2 fold in both transition and stationary phase in SL1344 *lrp*, which suggested that Lrp acts as both a positive and negative regulator for this gene. STM2800 was also identified as an Lrp-regulated gene during MOPS minimal medium microarray analyses: in logarithmic phase, STM2800 was upregulated by 4.8 fold in SL1344 *lrp*, and its expression was positively affected by > 10 fold during growth in 10 mM leucine. Expression of STM2800 was confirmed by

RT-PCR analysis in section 4.2.2.1.2, Figure 4.1, which showed that its expression was almost undetectable in SL1344, but was strongly enhanced by addition of leucine. In SL1344 *lrp*, expression was present in the absence of leucine, and unchanged in its presence, which suggested that Lrp negatively regulates STM2800, and this repression is relieved by leucine.

The genes STM1633, STM1635 and STM1636 were affected by leucine in SL1344 (Table S4.6). The putative operon, which includes the gene STM1634, shows homology to amino acid transport systems: the putative operon was downregulated by 2 to 5 fold by the addition of leucine. These genes did not pass through the statistical filter in SL1344 *lrp* in MOPS minimal medium, but may constitute an uncharacterised Lrp-regulated operon.

4.2.9 Regulation of gene clusters

It was observed that a number of Lrp-regulated genes identified from the LB and MOPS minimal broth microarrays appeared to be spatially related. The gene, *eco*, which encodes ecotin, a serine protease, is positioned diverging from the *nap* genes, both of which were affected by Lrp in LB (Fig. 4.7). The *opp* genes are negatively affected by Lrp and leucine. These genes were identified in the present study, as well as in studies of the Lrp regulon of *E. coli* (Hung *et al.*, 2002). The gene STM1747, which encodes a putative inner membrane protein, overlaps *oppA* at the 5' ends (Fig. 4.7). STM1747 was identified as leucine-regulated (affected by leucine list, in Table S4.6 in the supplementary data CD), as it was upregulated by 1.99 fold at logarithmic phase in SL1344, in the presence of 10 mM L-leucine.

STM2800, an uncharacterised Lrp-regulated gene identified by this study, was strongly affected by Lrp and leucine. Intriguingly, STM2800 diverges from *stpA*, another Lrp-regulated gene (Fig. 4.7). *stpA* did not pass through the statistical filter in MOPS minimal broth analysis with SL1344 *lrp*, although examination of total gene data suggests that this gene is downregulated in SL1344 *lrp*, suggesting that Lrp induces expression of this gene, and the expression of *stpA* was also unaffected by leucine. The expression of *stpA* is induced in minimal medium in *E. coli* and was also shown to be unaffected by leucine (Free and Dorman, 1997). It is tempting to hypothesise that the regulation of spatially related, particularly divergently transcribed, genes is linked.

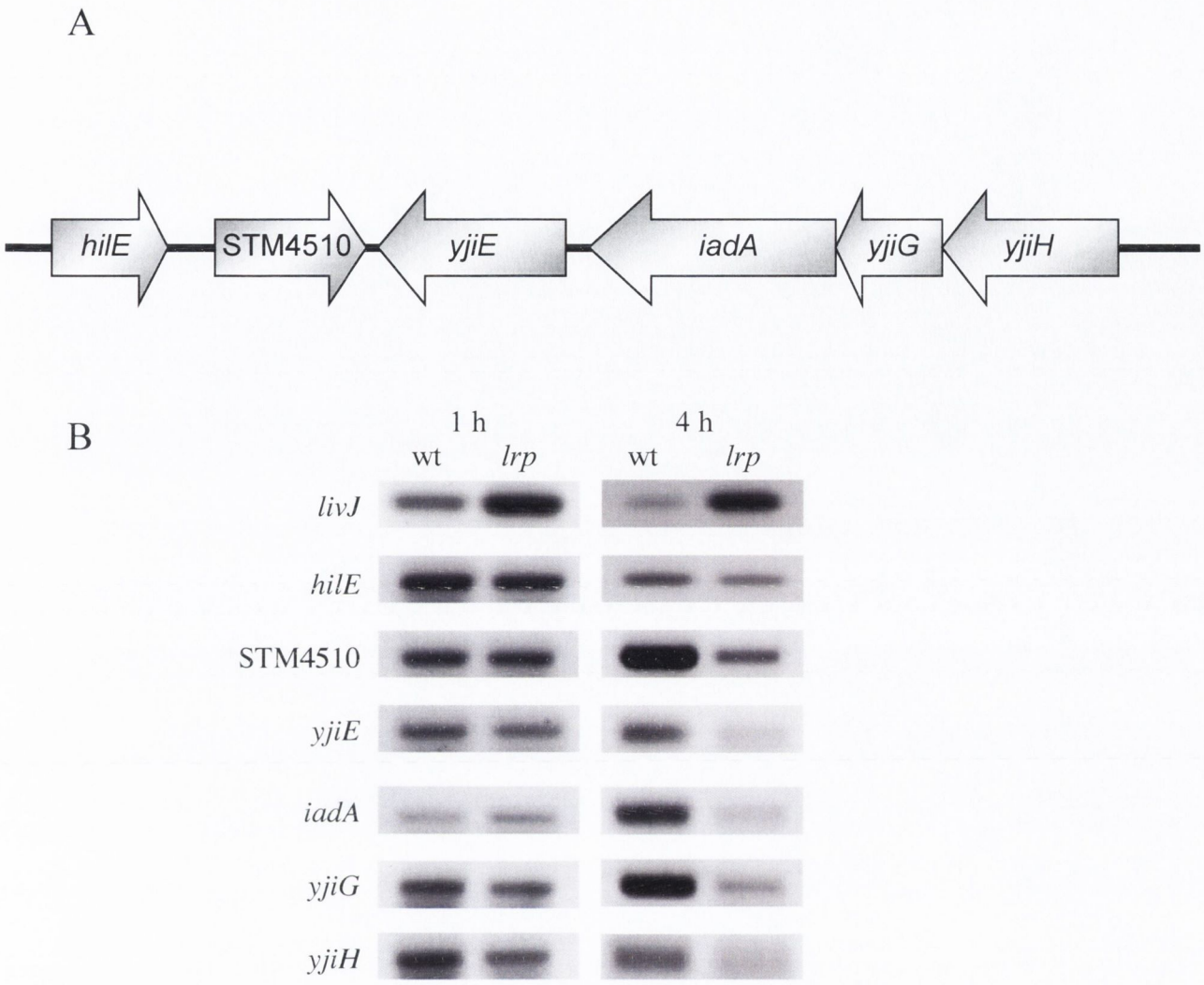


Fig. 4.6. A putative operon controlled by Lrp. The genes *yjiE-iadA-yjiGH* form a putative operon, which was strongly downregulated at logarithmic phase (4 h) in SL1344 *lrp* (A). Also downregulated at these timepoints were the genes *hilE* and STM4510. This was confirmed by RT-PCR analysis, including the control gene *livJ* (B). *livJ* is located elsewhere on the chromosome, and is negatively regulated by Lrp during growth in LB broth, so is upregulated in SL1344 *lrp*. This analysis was performed at least twice, with similar results.

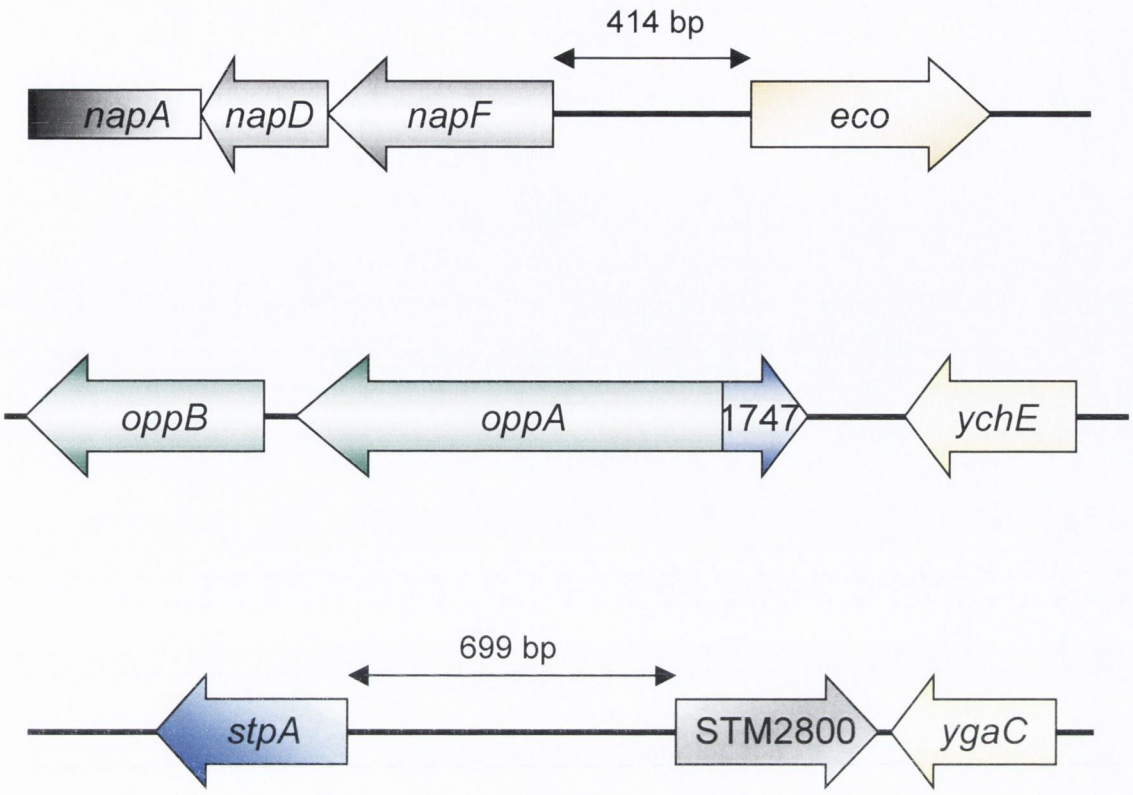


Fig. 4.7. Regulation of divergently transcribed genes by Lrp. Lrp appeared to regulate a number of gene clusters. Examples of these are shown above. The gene *eco* and the *nap* genes (top), both of which were affected by Lrp in LB broth, diverge and are their ORFs are separated by 414 bp. The *oppA* gene of the *opp* operon, and the gene STM1747 also diverge (middle), and are affected by Lrp/leucine, and leucine, respectively. The gene *stpA*, which is a known Lrp-regulated gene, diverges from the gene STM2800 (bottom), which was revealed in this study to be Lrp and leucine regulated. The ORFs of these two genes are separated by 699 bp. In E. *coli*, the upstream region of *stpA* is known to be required for its proper regulation in minimal medium (Free and Dorman, 1997).

4.3 Discussion

DNA microarray analysis was used to characterise the regulon of Lrp in *S.* Typhimurium, during growth in both rich and nutrient-poor media. The data revealed that Lrp in *S.* Typhimurium regulates a number of genes that are Lrp-regulated in *E. coli,* as well as *Salmonella*-specific genes, and previously uncharacterised genes.

Throughout this study, genes involved in anaerobic metabolism appeared to be regulated by Lrp/leucine. Previous studies have shown that the Lrp regulon of *E. coli* overlaps with that of FNR, the anaerobically-triggered transcription factor (Hung *et al.*, 2002; Salmon *et al.*, 2003). FNR coordinates carbon and energy metabolism during growth under anaerobic conditions, whereas Lrp coordinates metabolic activities with the nutritional and environmental growth state of the cell: the overlapping regulon of these two regulators suggests interactions between the two global regulatory networks (Salmon *et al.*, 2003). In this study, genes involved in anaerobic metabolism were identified as Lrp-regulated mainly during logarithmic growth in LB broth, and in stationary phase in MOPS minimal medium. The *nap* and *hyb* genes, which were affected by Lrp in LB, are FNR-dependent, anaerobically-regulated genes (Wei and Miller, 1999). The present study also identified the *frdBCD* genes, encoding anaerobically-induced fumarate reductase, as leucine-sensitive in MOPS minimal medium during logarithmic-phase growth: these genes were upregulated by 2.6 to 3.6 fold in the presence of leucine in SL1344. Interestingly, Tani *et al.* identified the *frdA* gene as 2.8-fold upregulated in the *lrp* mutant of *E. coli* (Tani *et al.*, 2002). Taken together, this suggests that the *frd* genes may be Lrp-regulated in *S.* Typhimurium. Fumarate reductase, which is encoded by the *frd* genes, allows *E. coli* to grow anaerobically with fumarate as terminal electron acceptor when the preferred electron acceptors oxygen and nitrate are not available. The *frdABCD* genes are thought to be regulated at the transcriptional level in response to the cellular availability of oxygen, nitrate and fumarate, and anaerobic induction of *frdABCD* requires FNR (Jones and Gunsalus, 1985, 1987).

Analyses by Hung *et al.* described functional categories of genes regulated by Lrp in *E. coli*. The category of small molecule transport included genes belonging to the *pot* operon, and the *art* operon, which had not previously been identified as Lrp-regulated (Hung *et al.*, 2002). These genes were identified as Lrp-regulated by the present study

(see Table 4.7), and are linked to the Ntr response in *E. coli*. Lrp is known to positively regulate the *gltBD* genes that control glutamate synthase, and is thus required for the Ntr response (Reitzer, 2003). The *gltBDF* operon is described as leucine-insensitive in *E. coli* (Ernsting *et al.*, 1993). Array data in the present study (Table 4.5) suggest that this operon is 5-fold repressed in the presence of leucine in SL1344. Prior to, and in agreement with the findings of the present study, Lrp has been shown to regulate a number of genes involved in the transport of oligopeptides and putrescine (Hung *et al.*, 2002), which are strongly linked to the Ntr response (Reitzer and Schneider, 2001). Surprisingly, the *glnALG* genes, which encode glutamine synthetase and are affected by *lrp* mutations (Calvo and Matthews, 1994), were not identified in this study. The Nac protein is a LysR-like regulator of the Ntr response in *E. coli* and *Klebsiella,* which is absent in *S.* Typhimurium. Nac regulates putrescine transport and *ompF,* which are also regulated by Lrp in *S.* Typhimurium and *E. coli* (Reitzer and Schneider, 2001). Nac also represses *serA* and *gltBD,* which are both activated by Lrp (Reitzer, 2003). The data suggest that Lrp may have a more important role in the Ntr response than previously thought, and may regulate additional Ntr response genes other than *gltBD,* such as those discussed in section 4.2.4. Alternatively, it is possible that the effects seen on the Ntr response genes were not a direct effect of Lrp, but a result of the effect of the *lrp* mutation on *gltB* expression. There is no discernible pattern of expression to suggest that the absence of Lrp in MOPS minimal broth culture mimics either nitrogen limitation or ammonia assimilation: Ntr response genes are both up and downregulated in the *lrp* mutant and in the presence of leucine.

A number of examples of divergently transcribed genes that are affected by Lrp and/or leucine were identified by this study. It is interesting to speculate that transcription of these genes may be linked. Most striking are the genes STM2800 and *stpA*, both of which are Lrp-regulated (Fig. 4.7). The STM2800 gene was shown in this study to be negatively regulated by Lrp in MOPS minimal broth, and this repression was relieved by the addition of leucine. It is unknown whether this is a direct or indirect effect of Lrp. STM2800 diverges from the Lrp-regulated *stpA* gene, which is directly positively regulated by Lrp and unaffected by leucine. The ATG start codons of STM2800 and *stpA* are separated by 699 bp in *S.* Typhimurium and 669 bp in *E. coli*. Interestingly, in *E. coli* the *stpA* gene requires hundreds of bp of upstream DNA to show regulation in minimal medium (Free and Dorman, 1997).

The stationary phase MOPS minimal medium data identified a large number of genes that were statistically different (FDR 0.05) between SL1344 and SL1344 *lrp*. Out of 4747 elements on the arrays used, over 60 % of genes were affected. Over 40 % of genes (1924 genes) were affected by ≥ 2 fold in SL1344 *lrp*. In the presence of leucine, only 595 gene transcripts were significantly changed by the *lrp* mutation, 5 times fewer changes than in the absence of leucine. A possible reason for this is the seemingly similar profile of SL1344 in the presence of leucine, and SL1344 *lrp*. Figure 4.3 shows the functional categories of genes affected by both SL1344 *lrp* and SL1344 in the presence of leucine. In all categories, the effect of SL1344 in the presence of leucine appears similar to the effect of SL1344 *lrp* on gene expression. Many changes were observed in SL1344 *lrp* by stationary phase in minimal medium. A number of genes identified in the *lrp* mutant were no longer behaving as expected by this phase of growth. Figure 4.3 shows that over 30 % of regulatory proteins of SL1344 were downregulated by ≥ 2 fold in the *lrp* mutant at this timepoint. This strongly suggests that ≥ 2 fold effects on 40 % of the genome of SL1344 shown in the *lrp* mutant are most likely to be indirect effects, and cumulative effects caused by the *lrp* mutation over time.

In this chapter, microarray analysis suggested that Lrp may be a positive regulator of *fim* genes, encoding type 1 fimbriae. This analysis also suggested that leucine may have a positive role in *fim* expression. Chapter 5 examines in more detail the effect of Lrp on type 1 fimbrial gene regulation in *S. enterica*. Genes of the SPI-2 pathogenicity island were also identified as putatively Lrp-regulated. These genes showed upregulation in the *lrp* mutant during logarithmic phase growth in MOPS minimal medium. The effect of Lrp on SPI-2 gene expression was more closely examined in Chapter 6.

Chapter 5

The regulation of type 1 fimbrial gene expression by Lrp in *Salmonella enterica* serovar Typhimurium

5.1 Introduction

Following ingestion of contaminated food or water, *S.* Typhimurium can colonise the intestine of the host, leading to invasion, and survival and replication within host cells. A critical initial step in colonisation is adhesion to the host mucosa. Bacterial surface appendages called fimbriae, or pili, interact with specific ligands on the host cell surface. The tropism of the bacterial fimbrial adhesin and the presence of these ligands together contribute to the process of adhesion. Whole-genome sequencing has revealed thirteen putative fimbrial operons in *Salmonella enterica* serovar Typhimurium (McClelland *et al.*, 2001), which may represent interactions with ligands of distinct epithelial cells. After an 8 h incubation in bovine ligated ileal loops, it is possible to detect the expression of the major antigens of nine of these fimbrial operons, including FimA of the *fim* operon (Humphries *et al.*, 2003). Type 1 fimbriae of the *fim* operon of *Salmonella* are characterized by their ability to bind mannosylated glycoproteins on eukaryotic cell surfaces. In *S.* Typhimurium, these phase variable fimbriae are expressed during infection of the rat (Ewen *et al.*, 1997), exhibit tropism for murine enterocytes (Thankavel *et al.*, 1999), and contribute to colonization of swine *in vivo* (Althouse *et al.*, 2003).

Lrp has been shown to regulate fimbrial genes, such as the *pef* (plasmid-encoded fimbriae) operon of *S.* Typhimurium (Nicholson and Low, 2000) and many fimbrial operons of *E. coli* including the *fim* gene cluster encoding type 1 fimbriae. Lrp regulates fimbrial genes by a number of methods. In the *pap* system, as in the *sfa, daa, fan, foo* and *clp* fimbrial operons of *E. coli* (Braaten *et al.*, 1992; Crost *et al.*, 2004; van der Woude and Low, 1994), and the *pef* system of *S. enterica* (Nicholson and Low, 2000), Lrp is required for methylation protection of GATC sites located within conserved DNA sequences in the regulatory regions of these operons. In the case of Pap (pyelonephritis-associated pili) fimbriae, the cis-located *papI* product enhances binding of Lrp to, and thus protection of, the sequence ACGATC, which contains the target GATC site for Dam methylase and is present in a promoter-proximal and promoter-distal site (Hernday *et al.*, 2003). Methylation of the promoter-proximal GATC site by Dam is required for transition to the phase ON state by specifically blocking PapI-dependent binding of Lrp to the promoter-proximal site, while occupation of the promoter-proximal site by Lrp is associated with a phase OFF state (Hernday *et al.*, 2003).

The regulatory mechanisms controlling expression of phase variable type 1 fimbriae (*fim*) of *E. coli* are distinct, but well characterized. This system consists of an invertible DNA element, *fimS,* containing a promoter for the polycistronic operon that encodes the fimbrial structural genes (Abraham *et al.*, 1985). Two site-specific recombinases, FimB and FimE, catalyse inversion of *fimS*, leading to phase variable transcription of type 1 fimbrial genes. FimB inverts the switch from phase ON-OFF and OFF-ON with approximately equal efficiency, while FimE preferentially switches from ON-OFF orientation. Accessory proteins also regulate the inversion of *fimS*, such as IHF and Lrp. Lrp binds to three sites within *fimS,* where it acts as an essential positive regulator of DNA inversion: leucine is known to potentiate this positive effect (Roesch and Blomfield, 1998).

The *fim* system of *Salmonella enterica* is comparatively poorly understood: in particular, no exogenous regulators have yet been identified. Although phase variable, *fim* of *S.* Typhimurium, unlike that of *Escherichia coli,* does not appear to contain an invertible element, as the *fimA* promoter region is only found in the orientation allowing transcription, regardless of fimbrial phenotype (Clegg *et al.*, 1996). In addition, no homologues of the FimB or FimE recombinases occur within the *S.* Typhimurium *fim* operon (Swenson and Clegg, 1992). Instead, expression of *S.* Typhimurium *fim* is controlled by a complex regulatory cascade. Expression of the major fimbrial subunit, encoded by *fimA*, is controlled at the transcriptional level by proteins encoded in the *fim* gene cluster, FimW, FimY, and FimZ, shown in Figure 5.1. These genes are located downstream of the structural genes and are transcribed in the opposite direction (Tinker *et al.*, 2001). FimZ, the best characterized of these regulators, is a DNA-binding protein with significant homology to the response regulator family of proteins, but no cognate sensor kinase has yet been discovered (Yeh *et al.*, 2002). FimZ has been shown to interact directly with the *fimA* promoter region to positively regulate transcription, and is essential for *fimA* transcription, as well as positively regulating its own transcription (Yeh *et al.*, 2002). FimZ has also been shown to have a negative effect on motility. When overexpressed, FimZ negatively affects *flhDC*, the master regulator of flagellar expression (Clegg and Hughes, 2002). In addition, FimZ has been shown by Baxter and Jones to positively affect the expression of HilE, a repressor of *hilA*, and thus SPI-1 expression (Baxter and Jones, 2005). HilE exerts this effect by interacting with HilD, to repress its function as a positive regulator of *hilA*.

FimY is essential for transcription of *fimA* as a co-activator with FimZ, although this is not through a direct interaction with *fimA* (Tinker and Clegg, 2000). FimW is a negative regulator of *fimA* transcription that does not interact directly with the *fimA* promoter (Fig. 5.1). FimW instead acts through protein-protein interactions with FimZ, and is thought to inhibit FimZ-mediated positive regulation of *fimA* (Tinker *et al.*, 2001). In addition, the translation of *fim* genes may be regulated by *fimU*, a rare arginine tRNA. A high frequency of rare arginine codons is found in the *fim* operon, with five in *fimY* alone. The *fimU* mutant strain has been shown to have inhibition of efficient *fimY* translation, as well as an afimbriate phenotype (Tinker and Clegg, 2001).

This study analysed the effect of Lrp on type 1 fimbriae of *Salmonella enterica* serovar Typhimurium. Microarray analysis (Chapter 4) showed that the mRNA levels of a number of *fim* genes are reduced in the *lrp* mutant. RT-PCR was used to analyse relative gene expression levels, while EMSA analyses demonstrated direct interactions of Lrp with *fim* promoter regions. DNase I footprinting was also used to further characterise these interactions.

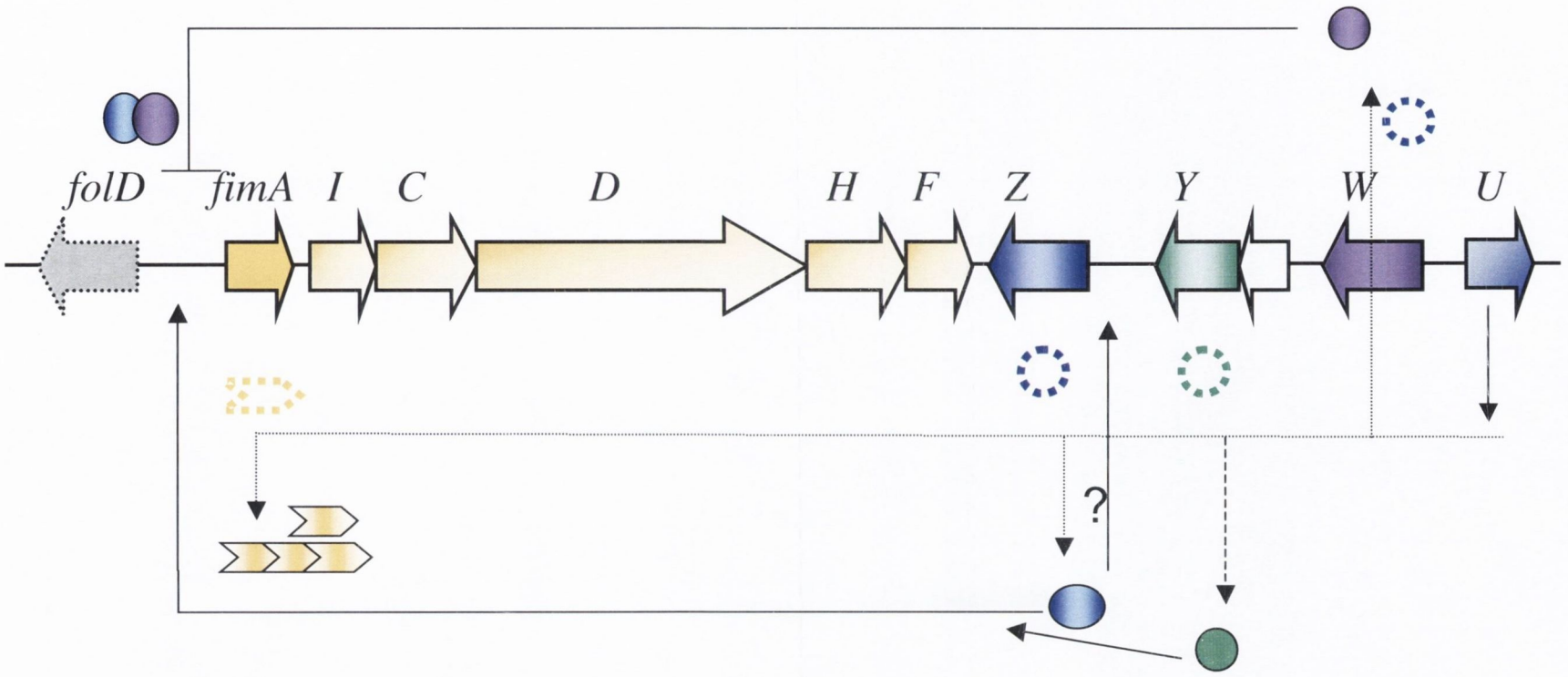


Fig. 5.1. Summary of the regulatory features controlling expression of the *fim* operon. The regulatory cascade controlling phase variation of *fim* expression is described in the text.

5.2 Results

5.2.1 An *lrp* mutant displays reduced haemagglutination activity

Previous studies have revealed that Lrp is a regulator of discrete fimbrial genes from a number of bacterial species. Prior to this study, Lrp has not been identified as a regulator of *fim* type 1 fimbriae of *Salmonella enterica*. The DNA microarray experiments detailed in Chapter 4 identified genes of the *fim* gene cluster as being downregulated in the *lrp* mutant, suggesting that Lrp acts as a positive regulator of *fim* gene expression. Type 1 fimbriae are responsible for mannose-sensitive haemagglutination of erythrocytes, by binding to mannosylated glycoproteins expressed on the erythrocyte cell surface. Type 1 fimbrial expression of the *lrp* mutant was therefore examined using mannose-sensitive haemagglutination assays (MSHAs) with guinea pig erythrocytes. In this phenotypic test, the *lrp* mutant displayed significantly decreased levels of haemagglutination, by comparison to the wild type (Fig. 5.2 (A)). Importantly, wild-type levels of haemagglutination were demonstrated when the *lrp* mutant was complemented *in trans* using pKMC102, which contains a functional *lrp* gene. In addition, erythrocyte agglutination by the wild type and complemented mutant was inhibited by the presence of 3 % mannose (Fig. 5.2 (B)).

5.2.2 Expression of *fim* genes in an *lrp* mutant

To analyse the expression of *fim* genes in the *lrp* mutant, RT-PCR analyses were undertaken. This served to confirm both the results of the LB microarray, and to examine the transcript levels of other *fim* genes not identified by microarray analysis. RNA was isolated under the same conditions as the LB microarray. The strains used were SL1344, SL1344 *lrp* and the complemented mutant. The *fim* genes chosen for analysis under LB microarray conditions were *fimA* (the major fimbrial subunit), *fimD* (outer membrane usher protein), *fimH* (the fimbrial adhesin), *fimF* (an uncharacterised putative fimbrial protein), *fimW* (a negative regulator), *fimY* (a positive regulator), and *fimZ* (a positive regulator).

The results of RT-PCR analysis showed that the transcript levels of *fimA* and *fimZ* were dramatically reduced in the *lrp* mutant at lag phase, by 5 and 10 fold respectively (Fig. 5.3 (A)). The *fimH, fimF and fimY* transcripts appeared downregulated by 2, 1.4 and 2 fold, respectively, in the *lrp* mutant under these conditions. Importantly *fimW*, a negative

regulator of fimbrial expression, did not show a significant difference in mutant transcript level compared to the wild type and complemented mutant.

Although the levels of *fimA* and *fimZ* were detectible, the conditions tested above were not conducive to fimbriation. Therefore, the transcript levels of the major fimbrial subunit and three essential regulators, *fimA*, *fimW*, *fimY*, and *fimZ* respectively, were analysed by RT-PCR using RNA isolated from cultures that were induced for expression of *fim*-encoded fimbriae. This analysis again showed that *fimA* and *fimZ* had decreased transcript levels in the *lrp* mutant (>3 fold each), whereas the complemented mutant showed little difference when compared to the wild type (Fig. 5.3 (B)). In addition, no significant effect was seen on the *fimW* or *fimY* transcript levels. Taken together, these data suggest that Lrp has a positive role in the regulation of *fimA* and *fimZ*.

5.2.3 The effect of leucine on *fim* gene expression

The regulation of some genes by Lrp is modulated by the effect of L-leucine. In *Escherichia coli*, inversion of the *fimS* DNA switch requires Lrp, and the presence of leucine increases the frequency of inversion through its effect on Lrp (Kelly *et al.*, 2006). DNA microarray analysis of the Lrp regulon in MOPS minimal medium demonstrated that the *fim* genes of *S.* Typhimurium SL1344 appeared to be up-regulated by 2 to 4 fold in the presence of 10 mM L-leucine, at mid-logarithmic phase (Fig. 5.4 (A)). To confirm this effect, SL1344, SL1344 *lrp*, and the complemented *lrp* mutant were grown in MOPS minimal medium to mid-logarithmic phase, in the presence and absence of 10 mM L-leucine. RT-PCR analyses of *fimA* and *fimZ* were performed on RNA isolated from these cultures.

These analyses showed that the wild type mRNA transcript levels of *fimA* and *fimZ* were increased 1.4 fold and 1.2 fold, respectively, during growth in the presence of 10 mM L-leucine (Fig. 5.4 (B)). The transcript levels of all genes were similar in the complemented *lrp* mutant. As expected, the mRNA levels of *fimA* and *fimZ* were significantly reduced in the *lrp* mutant and were not elevated in the presence of leucine. Another gene identified as leucine and Lrp insensitive, STM3038, was unaffected in all genetic backgrounds.

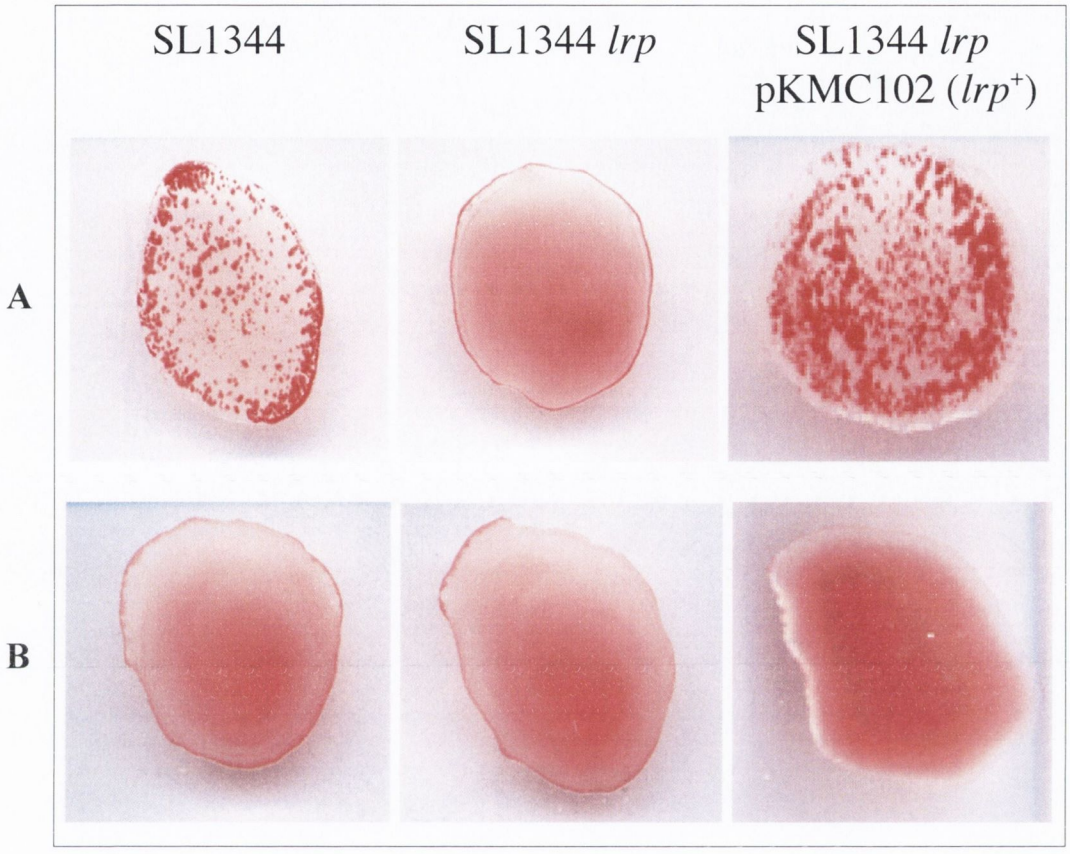


Fig. 5.2. Phenotypic analysis of the *lrp* mutant. SL1344, SL1344 *lrp* and the complemented *lrp* mutant were incubated with 3 % (v/v) RBCs on glass slides, in either the absence (row A) or the presence (row B) of 3 % (w/v) D-mannose. This was performed at least three times with similar results.

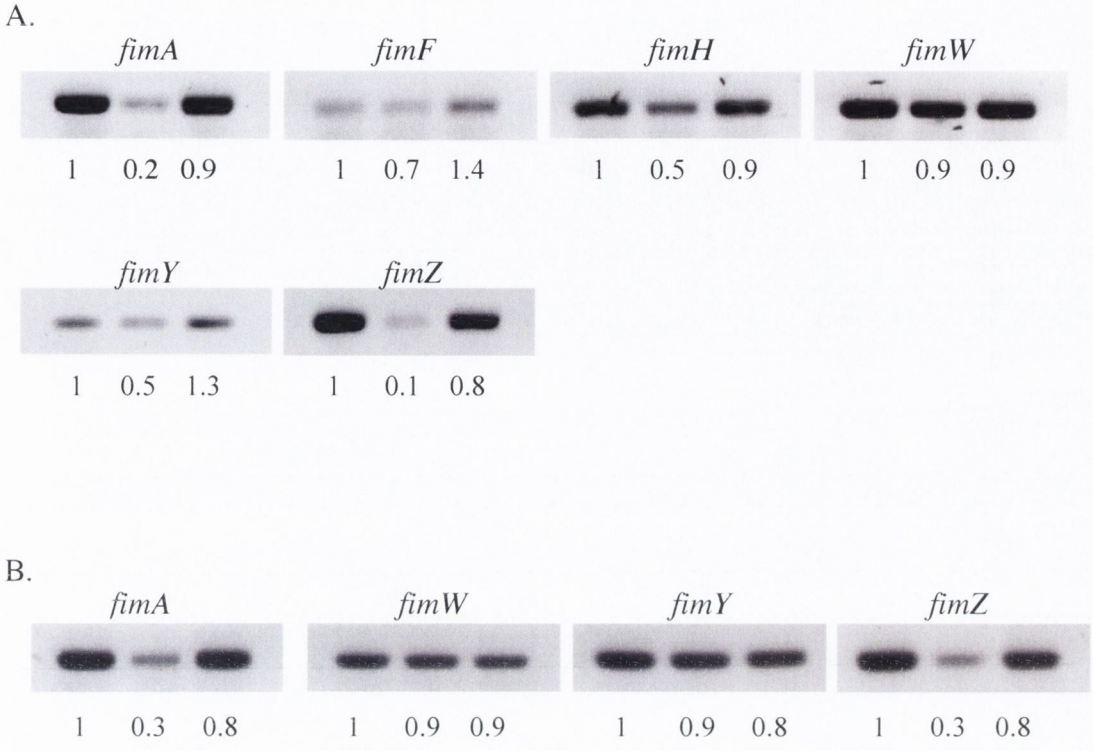


Fig. 5.3: RT-PCR analysis of *fim* gene expression. A: Analysis of mRNA taken from cultures grown to lag phase in LB medium. B: Analysis of mRNA taken from cultures induced for fimbriation. Lanes show data from SL1344, SL1344 *lrp* and the complemented *lrp* mutant, respectively. Numbers indicate values assigned by densitometry, to one decimal place, with data normalised to wild type values. Measurements were made on two separate occasions, and typical data are shown.

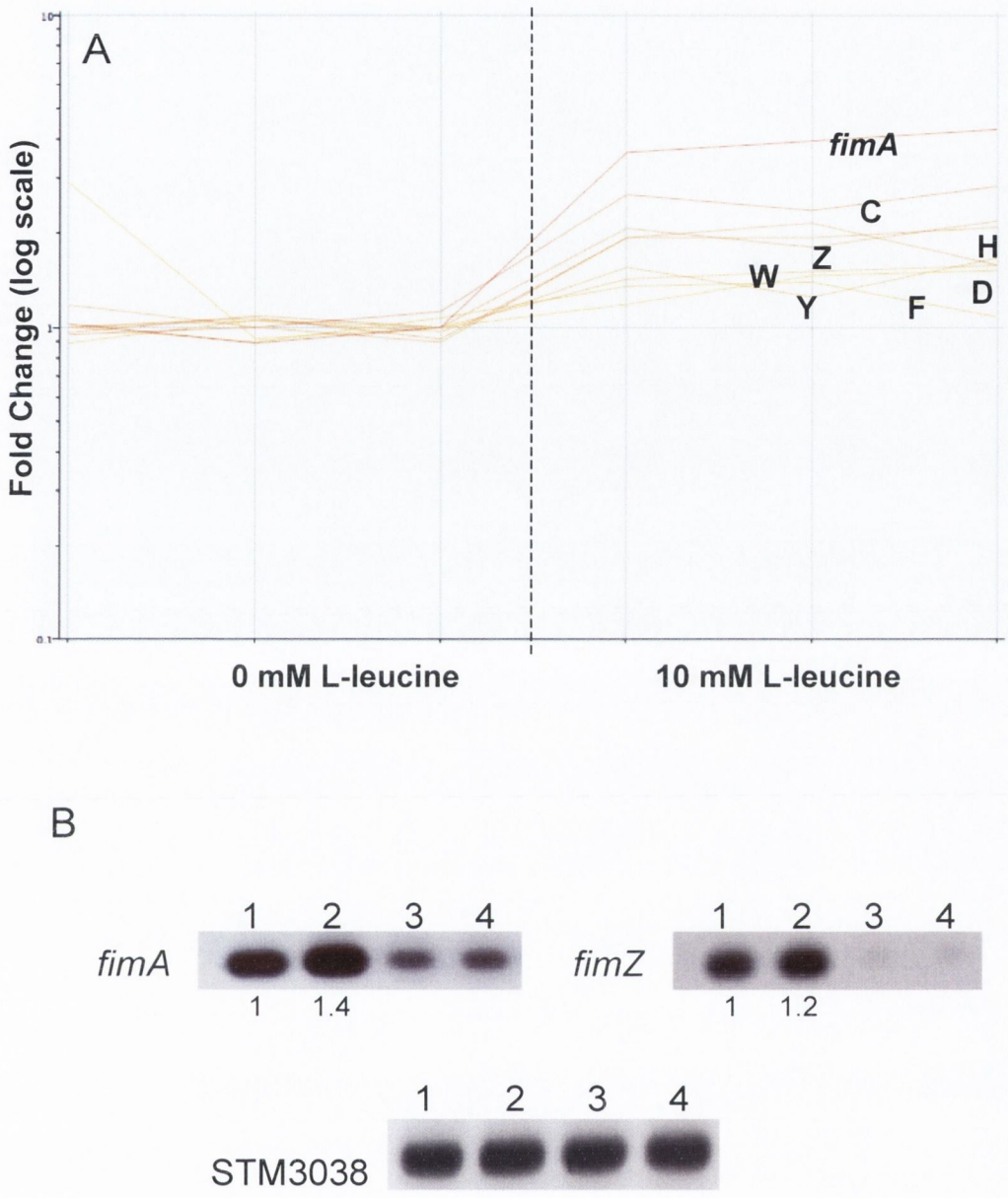


Fig. 5.4. The effect of leucine on *fim* gene expression. SL1344 was cultured in MOPS minimal medium in the absence and presence of 10 mM L-leucine. Microarray data obtained show the increase of mRNA levels of the *fim* genes in the presence of L-leucine, compared to its absence (A). RT-PCR analysis was used to confirm the increased level of mRNA in the presence of L-leucine (B). Lanes 1 and 3 show data from SL1344 and SL1344 *lrp*, respectively, grown in the absence of leucine, and lanes 2 and 4 show SL1344 and SL1344 *lrp*, respectively, cultured in the presence of 10 mM L-leucine.

5.2.4 Bioinformatic analysis of the *fim* operon

The transcription of *fimA*, the major fimbrial subunit, and *fimZ*, an essential positive regulator, were shown to be downregulated strongly in SL1344 *lrp*. In addition, agglutination of erythrocytes was undetectable in the *lrp* mutant by haemagglutination assay. The *fim* gene cluster was examined for the presence of sites resembling the Lrp binding site consensus sequence YAGHAWATTWTDCTR, using a web-based pattern searcher (colibase.bham.ac.uk). Bioinformatic analysis revealed nine putative binding sites, with a mismatch of three or less to the consensus sequence for Lrp binding sites, in the *fim* gene cluster (Fig. 5.5). Two of these sites were present in the *fimA* regulatory region centred at –363 and +114, one in the *fimZ* regulatory region centred at –80, and one in the *fimW-U* intergenic region, fourteen bases upstream from the *fimW* translation initiation codon.

5.2.5 Lrp does not interact with the *fimA*, or the *fimW-U* regulatory regions

Bioinformatic analysis revealed a number of putative Lrp binding sites in *fim* gene promoter regions. In order to determine whether the effect of Lrp on the transcription of *fim* genes was direct or indirect, electrophoretic mobility shift assays (EMSAs) were carried out. DNA fragments corresponding to the *folD-fimA* and *fimW-U* intergenic regions were PCR amplified using biotinylated primers (*fimA*-F and *fimA*-R.Bio, and *fimW*-F.Bio and *fimW*-R, generating fragments of 699 bp and 294 bp, respectively). The labelled promoter regions were incubated with increasing concentrations of Lrp protein.

The *fimA* region initially tested did not show any interaction with Lrp in either the presence or absence of leucine (Fig. 5.6). As a result of its large size, the *folD-fimA* intergenic region was divided into three overlapping probes, of 293 bp (amplified using primer pair *fimA*-F and *fimA*1/3-R), 236 bp (*fimA*2/3-F and *fimA*2/3-R), and 272 bp (*fimA*3/3-F and *fimA*-R.Bio). None of these *fimA* promoter region probes displayed any interaction with Lrp in either the presence or absence of leucine. In addition, the *fimW-U* intergenic region did not demonstrate an interaction with Lrp in the presence or absence of leucine (Fig. 5.6). Despite the presence of putative binding sites for Lrp in these probes, neither gene regulatory region appears to interact with Lrp. An *E. coli*-derived *fimS* probe, however, did show an interaction under the same conditions (Fig. 5.6)

5.2.6 The interaction of Lrp with the *fimZ* regulatory region

The mRNA transcript of *fimZ* showed strongly decreased levels in the *lrp* mutant. In addition, FimZ is a known positive regulator of *fimA*. As a result, the *fimZ* regulatory region was also examined by EMSA analysis. Probe I, of 372 bp, was generated by PCR amplification using primer pair *fimZ*-F.Bio and *fimZ*-R. This probe contains the transcriptional start site of *fimZ* (Yeh *et al.*, 2002), and 130 bp upstream. This probe also contains a putative site for Lrp binding, with three mismatches to the binding site consensus sequence, centred on –80. Despite this, the probe showed no interaction with increasing concentrations of Lrp in either the presence or absence of leucine (Fig. 5.7 (A)). To ensure that the entire regulatory region of *fimZ* was examined for a potential interaction with Lrp a second probe to the *fimZ* regulatory region, probe II, was designed, encompassing the same region as probe I but extended a further 166 bp upstream. This probe of 538 bp was PCR amplified using primer pair *fimZ*-F.Bio and *fimZ*-R.2. When probe II was investigated by EMSA analysis with increasing concentrations of Lrp (Fig. 5.7 (B)), one large complex was observed, suggesting that Lrp binds to at least one site in this region.

To demonstrate more clearly the region important for Lrp binding, a series of nested probes of the Lrp-binding region of *fimZ* was PCR amplified (Fig. 5.8 (A)). The probes, Z103 (amplified using primer pair *fimZ*-F.3 and *fimZ*-R.2), Z202 (primer pair *fimZ*-F.Bio-2 and *fimZ*-R.2) and Z299 (primer pair *fimZ*-F.5 and *fimZ*-R.2) increase in length by increments of approximately 100 bp.

EMSA analysis with increasing concentrations of Lrp demonstrated that Z103, located in the upstream region of the *fimZ* promoter region, did not show an interaction at 53.2, 133, or 266 nM Lrp (Fig. 5.8 (B)), either in the presence (lanes 3-1) or the absence (lanes 4-6) of 15 mM L-leucine. Probes Z202 and Z299 formed three, and two complexes, respectively (Fig. 5.8 (C, D)). Three complexes were observed in Z202, suggesting that Lrp bound to three sites within this region (Fig. 5.8 (C)). In the absence of leucine (lanes 1-5), complexes 1 and 2 predominated from 26.6 nM to 53.2 nM Lrp (lanes 2-3), with increasing formation of complex 3 at 26.6 nM to 266 nM (lanes 2-5). The stepwise complex formation suggested that cooperative Lrp binding may be important for the formation of complexes II and III. The addition of leucine had the effect of enhancing production of complexes 2 and 3, although complex 1 was still apparent at all concentrations of Lrp. This suggested that leucine affected the nature of

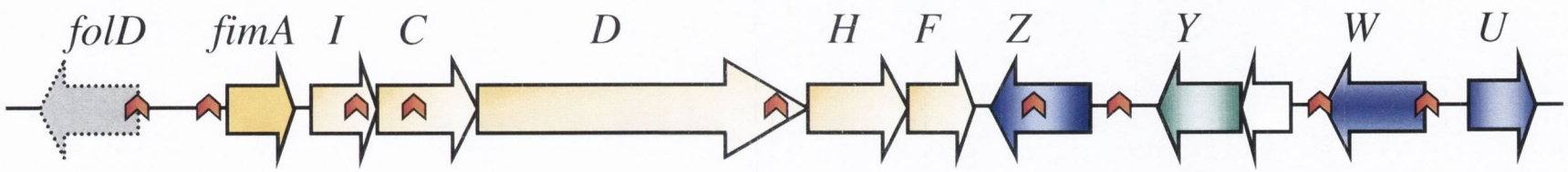
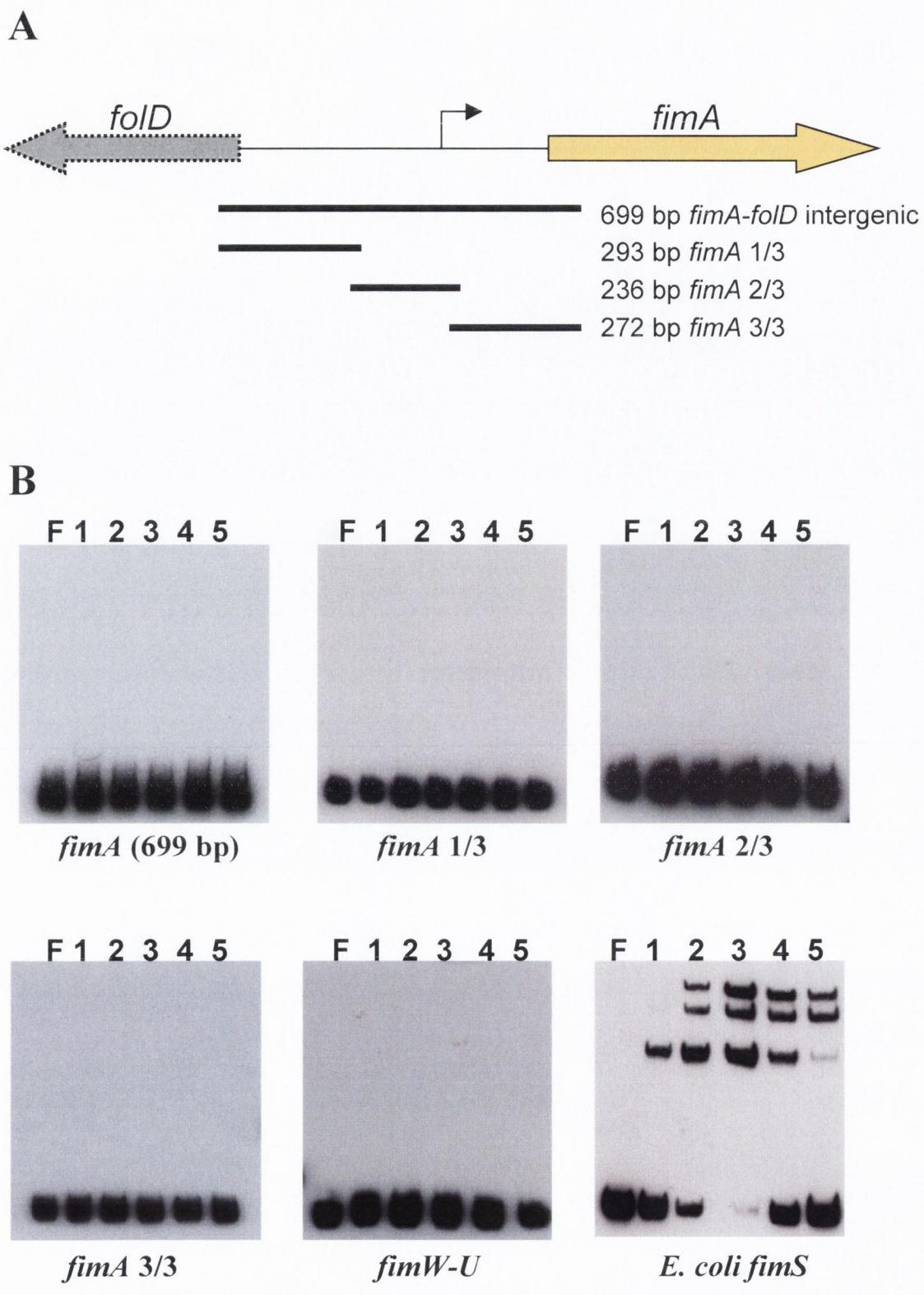


Fig. 5.5. Bioinformatic analysis of the *fim* operon. The approximate positions of putative Lrp binding sites on the *fim* operon, predicted by bioinformatics analysis using the Lrp consensus sequence, YAGHAWATTWTDCTR, are represented by orange chevrons.

Fig. 5.6. EMSA analysis of the interaction with *fimA* and *fimW-U* promoter regions. The sizes and relationships of the *fimA* probes to *fimA* and *folD* are shown (A). The *fimA* transcriptional start site is located 195 bp upstream of the *fimA* ORF (Yeh *et al.*, 2002). Increasing concentrations of Lrp were incubated with biotinylated DNA probes in the absence and presence of 10 mM L-leucine (B). The *fimS* fragment of *E. coli*, which interacts with Lrp, was used as a positive control. Lanes 1- 3 contain, 53, 133 and 266 nM Lrp, and lanes 4-5 contain 266 nM Lrp, with 5 and 20 mM L-leucine, respectively. F denotes free probe.


A
folD
fimA
699 bp fimA-folD intergenic
293 bp fimA 1/3
236 bp fimA 2/3
272 bp fimA 3/3
B
F 1 2 3 4 5
fimA (699 bp)
fimA 1/3
fimA 2/3
fimA 3/3
fimW-U
E. coli fimS

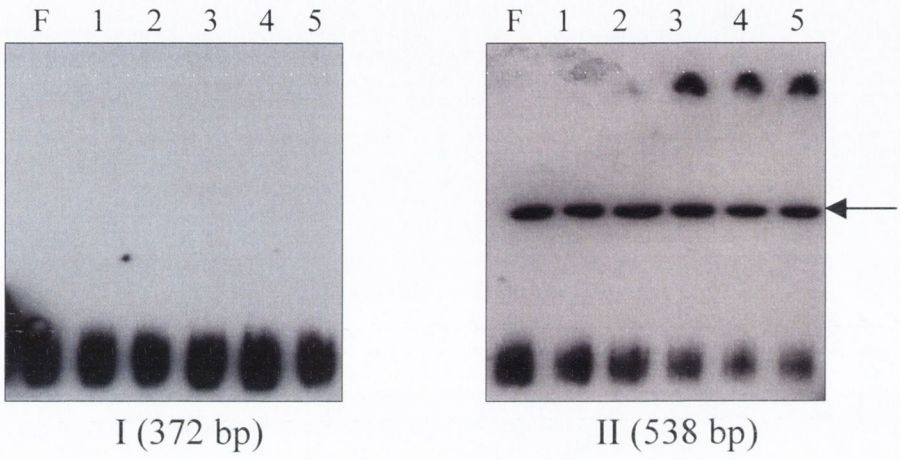
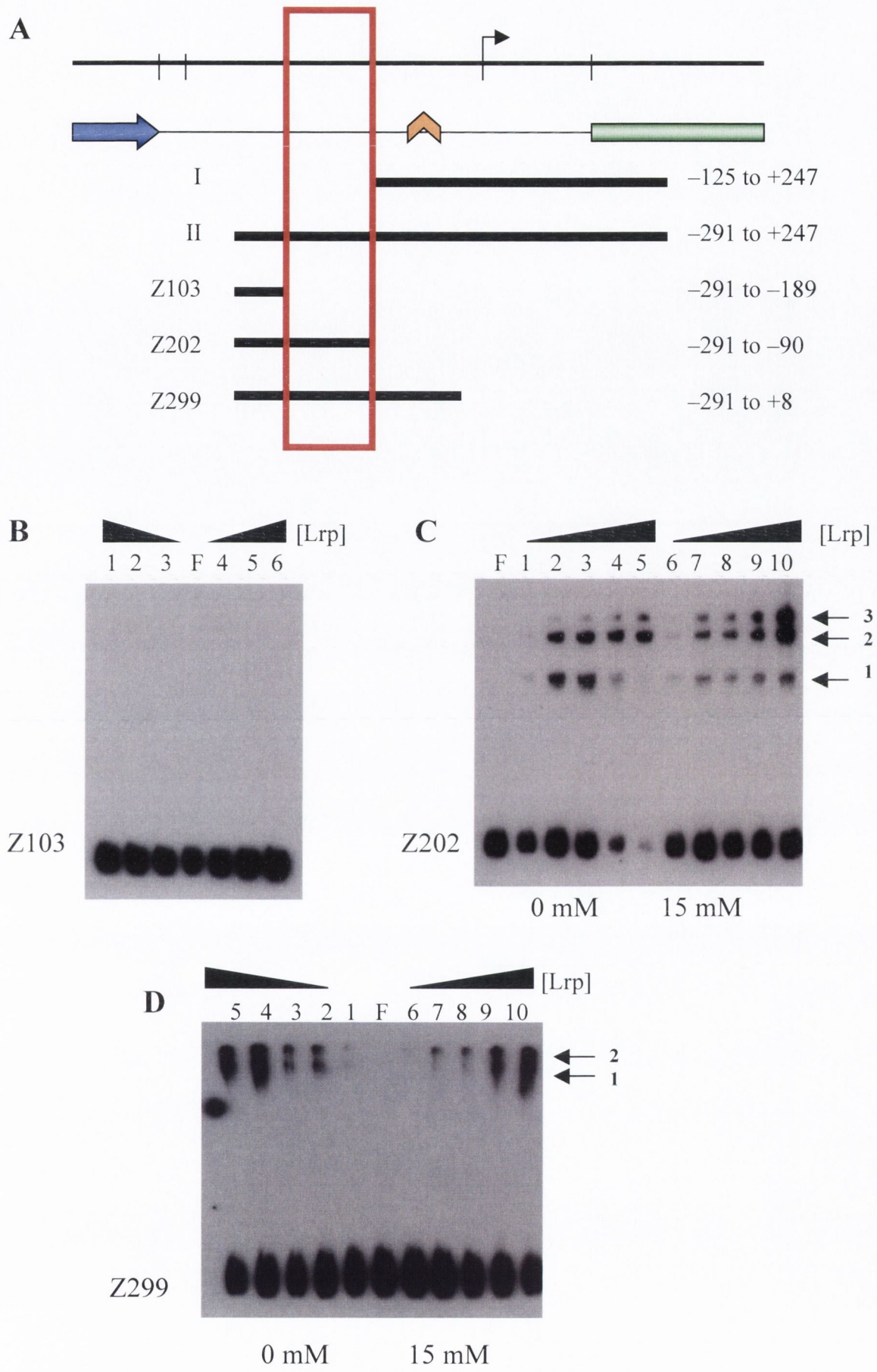


Fig. 5.7. EMSA analysis of the *fimZ* regulatory region with Lrp. Probe I, of 372 bp, showed no interaction with Lrp in the presence or absence of L-leucine. Lanes 1- 3 contain 53, 133 and 266 nM Lrp, and lanes 4-5 contain 266 nM Lrp, with 5 and 20 mM L-leucine, respectively. F denotes free probe. When probe I was extended by 166 bp to form probe II (538 bp), an interaction of Lrp with the *fimZ* promoter region in the presence and the absence of L-leucine was demonstrated. A non-specific DNA probe, arrowed, was unaffected.

Fig. 5.8. EMSA analyses of the *fimZ* promoter region. Electrophoretic mobility shift assays were performed to analyse the ability of Lrp to interact with a series of probes increasing in length by increments of approximately 100 bp. The chevron denotes the position of the putative Lrp binding site identified by bioinformatics analysis. The positions of the probes are shown, in relation to the *fimZ* ORF and promoter, and to probes I and II (A). The smallest probe of 103 bp, Z103, did not show any interaction with Lrp (B). Probes Z202 and Z299 showed the formation of 3 and 2 complexes respectively, when incubated with increasing concentrations of Lrp (lanes 1-5; 13.3, 26.6, 53.2, 133, 266 nM). Leucine appeared to change the profile of complex formation in Z202 (lanes 6-10), favouring the production of complexes 2 and 3, whereas in the absence of leucine, complex 2 appears to be favoured (C). Only two complexes appeared to be formed with probe Z299 (D). The EMSA analyses performed on the *fimZ* promoter identified a region important for Lrp-binding, between –190 and –90, highlighted in red (A).


A
I
II
Z103
Z202
Z299
–125 to +247
–291 to +247
–291 to –189
–291 to –90
–291 to +8
B
[Lrp]
1 2 3 F 4 5 6
Z103
C
[Lrp]
F 1 2 3 4 5 6 7 8 9 10
3
2
1
Z202
0 mM
15 mM
D
[Lrp]
5 4 3 2 1 F 6 7 8 9 10
2
1
Z299
0 mM
15 mM

the interaction of Lrp with *fimZ*, by appearing to enhance the ability of Lrp to form larger complexes with the regulatory region. Interestingly probe Z299, which encompasses probe Z202, shows the production of only two complexes (Fig. 5.8 (D)).

By alignment of the nucleotide sequences of probes either positive or negative for complex formation by Lrp, it was possible to identify a region important for the Lrp interaction, highlighted in Fig. 5.8 (A). This region was shown to be between approximately –190 and –90, with respect to the transcription initiation site of *fimZ* (+1).

5.2.7 Absence of 5'-GATC-3' sequences in the *fimZ* and *fimA* promoters

Lrp is known to regulate a number of P-regulatory fimbrial operons in conjunction with Dam methylase (Nicholson and Low, 2000; van der Woude *et al.*, 1992). As a result, the promoter region of *fimZ* was examined for the Dam methylase target site, GATC. No instances of the GATC sequence occur in the Lrp-binding *fimZ* regulatory region, suggesting that Dam methylase does not have a role in the regulation of *fimZ*. One GATC site was found in the *fimA* promoter, at –397, with respect to the transcription start site, which is located 195 bp upstream of the *fimA* ORF (Yeh *et al.*, 2002). P-regulatory fimbriae have two GATC sites separated by 102-103 bp (Harel and Martin, 1999). However, no second GATC site was found at the *fimA* promoter. This strongly suggested that *fim* fimbriae do not belong to the P-regulatory family of fimbriae.

5.2.8 Mapping the Lrp binding sites at the *fimZ* promoter

EMSA analysis enabled the identification of a region important for binding of the *fimZ* regulatory region by Lrp. It was also suggested that the interaction of Lrp occurs at three sites in the promoter region, and that leucine appeared to affect the binding preference of Lrp with these three sites. To further characterise this interaction, and to gain more information about the effect of leucine on this interaction, DNase I footprinting was performed. This technique is based on the ability of DNA-binding proteins to protect DNA from DNase I degradation. The DNA region of interest is examined by incubation with increasing protein concentrations. The phosphodiester backbone of DNA is subjected to digestion by DNase I, and is cleaved in regions where no protein is bound (Galas and Schmitz, 1978).

The region of *fimZ* that was shown to be important for Lrp binding, plus flanking DNA, was PCR amplified from SL1344 using primer pair *fimZ*-F.*Bam*HI and *fimZ*-R.*Eco*RI. This led to the amplification of a 317 bp fragment, flanked with *Bam*HI and *Eco*RI restriction sites. The fragment was digested and gel-purified, and ligated to *Bam*HI/ *Eco*RI-digested pBluescript. This vector, pBSK*fimZ*, was verified by commercial sequencing. pBSK*fimZ* was used as template for the amplification of the probes used in DNase I footprinting, *fimZ*-Fwd and *fimZ*-Rev. *fimZ*-Fwd, the non-coding top strand probe, was generated by PCR amplification using primer pair *fimZ*-F.*Bam*HI and pBSK-F. Primer pBSK-F is complementary to sequence in pBluescript, leading to generation of a 464-bp probe. T4 polynucleotide kinase was used to 5' end-label this probe with ^{32}P, resulting in labelling at both ends. To remove one labelled end, the probe was digested with *Eco*RI, leading to a 317-bp fragment, and a 147-bp fragment. The 317-bp fragment, which contained the region of interest, was 6% polyacrylamide gel-purified, extracted as described, and used to perform DNase I footprinting (section 2.17.4). pBSK*fimZ* was used as template for the sequencing reaction that was run alongside the DNase I footprinting products. The primer *fimZ*-Fwd.*Bam*HI was used to sequence the non-coding top strand (see section 2.17.1). *fimZ*-Rev, the coding strand probe, was generated in a similar fashion, using primer pair *fimZ*-R.*Eco*RI and pBSK-R. Primer pBSK-R is located in the pBluescript vector, leading to a 450-bp probe. T4 polynucleoide kinase was used to 5' end-label this probe with ^{32}P. To remove one labelled end, the probe was digested with *Bam*HI, leading to a 317-bp fragment, and a 133-bp fragment. The probe of interest, 317 bp, was 6% polyacrylamide gel-purified, extracted as described, and used to perform DNase I footprinting. For DNA sequencing of the coding strand, the primer *fimZ*-R.*Eco*RI was used with pBSK*fimZ*.

DNase I footprinting of the coding strand of the *fimZ* regulatory region revealed that Lrp was capable of protecting a 90-bp region on the coding strand, between –95 and –187 with respect to the transcriptional start site (Fig. 5.9). In addition, phased hypersensitive regions, which are indicative of bending of DNA and characteristic of Lrp, were shown in the protected region at –145 and –144, at –112 and –113, and at –84. This suggested that Lrp bends and wraps the DNA, forming a nucleoprotein complex. No apparent changes were observed by addition of leucine in the coding strand. DNase I footprinting of the top, non-coding strand of the *fimZ* regulatory region revealed a region of protection by Lrp, from –110 to –84, with respect to the transcriptional start site (Fig.

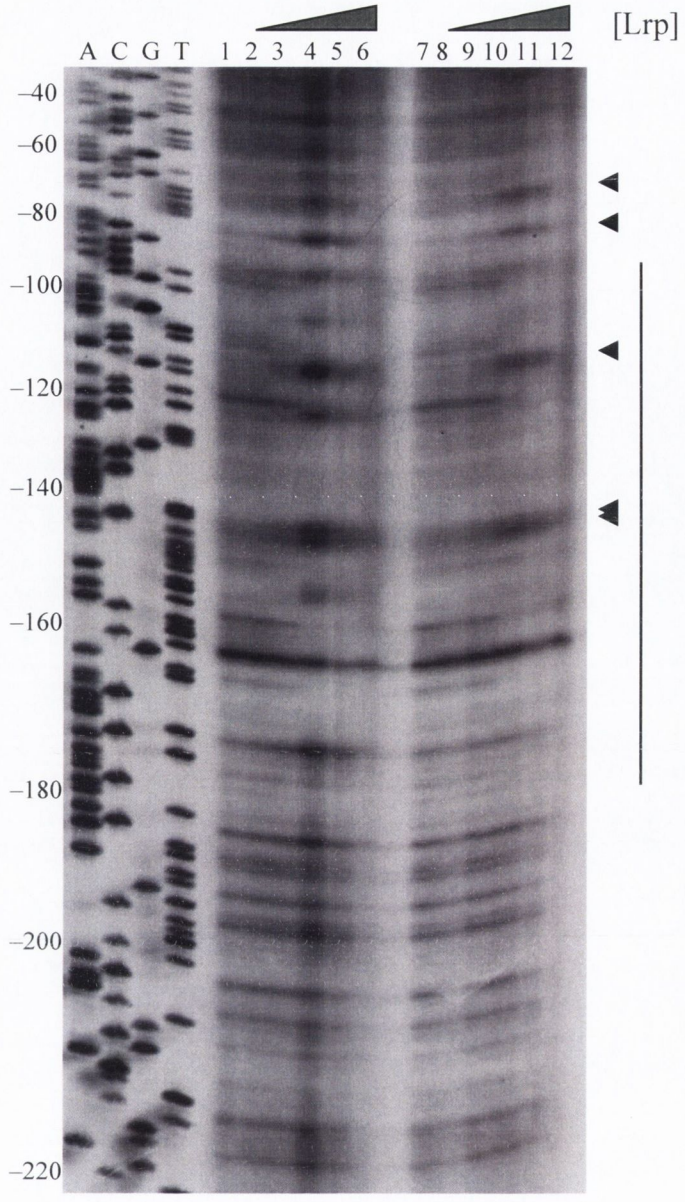


Fig. 5.9. DNase I footprint of Lrp on the coding strand of the *fimZ* promoter. Increasing concentrations of Lrp (0, 27, 53, 133, 266, 532 nM) were incubated with ^{32}P-labelled non-coding strand in the absence (lanes 1-6) or the presence of 15 mM L-leucine (lanes 7-12). The corresponding DNA sequence is alongside. Vertical lines signify regions of protection by Lrp, whereas arrowheads denote areas of hypersensitivity.

5.10). Hypersensitive bases were also visible in this strand, at –145 and –144, and –113. The presence of leucine had little effect in this assay, but affected protection and hypersensitivity of particular bases. In the absence of leucine, nucleotides –155 and –156 in the non-coding strand showed hypersensitivity and protection with increasing Lrp concentrations, respectively. In addition, –72 showed hypersensitivity in the presence, but not the absence of leucine. Interestingly, hypersensitive bases correlated well between both strands. It seemed likely that Lrp bound between hypersensitive bases, bending the DNA between binding sites as has been described in previous Lrp studies (Wiese *et al.*, 1997). This, along with EMSA analysis, would suggest three binding sites for Lrp between the hypersensitive bases in the protected region. These regions were examined for sequences with similarity to the Lrp binding site consensus sequence YAGHAWATTWTDCTR. Sites 1 and 2, located at –152 and –129, show a mismatch of three and four bases, respectively, compared to the consensus sequence (Fig. 5.11). The third site, centred at –100 and with a mismatch of six bases, is located in the region covered by probe I, which did not show any interaction with Lrp.

5.2.9 Construction of pBAD*fimZ*

In this study, it was shown that in the absence of Lrp, there were undetectable levels of haemagglutination activity by MSHA, and Lrp was shown to regulate positively the expression of *fimZ* and *fimA*. This study also showed that Lrp did not interact with the *fimA* or *fimW-U* regulatory regions, but did interact with the *fimZ* regulatory region. To examine whether the sole effect of Lrp on the expression of fimbriae is through its positive effect on *fimZ*, an arabinose-inducible *fimZ* expression plasmid, pBAD*fimZ*, was constructed.

The plasmid pBAD24, which encodes the ampicillin resistance cassette *bla*, contains the arabinose-inducible promoter of the *araBAD* operon, P_{BAD}. pBAD24 also contains the *araC* gene, the product of which acts as both a positive and negative regulator of transcription from P_{BAD}. In the presence of arabinose, AraC strongly induces transcription from P_{BAD}, whereas in the presence of glucose, transcription is tightly repressed. The *fimZ* open reading frame was put under the control of P_{BAD} in the pBAD24 plasmid, allowing regulation of its expression.

pBAD*fimZ* was constructed by PCR amplification of the *fimZ* open reading frame and a short downstream region, with primer pair *fimZ*-BAD-F and *fimZ*-BAD-R.*Hin*dIII, the latter containing a restriction site for *Hin*dIII. The use of the proof-reading *Pfu* polymerase leads to blunt-ended PCR products. The resulting product, 712 bp in length, was digested with *Hin*dIII and gel-purified. pBAD24 was, in order, digested with *Nco*I, treated with Klenow polymerase to create a blunt end, digested with *Hin*dIII and gel-purified. The *fimZ* ORF fragment was cloned directionally into this plasmid, creating pBAD*fimZ* (Fig. 5.12 (A)), which was subsequently verified by commercial sequencing.

5.2.10 Confirmation of the functionality of the pBAD*fimZ* construct

Overexpression of FimZ has been shown to inhibit motility by downregulation of the *flhDC* master regulatory genes, which are essential activators of flagellar expression (Clegg and Hughes, 2002). To confirm the functionality of the arabinose-inducible expression vector pBAD*fimZ*, the plasmid was transformed into wild type SL1344. pBAD*fimZ*-containing SL1344 were grown by overnight culture, and inoculated on swarm plates supplemented with carbenicillin, and either 0.2 % arabinose or 0.2 % glucose. As a control, SL1344 containing pBAD24 was also included.

In the presence of 0.2 % arabinose or 0.2% glucose the control plasmid, pBAD24, had no effect on motility of SL1344. pBAD*fimZ*-containing cells, however, clearly showed inhibition of motility in the presence of 0.2 % arabinose, whereas in 0.2 % glucose motility was unaffected (Fig. 5.12 (B)).

5.2.11 Overexpression of FimZ in SL1344 *lrp* restores fimbriation

The pBAD*fimZ* plasmid was constructed and confirmed for its functionality. To assess whether the effect of Lrp on expression of type 1 fimbriae is solely through its positive effect on *fimZ* expression, the pBAD*fimZ* plasmid was used to express *fimZ* in the *lrp* mutant of SL1344. Wild type SL1344 and SL1344 *lrp* were transformed with pBAD*fimZ* and grown for 48 h in LB broth supplemented with carbenicillin, and either 0.2 % arabinose, or 0.2 % glucose. After 48 h, bacteria were harvested, culture optical density equalised to OD_{600} 4.0 ml^{-1}, and MSHAs were performed. SL1344 displayed similar levels of agglutination in the presence of both arabinose and glucose, and this agglutination was inhibited by the presence of 3 % mannose (Fig. 5.13). The *lrp* mutant showed no agglutination after growth in 0.2 % glucose, which inhibits the transcription

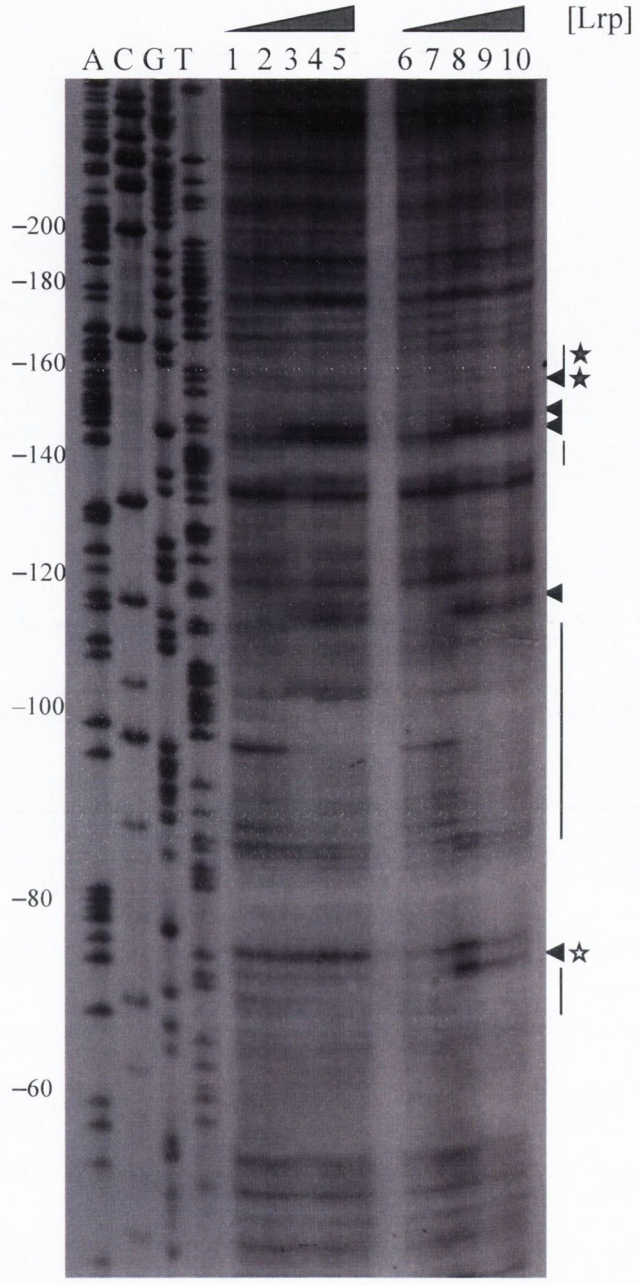


Fig. 5.10. DNase I footprint of Lrp on the non-coding strand of the *fimZ* promoter. Increasing concentrations of Lrp (0, 27, 53, 133, 266 nM) were incubated with ^{32}P-labelled non-coding strand in the absence (lanes 1-5) or the presence of 15 mM L-leucine (lanes 6-10). The corresponding DNA sequence is alongside. Vertical lines signify regions of protection by Lrp, whereas arrowheads denote areas of hypersensitivity. Notable differences in the presence of leucine, are highlighted with an open star; differences in the absence of leucine are denoted by a filled star.

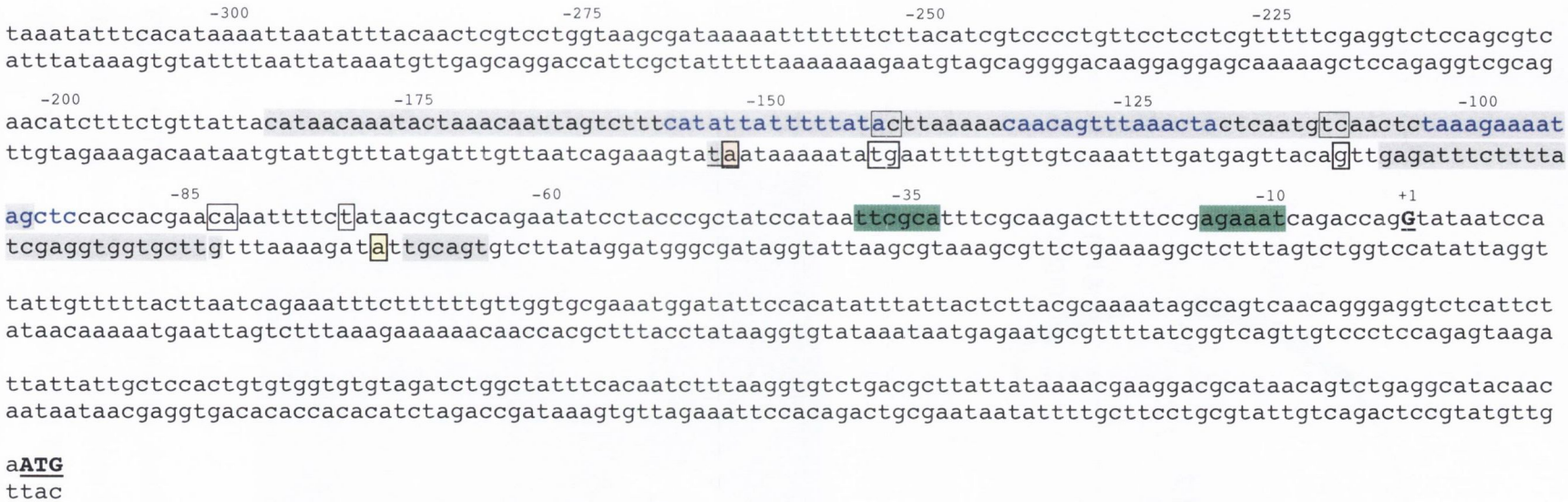


Fig. 5.11. The protection of the *fimZ* promoter region by Lrp. This figure depicts data collated from DNase I footprinting of both strands of the *fimZ* promoter region. The shaded area represents the region protected from DNase I cleavage by Lrp, whereas the boxed nucleotides signify regions of hypersensitivity, suggestive of DNA bending. Putative Lrp binding sites are highlighted in blue text. The putative –35 and –10 sites are highlighted in green. The transcriptional start site of *fimZ* (+1) is located 227 bp upstream from the initiation codon (**ATG**) of *fimZ* (Yeh *et al.*, 2002), and is denoted in bold, underlined script (**G**). Nucleotides highlighted in yellow denote Lrp-mediated differences in the presence of leucine that are absent in the absence of leucine, and orange denotes differences in the absence of leucine, not present when supplemented with leucine.

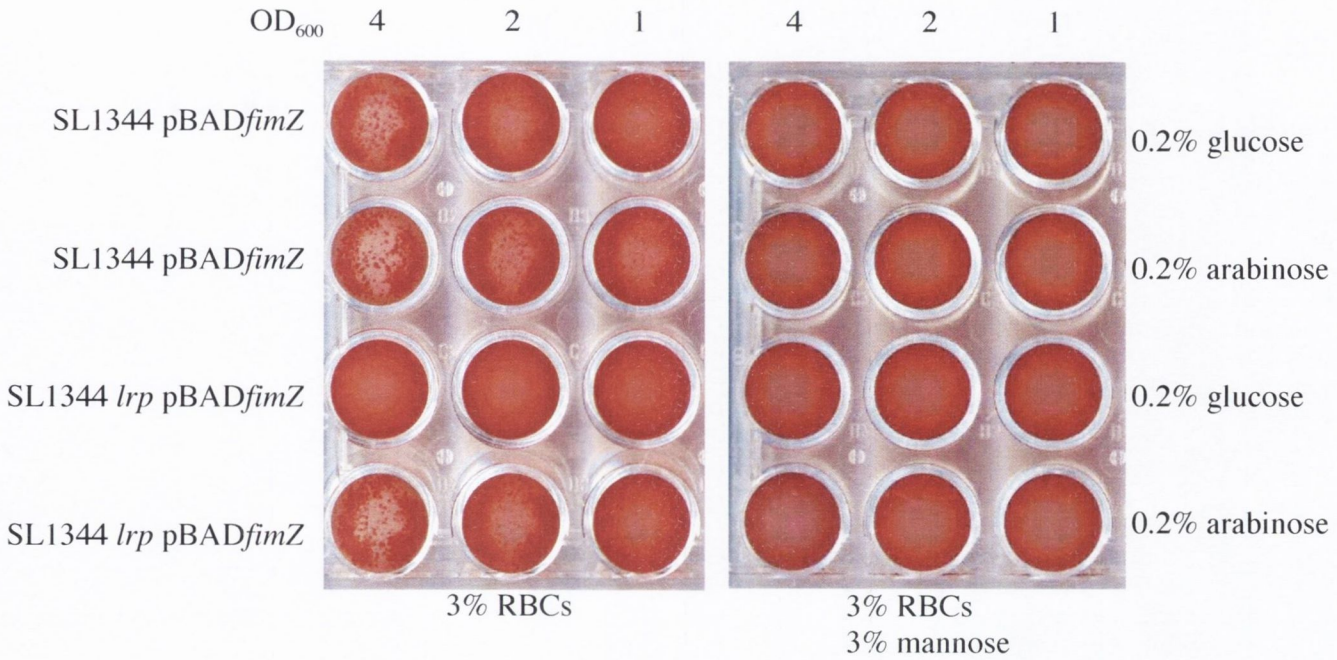


Fig. 5.13. Haemagglutination assays of SL1344 and SL1344 *lrp* containing the arabinose-inducible FimZ expression plasmid pBAD*fimZ*. FimZ production was induced from pBAD*fimZ* during growth in the presence of arabinose, but repressed in the presence of glucose. Culture optical densities were equalised by OD_{600}, and incubated with RBCs in the presence or absence of 3% mannose.

of *fimZ* from pBAD*fimZ*, whereas in the presence of arabinose, wild type levels of agglutination were observed (Fig. 5.13). This agglutination was also inhibited by the presence of 3 % mannose. This strongly suggested that the effect of Lrp on type 1 fimbriation was solely through its positive effect on *fimZ* expression.

5.2.12 Alignment of *fimZ* regulatory regions of *Salmonella* species

The role of Lrp on the expression of type 1 fimbriae in *Salmonella enterica* serovar Typhimurium was most likely to be solely through its positive effect on the expression of *fimZ*. To help determine if Lrp could regulate the expression of *fimZ* in other *Salmonella* species, BLAST analysis was performed using a 200-bp region of *fimZ* regulatory DNA, from –200 to +1 with respect to the transcription start site, including the region shown to interact with Lrp.

These data, depicted in alignment format (Fig. 5.14), showed that this sequence is 99 % conserved in the corresponding regions of five *Salmonella enterica* strains: the completely sequenced *Salmonella enterica* serovar Typhi strains CT18 and Ty2, serovar Paratyphi A strain ATCC 9150, serovar Choleraesuis strain SC-B67, and the preliminary genomic sequence data of *Salmonella enterica* serovar Enteritidis strain PT4 (www.sanger.ac.uk/Projects/Salmonella). The putative Lrp-binding sites identified by this study, and highlighted in blue in Fig. 5.14, were identical in the four strains.

5.2.13 Regulation of FimZ-regulated genes by Lrp

Recently, FimZ has been shown to have roles in regulation of other, non-*fim* genes. FimZ and FimY have together been shown to have a positive role in expression of *hilE*, the product of which is a negative regulator of *hilA*, and thus SPI-1 gene expression. As previously described (Clegg and Hughes, 2002), and demonstrated in this study (section 5.2.10), overexpression of FimZ also has a negative effect on motility of *S.* Typhimurium.

DNA microarray analysis of the Lrp regulon in LB medium undertaken in this study revealed that *hilE*, which is part of a cluster of genes that appear to be Lrp-regulated, showed reduced mRNA transcript levels in the *lrp* mutant in lag phase (Chapter 4). In addition, RT-PCR analysis was used to confirm that *hilE*, and the other members of the gene cluster, did indeed show reduced transcript levels in SL1344 *lrp* (Chapter 4). To

analyse whether inducing conditions for *fimZ* expression, with respect to the wild type, could lead to increased *hilE* transcript levels, and thus more decreased *hilE* levels in the *lrp* mutant, RT-PCR analysis was performed. RNA from SL1344, SL1344 *lrp* and SL1344 *lrp* pKMC102 (*lrp*$^{+}$), which had been induced for fimbriation, were used to perform RT-PCR analysis on the *hilE* gene. This analysis showed that there was no significant difference in the level of *hilE* expression between the wild type, *lrp* mutant and complemented mutant, under the conditions tested (Fig. 5.15).

5.2.14 Identification of regulatory mutants with decreased fimbriation

Type 1 fimbriation of *E. coli* is well studied, many important regulators have been identified, and the mechanisms of DNA switching are well defined. Comparatively little is known about type 1 fimbriation of *S. enterica*. To identify more regulators of type 1 fimbriation of *S. enterica,* a number of regulatory mutants were examined for their ability to agglutinate erythrocytes. Cells were induced for fimbriation, culture optical densities equalised by OD_{600}, and incubated with 3 % RBCs in the presence and absence of 3 % D-mannose.

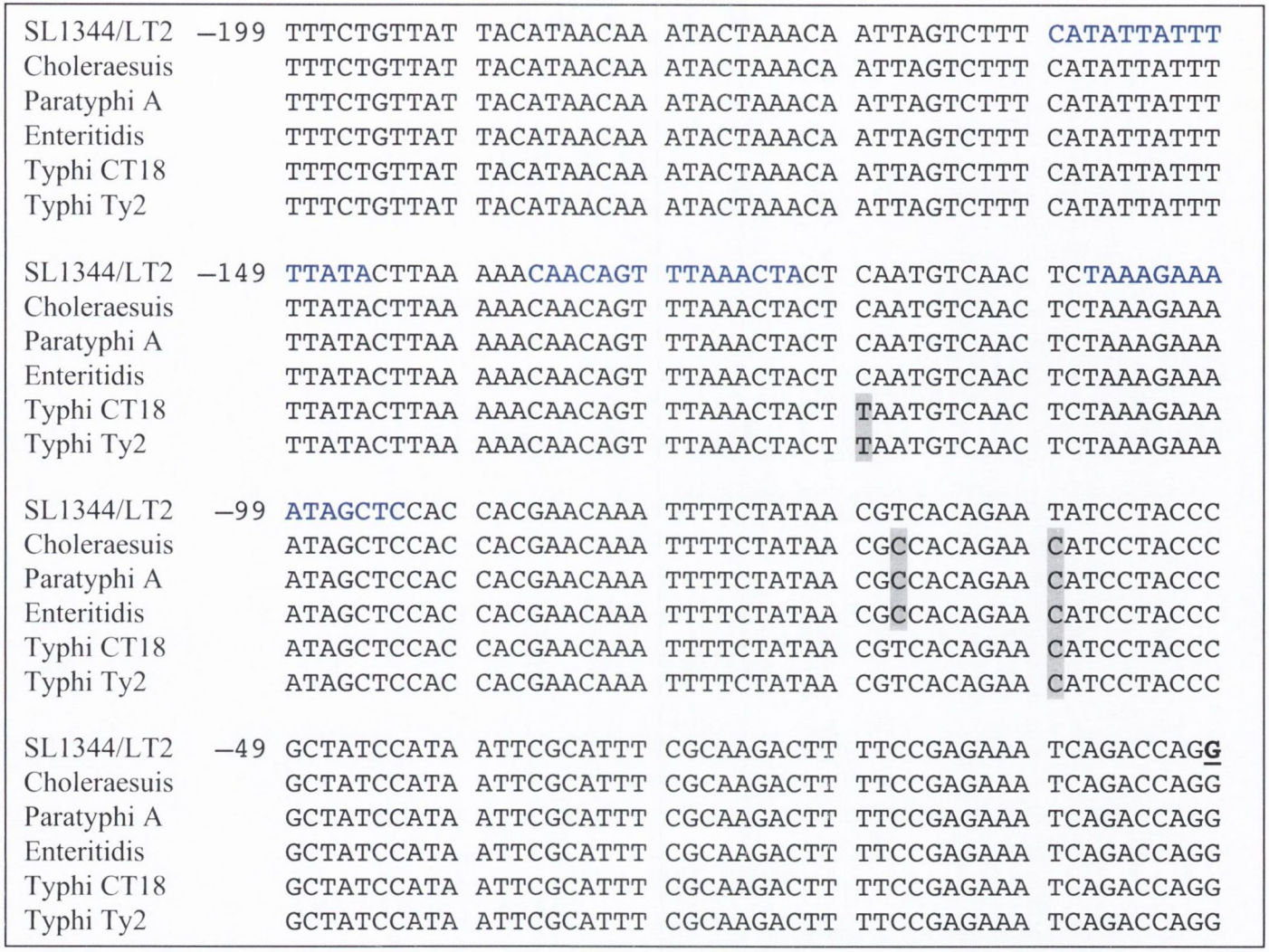
As expected, SL1344 strongly agglutinated RBCs, whereas agglutination by the *lrp* mutant was undetectable. SL1344 *fis* displayed strongly reduced levels of activity, whereas the *hns* and *rpoS* mutants appeared to show slightly increased levels of HA activity (Fig. 5.16). The SL1344 *ompR* mutant did not show any detectable changes in haemagglutination activity, with respect to the wild type (Fig. 5.16). In all cases, haemagglutination was inhibited by the presence of 3 % D-mannose.

5.2.15 H-NS may negatively regulate *fimZ* expression

Navarre *et al.* have shown that H-NS of *S.* Typhimurium preferentially binds and silences regions of low G+C DNA content. In their study, they also show that the *fim* gene cluster possesses A+T-rich DNA with the highest present in the *fimZ* promoter region (Navarre *et al.*, 2006). These data were supported by Lucchini *et al.,* who showed by chromatin immunoprecipitation that DNA in the *fimZ* region is bound by H-NS (Lucchini *et al.*, 2006).

The presence of intrinsic DNA curvature, caused by A+T tracts, is suggestive of possible H-NS binding. The *fimZ* promoter was analysed for low G+C DNA curvature using the

Fig. 5.14. Alignment of the *fimZ* regulatory region of six *S. enterica* strains. Alignment of –199 to +1 of the *fimZ* regulatory region of SL1344/LT2 with the corresponding sequences in sequenced *Salmonella enterica* serovar Typhi strains CT18 and Ty2, serovar Paratyphi A strain ATCC 9150, serovar Choleraesuis strain SC-B67, and the preliminary genomic sequence data of *Salmonella enterica* serovar Enteritidis strain PT4. The putative Lrp binding sites are highlighted in blue, and variations from the SL1344 sequence are shaded.


SL1344/LT2 −199 TTTCTGTTAT TACATAACAA ATACTAAACA ATTAGTCTTT CATATTATTT
Choleraesuis TTTCTGTTAT TACATAACAA ATACTAAACA ATTAGTCTTT CATATTATTT
Paratyphi A TTTCTGTTAT TACATAACAA ATACTAAACA ATTAGTCTTT CATATTATTT
Enteritidis TTTCTGTTAT TACATAACAA ATACTAAACA ATTAGTCTTT CATATTATTT
Typhi CT18 TTTCTGTTAT TACATAACAA ATACTAAACA ATTAGTCTTT CATATTATTT
Typhi Ty2 TTTCTGTTAT TACATAACAA ATACTAAACA ATTAGTCTTT CATATTATTT
SL1344/LT2 −149 TTATACTTAA AAACAACAGT TTAAACTACT CAATGTCAAC TCTAAAGAAA
Choleraesuis TTATACTTAA AAACAACAGT TTAAACTACT CAATGTCAAC TCTAAAGAAA
Paratyphi A TTATACTTAA AAACAACAGT TTAAACTACT CAATGTCAAC TCTAAAGAAA
Enteritidis TTATACTTAA AAACAACAGT TTAAACTACT CAATGTCAAC TCTAAAGAAA
Typhi CT18 TTATACTTAA AAACAACAGT TTAAACTACT TAATGTCAAC TCTAAAGAAA
Typhi Ty2 TTATACTTAA AAACAACAGT TTAAACTACT TAATGTCAAC TCTAAAGAAA
SL1344/LT2 −99 ATAGCTCCAC CACGAACAAA TTTTCTATAA CGTCACAGAA TATCCTACCC
Choleraesuis ATAGCTCCAC CACGAACAAA TTTTCTATAA CGCCACAGAA CATCCTACCC
Paratyphi A ATAGCTCCAC CACGAACAAA TTTTCTATAA CGCCACAGAA CATCCTACCC
Enteritidis ATAGCTCCAC CACGAACAAA TTTTCTATAA CGCCACAGAA CATCCTACCC
Typhi CT18 ATAGCTCCAC CACGAACAAA TTTTCTATAA CGTCACAGAA CATCCTACCC
Typhi Ty2 ATAGCTCCAC CACGAACAAA TTTTCTATAA CGTCACAGAA CATCCTACCC
SL1344/LT2 −49 GCTATCCATA ATTCGCATTT CGCAAGACTT TTCCGAGAAA TCAGACCAGG
Choleraesuis GCTATCCATA ATTCGCATTT CGCAAGACTT TTCCGAGAAA TCAGACCAGG
Paratyphi A GCTATCCATA ATTCGCATTT CGCAAGACTT TTCCGAGAAA TCAGACCAGG
Enteritidis GCTATCCATA ATTCGCATTT CGCAAGACTT TTCCGAGAAA TCAGACCAGG
Typhi CT18 GCTATCCATA ATTCGCATTT CGCAAGACTT TTCCGAGAAA TCAGACCAGG
Typhi Ty2 GCTATCCATA ATTCGCATTT CGCAAGACTT TTCCGAGAAA TCAGACCAGG

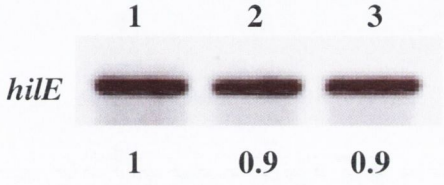


Fig. 5.15. The effect of Lrp on *hilE* expression, under conditions inducing for fimbriation. RT-PCR analysis was used to examine relative mRNA levels of *hilE* under conditions inducing for fimbriation. Lane 1 denotes the wild type SL1344; lane 2, SL1344 *lrp;* lane 3, the complemented *lrp* mutant. Densitometry was used to quantify band intensity relative to wild type levels, shown below the corresponding band.

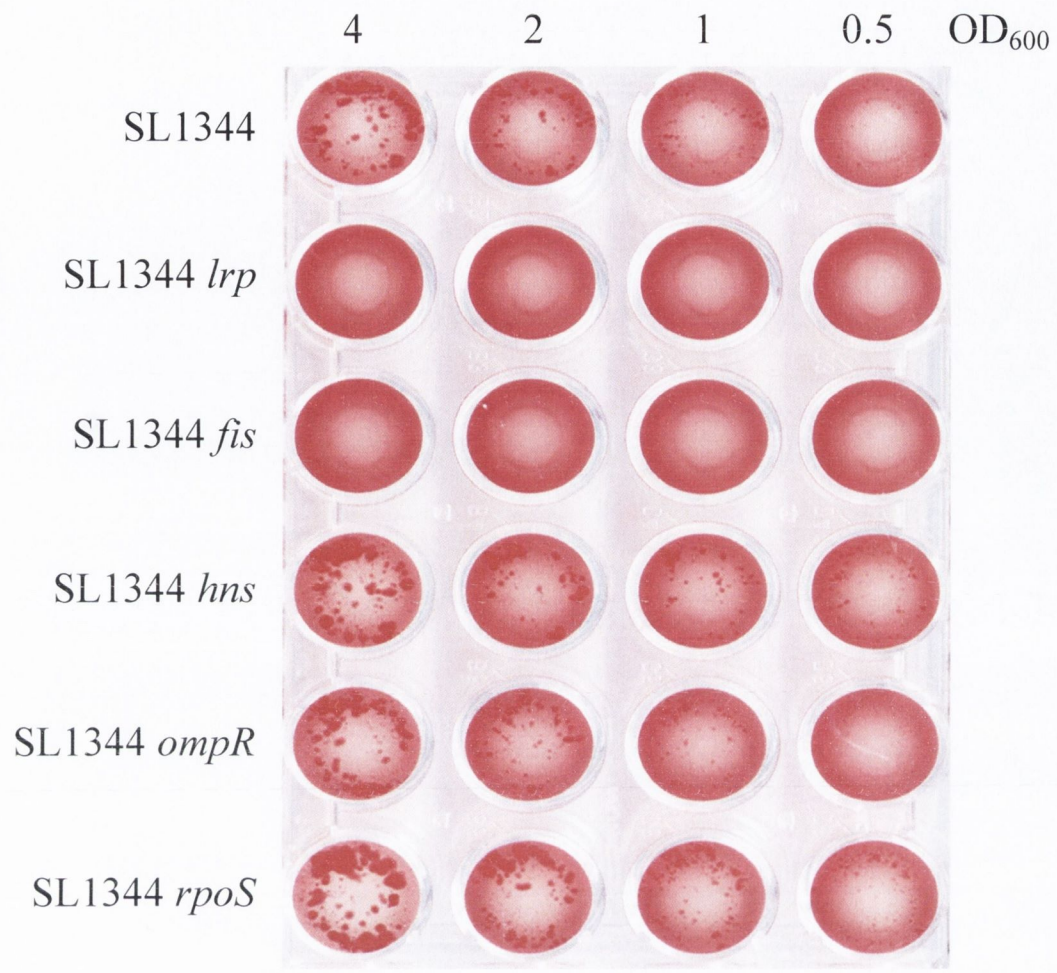


Fig. 5.16. Haemagglutination assays of regulatory mutants of *Salmonella* Typhimurium. Equal OD_{600} values of bacterial grown under fimbriae-inducing conditions were incubated with 3 % (v/v) RBCs and incubated at room temperature. This was performed twice with similar results.

Bend.It program (Figure 5.17). This showed that curvature is present in the –166 to –126 region. Taken with the MSHAs of the *hns* mutant shown in section 5.2.14, this supports the idea that H-NS may act as a negative regulator of *fimZ* expression.

5.3 Discussion

DNA microarray analysis performed in this study identified a role for Lrp in the regulation of type 1 fimbriae of *S.* Typhimurium. This was interesting, as Lrp is a known regulator of a number of fimbrial operons. Haemagglutination assays were used to examine the phenotype of the *lrp* mutant. This confirmed that surface expression of type 1 fimbriae was strongly decreased in the *lrp* mutant, whereas the complemented mutant displayed wild type levels of fimbrial expression (Fig. 5.2). Additionally, RT-PCR analysis was used to show the downregulation of the *fimA* and *fimZ* genes, both of which are essential for fimbrial expression. Interestingly, RT-PCR analyses of RNA taken from lag phase-grown cells suggest a slight repression of *fimY*, a positive regulator of *fim* expression, and the structural genes, *fimD, fimF, and fimH.* This down-regulation is likely to be a result of the decreased expression of *fimZ*, since FimZ positively regulates *fimA* and *fimY* expression (Tinker and Clegg, 2000). The down-regulation of the structural genes can possibly be explained by the fact that the *fim* structural genes form an operon, starting with *fimA* (Klemm, 1994). The regulation of *fimW* expression has not been examined in detail, but it is thought to regulate its own expression negatively (Tinker *et al.*, 2001). Interestingly, the *fimW* transcript did not show changes under any of the conditions examined, suggesting that it is not regulated by Lrp. In conditions that induce fimbriation, only the *fimA* and *fimZ* transcripts were downregulated in comparisons between wild type and mutant, suggesting strongly that Lrp is a positive regulator of *fimA* and *fimZ* expression (Fig. 5.3). Microarray analysis suggested that L-leucine, which is known to affect the regulation by Lrp of a number of genes, increases the expression of a number of *fim* genes. As a result, the expression of *fimA, fimZ, fimY* and *fimW* was examined by RT-PCR analysis on RNA samples taken from cultures grown in MOPS minimal broth in the presence and absence of 10 mM L-leucine. These analyses showed a slight increase in the expression of *fimA* and *fimZ* in the presence of leucine, but no change in the expression of *fimW* or *fimY* (Fig. 5.4). RT-PCR analysis was generally employed in this study to examine trends of gene expression, and to confirm microarray data. A more sensitive technique, such as quantitative real-time PCR (QRT-PCR) could be used to more accurately examine details of leucine sensitivity.

Bioinformatic analysis was employed to identify putative Lrp binding sites in the *fim* gene cluster. This identified putative sites within the *fimA* and *fimZ* regulatory regions (Fig. 5.5). The regulatory regions of three *fim* genes were examined for the ability of Lrp

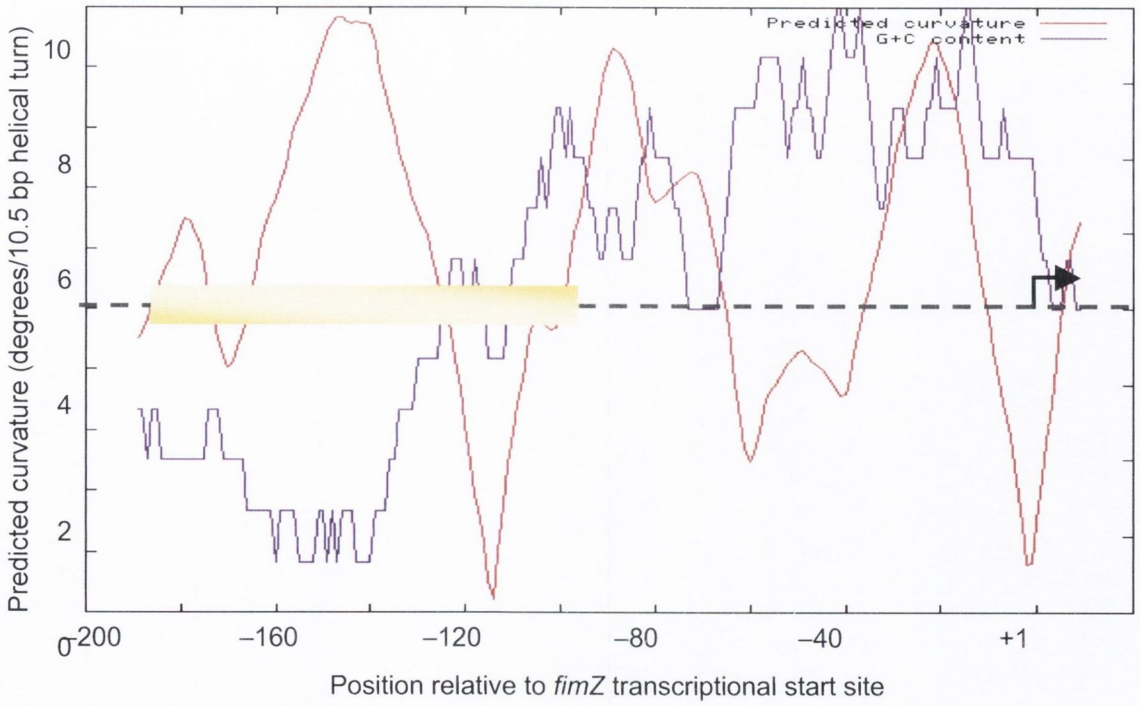


Fig. 5.17. Analysis of DNA curvature of the *fimZ* regulatory region. DNA curvature was analysed using the web-based Bend.it program. DNA with over 5° curvature per 10.5 bp helical turn (dashed line) is predicted to be curved. H-NS binds preferentially to A+T-rich, curved DNA, and may interact with the *fimZ* promoter to repress *fimZ* transcription. Interestingly, the region protected by Lrp on the coding strand of *fimZ* as revealed by DNase I footprinting analyses, shown in yellow, overlaps the region with the lowest G+C content, and thus, the putative H-NS-binding region. The DNA sequence is numbered with respect to the *fimZ* transcriptional start site (Yeh *et al.*, 2002).

to interact. A large *fimA* promoter probe showed no interaction with Lrp. To examine whether Lrp-binding of the 700-bp probe resulted in impeded migration in the gel, the probe was divided into smaller sections, but still showed no complex formation. This was interesting, considering the strong repression of *fimA* transcription seen in the *lrp* mutant, and the presence of two sequences with high homology to the Lrp consensus sequence within the *fimA* upstream region. *fimW*, whose transcription was unaffected in the *lrp* mutant, was also assayed for an interaction with Lrp (Fig. 5.6). As expected, no complex was formed. Two *fimZ* promoter region probes were also examined for an Lrp interaction. The smallest of these probes contained a putative Lrp binding site, identified by bioinformatics. This probe did not show an interaction with Lrp, but when the probe was extended further upstream, a complex was formed (Fig. 5.7). Deletion analysis was used to identify a region important for Lrp-binding between –90 and –190, with respect to the transcriptional start site of *fimZ* (Fig. 5.8). The binding of Lrp to nested *fimZ* probes was examined in the presence and absence of L-leucine. The probe Z202 formed three complexes in both the presence and absence of L-leucine, but L-leucine presence did appear to favour formation of the larger complexes, 2 and 3. How does Lrp bind to the *fimZ* promoter to form these complexes? The production of three binding sites on the Z202 probe suggests three Lrp binding sites. Interestingly, the larger probe Z299 showed formation of two less well-resolved complexes. The presence of L-leucine gave a less dramatic change to complex formation with Z299 than Z202, appearing to favour the formation of both complexes 1 and 2, whereas complex 2 was dominant in the absence of L-leucine. This may be a result of the additional length of this probe, which may somehow affect the ability of Lrp to interact with its target sequence. Alternatively, the increased size may affect the resolution of the complexes, as it was found throughout this study that performance of EMSA analyses using smaller probes improves complex resolution. If this were so, perhaps complex 2 contains both unresolved complexes 2 and 3, seen with probe Z202 (Fig. 5.8).

Lrp has been shown to protect regions of over 100 bp in regulatory regions of its target genes (Jafri *et al.*, 2002; Mathew *et al.*, 1996). Therefore a large, 317-bp region, encompassing the entire region of probe Z299, was further analysed by DNase I footprinting (Figs. 5.9 and 5.10). The presence of areas of protection from DNase I, and intermittent hypersensitive regions revealed that Lrp binds and wraps the DNA. The presence of leucine did not have a dramatic effect on Lrp binding, but increased

hypersensitivity of certain bases was visible. Interestingly, regions of hypersensitivity correlated well between both strands in three places, suggesting that Lrp binds between those sites, bending the DNA between binding sites. This would suggest three binding sites for Lrp, centred between the hypersensitive regions along the region of protection. Analysis of these regions has identified three sites with homology to the Lrp binding site consensus sequence in the region of protection on the *fimZ* coding strand highlighted in Figure 5.11. Sites 1 and 2, located at –152 and –129, show a mismatch of three and four bases, respectively, compared to the consensus sequence, while the third site is centred at –100, with a mismatch of six bases. Interestingly, sites 1, 2 and 3 are roughly equidistant, which appears to be a characteristic of Lrp binding sites: Lrp binding sites are often spaced by approximately 30 bp, for example in the *dadAX, ilvIH* and *pap* operons (de los Rios and Perona, 2007; Jafri *et al.*, 2002; Zhi *et al.*, 1999). According to the recently solved crystal structure of *E. coli* Lrp, 30 bp of DNA corresponds well with the separation of the intradimer two-fold axes of the Lrp octamer (de los Rios and Perona, 2007) (Fig. 1.4). In addition, 30 bp is roughly equivalent to three helical turns of DNA, suggesting that Lrp binding is likely to occur on one plane. An Lrp dimer bound to one Lrp binding site has been shown to induce a bend of 52°: when two dimers are bound to adjacent sites, a bend of at least 135° is induced (Wang and Calvo, 1993). These data were supported by a model generated using the crystal structure of *E. coli* Lrp, which also suggested that three dimers produce a bend of at least 180° over three binding sites (de los Rios and Perona, 2007) (Fig. 1.4). A model of binding, bending and complex formation as observed by EMSA analysis is suggested in Figure 5.18. In this model, it is assumed that the occupation of putative Lrp binding sites by the Lrp dimer begins at sites with the highest identity to the binding site consensus sequence, and extends to those with lower identity as Lrp concentration increases. The presence of leucine appears to enhance the formation of complex 3, which is expected to represent the interaction of Lrp with all three sites. In the *ilvIH* operon of *E. coli*, leucine has been shown to negatively impact *ilvIH* transcription by enhancing Lrp dimer-dimer interactions that contribute to octamer formation, while reducing the long-range interactions that are required for *ilvIH* expression. Perhaps at the *fimZ* promoter, the role of leucine is to enhance cooperative binding of Lrp to its putative sites and to increase the efficiency of formation of complex 3, by enhancing occupation of site 3 (centred at –100), which is relatively degenerate with six mismatches to the consensus sequence. If leucine does increase Lrp-mediated positive regulation of *fimZ in vivo*, as suggested by

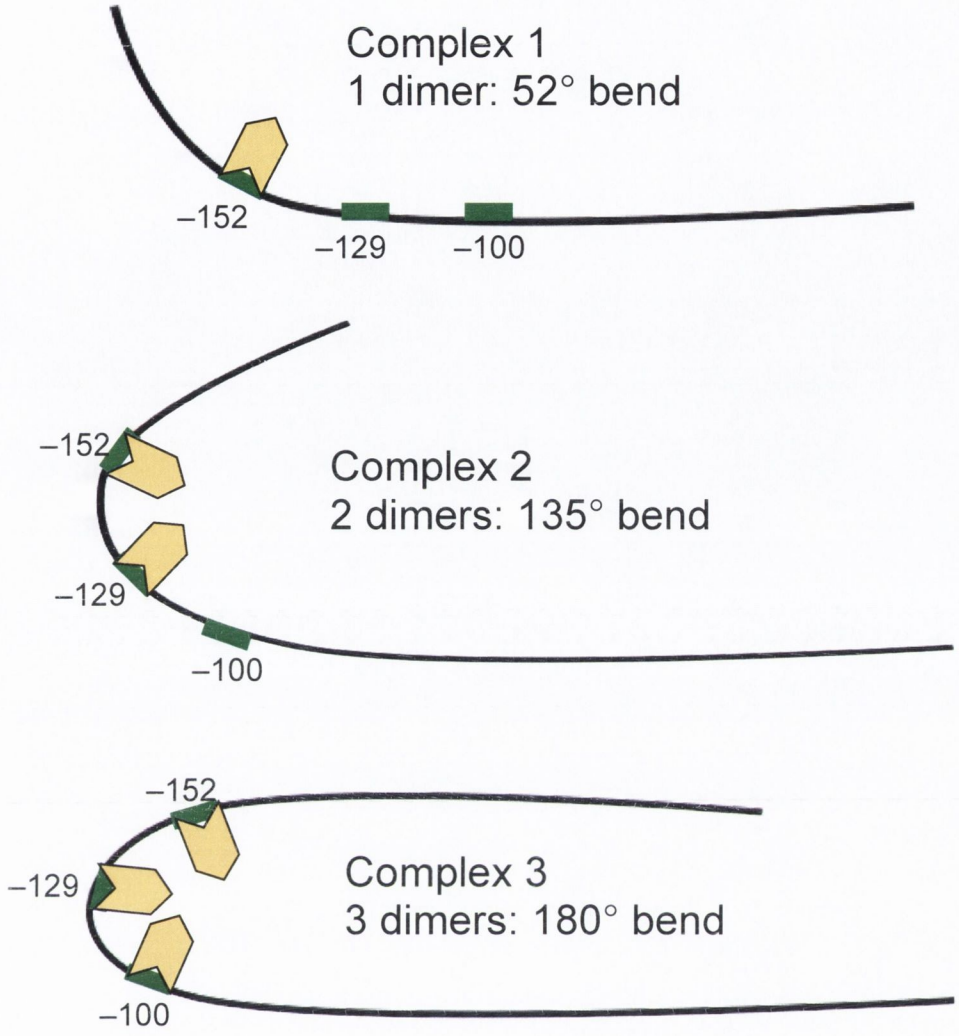


Fig. 5.18. Model of binding, bending and complex formation by Lrp on the *fimZ* promoter. The putative Lrp binding sites are represented by green boxes, and labeled with their centre positions. It is assumed that the primary interaction to form complex 1 would occur at the site with the highest identity to the Lrp consensus binding site sequence, at –152. Cooperative binding of Lrp may be important for formation of complexes 2 and 3. Upon binding, one dimer induces a bend of 52°, two adjacent dimers bend DNA approximately 135°, and three dimers on adjacent sites are estimated to bend DNA approximately 180° (de los Rios and Perona, 2006). Presumably, the binding of another dimer, leading to octamer formation, would stabilise this interaction *in vivo*.

RT-PCR and microarray data, presumably the occupation of Lrp at all three sites, and hence the generation of a bend of 180°, is most favourable for *fimZ* transcription (See Fig. 5.18). It is not fully understood how leucine is capable of affecting Lrp dimer binding of the *fimZ* promoter, given that dimer structure is not believed to be affected by leucine. If an Lrp octamer were to form on the *fimZ* promoter, it would be expected that four complexes, each representing the stepwise addition of four dimers to the overall molecular mass, would be observed. Instead, only three complexes were observed by EMSA analysis, which suggest that three dimers were bound to the region, and that the octamer form of Lrp was not present (Fig. 5.8 (C)). This may represent the events *in vitro*, whereas *in vivo*, unknown factors that lead to the addition of another non-DNA-binding dimer to form the Lrp octamer may stabilise the interaction of Lrp with the *fimZ* promoter.

Comparison of EMSA analyses of probes Z202 and Z299 shows that the length of the probe has an effect on complex formation. It is unclear whether this effect is a result of complex migration and resolution, or hindrance of Lrp-binding caused by secondary structure. If this effect was caused by secondary structure, it is interesting to hypothesise that the effect of leucine on Lrp binding would have been shown more clearly using a smaller probe for DNase I footprinting. Despite three putative Lrp binding sites in the *fimZ* regulatory region, at the highest concentration of Lrp (Figure 5.9: lanes 6 and 12) the protection appears to extend to the entire fragment, and hypersensitive regions are no longer as prominent. Perhaps this represents the formation of a higher-order nucleoprotein complex, where the DNA probe is wrapped non-specifically around higher oligomers of Lrp, and thus is protected from DNase I cleavage.

This study has shown that Lrp is a positive regulator of *fimZ* expression, but it is unknown how Lrp enhances the expression of *fimZ*. It is tempting to hypothesise that Lrp interacts with RNA polymerase at the *fimZ* promoter to enhance its transcription: Lrp has never been shown to interact with RNA polymerase, but it is hypothesised to do so in some cases (Brinkman *et al.*, 2003). Lrp protects a region from –95 to –187 on the coding strand of the *fimZ* regulatory region, and from –84 to –110 on the non-coding strand, a considerable distance from the promoter. An unknown protein may interact with Lrp on the *fimZ* promoter, as seen in the *pap* system (Kaltenbach *et al.*, 1995), or may be brought into contact with RNA polymerase by virtue of DNA-bending by Lrp, as

seen with GcvA in regulation of the *gcvTHP* operon (Stauffer and Stauffer, 1999). FimZ appears to regulate its own transcription positively. Interestingly, the sequence AATAAGA is found in an 11-bp region essential for the interaction of FimZ with the *fimA* promoter, and FimZ-mediated DNase I-protected region of the *fimA* promoter. This sequence is also found in the *fimZ* promoter, raising the possibility that FimZ binds to this region (Yeh *et al.*, 2002). At –352, this putative site is relatively far upstream, perhaps requiring Lrp for DNA-bending to allow the interaction of FimZ with RNA polymerase. The position of Lrp binding is putatively placed between –152 and –100, potentially placing a bend of 180° approximately halfway between the putative FimZ binding site, and the RNA polymerase binding sites of *fimZ*. Another possibility is the role of Lrp as a derepressor, acting to displace a negative regulator, e.g. H-NS, from the *fimZ* promoter. Examples of H-NS antagonists are LeuO, SlyA and VirB, all of which are believed to displace H-NS from DNA by remodelling local DNA structure (Madhusudan *et al.*, 2005; Turner and Dorman, 2007; Wyborn *et al.*, 2004). Analysis of the *fimZ* promoter reveals the presence of intrinsic DNA curvature, indicative of H-NS binding. Recent reports by Navarre *et al.* and Lucchini *et al.* have shown that H-NS of *S.* Typhimurium preferentially binds and silences regions of low G+C DNA content. In these studies, they show that the *fim* gene cluster possesses A+T-rich DNA with the highest levels present in the *fimZ* promoter region (Navarre *et al.*, 2006), and that H-NS interacts with the *fimZ* region (Lucchini *et al.*, 2006). Analysis of DNA curvature using the Bend.it program showed that the low G+C region of curvature is present in the –166 to –126 region, overlapping the Lrp-binding region, supporting the idea that H-NS acts as a negative regulator of *fimZ* expression (Figure 5.17). MSHAs also suggest a slight increase in agglutination in the *hns* mutant (Figure 5.16) although this effect is difficult to quantify. MSHAs did identify a putative positive regulatory role for Fis in *fim* expression. A model for *fimZ* regulation by Lrp is hypothesised in Fig. 5.19.

This study showed that fimbriation could be restored in an *lrp* mutant by over-expression of FimZ. This strongly suggests that the role of Lrp in the *fim* system is to act as a positive regulator of *fimZ*, and that the absence of Lrp leads to reduced levels of FimZ, which is essential for the expression of *fimA* and the remaining structural *fim* genes. Under the conditions examined in this study, Lrp did not appear to have a significant effect on FimZ-regulated genes other than *fimA* (Figure 5.15), and under some conditions, *fimY* (Fig. 5.3). How Lrp exerts its positive effect on FimZ expression

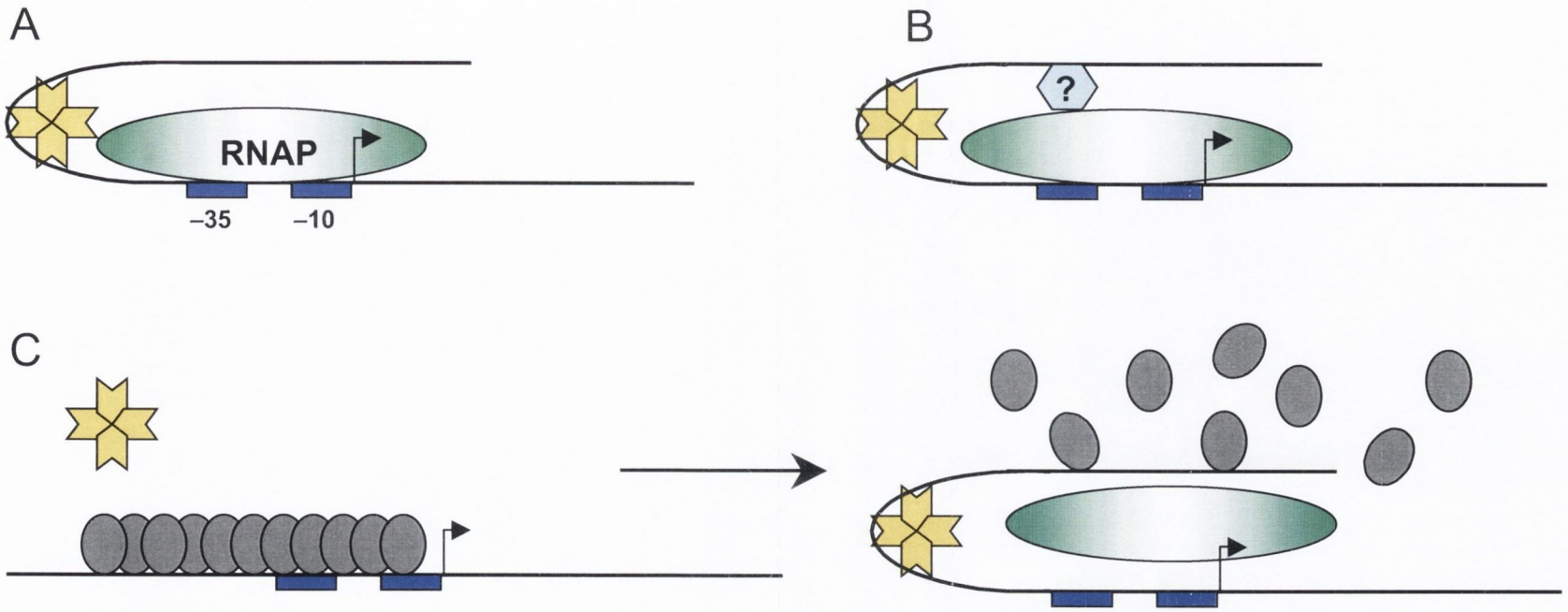


Fig. 5.19. Model of *fimZ* regulation by Lrp. Lrp is a positive regulator of *fimZ* expression, but the way in which Lrp exerts this effect is unknown. This model hypothesises that Lrp (yellow chevrons) may interact directly with RNA polymerase to enhance transcription of *fimZ* (A) or, by bending DNA, allow an unknown upstream enhancer (blue hexagon) to come into contact with RNA polymerase, thus enhancing transcription (B). Alternatively, Lrp may act as a derepressor of *fimZ*, by displacing a negative regulator (grey ovals) from the *fimZ* promoter, and allowing access of RNA polymerase.

is unknown, but has many interesting implications as FimZ has been shown to be a co-ordinator of motility, adhesion and invasion (Baxter and Jones, 2005). Why does Lrp have a role in the expression of these fimbriae? Lrp has been described as a feast/famine regulatory protein, as its expression is regulated by nutrient availability, and it is capable of sensing nutrient up- or down-shift due to the modulatory effect of leucine on Lrp-mediated regulation (Yokoyama *et al.*, 2006). This suggests that nutrient concentration may be an important environmental signal for the expression of *fimZ* and, in combination with other factors, indicates gut-like conditions under which expression of fimbriae would be favourable to the bacterium, allowing host cell interactions.

The importance of type 1 fimbriae of *S.* Typhimurium in virulence is disputed. Previous studies have shown that type 1 fimbriae mediate tropism of *S.* Typhimurium to mouse (Thankavel *et al.*, 1999) and rat enterocytes (Ewen *et al.*, 1997), and human HeLa cells (Baumler *et al.*, 1996). Studies have shown evidence for and against the adhesion of *S.* Typhimurium type 1 fimbriae to HEp-2 cells (Baumler *et al.*, 1996; Ernst *et al.*, 1990; Tavendale *et al.*, 1983). A potential molecular basis for these discrepancies has been shown by Boddicker *et al.*, who observed that amino acid polymorphisms in FimH, the fimbrial adhesin, account for an eight-fold decrease in the ability of *S.* Typhimurium strain SL1344 to bind HEp-2 cells, compared to strain LT2 (Boddicker *et al.*, 2002). This suggests that the tropism of type 1 fimbriae for target tissue is, at least in part, mediated by the FimH subunit. With this taken into account, it seems plausible that in order to study the tropism of type 1 fimbriae of SL1344, individual strain differences should be taken into account. The 200-bp region, including the Lrp binding regions, of the *fimZ* gene of type 1 fimbriae in the genomes of a number of sequenced *Salmonella* strains, and the partially sequenced Enteritidis PT4 strain, have shown 99 % homology to that of SL1344/LT2. In each case, the putative Lrp-binding sites are 100 % identical. This suggests that Lrp may also regulate *fimZ* genes of clinically and agriculturally important *Salmonella* strains. A study by De Buck *et al.* showed that type 1 fimbriae mediate binding of *S.* Enteritidis PT4 to isthmal secretions in the laying hen, which are incorporated into the forming egg, and thus are thought to play a role in the contamination of eggs by bacteria (De Buck *et al.*, 2003).

The expression of fimbriae is often phase variable, presumably to prevent inappropriate expression of antigenic proteins. In *E. coli,* the inversion of *fimS* controls phase

variation. The regulatory mechanisms controlling expression of phase variable type 1 fimbriae (*fim*) of *E. coli* are well characterized, and are known to require Lrp (Kelly *et al.*, 2006). Although phase variable, the *fim* system of *S.* Typhimurium does not appear to contain an invertible element, as the *fimA* promoter region is only found in the orientation allowing transcription, regardless of fimbrial phenotype (Clegg *et al.*, 1996) and no recombinases occur within the *S.* Typhimurium *fim* operon (Swenson and Clegg, 1992). In addition, no GATC sequences indicative of P-regulatory fimbriae were found in the *fimZ* promoter region, and only one GATC sequence was identified in the *fimA* promoter region (section 5.2.7). Instead, phase variation of *S.* Typhimurium type 1 fimbriae is controlled by a regulatory cascade. This study has revealed that Lrp constitutes in a novel way another level of control of this complex regulatory system by regulation of *fimZ,* an essential positive regulator of *fim* gene expression.

Chapter 6

The effect of Lrp on *ompR* expression in *Salmonella enterica* serovar Typhimurium

6.1 Introduction

The SPI-2 pathogenicity island of *S. enterica* encodes a type three secretion system (TTSS), which is a needle-like organelle that traverses the vacuolar membrane of the SCV, and secretes effector proteins (Shea *et al.*, 1996). The translocated effector proteins enter the host cell, and modify host cell functions, and cellular trafficking (Abrahams and Hensel, 2006). SPI-2 is required for systemic infection, and for the survival and replication of *Salmonella* in macrophages and epithelial cells (Cirillo *et al.*, 1998; Hensel *et al.*, 1995; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Vazquez-Torres *et al.*, 1999).

A variety of environmental signals influences SPI-2 gene expression in the phagosome. In the murine macrophage, most SCVs undergo acidification to pH 4.0-5.0 within 20-30 min of formation (Rathman *et al.*, 1996), which suggests that pH may be an important signal for gene expression. *In vitro* analyses suggested that minimal media with low concentrations of Mg^{2+}, and phosphate starvation led to the induction of SPI-2 gene fusions (Deiwick *et al.*, 1999). However, Miao *et al.* reported that low pH, or limitation of Mg^{2+} or phosphate, were not required for induction of the SPI-2 effector gene *sspH2* (Miao *et al.*, 2002). Garmendia *et al.* have shown that low osmolarity and absence of Ca^{2+} are major signals that affect SPI-2 gene expression during growth in magnesium minimal medium (Garmendia *et al.*, 2003). In addition, Lee *et al.* showed that acidic pH induced the expression of SPI-2 genes *ssrA* and *ssaH* in wild type SL1344. They describe induction of *ssrA* and *ssaB* in minimal media at pH 4.5 in the presence of both low (1 μM) and high concentrations (1 mM) of Mg^{2+}. No change in *ssrA* expression was seen at either low or high Mg^{2+} concentrations at pH 7.2, which therefore failed to reproduce the effect of low Mg^{2+} concentrations, and strongly suggested that pH is an important signal (Lee *et al.*, 2000). In support of this, immunoblot analysis detected that the effector proteins SseA and SseD were secreted under conditions of acidic pH in minimal medium, indicating that the SPI-2 machinery is likely to be expressed under these conditions. In agreement with the above study, Rappl *et al.* demonstrated that acidic pH is required for the functional assembly of the SPI-2 TTSS (Rappl *et al.*, 2003). More recently, the same group showed that exposure of bacteria to minimal media with acidic pH of 6.2 or below resulted in a rapid induction of SPI2 expression (Löber *et al.* 2006).

The SPI-2-encoded two-component system SsrA-B controls SPI-2 TTSS gene expression, as well as the expression of SPI-2-secreted effector molecules located outside the SPI-2 pathogenicity island (Cirillo *et al.*, 1998). SsrA-B responds to low osmolarity, the absence of Ca^{2+} and acidic pH (Garmendia *et al.*, 2003). OmpR directly regulates *ssrA* expression by interaction with the *ssrA* promoter region: in addition, expression of *ssrA* was shown to increase upon successive deletion of parts of the promoter region suggesting that there is a region of DNA important for the repression of *ssrA* (Lee *et al.*, 2000). DNA relaxation has also been shown to positively affect the expression of *ssrA* during survival in macrophages, and the nucleoid associated protein Fis has a role in this (Ó Cróinín *et al.*, 2006).

OmpR-EnvZ is a two-component system in which EnvZ is the sensor kinase, and OmpR is the response regulator. OmpR-EnvZ regulates the expression of SsrA-B, and it is required for *Salmonella* survival and replication in macrophages (Lee *et al.*, 2000). In *Salmonella*, OmpR levels are induced by acid shock, but reduced by the presence of osmotic stress (Bang *et al.*, 2000; Bang *et al.*, 2002). Acid shock leads to induction of *ompR* transcription from a second promoter, P2, located approximately 12 bp upstream of the constitutively active promoter, P1 (Bang *et al.*, 2002). OmpR is essential for the stationary phase acid tolerance response (ATR), but not the log phase ATR (Bang *et al.*, 2000). It is thought that changes in DNA topology lead to the acid induction of *ompR* P2, as acid stress decreases negative supercoiling (Karem and Foster, 1993): increasing concentrations of the gyrase inhibitor novobiocin, which also decreases negative supercoiling, led to increased *ompR* expression (Bang *et al.*, 2002). H-NS is a negative regulator of *ompR* expression from P2 at the transcriptional level. In the *S.* Typhimurium strain UK1 *hns* mutant, *ompR* expression levels from the P2 promoter are constitutively high, in both inducing (acidic pH) and non-inducing conditions (neutral pH). Phosphorylated OmpR is required for its own expression by counteracting H-NS-mediated repression: in the *hns* mutant of UK1, OmpR-P is not required, indicating that its effect is dependent on H-NS (Bang *et al.*, 2002).

DNA microarray analysis suggested that Lrp acts as a negative regulator of SPI-2 genes during growth in MOPS minimal medium (Chapter 4). In this chapter, the effect of Lrp on *ssrA* and *ompR* gene expression was examined using *gfp* reporter fusions, EMSA analyses, and DNase I footprinting. Lrp was shown to be an indirect negative regulator

of *ssrA,* which encodes the sensor kinase of the SsrA-B two component system that regulates SPI-2 gene expression. It was also shown that Lrp regulates *ompR* expression in response to acidic pH in a positive manner, and that Lrp interacts directly with the *ompR* promoter.

6.2 Results

6.2.1 The effect of the *lrp* mutation on expression of SPI-2 genes

DNA microarray analysis in Chapter 4 had established that during growth in MOPS minimal medium, six *Salmonella*-specific SPI-2 genes appeared to be affected by the *lrp* mutation. In the logarithmic phase of growth, SPI-2 gene expression was increased in SL1344 *lrp*, whereas in the stationary phase of growth in MOPS minimal broth, SPI-2 genes were downregulated in SL1344 *lrp*. This apparent reversal of regulation was examined using RT-PCR from RNA extracted from SL1344, SL1344 *lrp* and the complemented *lrp* mutant during growth in MOPS minimal medium. Four SPI-2-encoded genes were chosen for analysis: three of the genes, *ssaG, sseA,* and *ssaB,* are each the primary genes in three SPI-2 operons. The *ssrA* gene encodes an important regulator of SPI-2. RT-PCR analyses verified that the expression of the four SPI-2 genes examined was increased during logarithmic growth, but reduced in stationary phase growth in SL1344 *lrp* in MOPS minimal broth (Fig. 6.1). The transcript levels were similar between SL1344 and the complemented mutant in all cases. RT-PCRs were carried out at least twice with similar results.

6.2.2 The expression of *ssrA* in SL1344 *lrp*

SsrA-B is a two-component system that is required for SPI-2 TTSS gene expression. SsrA-B also regulates the expression of SPI-2-encoded effectors and SPI-2 effectors that are in other locations on the chromosome. The gene *sifB* encodes a SPI-2 effector, located outside of SPI-2, and regulated by SsrA-B. The expression of *sifB* was found by microarray analysis (Chapter 4) to be affected by the *lrp* mutation in MOPS minimal broth. RT-PCR analysis verified that *ssrA* transcription was increased in SL1344 *lrp* during logarithmic phase, yet reduced in stationary phase in MOPS minimal broth. As SsrA is an important regulator of SPI-2 genes, and was affected by the *lrp* mutation, it was possible that the effects on SPI-2 were a result of the effect of Lrp on *ssrA*. Therefore, it was decided to examine *ssrA* expression in SL1344 *lrp*. A plasmid, pZEP*ssrA,* containing a transcriptional *gfp* fusion to *ssrA* was used to examine *ssrA* expression in SL1344 *lrp* (Ó Cróinín *et al.,* 2006). This plasmid was transformed into SL1344, SL1344 *lrp* and CJD365 (SL1344 *ompR*). In an *ompR* mutant, *ssrA* expression has been shown to be strongly reduced (Lee *et al.*, 2000). Bacteria were cultured in MOPS minimal medium at both pH 4.5 and pH 7.2, as described in section 2.2.3. Acidic

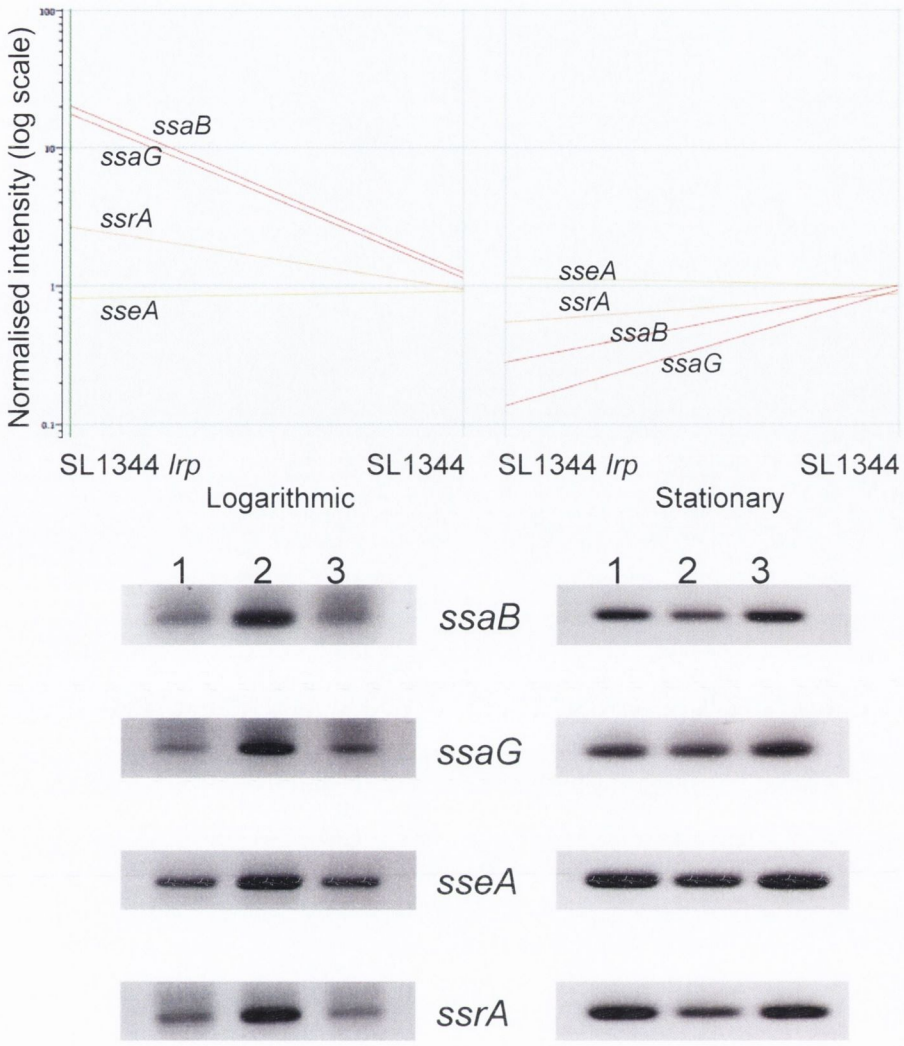


Fig. 6.1. Confirmation of SPI-2 gene expression from microarray analysis. Microarray analysis suggested that in SL1334 *lrp*, a number of SPI-2 genes had increased expression levels at logarithmic phase and decreased levels of expression at stationary phase, compared to SL1344 (top). Colours of lines denote expression in logarithmic phase in SL1344 *lrp*: yellow signifies unchanged expression, whereas red signifies increased expression. Analysis was carried out on four SPI-2 genes, *ssaB, ssaG, sseA* and *ssrA,* which are the primary genes in four SPI-2 operons. RT-PCR was carried out on RNA isolated from SL1344, SL1344 *lrp* and the complemented *lrp* mutant (lanes 1, 2 and 3, respectively) grown to logarithmic phase and stationary phase in MOPS minimal medium. This verified the reversal of expression identified in the microarray. RT-PCRs were performed twice with similar results.

pH has been shown to increase the expression of *ssrA* in minimal medium (Lee *et al.*, 2000). After 3 h at 37 °C, samples were taken for examination by flow cytometry. The results showed that *ssrA* expression was induced at pH 4.5 compared with pH 7.2 in SL1344 (Fig. 6.2). In the *lrp* mutant, *ssrA* expression at pH 7.2 was relatively high, compared with that of wild type SL1344. In addition, *ssrA* was expressed at a similar level at both pH 4.5 and pH 7.2 in SL1344 *lrp*, which suggested that in the *lrp* mutant, *ssrA* expression did not respond to pH. In the *ompR* mutant, *ssrA* expression was strongly decreased at both pH 4.5 and pH 7.2, and did not show pH-mediated induction, as was expected. This experiment was performed in triplicate at least three times with similar results.

6.2.3 Lrp does not interact with the promoter region of *ssrA*

In this study, *ssrA* expression levels appeared to be increased under normally non-*ssrA*-inducing conditions (minimal medium at pH 7.2) in the *lrp* mutant compared with the wild type, supporting the microarray results. In addition, the pH sensitivity of *ssrA* transcription appeared to be reduced in the *lrp* mutant. This suggested that Lrp has a role in the response of *ssrA* transcription to pH, perhaps by direct interaction with the *ssrA* promoter region.

To examine whether Lrp could directly interact with the *ssrA* promoter, EMSA analyses were undertaken. A biotinylated *ssrA* promoter region probe of 331 bp was PCR amplified from the SL1344 genome using primer pair *ssrA*-F.1 and *ssrA*-R-Bio. This probe encompassed 293 bp upstream to 38 bp downstream of the *ssrA* ATG start codon. An additional probe of 632 bp, from 594 bases upstream, containing 194 bp of the *ssaB* coding region, to 38 bp downstream of the *ssrA* ORF, was also amplified (Fig. 6.3 (A)), using primer pair *ssrA*-F.2 and *ssrA*-R-Bio. The larger 632-bp probe was amplified because Lrp can bind to DNA relatively far upstream of certain promoters (Wiese *et al.*, 1997). Both probes were incubated with increasing concentrations (0-266 nM) of Lrp. Neither probe demonstrated complex formation in the presence of Lrp, with or without leucine supplementation (Fig. 6.3 (B)). This suggested that Lrp does not interact with the promoter of *ssrA*, indicating that its effect on *ssrA* expression was likely to be indirect. An EMSA using the *fimS* fragment of *E. coli* as a positive control showed complex formation as expected (data not shown).

6.2.4 Expression of *ssrA* in SL1344 *lrp ompR*

The expression of *ssrA* was strongly reduced in the *ompR* mutant, but was expressed at a relatively high level in pH 7.2 and was insensitive to acidic pH in the *lrp* mutant (Fig. 6.2). To examine the effect of an *lrp ompR* mutant on *ssrA* expression, the *ompR* lesion was moved into the *lrp* mutant using P22-mediated transduction. The presence of the *lrp* and *ompR* mutations in the double mutant was confirmed using PCR analysis using primer pairs Fwd1.3 and Rev1.3, and *ompR-gfp*-F and *ompR-gfp*-R, respectively (Fig. 6.4 (A)).

Analysis of *ssrA* expression was performed as described in section 6.2.3, using SL1344, SL1344 *lrp*, SL1344 *ompR*, and SL1344 *lrp ompR*. Expression profiles of SL1344, SL1344 *lrp* and SL1344 *ompR* were similar to those shown in Fig. 6.2. The *lrp ompR* double mutant showed no significant difference in expression to that of the *ompR* single mutant (Fig. 6.4 (B)). This suggested that in SL1344, the Lrp-associated increase of *ssrA* expression at pH 4.5 was OmpR-dependent.

6.2.5 Construction of an *ompR* transcriptional fusion

Previous work has shown that *ssrA* is induced by acidic pH in wild type *S. enterica* and that OmpR, the sensor of the two-component system OmpR-EnvZ, is acid-induced and regulates the acid induction of *ssrAB* (Lee *et al.*, 2000). In the present study, *ssrA* was induced in SL1344 at pH 4.5 compared with pH 7.2 (Fig. 6.2) and, as expected, was not induced in the *ompR* mutant. Interestingly, *ssrA* expression was not induced by acidic pH in SL1344 *lrp*, but had relatively high expression levels at both pH 4.5 and pH 7.2. Lrp did not appear to interact directly with *ssrA* (Fig. 6.3), so it was likely that the effect of Lrp on *ssrA* was indirect. As pH-mediated *ssrA* induction was compromised in SL1344 *lrp*, it was decided to examine whether Lrp had an effect on OmpR, as OmpR regulates the acid induction of *ssrA* (Lee *et al.*, 2000).

To examine the effect of Lrp on *ompR* expression, a transcriptional *gfp* fusion to *ompR* was constructed. Using primer pair *ompR-gfp*-F and *ompR-gfp*-R, a 363-bp fragment was amplified from the SL1344 genome. This fragment included all DNA regions believed to be involved in *ompR* regulatory control (Bang *et al.*, 2002). The 363-bp fragment was digested with *Not*I and *Xba*I, and ligated into the same restriction sites in

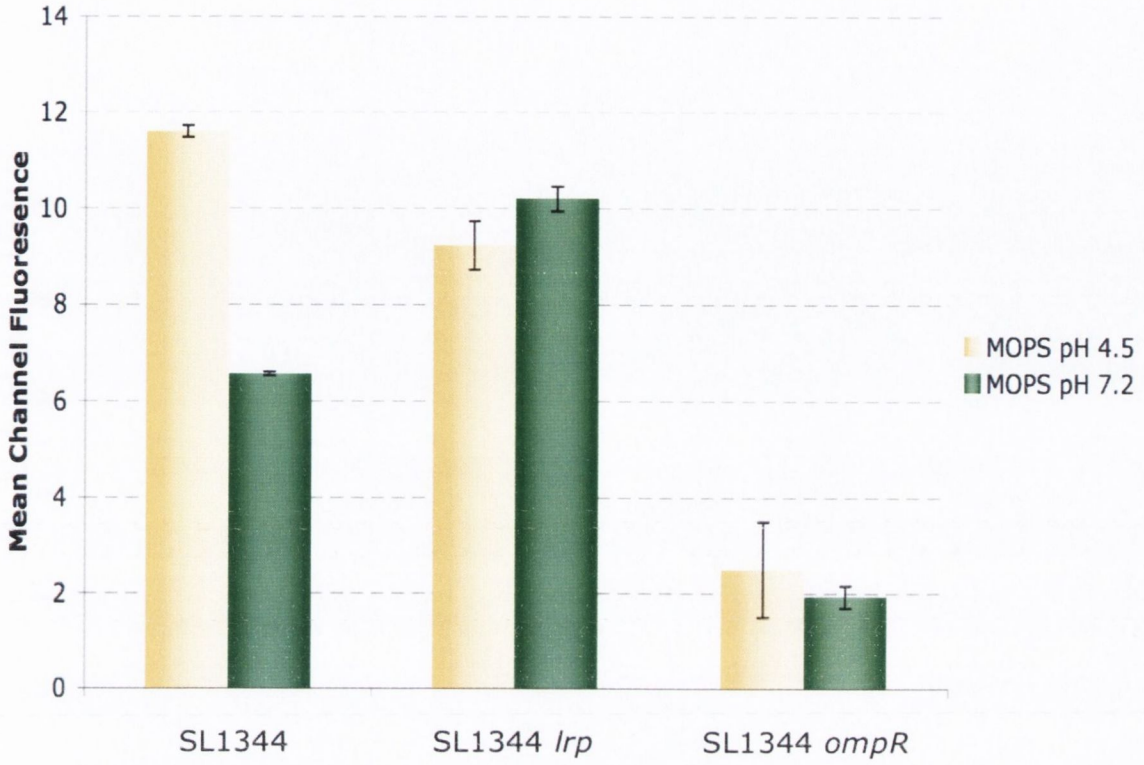


Fig. 6.2. Expression of *ssrA* in SL1344 *lrp*. Expression of *ssrA* in SL1344, SL1344 *lrp* and SL1344 *ompR* after 3 h incubation in MOPS minimal medium at pH 4.5 (yellow bars) and pH 7.2 (green bars). Error bars denote ± SEM.

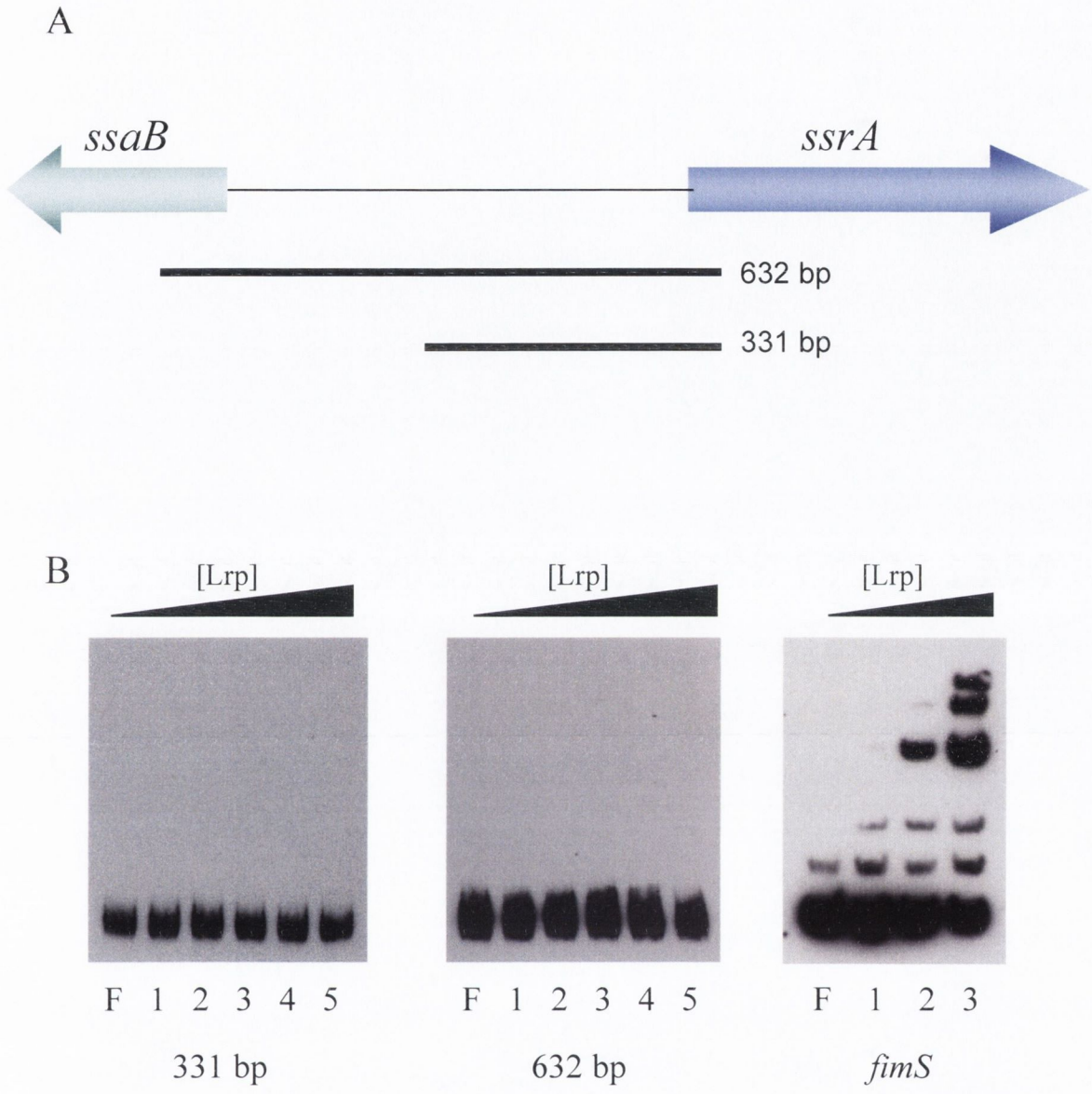


Fig. 6.3. EMSA analysis of the *ssrA* promoter region with Lrp. Two biotinylated probes, of 331 bp and 632 bp, were PCR amplified from the *ompR* promoter. The probes were incubated with increasing concentrations of Lrp protein in the absence (53.2, 133, 266 nM: lanes 1-3) and presence of 5 and 20 mM L-leucine (266 nM Lrp, lanes 4 and 5). No complexes were formed, indicating that Lrp is unlikely to interact directly with the *ssrA* promoter. F denotes free probe. The *fimS* fragment of *E. coli*, used as a positive control, showed complex formation.

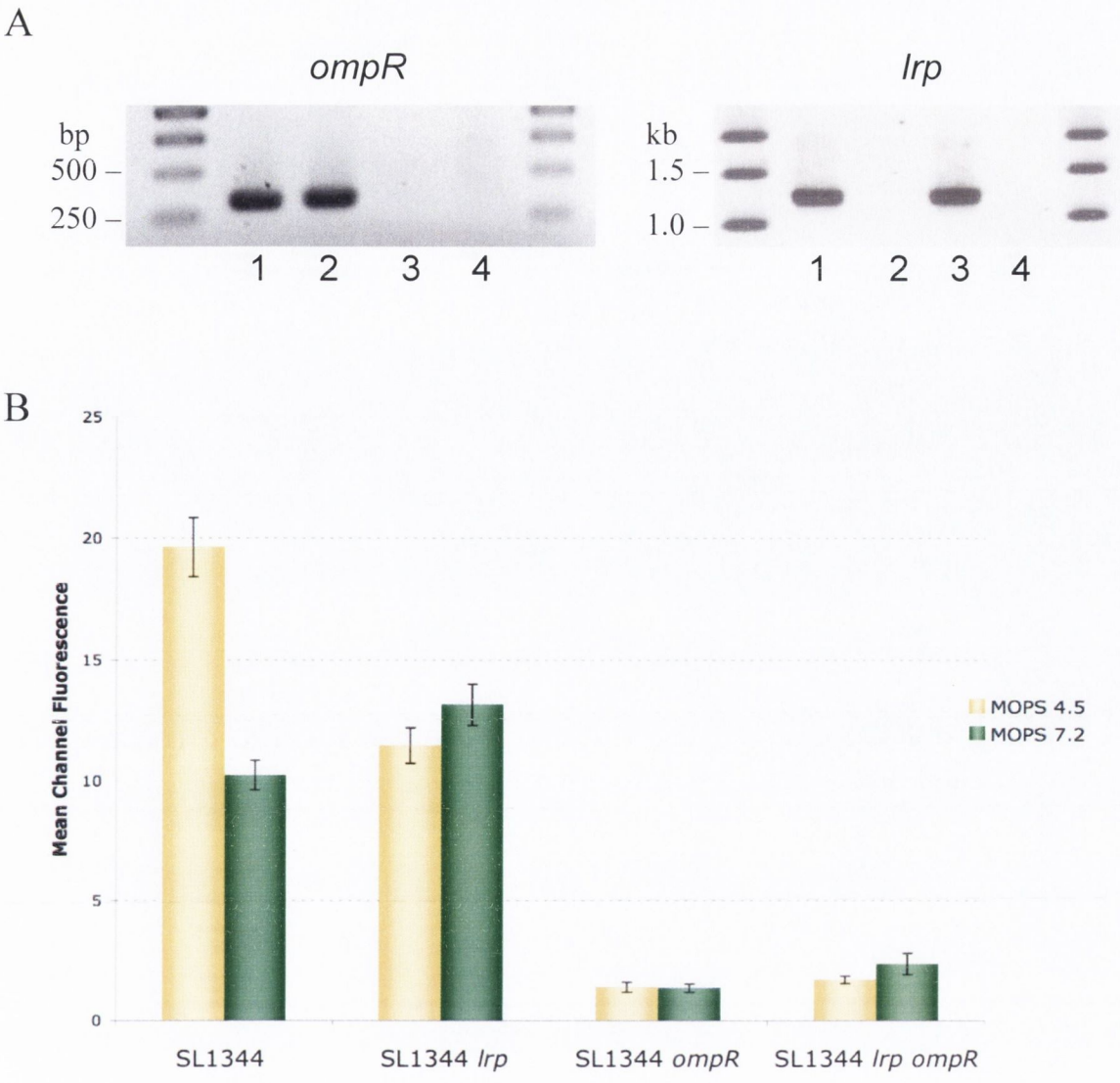


Fig. 6.4. Expression of *ssrA* in SL1344 *lrp ompR*. The *lrp ompR* double mutant construction was verified by PCR analysis (A). Primers to *ompR* and *lrp* were used to amplify products from SL1344, SL1344 *lrp*, SL1344 *ompR* and SL1344 *lrp ompR* genomic DNA (lanes 1-4). The *ompR* product could not be amplified from either the *ompR* (lane 3) or double mutant (lane 4). Similarly, the *lrp* product could not be amplified from either the *lrp* (lane 2) or double mutant (lane 4). Flow cytometry was used to examine the expression of *ssrA* in SL1344, SL1344 *lrp*, SL1344 *ompR* and SL1344 *lrp ompR* (B). Expression levels were as described previously in SL1344, SL1344 *lrp* and SL1344 *ompR:* the *lrp ompR* mutant showed no significant difference in expression to that of the *ompR* single mutant. Error bars denote ± SEM. Experiments were performed in triplicate at least three times: this figure shows typical results.

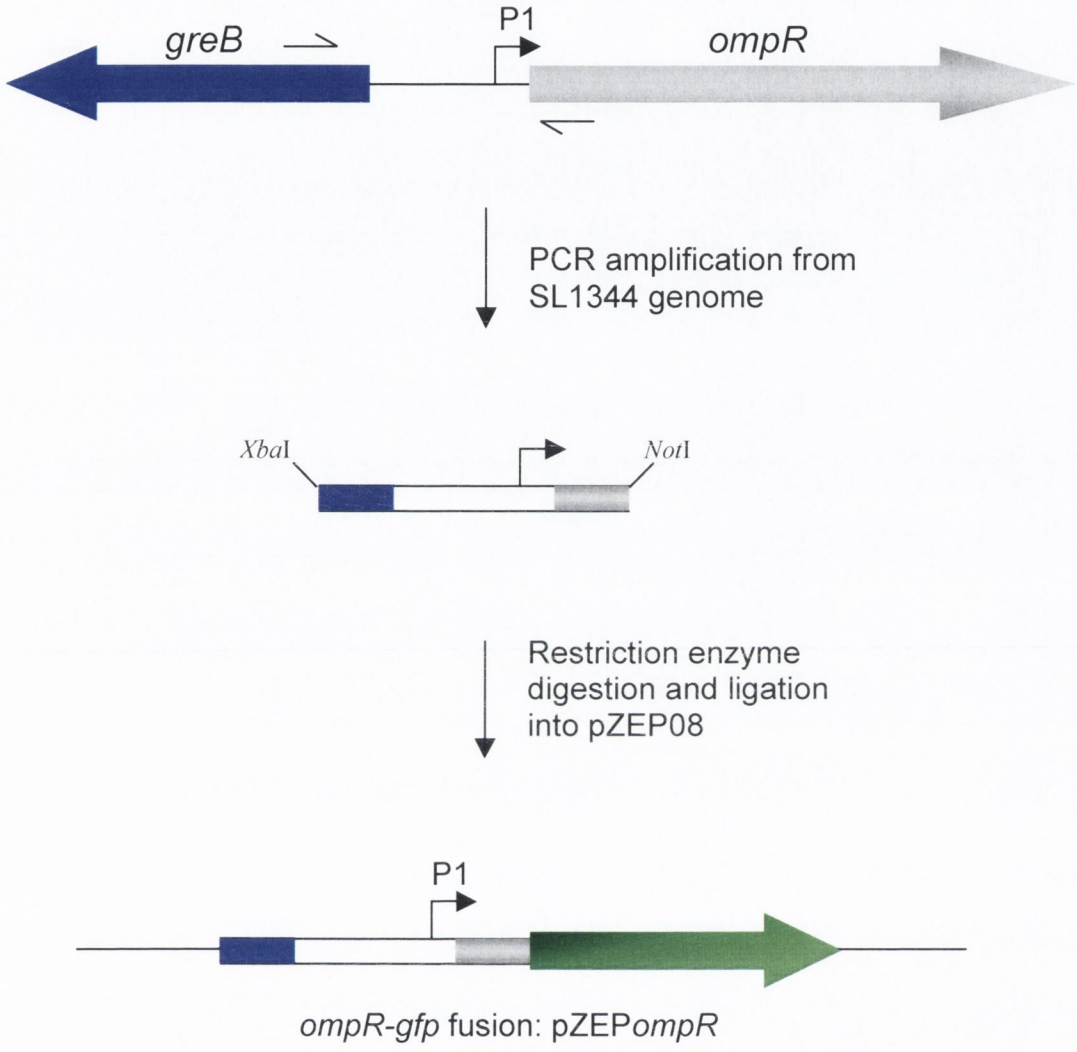


Fig. 6.5. Construction of the *ompR-gfp* reporter plasmid, pZEP*ompR*. The *ompR* regulatory region was PCR amplified from SL1344, digested with the appropriate restriction enzymes, and ligated into the same restriction sites in pZEP08, to form pZEP*ompR*.

the plasmid pZEP08. The structure of the resulting plasmid, pZEP*ompR* (Fig. 6.5), was verified by PCR and commercial sequencing.

6.2.6 The insensitivity of *ompR* to acidic pH in SL1344 *lrp*

To examine whether Lrp has an effect on *ompR* expression, bacteria containing the *ompR-gfp* transcriptional fusion were cultured as described in section 2.2.3. The bacteria were sampled for analysis by flow cytometry after 3 h growth in MOPS minimal broth at pH 4.5 or pH 7.2. *ompR* expression has been shown to be induced by 4 fold after incubation in acidic media (Bang *et al.*, 2002). Under the conditions in this assay, *ompR* was induced by 2 fold in SL1344 in pH 4.5 compared with pH 7.2. The reduced pH had no significant effect on *ompR* expression in SL1344 *lrp* (Fig. 6.6). From this experiment, it seemed that in SL1344 *lrp, ompR* was not regulated by acidic pH in the same way as in SL1344, where *ompR* displayed at least a 2-fold induction compared to neutral pH. Importantly, *ompR* expression levels were at the same level in SL1344 and SL1344 *lrp* at pH 7.2. This suggested that the increased level of *ssrA* expression in the *lrp* mutant was not a result of OmpR, since *ssrA* and not *ompR* levels were affected at pH 7.2. This raised the possibility that the lack of *ssrA* induction in SL1344 *lrp* in acidic pH, and the increased level of *ssrA* in SL1344 *lrp* at pH 7.2 were caused by different regulatory networks.

6.2.7 The effect of acidic pH on *lrp* expression

It was unclear how Lrp might affect the expression of *ssrA* and *ompR*. This was because the levels of *ssrA* were increased in the *lrp* mutant in pH 7.2 compared with the wild type, despite *ompR* levels remaining the same between the two strains. However, neither *ssrA* nor *ompR* expression levels were pH-responsive in the *lrp* mutant, which suggested that Lrp might have a role in the response of gene expression to acidic conditions.

To examine the *lrp* promoter activity in response to acidic pH, the *lrp-gfp* transcriptional fusion plasmid pZEP*lrp* was transformed into SL1344 and SL1344 *lrp*. The bacteria were cultured as before, and after 3 and 5 h incubation were analysed by flow cytometry for *gfp* reporter expression. This experiment, performed at least three times, showed that at pH 4.5, *lrp* promoter activity was strongly reduced compared with pH 7.2 (Fig. 6.7), which suggested that in SL1344, *lrp* expression was repressed at pH 4.5, compared to pH 7.2. This result was surprising, as Lrp is an important regulator during growth in

minimal medium conditions at neutral pH, where it is highly expressed. It appeared that acidic pH in minimal medium conditions led to > 2-fold repressed *lrp* expression levels in SL1344. As expected, at pH 7.2 the *lrp* promoter, which is subject to negative autoregulation, showed higher levels of activity in the *lrp* mutant than in SL1344 (shown in Chapter 3). However, at pH 4.5, the *lrp* promoter was 4-fold less active in SL1344 *lrp* than at pH 7.2. Strikingly, at pH 4.5 the *lrp* expression level was lower in SL1344 *lrp* compared with SL1344. This suggested that a factor other than Lrp strongly repressed *lrp* promoter activity at pH 4.5.

6.2.8 Lrp interacts directly with the *ompR* promoter

It had been shown that the *lrp* mutant did not show wild-type levels of *ompR* induction at an acidic pH. To examine whether Lrp could directly interact with the *ompR* promoter, EMSA analyses were performed. The *ompR* promoter probe was amplified from the SL1344 genome using biotinylated primer pair *ompR*-EMSA-F1 and *ompR*-EMSA-RBio.1. This produced a 335-bp fragment, called *ompR*-1 (Fig. 6.8 (A)). This fragment was incubated with increasing concentrations of Lrp protein, as described in section 2.15. An interaction of Lrp with this fragment was shown by complex formation, shown in Figure 6.8 (B).

To determine more accurately the position of Lrp binding, deletion analysis was employed. Two fragments of 163 bp, and 216 bp were amplified using primer pair *ompR*-EMSA-F2 and *ompR*-EMSA-RBio.1, and primer pair *ompR*-EMSA-F1 and *ompR*-EMSA-RBio.2, respectively. The positions of these fragments are shown relative to fragment *ompR*-1 in Figure 6.9 (A). The 163-bp fragment did not show an interaction with Lrp at any concentration tested, whereas the 216-bp fragment showed formation of two complexes (Fig. 6.9 (B)). A 124-bp fragment that was encompassed by the 216-bp fragment was PCR amplified using primer pair *ompR*-EMSA-F3 and *ompR*-EMSA-RBio.2. This fragment was incubated with increasing concentrations of Lrp, forming three complexes, and a putative fourth complex (lowest band: Fig. 6.9 (B)).

6.2.9 Mapping the Lrp binding sites at the *ompR* promoter

DNase I footprinting was employed to examine further the interaction of Lrp with the *ompR* promoter. Both coding and non-coding strands of the *ompR* promoter were analysed. The template plasmid for DNA sequencing and probe amplification was

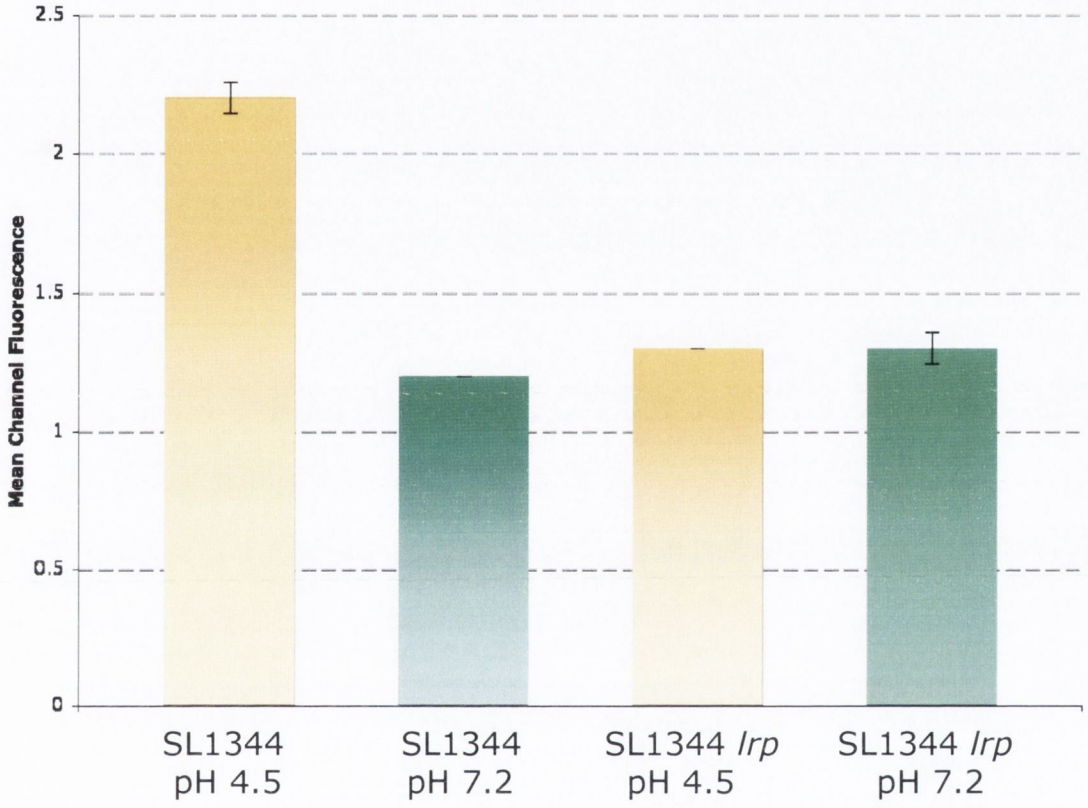


Fig. 6.6. The expression of *ompR* in SL1344 *lrp*. The ability of *ompR* to respond to acidic pH was examined in SL1344 and SL1344 *lrp*, in MOPS minimal medium at pH 4.5 and pH 7.2. Error bars denotes ± SEM. Experiments were performed in triplicate at least three times with similar trends. In SL1344 *lrp, ompR* expression displayed pH-insensitivity, and was not induced at acidic pH, whereas *ompR* expression increased by approximately 2 fold in SL1344.

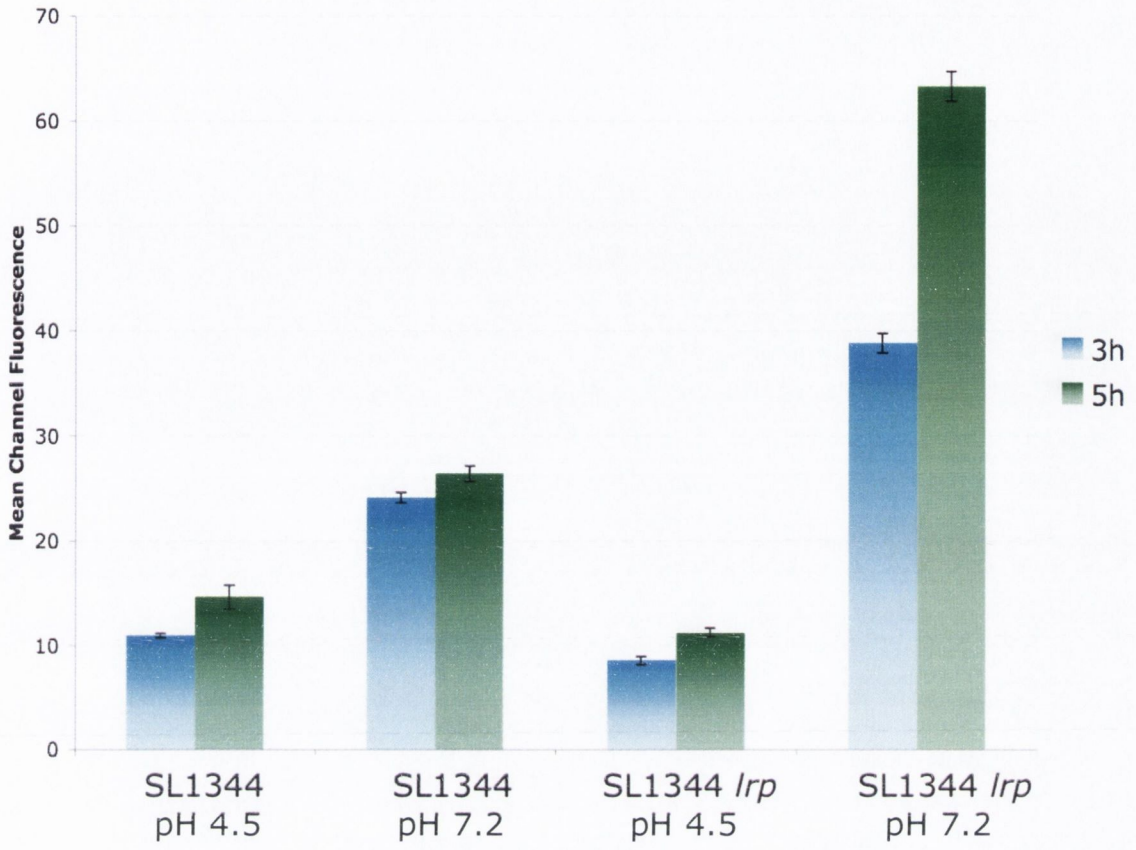


Fig. 6.7. The effect of acidic pH conditions on *lrp* expression. SL1344 and SL1344 *lrp* containing the *lrp-gfp* reporter fusion plasmid were grown in MOPS minimal media at pH 4.5 and pH 7.2, as described in Chapter 2, and sampled for analysis by flow cytometry after 3 and 5 h. Error bars denote ± SEM of triplicate samples. Experiments were performed at least three times. This experiment showed that *lrp* promoter activity is repressed by acidic pH.

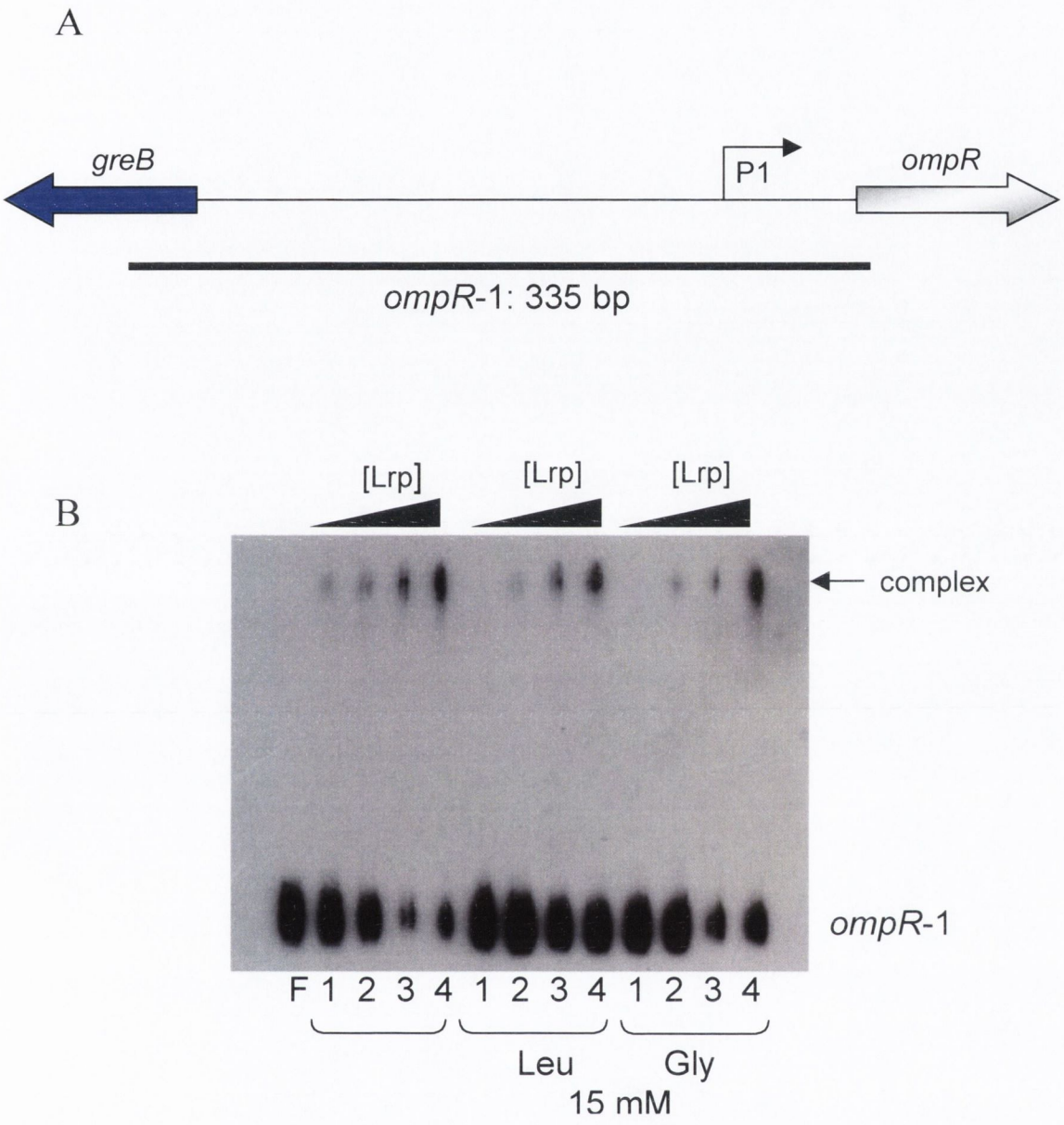


Fig. 6.8. EMSA analysis of the *ompR* promoter with Lrp. The fragment *ompR*-1, a 335-bp fragment encompassing the entire regulatory region of *ompR* (A), was incubated with increasing concentrations of Lrp protein (lanes 1-4: 66, 133, 266 and 332 nM) in the absence and presence of 15 mM L-leucine and glycine (B). Complex formation (arrowed) suggested a direct interaction of Lrp with the *ompR* promoter. The presence of amino acids did not appear to affect complex formation significantly. F denotes probe with no added amino acids or protein.

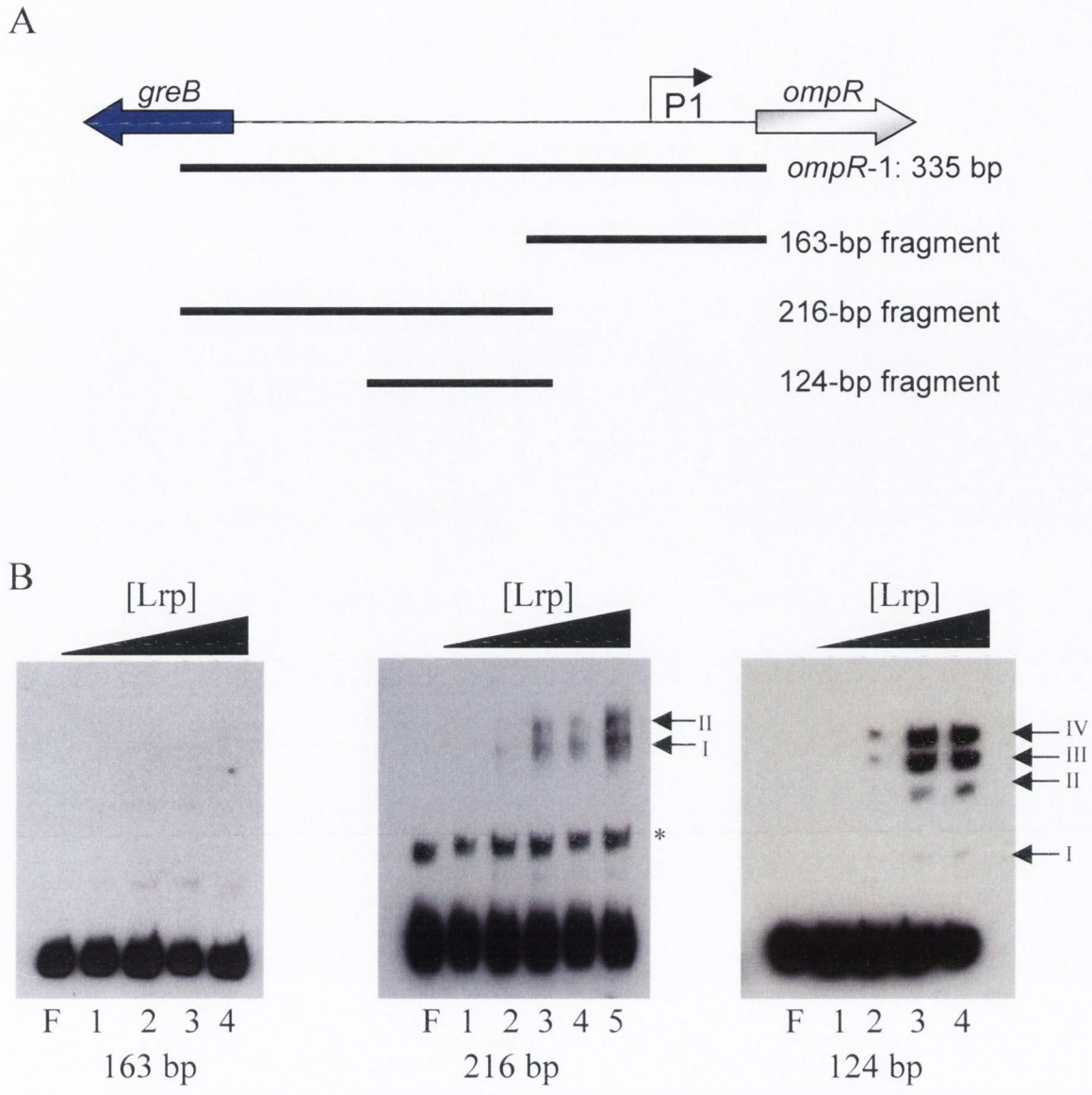


Fig. 6.9. Deletion analysis of the *ompR* promoter. EMSA analysis was performed on a series of *ompR* promoter probes, which were incubated with increasing concentrations of Lrp. The relation of these probes to *ompR*-1 is shown in A. The 163-bp fragment did not show any interaction (B) when incubated with 53, 133, 266 and 332 nM of Lrp (lanes 1-4). The 216-bp fragment showed formation of two complexes in the presence of 53, 133, 266, 332 and 400 nM Lrp, (lanes 1-5: arrowed) and a non-specific band (asterisk) was not affected. Three complexes (II-IV), and a putative fourth (I), were identified when the 124-bp fragment was incubated with 66, 133, 266 and 332 nM Lrp (lanes 1-4: arrowed). F denotes no added protein.

constructed by amplification of the region of interest from SL1344 genomic DNA using primer pair *ompR*-F.*Bam*HI and *ompR*-R.*Eco*RI, followed by restriction digestion with *Bam*HI and *Eco*RI. The resulting 290-bp fragment was ligated into pBluescript SK (–), forming plasmid pBSK*ompR*, the sequence integrity of which was verified by commercial sequencing. The coding strand was produced by amplification of a 435-bp fragment from pBSK*ompR* using primer pair *ompR*-R.*Eco*RI and pBSK-R. This was labelled at both ends with ^{32}P using T4 polynucleotide kinase, as described in section 2.17.2. One end of this probe was removed by restriction digestion with *Bam*HI, resulting in a 302-bp probe that was purified by polyacrylamide gel electrophoresis. The non-coding strand was produced by amplification of a 449-bp fragment from pBSK*ompR* using primer pair *ompR*-F.*Bam*HI and pBSK-F. This was labelled at both ends with ^{32}P using T4 polynucleotide kinase, followed by restriction digestion with *Eco*RI, resulting in a 302-bp probe that was also purified by polyacrylamide gel electrophoresis.

DNase I footprinting of the *ompR* coding strand revealed two small regions of protection, and some hypersensitive sites, which suggested DNA bending by Lrp (Fig. 6.10 (A)). The short protected regions occurred at –136 to –138, –117 and –118, and at –71 to –73. Hypersensitive bases, which became more intense with increasing concentrations of Lrp, occurred at –134, –91 and –82. Overall, the Lrp-mediated effect on the coding strand of the *ompR* promoter appeared to be limited to the region approximately between –70 and –140 on the coding strand, which correlated well with the EMSA analysis. The 124-bp fragment, which spans from –62 to –185, showed formation of three to four complexes with Lrp. Further upstream of –140, no visible Lrp-mediated differences were seen. By the highest concentration of Lrp (332 nM: far-right lane), the region between –70 and –140 appears to have increased protection, which can be seen by the disappearance of the labelled hypersensitive bases.

The non-coding strand of the *ompR* promoter did not show any visible regions of protection, but did show many hypersensitive bases, more so than the coding strand (Fig. 6.10 (B)). Hypersensitive sites were observed at –90, –124 and –123, –134, –177 and –178, –183, –192, –205, –224 and –225, and –234. The majority of the non-coding strand appeared to become more intense: this could have been due to slight overloading

of lanes, although some bands did not appear more intense (identified by asterisks in Fig. 6.10 (B)), which does not support overloading of lanes.

DNase I footprinting showed that the Lrp effect appeared to correlate well with the EMSA analysis. The irregular phasing of hypersensitive sites on both strands makes interpretation of the DNase I footprinting data difficult. In addition, large regions of protection are not visible in either strand: instead, short regions of protection are seen in the coding strand analysis only. However, this analysis, along with the supporting EMSA data, clearly showed an interaction of Lrp with the *ompR* promoter.

6.2.10 Examination of regulatory influences at the *ompR* promoter

H-NS is a negative regulator of *ompR* expression from the acid-inducible P2 promoter (Bang *et al.*, 2002). The position of H-NS binding on the *ompR* promoter is unknown, however. H-NS is known to bind preferentially to intrinsically curved DNA. Analysis of the intrinsic curvature of the *ompR* promoter using the Bend.it web-based program was used to identify regions that are likely to bind H-NS (Fig. 6.11). The positions of OmpR-P binding overlap the putative H-NS binding regions. OmpR-P is required for *ompR* expression from the P2 promoter, and it is thought to act by causing local DNA structural remodelling, leading to H-NS displacement (Bang *et al.*, 2002). The position of the Lrp-binding region of the *ompR* promoter is also shown in Fig. 6.11.

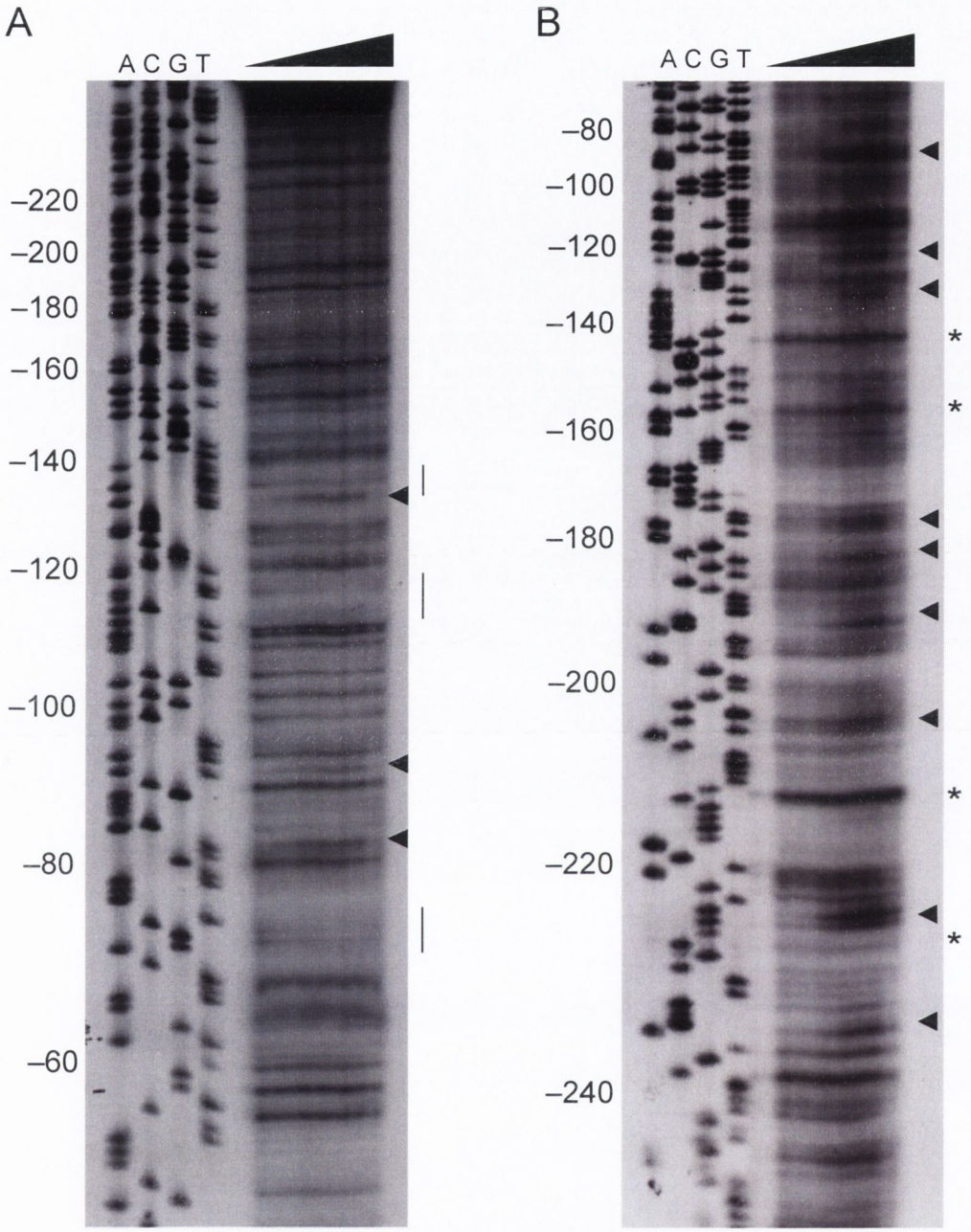
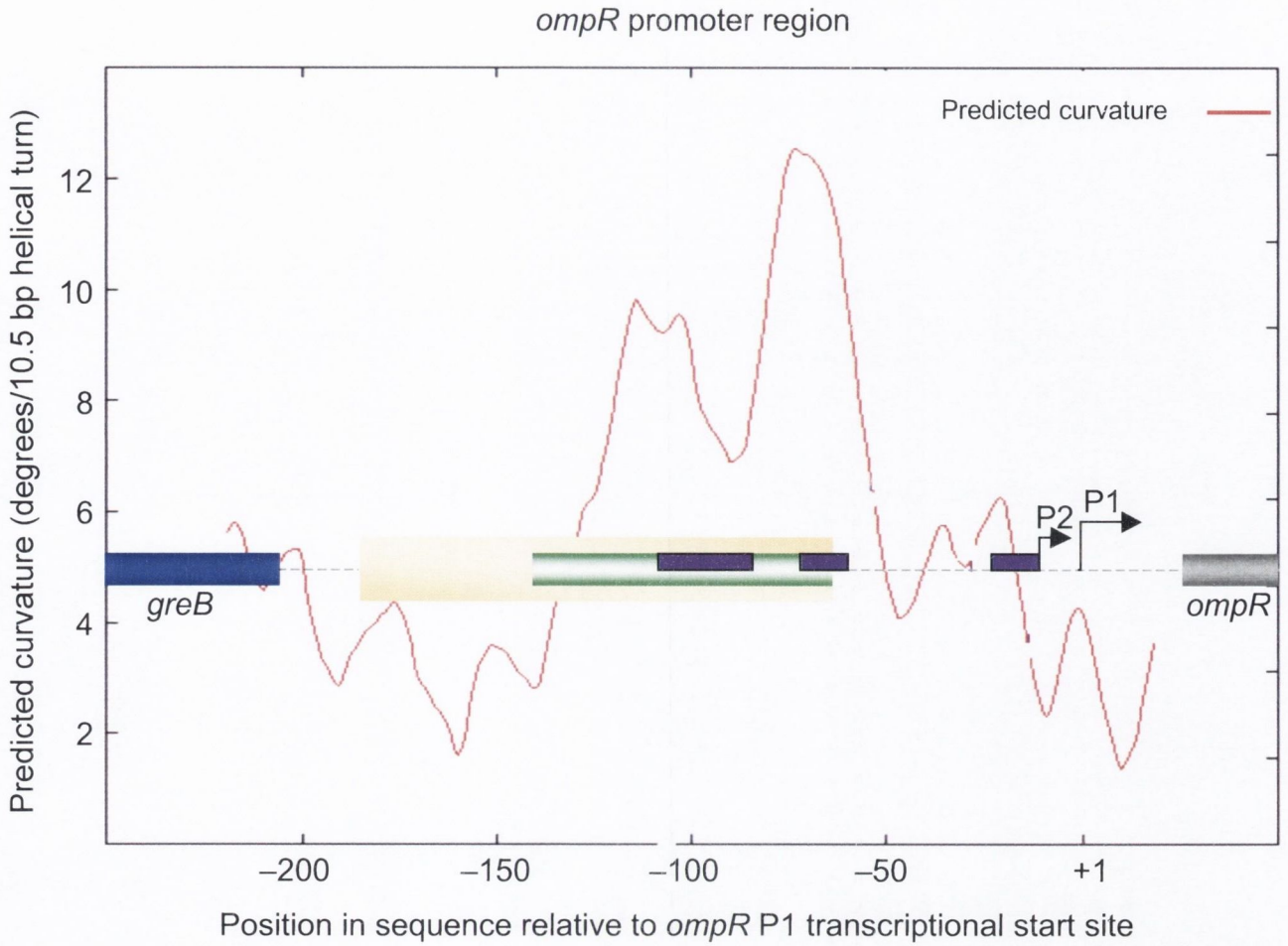


Fig. 6.10. DNase I footprinting of Lrp on the *ompR* promoter. Both coding (A) and non-coding (B) strands were analysed. Increasing concentrations of Lrp (0, 66, 133, 200, 266, 332 nM) were incubated with the corresponding probe and treated as described in Chapter 2. Protected regions are shown by vertical lines, hypersensitive bases are denoted by arrowheads, and unaffected bases (see text) are highlighted with an asterisk. The corresponding DNA sequence, in both cases, is alongside.

Fig. 6.11. Analysis of regulatory factors at the *ompR* promoter. The DNA sequence is numbered with respect to the P1 transcriptional start site (+1). OmpR binding-sites are shown by purple bars, the large arrow indicates the *ompR* promoter (P1) start site, while the small arrow indicates the position of the acid-responsive P2 promoter (Bang *et al.*, 2002). The green bar denotes the *ompR* promoter region on the coding strand that shows an interaction with Lrp, as demonstrated by DNase I footprinting analysis. The yellow bar highlights the region encompassed by the 124-bp fragment of the *ompR* promoter, demonstrated by EMSA analysis to form four complexes with Lrp. H-NS has been shown to regulate the P2 *ompR* promoter negatively. Predicted curvature (red trace) of the *ompR* promoter sequence of over 5 ° per 10.5 bp helical turn (dashed line) suggests an intrinsic curve in DNA structure, suggestive of DNA binding by H-NS, which may elucidate the position of H-NS binding on the *ompR* promoter.


ompR promoter region
Predicted curvature
Predicted curvature (degrees/10.5 bp helical turn)
12
10
8
6
4
2
P2
P1
greB
ompR
–200
–150
–100
–50
+1
Position in sequence relative to *ompR* P1 transcriptional start site

6.3 Discussion

DNA microarray analysis suggested that the expression of some SPI-2 genes was increased in logarithmic phase, and decreased at stationary phase, during growth in MOPS minimal media in SL1344 *lrp* compared with SL1344 (Chapter 4). RT-PCR analyses confirmed this data and showed that *ssrA,* which encodes an essential positive regulator of SPI-2 genes, was also affected in this way (Fig. 6.1). *ssrA* expression was induced by acidic pH in minimal media in SL1344, as had been described previously (Lee *et al.*, 2000), however, in the *lrp* mutant, the levels of *ssrA* appeared high at both neutral and acidic pH (Fig. 6.2). This suggested two hypotheses, which revealed two novel putative roles for Lrp in SPI-2 gene regulation.

Firstly, the *ssrA* promoter appeared to be insensitive to acidic pH in the *lrp* mutant, as it did not show wild type levels of induction in pH 4.5 compared with pH 7.2 (Fig. 6.2). Examination of *ompR* and *lrp ompR* mutants showed that the level of *ssrA* expression was not significantly different between the two strains, which suggested that the effect of Lrp on *ssrA* acid insensitivity might be dependent on OmpR. OmpR is an acid-sensitive response regulator that is important for the expression of *ssrA* (Lee *et al.*, 2000). Comparisons of *ompR* expression in minimal media revealed that *ompR* promoter activity increased approximately 2 fold at acidic pH compared with neutral pH in SL1344, whereas in SL1344 *lrp, ompR* expression levels were unaffected by pH (Fig. 6.6). This was consistent with the lack of induction of *ssrA* in acidic pH in SL1344 *lrp.* Taken together, it appeared that the *lrp* mutant could negatively affect the sensitivity of *ompR* expression to acidic pH, which in turn, might have been responsible, at least in part, for the insensitivity of *ssrA* to acidic pH.

This suggested that Lrp might have an important role in the response of *ompR* to acid. It is known that *ompR* has two promoters: a constitutive promoter, P1, and an acid inducible promoter, P2 (Bang *et al.*, 2002). It was considered possible that Lrp might directly interact with the *ompR* promoter, to affect positively the transcription of the acid inducible promoter. EMSA analysis was performed with the *ompR* promoter, and this showed that Lrp could interact with a 124-bp region from –185 to –62 from the *ompR* P1 transcription start site, which is positioned approximately 21 bp from the *ompR* ORF start site (Bang *et al.*, 2002), shown in Figure 6.9. Lrp did not interact with another *ompR* promoter probe spanning from –105 to +58 (163-bp probe), which suggested that

the 80 bp region from –185 to –105 was essential for Lrp binding. Between three and four complexes were formed with the 124-bp probe, which suggested the interaction of three to four Lrp dimers with the *ompR* promoter region (Fig. 6.9). This suggested that the Lrp octamer is capable of binding to a 124-bp fragment, in a manner similar to that shown in Chapter 3 (Fig. 3.16), whereby the fourth dimer may be involved in non-specific interactions with DNA. DNase I footprinting analysis was employed to understand further the interaction of Lrp with the *ompR* promoter (Fig. 6.10). DNase I footprinting showed that the Lrp effect appeared to correlate well with the EMSA analysis. The irregular phasing of hypersensitive sites on both strands makes interpretation of the DNase I footprinting data difficult. However, this irregular phasing was also observed in DNase I footprinting of the *lrp* non-coding strand (Chapter 3), and had been described previously by Wang *et al.* who performed DNase I footprinting on the *lrp* promoter in *E. coli* (Wang *et al.*, 1994). In addition, large regions of protection are not visible in either *ompR* promoter strand: instead, short regions of protection are seen in the coding strand analysis only.

In this study, it is hypothesised that Lrp acts as a regulator of *ompR* expression under acidic conditions, by binding upstream of the *ompR* promoter region to influence transcription. How Lrp might regulate *ompR* is not understood: *ompR* appears to require Lrp for its expression as the *lrp* mutant does not show induction of *ompR* at acidic pH (Fig. 6.6). However, comparisons of *lrp* and *ompR* expression at pH 4.5 in SL1344 contradict this. At pH 4.5 in SL1344 the decrease in *lrp* expression appears to coincide with an increase in *ompR* expression (Figs. 6.6 and 6.7). This hints that the decreased level of *lrp* could account for the increased level of *ompR* promoter activity at pH 4.5, and that Lrp acts as a negative regulator: however, in the *lrp* mutant, where Lrp is absent, the levels of *ompR* expression are not constitutively high as would be expected if this were the case. Clearly, this regulation is more complex than first expected. It is thought that the *ompR* promoter may respond sensitively to levels of Lrp and other signals to accurately regulate the *ompR* gene in response to acidic pH.

H-NS acts as a negative regulator of *ompR* expression from the acid-inducible P2 promoter (Bang *et al.*, 2002). OmpR-P is required for *ompR* expression from the P2 promoter, and is thought to act by causing local DNA structural remodelling, leading to H-NS displacement (Bang *et al.*, 2002). The position of H-NS binding is unknown,

however, H-NS binds preferentially to curved DNA. Analysis of the intrinsic curvature of the *ompR* promoter can identify regions that are likely to bind H-NS (Fig. 6.11). Analysis of the *ompR* promoter places the Lrp binding region in region overlapping the H-NS binding region (Fig. 6.11). Perhaps Lrp has a role in the displacement of H-NS at the *ompR* promoter. Presumably, given that *ompR* does not respond to acidic pH in the *lrp* mutant, the effect of Lrp would be in promoter P2. It is possible that Lrp could affect *ompR* expression more dramatically from either the P1 or P2 promoter, under other conditions not identified in this study. CRP, the catabolite repressor protein, is a regulatory protein that senses glucose starvation. Like Lrp, CRP responds to nutritional signals and regulates metabolism accordingly. Cyclic AMP (cAMP) is synthesised from ATP by adenylate cyclase in response to the absence of glucose. The binding of cAMP leads to CRP activation. In *E. coli,* CRP is a dual regulator of *ompR* expression, where two transcripts are regulated positively in a CRP-dependent manner in response to cAMP, and two are regulated negatively in response to cAMP-CRP (Huang *et al.*, 1992). This gives an example of *ompR* regulation in response to nutrient availability.

The second hypothesis highlighted by this study suggested that Lrp might act as a negative regulator of *ssrA* expression at pH 7.2. However, EMSA analyses revealed that Lrp did not directly interact with the *ssrA* promoter (Fig. 6.3), which suggested that the effect of Lrp on *ssrA* was indirect. It became apparent that the effect of Lrp on the acid sensitivity of *ompR* could not explain why *ssrA* levels were higher in the *lrp* mutant at neutral pH than in SL1344: *ompR* levels were comparable in SL1344 and SL1344 *lrp* at pH 7.2 (Fig. 6.6). Thus, increased *ssrA* expression at pH 7.2 was unlikely to be dependent on OmpR. This suggested that Lrp is a negative regulator of *ssrA* at pH 7.2, acting indirectly, as Lrp did not interact with the *ssrA* promoter. An alternative explanation is that SL1344 *lrp* has increased phosphorylation of OmpR at pH 7.2, which could increase the levels of active OmpR-P in the cell, and lead to an increase in *ssrA* expression. This possibility has not been examined. DNA supercoiling has been shown to affect *ssrA* expression: more relaxed DNA leads to increased expression of *ssrA* (Ó Cróinín *et al.*, 2006). Another possibility is that Lrp requires another factor to allow binding at the *ssrA* promoter. Alternatively, Lrp may regulate positively a repressor of *ssrA* expression, or may negatively regulate an activator of *ssrA* other than OmpR. A potential candidate for this is SlyA, which has been shown to act positively on SPI-2 gene expression through *ssrAB* (Linehan *et al.*, 2005).

It is noteworthy that *lrp* expression itself was also affected by acidic pH. At pH 4.5 the level of *lrp* promoter activity was repressed by >2 fold in SL1344, and by > 4 fold in SL1344 *lrp*, compared to the levels at pH 7.2 at both 3 h and 5 h (Fig. 6.7). In addition, the level of *lrp* activity was repressed more strongly at pH 4.5 in the *lrp* mutant than in the wild type, which suggested that a factor other than Lrp was leading to the strong repression of the *lrp* promoter. This was a very interesting result, as Lrp is an important regulatory protein during growth in minimal media. As shown in Chapter 3, levels of Lrp are highest during growth in nutrient-poor media, and the *lrp* mutant doubled 1.4 times more slowly than SL1344, in MOPS minimal medium. It is intriguing that at an acidic pH, the level of *lrp* expression would be reduced. Perhaps the acidification and subsequent reduction of *lrp* expression represents an important signal during growth in nutrient-poor conditions, such as that of the intracellular environment, with particular reference to the phagolysosome. The impact that acidic pH on *lrp* expression in minimal media would have on global gene expression is unknown. A previous study that examined the expression profile of *S.* Typhimurium during macrophage infection showed that the expression of acid response genes, *cadB, cysB* and *adiY* were increased, whereas *lrp* gene expression was decreased, compared to cells grown in cell culture media (Eriksson *et al.*, 2003). It is tempting to hypothesise that this down-regulation of *lrp* may represent the effect of acidic pH on *lrp* expression. It is also not known how acidic pH exerts its effect on *lrp* expression. A study examining reporter plasmids has shown that external pH affects DNA supercoiling in *S.* Typhimurium, resulting in decreased DNA supercoiling (Karem and Foster, 1993), therefore, it may be possible that DNA supercoiling changes may affect *lrp* expression.

Interestingly, this was not the first time that Lrp has been linked with genes involved with acidic conditions. In *Vibrio vulnificus*, CadC is an activator of pH-dependent expression of the *cadBA* operon, which encodes a lysine/ cadaverine antiporter and a lysine decarboxylase, which counteract acidic conditions in the cell. Lrp has been implicated as a coregulator of *cadBA,* as putative Lrp binding sites have been identified in the *cadBA* promoter region (Rhee *et al.*, 2005).

GadE (previously called YhiE) of *E. coli* is a regulator of gene expression in the acid response, and when overexpressed leads to increased resistance of *E. coli* to highly acidic conditions (Hommais *et al.*, 2004). Overexpression of GadE at pH 5.5 induced

genes that are known to be involved in the acid response of *E. coli*, such as *hdeAB, gadA* and *gadX,* but also induced the expression of *lrp* by approximately 3 fold (Hommais *et al.*, 2004). A putative binding site for GadE at the *lrp* promoter was assigned by Hommais *et al.* However, there is no GadE homolog in *S.* Typhimurium. Microarray analysis was performed in *E. coli* W3110 and the isogenic *lrp* mutant by Tani *et al.* This study was performed in MOPS minimal medium with bacterial cells grown to early logarithmic phase. Tani *et al.* found that a number of acid stress genes were affected by the *lrp* mutation. These genes included *hdeAB, osmY, dps, gadA* and *sodC*, which were all induced in the *lrp* mutant under the conditions tested (Tani *et al.*, 2002).

What is the physiological relevance of Lrp as a regulator of virulence factors? Lrp is traditionally known as a regulator of amino acid metabolism: however, it also regulates virulence factors such as fimbriae. Lrp is capable of sensing nutrient availability, through binding of effector molecules such as leucine and alanine, and it is positively regulated in response to low nutrient availability and low growth rate. Nutrient availability is an important environmental signal, enabling the bacterium to adjust its metabolism accordingly. In the host, the ability to sense nutrient content, along with other environmental conditions such as temperature and pH, allows the bacterium to regulate the appropriate virulence factors. Given the diverse niches that are occupied by *S. enterica,* it is likely to have a discrete set of environmental cues: perhaps during growth in minimal medium, allowing *lrp* expression to be reduced by acidic pH acts as a unique signal in a particular niche. Presumably, Lrp acts in concert with other regulatory proteins to coordinate gene expression efficiently.

Chapter 7

Phage-mediated lysis of *Salmonella enterica* serovar Typhimurium during growth in nutrient-poor medium

7.1 Introduction

When a temperate phage enters a bacterial cell, the cell encounters two pathways. One pathway leads to phage replication and bacterial cell lysis, termed the lytic cycle. The other pathway leads to lysogeny: the phage DNA may enter the bacterial chromosome and become stably inherited, where it exists in an autorepressed state. Most temperate phages express functions that prevent superinfection of the lysogenic cell. Autorepressed lysogenic phages are also called prophages. Some prophages are not integrated into the bacterial chromosome but are maintained as plasmid molecules, such as phage P1 of *E. coli* (Li *et al.*, 2004) or, rarely, as linear plasmids with covalently closed ends, such as the *E. coli* phage N15 (Ravin, 2003). Prophages have the ability to enter the lytic cycle, leading to phage replication and bacterial cell lysis. Lysogeny can be terminated if the lysogenic bacterium undergoes certain stresses. This change from the lysogenic to lytic cycle is called induction. Induction can be caused by environmental stress, UV exposure and DNA-damaging agents that induce the SOS response, for example mitomycin C. In addition, induction can also be caused by starvation stress: lambdoid prophages can be induced in auxotrophic bacteria by omission of amino acids and thymine (Melechen and Go, 1980; Rudin and Lindberg, 1975).

S. Typhimurium carries a number of prophages in its genome. The *S.* Typhimurium strain LT2 contains the prophages *Fels*-1 and -2, and *Gifsy*-1 and -2 (Figueroa-Bossi *et al.*, 2001). The newly sequenced strain SL1344 is known to contain the phages *Gifsy*-1 and -2, *sopE*Φ and ST64B (Alonso *et al.*, 2005; Figueroa-Bossi *et al.*, 2001). ST64B is a lambdoid phage that is lysogenic in SL1344 and is integrated within the *serU* serine tRNA gene (Figueroa-Bossi and Bossi, 2004). It is a mosaic phage which is composed of genes from other phages of diverse bacterial groups (Mmolawa *et al.*, 2003). ST64B can be induced by treatment with mitomycin C, causing lysis of the bacterial host. However, ST64B is tailless and is therefore unable to produce infectious virions, but it can occasionally revert to produce functional phage particles (Mmolawa *et al.*, 2003). The ORFs SB13-25 of ST64B form the tail operon. The contiguous ORFs of SB21 and SB22 were shown to be similar to two halves of an uninterrupted ORF in phages P27 and Mu (Figueroa-Bossi and Bossi, 2004). It is believed that a frameshift mutation in a G:C run within the SB21 ORF leads to a stop codon, which terminates SB21 translation. Reversion of this frameshift mutation leads to translation of the SB21 and SB22 genes as

one ORF, SB21*, which leads to the production of infectious phage particles (Figueroa-Bossi and Bossi, 2004).

P22 is a well characterised, temperate lambdoid phage of *S.* Typhimurium that is widely used in *Salmonella* molecular genetics studies as it is capable of generalised and specialised transduction of bacterial DNA (Susskind and Botstein, 1978). Generalised transduction involves the uptake of host chromosome rather than the phage concatamer. Generalised transduction is more extreme in HT (high transducing) mutants of P22 (see Chapter 2). Specialised transduction occurs when bacterial genes are incorporated into the genome of the temperate phage, and carried from one host to another.

This chapter reports on the apparent induction and isolation of a hybrid P22-like phage from *S.* Typhimurium strain SL1344, during growth in minimal medium.

7.2 Results

7.2.1 Autolysis of SL1344 during growth in minimal media

In order to examine the effect of Lrp on gene expression in *S.* Typhimurium, it was necessary to perform microarray analysis on SL1344 and SL1344 *lrp* in both rich medium (Luria-Bertani) and nutrient-poor minimal medium (MOPS minimal medium). This is because Lrp is a known regulator of many genes involved in metabolism and nutrient availability, and in particular, the availability of amino acids. SL1344 and the *lrp* mutant were grown overnight in 3 ml L broth. This was to reduce stress on the bacteria, and thus the likelihood of compensatory mutations in SL1344 *lrp*. The cells were washed in MOPS minimal medium (MMM) twice to remove residual nutrients, the optical density equalised by OD_{600}, and then used to inoculate 25 ml MMM by 1:100 dilution. Measurements of turbidity of bacterial growth in MOPS minimal medium were taken at OD_{420} as described previously (Neidhardt *et al.*, 1974).

It was observed that the cultures grew as expected occasionally, with the *lrp* mutant displaying a strong growth defect in MMM (Fig. 7.1 (A)). More often, however, with the same batch of MMM (stored overnight at 4 °C, without addition of glucose) and the same source of bacteria, an apparent lysis of both SL1344 and SL1344 *lrp* was observed (Fig. 7.1 (B)). This result, described as autolysis, had been observed previously by collaborators working with SL1344 (S. Lucchini, personal communication). This was repeated a number of times, using freshly made media and freshly recovered bacteria from frozen stocks. Each time, at different timepoints in logarithmic phase, lysis was observed.

7.2.2 Confirmation of the presence of bacteriophage

SL1344 was grown to exponential phase in LB medium and 500-µl volumes were lawned onto L plates and dried. Spent broth from lysed MMM cultures was cleared of cell debris by centrifugation at 16,000 x g for 5 min. Ten-fold dilutions of the supernatant from 10^{-1} to 10^{-6} were made in sterile L broth and 20-µl volumes of each dilution were plated onto the lawned SL1344. Plates were incubated at 37 °C overnight. Plates were examined the following day: the presence of plaques on the lawned SL1344 identified the presence of phage particles in the spent MMM broth. This showed that the phage suspension contained 10^7 p.f.u. ml^{-1}. Titrations were also performed on *S.*

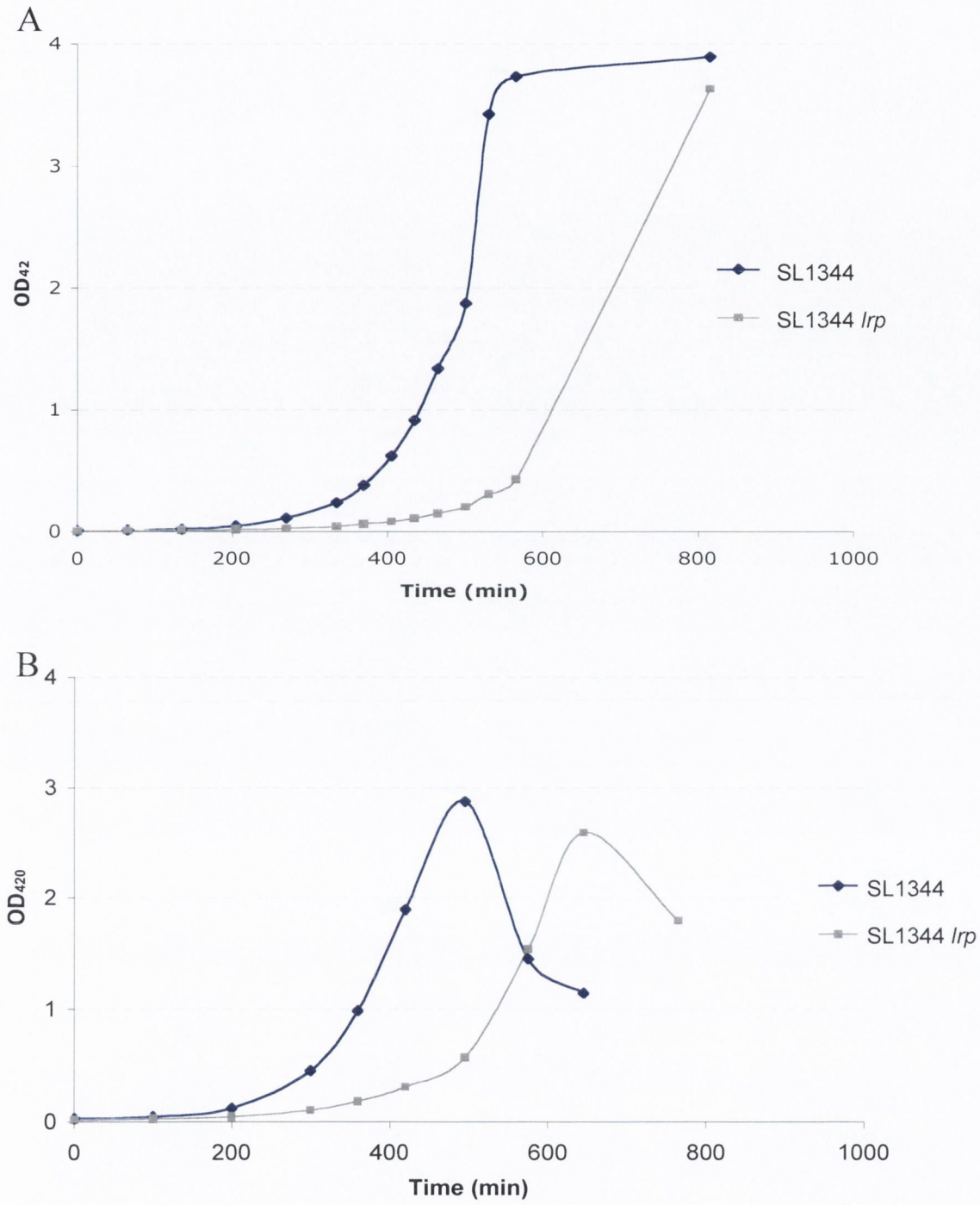


Fig. 7.1. Lysis of SL1344 and SL1344 *lrp* in MOPS minimal medium. Growth in MOPS minimal medium growth curve was sometimes successful (A), but attempts to reproduce the growth curves showed dropping OD_{420} values, indicative of bacterial cell lysis (B).

Typhimurium strains LT2 and CH133 (*galE*). This showed that the same phage suspension gave 10^{10} p.f.u. ml^{-1} on lawned LT2, suggesting that LT2 was more sensitive to the phage. Interestingly, CH133 did not support plaques, which suggested that the *galE* mutant was resistant to phage infection. Mutants in *galE* lack the O-antigen of LPS, which is a known receptor for some *Salmonella* phages, including P22 (Susskind and Botstein, 1978).

7.2.3 Analysis of phage contamination causing SL1344 lysis in MMM

Plaque assays confirmed the presence of phage particles in lysing SL1344 cultures. A number of measures were undertaken to find the source of contamination. Firstly, to examine whether the glassware being used for growth curves was the source of contamination, sterile irradiated 50 ml polypropylene tubes (BD Biosciences) were used for bacterial growth. Growth curves were plotted as before, and showed similar trends, with lysis occurring in the logarithmic phase. To examine the possibility of phage contamination of the frozen bacterial stocks, single colonies were used to produce 3 ml overnight cultures, which were then used to inoculate either MMM, or LB medium, as before. This led to lysis in MMM, but not in LB medium (Fig. 7.2 (A)). This suggested that the phage were endogenous and induced in MMM, but not LB. An alternative possibility is that phage particles associated with the bacterial cells could gain entry to the cell through a porin or other cell surface appendage only expressed during bacterial cell growth in MMM. To examine this, phage lysates from spent MMM cultures were inoculated into LB broth cultures of SL1344. These exponentially growing LB cultures cleared of turbidity, indicative of phage lysis. This suggested that the phage was capable of entry to the bacterial cell during growth in either LB or MOPS minimal media.

Two methods were used to examine whether the minimal medium being used was contaminated with phage particles. Firstly, M9 minimal medium salts obtained from another source were used in growth experiments. These results show that lysis of SL1344 also occurred in M9 minimal medium (Fig. 7.2 (B)). To examine whether components of the minimal media used were contaminated with exogenous phage, MMM was prepared as before. Bacterial cells were grown in either LB medium inoculated with MMM, or in MMM medium. Bacterial cells grown in LB medium did not lyse, whereas those cultured in MMM displayed lysis. This suggested strongly that the MMM medium does not contain phage particles, and that contamination by phage is

not supported by the above results. However, the lysis observed appeared to be limited to growth in minimal medium salts.

7.2.4 Purification of bacteriophage particles

In order to identify the phage isolated from lysed broth cultures of SL1344, large-scale batch culture was grown and, when bacterial lysis was observed, the culture was treated as described in section 2.21. Purified phage particles were analysed by SDS-PAGE for their protein content (Fig. 7.3 (A)). This identified a major band of approximately 32.5 kDa, and three smaller, less prominent bands from between 32 and 25 kDa. Two bands with a higher molecular mass were also identified, at approximately 55 and 60 kDa.

7.2.5 Analysis of bacteriophage DNA

Bacteriophage DNA was isolated as described in section 2.21.2. To examine the genome size of the isolated phage DNA, the phage DNA was analysed by agarose gel electrophoresis. This indicated that the phage genome was over 10 kb in size (Fig. 7.3 (B)). Therefore, pulsed-field gel electrophoresis (PFGE) analysis was employed as described in section 2.21.3. PFGE is used to analyse the length of DNA fragments of large sizes. During PFGE, DNA is forced to change direction during electrophoresis, which allows large DNA fragments to separate from each other (Schwartz and Cantor, 1984).

PFGE analysis demonstrated that the genome size of the phage isolated from SL1344 was greater than 38 kb (Fig. 7.3 (C)). The phage DNA was also examined by restriction enzyme digestion using a range of enzymes; *Eco*RI, *Eco*RV, *Bam*HI, *Hin*dIII, *Sac*II and *Sau*3AI; and analysed by agarose gel electrophoresis. This demonstrated that *Eco*RV digested the phage genome into segments of under 10 kb (Lane 2: Fig. 7.4 (A)).

7.2.6 Sequence analysis of bacteriophage DNA fragments

To examine the bacteriophage DNA sequence in more detail, the *Eco*RV restriction digestion fragments of purified phage DNA were ligated into the *Eco*RV restriction site in pBluescript SK II (–), which had been phosphatase-treated. The ligation reaction was transformed into competent XL1-Blue cells, examined by blue-and-white screening, and 20 plasmid products purified. The insert sizes were examined by PCR using primer pair pBSK-F and pBSK-R (Fig. 7.4 (B)). Ten plasmids with different sized inserts, pPHI-1,

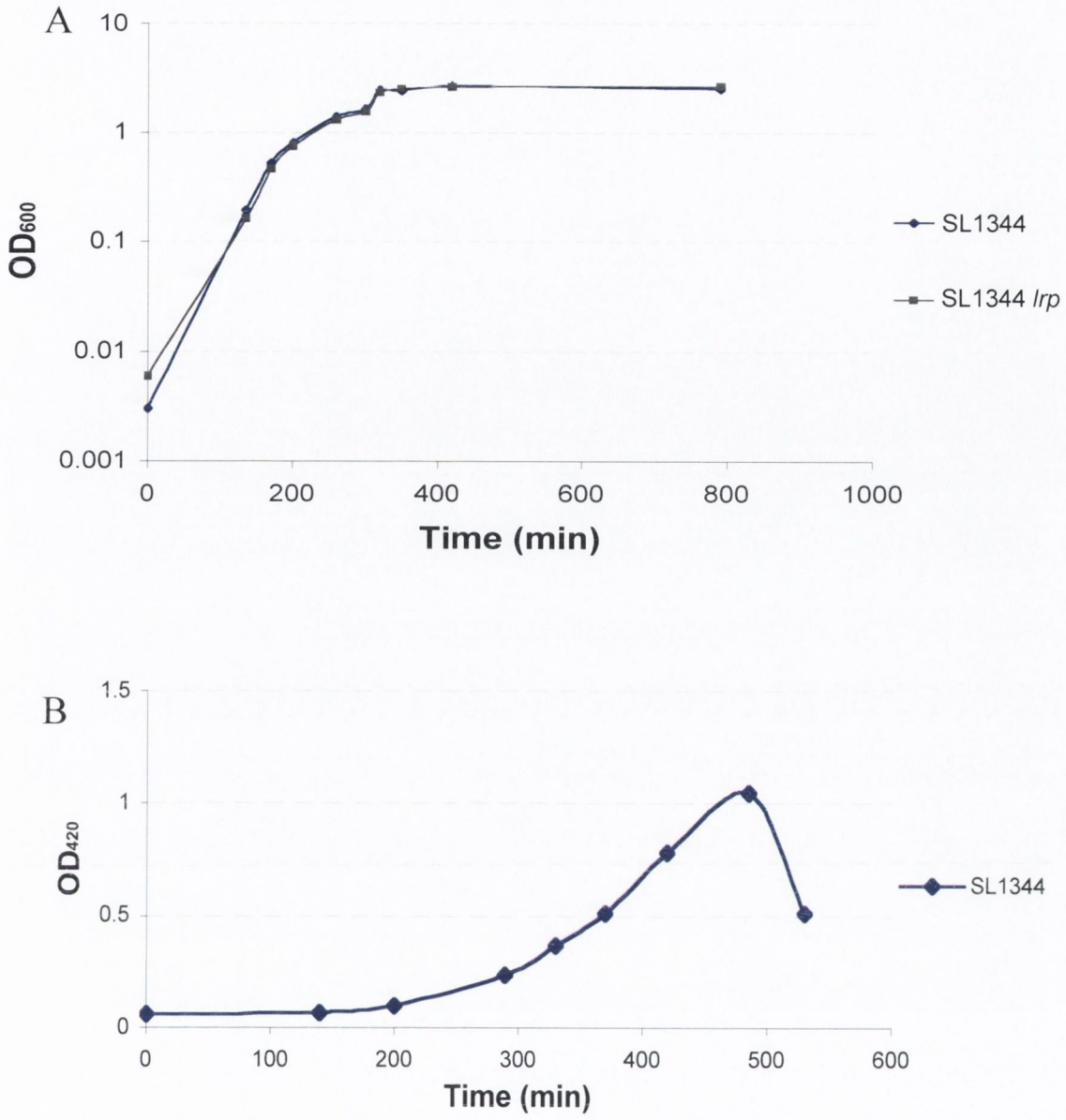


Fig. 7.2. Autolysis of SL1344. SL1344 and SL1344 *lrp* from a source that had previously been shown to lyse were inoculated into L broth, and showed normal growth (A). SL1344 was also grown in M9 minimal medium and showed lysis (B), as had also been shown in MOPS minimal medium (Fig. 7.1).

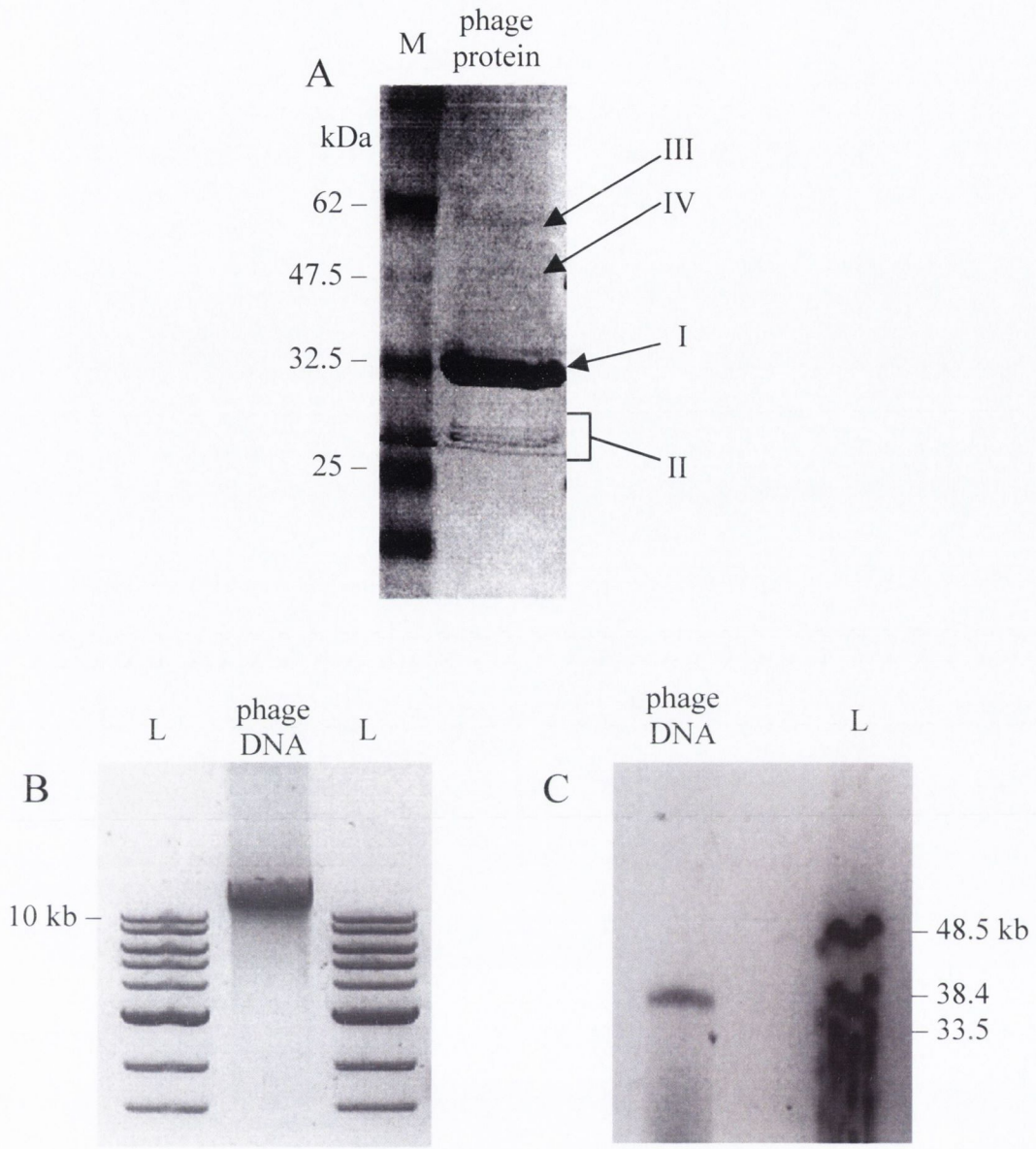


Fig. 7.3. Analysis of isolated phage. Purified phage was analysed by SDS-PAGE (A), which identified a major band at approximately 32.5 kDa (I), and three smaller bands between 32 and 25 kDa (II). Two bands approximately corresponding to sizes of 55 and 60 kDa were also identified (III and IV, respectively). M denotes molecular weight marker. Agarose gel electrophoresis showed that the phage genomic DNA was greater than 10 kb (B). PFGE analysis showed that the phage genome was greater than 38 kb in length (C). L denotes DNA standards.

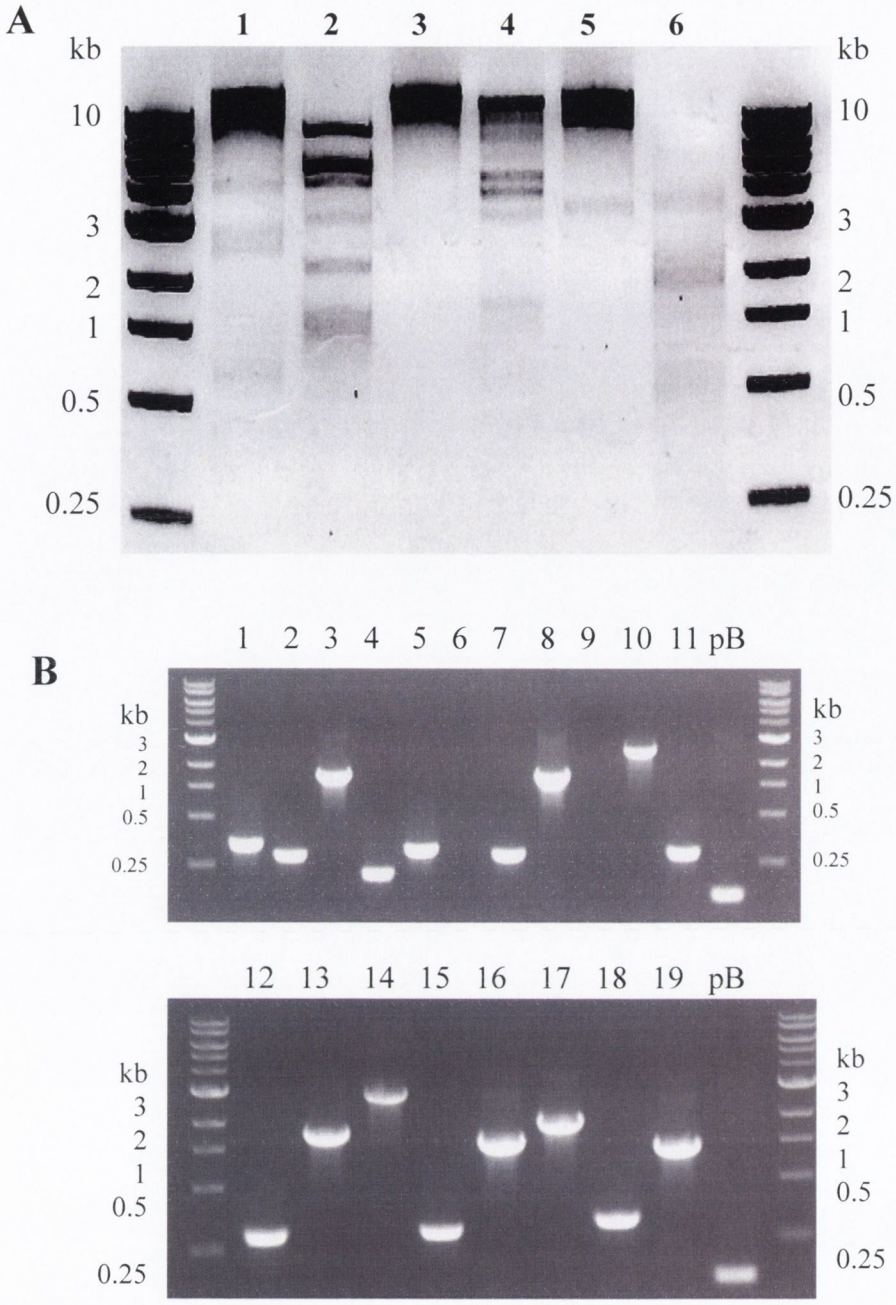


Fig. 7.4. Analysis of phage DNA. Phage DNA was digested with restriction enzymes, *Eco*RI, *Eco*RV, *Bam*HI, *Hin*dIII, *Sac*II, or *Sau*3AI (lanes 1 to 6, respectively). *Eco*RV digested the phage DNA into fragments with sizes of below 10 kb (A). *Eco*RV fragments of phage DNA were ligated into pBluescript SK (–) and the inserts sizes of 20 plasmids, called pPHI plasmids, were examined by PCR (B: lanes 1-19) alongside a DNA ladder. Lane pB contains pBluescript as template DNA.

2, 4, 8, 10, 12, 14, 16, 17, and 18, were sequenced commercially. Sequencing showed that the majority of constructs had between 98 to 100 % matches to parts of the genome of wild type bacteriophage P22 (Table 7.1). However, pPHI-4 had a 100 % match to a portion of ST64B DNA, a phage of SL1344. This suggested that the phage might be a hybrid phage consisting of parts of *Salmonella* phages. It is important to note that parts of the sequenced phage DNA identified as P22-like also had over 90 % identity to the phages ST64B and ST64T. ST64T is a *Salmonella* phage lysogenic in strain DT64: it has no significant sequence similarity to ST64B, which is also found in DT64 (Mmolawa *et al.*, 2003).

7.2.7 PCR analysis of phage DNA in SL1344

Sequencing of phage DNA revealed that the inserts in plasmids pPHI-1 and pPHI-8 were contiguous in the P22 genome. Oligonucleotides P22-F and P22-R were designed to amplify a region of 1,056 bp spanning the two inserts to determine if they were also contiguous in the identified phage genome. Using the phage DNA as template, PCR analysis showed that these fragments were also contiguous in the isolated phage genome. The oligonucleotides were also used to try to amplify the product from the SL1344 genome. This was unsuccessful even after several attempts.

7.2.8 Apparent recombination events of phage with genomic DNA

SL1344 growing in MMM broth was inoculated with purified phage particles in order to propagate more phage DNA for analysis. The resulting phage lysate was purified and the DNA isolated, as described in section 2.21. Primer pair *sb21*-F and *sb21*-R complementary to the gene *sb21* of ST64B, and primer pair P22-F and P22-R were used to amplify their respective products from template DNA from the first isolated phage, and from the newly propagated phage. In addition, genomic DNA from SL1344 was used as a DNA template. The results (Fig. 7.5) showed that the 387 bp *sb21* fragment could be amplified from the SL1344 and newly propagated phage genomic DNA. However, the first phage did not appear to contain the primer target, as no product was produced. The P22 DNA fragment was amplified from both phage, but was not amplified from the SL1344 genome, as expected. This suggested that propagation of the phage DNA may have led to additional recombination events with SL1344 genomic DNA.

7.2.9 Identification of growth conditions that reduce/prevent lysis

Autolysis of bacteria in nutrient-limiting media suggested that nutrient stress was likely to be a key signal for lysis. This was supported by the lack of lysis seen in rich broth cultures. It also appeared, from the steps taken to prevent lysis during growth in MMM, that other stresses on bacterial growth might increase the frequency of lysis. Certain environmental stresses are known to induce prophages (Lunde *et al.*, 2005; Melechen and Go, 1980; Rudin and Lindberg, 1975).

Cultures were washed to remove residual nutrients with warmed MMM broth, and bacteria were cultured in a waterbath incubator at 37 °C with shaking without removal of flasks for sampling, to reduce temperature fluctuations. Overnight bacterial culture concentrations were made equal to an OD_{600} of 2.5, and inoculated at 1:50 (500 µl in 25 ml MMM). Media was made freshly each day, as storage at 4 °C led to fluctuations in pH. Employing these conditions together prevented lysis from occurring, and allowed further experimentation as described in the previous chapters.

Plasmid	Insert size	Sequence match
pPHI-1 *	404 bp	100 % Enterobacteria phage P22 section C
pPHI-2/12	300 bp	100 % Enterobacteria phage P22 section F
pPHI-4	155 bp	100 % Salmonella typhimurium phage ST64B
pPHI-8 *	699 bp	100 % Bacteriophage P22 complete genome
pPHI-10/17	717 bp	99 % Enterobacteria phage P22 section A
pPHI-16	617 bp	98 % Bacteriophage P22 *eaA* - *eaI* genes
pPHI-14	460 bp	100 % Bacteriophage P22 packaging genes
pPHI-18	361 bp	100 % Bacteriophage P22 proteins 15 and 19 (3' end)

Table 7.1. Sequence analysis of phage DNA. Constructs containing phage-derived *Eco*RV fragments were ligated into pBluescript SK (–) and analysed by commercial sequencing. This identified an overall high degree of similarity to phage P22 DNA, but one construct, pPHI-4, comprised of 100% ST64B DNA. ST64B is a phage that is present in the genome of SL1344. The plasmids containing contiguous inserts are highlighted with an asterisk.

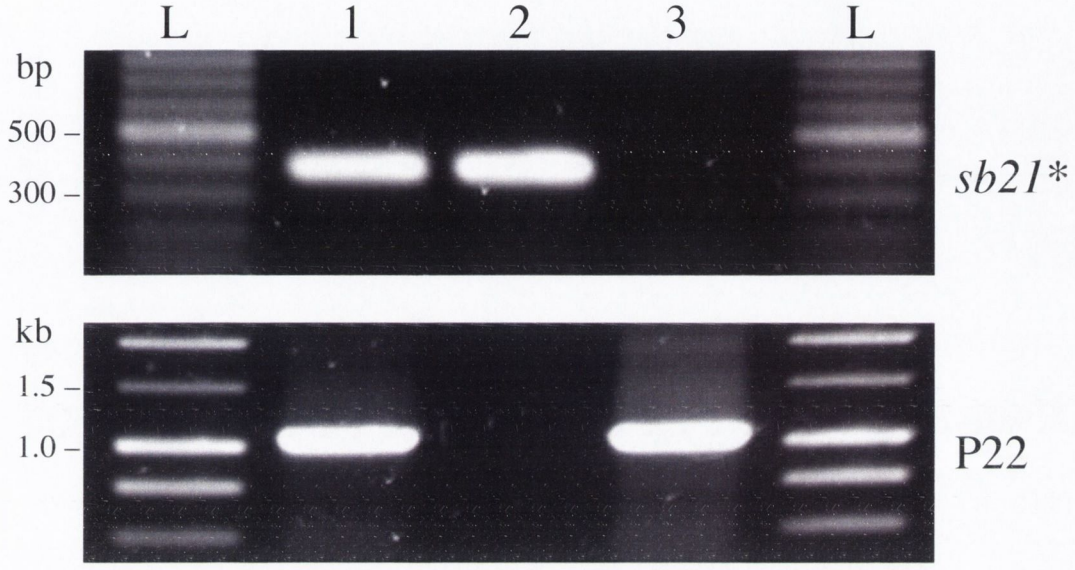


Fig. 7.5. PCR analysis of *sb21 and P22 DNA.** Primer pairs complementary to the *sb21** gene of ST64B, and to previously sequenced P22 DNA were used to PCR amplify products from DNA templates. Template DNA was isolated from the newly propagated phage (lane 1), from SL1344 (lane 2), and the original isolated phage (lane 3). DNA standards are shown in lane L.

7.3 Discussion

In this study, a novel phage, seemingly composed of parts of P22 and the defective phage ST64B, of SL1344 appeared to become lytic in minimal medium, causing the lysis of the bacterial population.

This phenomenon has been previously described. This report, with results that are strikingly similar to those in this chapter, describes phage induction in *S.* Typhimurium LT2 grown in minimal medium (Downs and Roth, 1987). Those phage seemed to be induced under conditions of purine starvation. The phage that were released had identical restriction digestion patterns to wild type P22 (Downs and Roth, 1987), and sequenced parts of the phage DNA were identical to P22 (John Roth, unpublished data). As is shown in this chapter, the results point to the phage coming from inside the bacterial cell, as opposed to contamination. One very clear problem with the observations presented in this chapter, is as follows: the SL1344 and LT2 genomes have been sequenced and there are no P22 phage sequences present. Downs and Roth explained this phenomenon as a new kind of lysogenic state called 'archived', suggesting that the P22 DNA is held in a highly modified state, leaving it unamenable to molecular biological techniques (Downs and Roth, 1987). This proposal was made before the genome sequences of *S.* Typhimurium were available.

One of the most striking features of the phage described in the present study is their ability to form plaques on the strain from which they were released. Superinfection immunity is used by bacteriophage that are lysogenic in a bacterial chromosome to render the bacterium unable to become infected by another phage of the same type. Therefore, phages that are released from cells are not expected to plaque on the immune lysogenic strain. Like the phage described in the present study, the released phage described by Downs and Roth were able to form plaques on the strains from which they were released, therefore showing no immunity to the released phage (Downs and Roth, 1987). In the present study, sequencing of parts of the phage DNA identified the presence of DNA from both P22 and ST64B. Novel phage variants are known to arise in cells after phage infection, which often differ in immunity from the original infecting phage. Perhaps the recombination of P22 and ST64B DNA sequences created mutant or otherwise defective superinfection immunity. Alternatively, if the phage is indeed coming from inside the cell, as suggested above, perhaps the archiving process holds the

phage DNA modified or encrypted in some way, which leads to inefficient expression of the superinfection exclusion machinery.

A subsequent phage propagation using the original isolated phage on SL1344 in MOPS minimal medium led to the uptake of more ST64B DNA, which was not present in the original phage (Fig. 7.5). Thus, the original isolated phage appeared to be able to recombine with the SL1344 genome, and specifically with defective ST64B phage sequences, a fragment of which was identified in the original phage DNA during sequencing. Downs and Roth described a number of phages with a wide range of phenotypes (Downs and Roth, 1987). They suggest that the 'de-archiving' process could be mutagenic. However, the present study showed evidence of phage recombination with the SL1344 chromosome. Perhaps the observation by Downs and Roth of phage with different morphologies could represent recombination of P22 with defective phage DNA in the bacterial genome, to form discrete phage types. In addition, SDS-PAGE analysis of phage proteins indicated that the major protein species migrated at approximately 32.5 kDa. However, the major P22 virion protein species is expected to be a 55-kDa polypeptide, which is the major component of the head and accounts for 85 % of the total protein of the virion (Susskind and Botstein, 1978). This suggests that the isolated phage does not express wild type P22 head protein.

P22 phage DNA could not be PCR amplified from SL1344; however, ST64B-encoded fragments were amplified as expected (Fig. 7.5). The inability to access phage DNA from SL1344 for analysis strongly suggests contamination, although the experiments outlined in section 7.2.3 appear to rule out contamination. If phage P22 is archived in the bacterial cell, the hypothesis that phage DNA can be held in a highly modified state in the cell could explain why PCR analysis is unsuccessful. However, the statement that undetectable DNA sequences exist in *Salmonella* is not one that can be made lightly. The possibility of the existence of phage 'archiving' would mean that DNA could be hidden from molecular genetics techniques inside the cell. This would have far reaching implications, not only for *Salmonella* genetics, but for the study of genetics as a whole.

The triggers that lead to lysis were poorly understood; this makes reproducibility of this phenomenon a problem. It was clear from autolysis of bacteria that nutrient stress encountered in MMM was likely to be a key signal for lysis. This was supported by the

lack of lysis seen in rich broth culture: nevertheless, bacteria growing in L broth were still capable of lysis if phage suspension was added. It also appeared, from the steps taken to prevent lysis during growth in MOPS minimal medium, that other stresses on bacterial growth might increase the frequency of lysis. Although the exact triggers for lysis are unknown, some steps that were taken may have the effect of reducing these stresses. Washing of cultures to remove residual nutrients was performed using warmed MOPS minimal broth, and bacteria were cultured in a waterbath incubator without removal of flasks for sampling: this reduced temperature fluctuations. Inoculating with higher concentrations of bacteria as the initial inoculum significantly reduced the time required for the culture to become saturated in MOPS minimal medium: this also seemed to reduce the likelihood of lysis.

Chapter 8

General Discussion

8.1 Discussion

Lrp is a transcriptional regulatory protein found in many diverse bacterial species. Lrp of *E. coli* is the best-characterised member of the Lrp/AsnC family of proteins, but its homolog in *S.* Typhimurium had not been studied in such depth. This study set about characterising an *lrp* mutant of *S.* Typhimurium, to allow a comparative analysis with that of *E. coli* as well as identifying the *lrp* regulon in *S.* Typhimurium.

The *E. coli lrp* mutant has been well characterised. It was reported that the *lrp* mutant displayed a similar growth profile to the wild type in rich broth, but that a growth defect of the *lrp* mutant, compared with wild type, was apparent during growth in nutrient-poor media (Ambartsoumian *et al.*, 1994; Lin *et al.*, 1990). These analyses had never been performed on an *lrp* mutant in *S.* Typhimurium. In this study, an *lrp* knockout mutant of the *S.* Typhimurium strain SL1344 was constructed, and verified by Southern blotting (Fig. 3.2) and its inability to utilise the *gcv* operon for glycine cleavage. Growth experiments were conducted to examine whether the *lrp* mutant in *S.* Typhimurium would have a similar growth phenotype to that of the *E. coli lrp* mutant. Analyses in rich and nutrient-poor media showed that both mutants had similar growth profiles (Figs. 3.3 and 3.4). Interestingly, the *E. coli lrp* mutant is hypersensitive to the addition of L-leucine (Ambartsoumian *et al.*, 1994), whereas SL1344 *lrp* growth was enhanced by the addition of L-leucine to MOPS minimal medium. The reason for this discrepancy is unknown.

In this study, bioinformatics showed that the regulatory regions of the *lrp* genes from *E. coli* and *S.* Typhimurium were 83.3 % identical (Fig. 3.6). In *E. coli,* Wang *et al.* showed that *lrp* transcription initiates 267 bp upstream of the ORF initiation codon (Wang *et al.*, 1994). A putative transcriptional start site of *lrp* in *S.* Typhimurium had been predicted, by bioinformatic analysis, to be positioned approximately 29 bp upstream of the *lrp* ORF start site, but this transcriptional start site had never been shown experimentally (McClelland *et al.*, 2001). The present study demonstrated by primer extension analysis that the transcriptional start site of *lrp* of *S.* Typhimurium lies between 258 and 263 bp upstream of the ORF initiation codon, placing it over 200 bp upstream of the predicted start site and between 4-9 bp downstream of the start site in *E. coli* (Fig. 3.7). The function of the long 5′ UTR is unknown.

The *lrp* gene was known to be regulated by nutrient availability in *E. coli*: the *lrp* gene is maximally expressed in nutrient-poor media, compared with rich media (Landgraf *et al.*, 1996). This had never been examined in *S.* Typhimurium, so an examination of the regulation by nutrient availability of the *lrp* gene of *S.* Typhimurium was performed. The expression of *lrp* increased by 3-4 fold in *E. coli* following nutrient downshift: a similar 3-5-fold increase in *lrp* expression was shown in *S.* Typhimurium in this study (Fig. 3.10). This study also confirmed that *lrp* is negatively regulated in *S.* Typhimurium, as has also been shown in *E. coli*: reporter fusions showed that the *lrp* mutant had increased levels of *lrp* expression. This suggested that the *lrp* gene of *E. coli* and the *lrp* gene of *S.* Typhimurium were regulated similarly in response to nutrient availability. There are conflicting reports of the regulation of *lrp* in response to L-leucine. A number of studies have described the autoregulation of *lrp* of *E. coli* as leucine-insensitive, while Borst *et al.* report a slight increase in Lrp levels in the presence of leucine (Borst *et al.*, 1996). The present study showed that in the presence of 10 mM L-leucine, *lrp* expression was increased by between 1.5-2 fold, in agreement with the latter *E. coli* study. Importantly, the presence of L-leucine had no effect on *lrp* expression in SL1344 *lrp*, which suggested that the effect of L-leucine on *S.* Typhimurium *lrp* expression is Lrp-dependent.

Direct autoregulation of *lrp* in *E. coli* has previously been described (Wang *et al.*, 1994). In the present study, it was verified by EMSA and DNase I footprinting analyses that Lrp of *S.* Typhimurium also regulates its own transcription by direct interaction with the *lrp* promoter. Previous *E. coli* studies characterised an Lrp binding site on the *lrp* promoter centred at –61, which is conserved in *S.* Typhimurium (Fig. 3.6). In *S.* Typhimurium, Lrp was shown to interact with the *lrp* promoter to form between 3 and 4 complexes. The second site was hypothesised to be positioned between –98 and +1. A third site was proposed between +1 and +46, suggesting that Lrp may occlude its own promoter and thus suggesting a method by which Lrp acts as a negative regulator of its own expression (Fig. 3.16). A putative fourth complex that was identified may represent non-specific binding by Lrp to the *lrp* promoter: this is discussed in more detail later.

In *E. coli*, Lrp has been identified as a regulator of a number of genes and operons involved in amino acid transport and biosynthesis and fimbriation, and microarray analyses on *E. coli lrp* mutants has been performed (Hung *et al.*, 2002; Tani *et al.*,

2002). No such elucidation of the regulon of Lrp in *S.* Typhimurium had been performed. In *S.* Typhimurium, Lrp had previously been identified as a regulator of genes involved in metabolism, such as *dadAX* and *ilvIH*, as well as *traJ,* for conjugal transfer, *pef* fimbriae, and the *spv* virulence locus of the large virulence plasmid, pSLT (Camacho *et al.*, 2005; Hecht *et al.*, 1996; Marshall *et al.*, 1999; Nicholson and Low, 2000; Wang *et al.*, 1993). The effect of Lrp on *spv* genes, which are essential for efficient systemic infection beyond the intestines in orally inoculated mice, was more severe during growth in intracellular salts medium, which mimics some of the conditions encountered by bacteria in the intracellular niche (Libby *et al.*, 1997; Marshall *et al.*, 1999). This was the first indication that Lrp is a regulator of genes that are important in the intracellular environment. *Salmonella* is capable of inhabiting several unique niches, such as that of the intracellular environment, in which it is expected to experience nutrient-poor conditions. Considering that Lrp is capable of sensing nutritional status of the bacterium, it was tempting to hypothesise that Lrp could have a role in intracellular virulence gene expression in *S.* Typhimurium.

In this study, SL1344 *lrp* was subjected to DNA microarray analysis, to analyse the Lrp regulon, which was one of the primary objectives of the study. To fully elucidate the Lrp regulon, analysis was performed in rich media and nutrient poor media. Microarray analysis had been performed on *E. coli lrp* mutants in MOPS minimal medium, and in addition, known Lrp-regulated genes in *E. coli* were well characterised. This allowed further comparisons with the Lrp regulons between the two *Enterobacteriaceae*. Briefly, analysis of the Lrp regulon in LB broth identified that few changes resulted from the *lrp* mutation. Examination of data revealed that known Lrp-regulated genes and operons in *E. coli* were also regulated by Lrp in *S.* Typhimurium, namely *gltBDF*, *glyA*, *ilvGMEDA*, *livJ* and *livK* (Table 4.2). Interestingly, the *fimA* and *fimD* genes of the type 1 fimbrial operon were identified as Lrp-regulated in LB broth, which had not been previously identified. Lrp-regulated genes in *E. coli* are known to differ in sensitivity to Lrp concentration, and have differing Lrp-DNA dissociation constants (Borst *et al.*, 1996; Chen and Newman, 1998; Wang *et al.*, 1994). It is believed that the varying sensitivities of Lrp-regulated genes may reflect the varying levels of Lrp in response to nutrient availability (Ernsting *et al.*, 1993). As a result, it was assumed that genes identified in studies in LB broth might represent those genes with a high affinity for Lrp, as Lrp levels are at their lowest during growth in rich media.

Analyses of growth of SL1344 in MOPS minimal medium identified an autolysis phenomenon, making it impossible to study the growth, and consequently gene expression profiles, in MOPS minimal medium. Experimentation showed that autolysis was associated with minimal nutrient availability. Conditions were elucidated that reduced the stress encountered by the bacteria, and this reduced the likelihood of lysis: these involved incubation of cultures in a 37 °C shaking waterbath, without removal for sampling, and the cells were washed with warm MOPS medium to remove residual nutrients. Complete growth curves were obtained in this way, without the appearance of lysis.

Analysis of the Lrp regulon in MOPS minimal medium was also performed at logarithmic and stationary phase. Data from comparisons of SL1344 and SL1344 *lrp* at logarithmic phase showed that 25 of 57 genes shown to be differentially regulated by ≥ 2 fold had previously been associated with Lrp in *E. coli*, including genes of the *leu* operon, *serA, ilvI* and *gltB* (Table 4.4). The *fimA* and *fimZ* genes of type 1 fimbriae were identified as down regulated in SL1344 *lrp* by this study. In addition to these genes, *Salmonella*-specific intracellular virulence determinants were also identified as Lrp regulated. Six genes belonging to the TTSS structural II operon of SPI-2, the translocon gene *sseD*, and the effector protein *sifB*, were upregulated in SL1344 *lrp* by between 2-16 fold. Importantly, the *sifB* gene is located outside SPI-2, but responds to SPI-2 inducing signals in an SsrA-B-dependent manner (Miao and Miller, 2000). MOPS minimal arrays were also examined in the presence of leucine. Leucine-responsive genes included *kbl-tdh*, *fimA,* and *dadAX,* which were upregulated by the presence of leucine in SL1344 compared to its absence, at logarithmic phase. The *livKHMGF*, *leu* and *serA* genes were downregulated by the presence of leucine at logarithmic phase in SL1344 (Fig. 4.5).

Exogenous L-leucine can modulate the regulatory effect of Lrp in six ways: where Lrp is a positive regulator of expression, leucine may antagonise the effect, be required for the effect, or may have no effect. Where Lrp is a negative regulator of expression, leucine may antagonise or potentiate the negative effect, or may have no effect. In the microarray analysis, comparison of gene profiles in SL1344 *lrp*, SL1344 in the absence of leucine, and SL1344 in the presence of leucine were performed (Fig. 4.1). This

identified examples of Lrp-regulated genes and operons that show reduced activation or repression in the presence of leucine, that require leucine for activation or repression, and genes that are not affected by leucine. It is proposed that these examples must preferentially respond to Lrp that does not contain leucine, respond to Lrp as a leucine-bound octamer, and respond to both, respectively (Chen and Calvo, 2002).

Type 1 fimbriae of the *fim* operon are phase variable, but unlike the *fim* operon of *E. coli*, they do not contain an invertible DNA element or DNA recombinases. The phase variation is instead controlled by a complex regulatory network, where FimZ and FimY are essential positive regulators of *fimA*, the major fimbrial subunit, and the remaining structural genes. FimW is a negative regulator of *fimA* that acts through protein:protein interactions to prevent the positive effect of FimZ on *fimA* expression. *fim* gene expression is also controlled at the level of translation by *fimU*, which encodes a rare arginine tRNA. The translation of FimY is particularly affected by *fimU*. Microarray analysis identified the *fimA* gene as downregulated in SL1344 *lrp*. The effect of Lrp on *fimA* and *fimZ* was confirmed by RT-PCR analysis, and phenotypically, by haemagglutination assay (Figs. 5.2 and 5.3). These results suggested that Lrp is a positive regulator of type 1 fimbrial expression, as the *lrp* mutant had no detectable haemagglutination activity, and strongly reduced *fimA* and *fimZ* expression. RT-PCR analysis also confirmed DNA microarray data that showed that the addition of leucine to MOPS minimal medium had the effect of increasing *fimA* and *fimZ* gene expression. These are therefore examples of genes that are positively regulated by Lrp, and leucine increases the positive regulation. Bioinformatics using the Lrp consensus binding site sequence identified putative Lrp binding sites in the promoter regions of *fimA*, *fimW-U*, and *fimZ*. However, EMSA analyses showed that Lrp did not bind to the *fimA* or *fimW-U* promoter regions, and did not bind to a *fimZ* DNA probe containing the putative binding site. The Lrp consensus binding site sequence will be discussed in a later section. Further EMSA analyses, and deletion analysis of the *fimZ* promoter showed an Lrp-*fimZ* interaction, and a region important for Lrp binding was identified between –90 and –190, with respect to the *fimZ* transcriptional start site. Together, the results reveal that Lrp regulates *fimZ*, an essential positive regulator of *fimA*. Furthermore, EMSA analysis showed cooperative binding of Lrp to the *fimZ* promoter (Fig. 5.8 (C)).

Cooperative binding, where binding of Lrp to one site strongly influences the binding of Lrp to additional adjacent sites, is a characteristic of Lrp-DNA interactions. In EMSA analyses, cooperativity can sometimes be identified by stepwise complex formation: presence of a smaller molecular weight complex that forms before the larger molecular weight complex, indicating that one complex, presumably Lrp dimer-DNA, must initially form to allow the second complex to form, cooperatively (Chen *et al.*, 2005). Highly cooperative Lrp-DNA binding sometimes results in the absence of the primary complex: this is because the primary complex is so attractive for subsequent Lrp binding that it is not seen (Chen *et al.*, 2005). A recent study has demonstrated that Lrp is capable of non-specific binding to DNA with a high affinity, and it is believed that Lrp dimers can interact with DNA in a non-specific, cooperative manner (Peterson *et al.*, 2007). It is possible to incubate DNA with increasing concentrations of Lrp without observing complex formation, as has been shown in this, and other studies. Presumably, Lrp is capable of interaction with non-specific DNA through cooperative interactions at adjacent, specific sites. If this is the case, it would be difficult to analyse accurately the number of Lrp-binding sites at a promoter. Cooperative binding has been described at the *E. coli ilvIH* operon (Chen *et al.*, 2001a; Chen *et al.*, 2001b; Chen and Calvo, 2002; Chen *et al.*, 2005; Wang and Calvo, 1993), but has also been demonstrated in most other Lrp-regulated genes and operons (Kaltenbach *et al.*, 1995; Rhee *et al.*, 1996; Wiese *et al.*, 1997). The presence of putative fourth complexes at the *lrp* and *ompR* promoters may be a result of DNA binding by a fourth dimer, either non-specifically or specifically, with octamer formation. Alternatively, a fourth dimer-DNA interaction may be absent, but an increase in size corresponding to the increased molecular mass of octameric Lrp on the Lrp-DNA complex would be seen. Theoretically, either of these possibilities would be expected to give a fourth complex of identical molecular mass.

At the *fimZ* promoter, leucine appeared to change the profile of complex formation, which suggested that cooperative binding by Lrp was affected by the addition of leucine (Fig. 5.8 (C)). Complex 1 appeared reduced in the presence of leucine, and formation of complex 3 appeared to be enhanced, compared to the absence of leucine. This suggested that leucine might increase the cooperativity of Lrp binding at the *fimZ* promoter, which through unknown means increases *fimZ* expression. DNase I footprinting performed on the *fimZ* promoter showed that Lrp wrapped the DNA, causing protection and hypersensitivity. The binding of multiple sites, and subsequent bending and wrapping of

DNA by Lrp leads to the protection of large regions, often over 100 bp, from DNase I-mediated cleavage (Jafri *et al.*, 2002; Mathew *et al.*, 1996; Wiese *et al.*, 1997). Protected regions show phased hypersensitivity, which is a characteristic of Lrp binding and bending: as Lrp binds DNA, the minor groove of DNA becomes widened, facilitating DNA cleavage by DNase I (Drew and Travers, 1984). Hypersensitive sites were phased by approximately 30 bp (Fig. 5.10), which is supported by the crystal structure model of Lrp-*pap* DNA (Fig. 1.4) (de los Rios and Perona, 2007). It seemed likely that the Lrp binding sites would be located between the hypersensitive sites, as has been described in the Lrp-*gltB* system (Wiese *et al.*, 1997). This placed Lrp binding sites, 1, 2 and 3 at the *fimZ* promoter, centred at –152, –129 and –100, respectively. Site 1, with a high degree of similarity to the Lrp consensus binding sequence, seems likely to be the site with highest affinity for Lrp, and the first occupied site. It is expected that site 2 is occupied next, in a cooperative manner, followed by site 3, which has the lowest degree of similarity to the consensus binding sequence.

It had been shown that Lrp was required for the transcription of *fimA* and *fimZ*, and was required for haemagglutination activity. Lrp was shown to interact with the *fimZ* promoter, presumably to promote transcription. It seemed likely that the effect of Lrp on the expression of type 1 fimbriae was solely through its effect on *fimZ*. By the expression of *fimZ* in SL1344 *lrp*, with an arabinose-inducible promoter, haemagglutination activity was restored, indicating that it was likely that the effect of Lrp was indeed solely through its effect on *fimZ* expression. Most known Lrp-regulated fimbriae, with the exception of *fim* of *E. coli*, belong to the P-regulatory family of fimbriae (Braaten *et al.*, 1992; Crost *et al.*, 2004; Nicholson and Low, 2000). P-regulatory fimbriae are characterised by phase variable expression, regulation by Lrp and a PapI homolog, the spacing of the two GATC sites by 102-103 bp, and the influence of differential methylation status of these GATC sites (Harel and Martin, 1999). It appears that *fim* of *S.* Typhimurium may constitute a new method of fimbrial gene regulation.

The Lrp consensus binding site sequence is a 15-bp part palindromic A+T-rich sequence, which binds an Lrp dimer (Cui *et al.*, 1995; Cui *et al.*, 1996). The reliability of the Lrp consensus binding site sequence is questionable. It has been used to successfully identify Lrp binding sites in promoters (Lahooti *et al.*, 2005; Rhee *et al.*, 1996).

However, at the *serA* promoter in *E. coli*, the Lrp binding site consensus sequence was not present, yet Lrp was later shown to interact directly with the *serA* promoter (Rex *et al.*, 1991; Yang *et al.*, 2002). In the present study, the Lrp consensus binding sequence identified putative binding sites in the *fimA* and *fimW* regulatory regions: however, when these regions were examined by EMSA analysis, they failed to show an interaction with Lrp (Figs. 5.6). It has been suggested that Lrp may recognise DNA secondary structure in combination with a degenerate recognition sequence (Chen and Newman, 1998; Pul *et al.*, 2007).

Microarray analysis identified that the expression of SPI-2 genes were affected by the *lrp* mutation in logarithmic and stationary phase growth in MOPS minimal medium. A role for Lrp in the expression of SPI-2 had not been previously identified, although Lrp has been shown to regulate negatively the expression of the *spv* genes of pSLT, which are required for intracellular virulence (Libby *et al.*, 1997). In logarithmic phase, it appeared that SPI-2 expression was increased in the *lrp* mutant, whereas at stationary phase, this effect was reversed. RT-PCR analysis was used to confirm the effect of the *lrp* mutation (Fig. 6.1). The ability of SL1344, SL1344 *lrp* and SL1344 *ompR* to induce the expression of *ssrA* was examined in medium with an acidic pH, compared to the levels at neutral pH. This showed that *ssrA* expression was induced in SL1344 at pH 4.5 compared with pH 7.2, by approximately 2 fold (Fig. 6.2). SL1344 *lrp* showed high *ssrA* expression at pH 7.2, and the level of *ssrA* did not increase in response to acidic pH in SL1344 *lrp.* As expected, *ssrA* levels were induced in SL1344 *ompR* at neither pH 4.5 nor pH 7.2, as the sensitivity of *ssrA* expression to acidic pH is OmpR-dependent (Lee *et al.*, 2000). In our hands, Lrp did not directly interact with the *ssrA* promoter, and so was presumed to affect the expression of *ssrA* in an indirect manner (Fig. 6.3).

To examine whether the insensitivity of *ssrA* to acidic pH in SL1344 *lrp* was a result of reduced *ompR* expression, an *ompR-gfp* transcriptional fusion was constructed. This showed that the level of *ompR* expression was doubled in acidic pH compared with neutral pH, in SL1344. In SL1344 *lrp*, however, acidic pH had no effect on *ompR* expression (Fig. 6.6). This suggested that Lrp in some way contributes to the ability of *ompR* and, thus, *ssrA* to respond to acidic pH. SPI-2 responds to acidic pH in an SsrA-B-dependent manner, which is also partially dependent on OmpR-EnvZ (Garmendia *et al.*, 2003), indicating that the effect of *ompR* may be partly responsible for the effect on

ssrA. It also implied that the effect of Lrp on *ssrA* expression at neutral pH was independent of *ompR*, as *ompR* levels were unchanged in SL1344 *lrp* at both pHs. Acidic pH was also shown to have a negative effect on *lrp* expression, with a 2-fold decrease in *lrp* promoter activity at acidic pH, compared with pH 7.2 (Fig. 6.7). Lrp was shown, by EMSA and DNase I footprinting analyses, to interact with the *ompR* promoter to form three to four complexes (Figs. 6.9 and 6.10). The position of Lrp binding overlaps the predicted H-NS binding region of the *ompR* promoter, although the precise position of H-NS binding is unknown (Fig. 6.11). Together these results suggest that Lrp has a role in the expression of *ompR* from the P2 promoter, which is responsible for the acid induction of *ompR* (Bang *et al.*, 2002).

To summarise, it appears that Lrp has a direct role in the acid-sensitivity of *ompR* expression, and an indirect role in the acid-sensitivity of *ssrA* expression, and is important for the correct expression level of these genes at pH 4.5 in wild type SL1344. It also appears that Lrp is a negative regulator of *ssrA* expression at pH 7.2, but as Lrp did not interact with the *ssrA* promoter under the conditions examined, it is expected that it is also through indirect means. Lrp has previously been shown to regulate the intracellular virulence genes, *spv*, so it is perhaps unsurprising that Lrp might have an effect on SPI-2 genes. Given that the effect of Lrp on *spv* expression was more severe during growth in intracellular salts medium, which mimics some of the conditions encountered by bacteria in the intracellular niche, than in LB (Marshall *et al.*, 1999), it suggests that Lrp may have a more significant role in virulence gene expression *in vivo* than *in vitro*.

Lrp does not appear to act alone at promoters that it regulates, and it is expected that the Lrp-regulated promoters identified in this study are no exception. In this study, Lrp was shown to regulate the expression of the *fimA* and *fimZ* genes, indirectly, and directly, respectively. It was suggested that Lrp may interact with FimZ itself, which has a putative binding site upstream of its promoter, or may be involved in the bending of DNA to allow contact of FimZ with RNA polymerase, to promote *fimZ* transcription, as has been described at the *gcvTHP* operon (Stauffer and Stauffer, 1999). In addition, a putative role for H-NS was identified at the *fimZ* promoter. Importantly, Lrp appears to recognise and bind to an A+T-rich consensus sequence, which may place it in close proximity to H-NS binding sites. A putative role for H-NS in *fimZ* regulation was

considered. H-NS also regulates *ompR* expression from the acid-inducible P1 promoter negatively (Bang *et al.*, 2002), and the Lrp binding region and putative H-NS binding sites overlap in the *ompR* regulatory region (Fig. 6.11). Lrp and H-NS interact at the *rrnB* promoter in *E. coli*: here H-NS makes transient DNA contacts that enhance Lrp-binding (Pul *et al.*, 2005; Pul *et al.*, 2007). Perhaps Lrp is also able to bind to H-NS-bound DNA to displace H-NS, thereby removing its regulatory effects. Displacement of H-NS by regulatory protein-mediated DNA structural remodelling has been described in previous reports (Madhusudan *et al.*, 2005; Turner and Dorman, 2007; Wyborn *et al.*, 2004). It is also likely that other regulatory proteins have additional roles in the regulation of these promoters.

The characteristics of Lrp discussed in this chapter raise another important question: is Lrp a nucleoid-associated protein (NAP)? NAPs are characterised as structural proteins that wrap, package, and bend or coil DNA (Drlica and Rouviere-Yaniv, 1987). Lrp is well known as a DNA binding and bending protein, shown in this work and in previous studies (Gazeau *et al.*, 1994; Nou *et al.*, 1995; Wiese *et al.*, 1997), and a model of the interaction of Lrp-*pap* DNA, based on the crystal structure of Lrp (Fig. 1.4), strongly suggests DNA wrapping around an Lrp protein core (de los Rios and Perona, 2007). NAPs not only influence the conformation of DNA, but also DNA transactions, such as transcription. Lrp is a global regulator of transcription: taken together, these factors support the possibility that Lrp is a NAP. To confirm this, and to identify Lrp as a NAP as well as a transcriptional regulator, it would be important to show chromosome condensation by Lrp. The Lrp homolog, LrpC of *Bacillis subtilis* has recently been shown by electron microscopy, to form DNA duplex bridges as well as DNA wrapping (Beloin *et al.*, 2003). It is also believed that Lrp may have a role in loop formation, thereby contributing to local organisation and compaction of DNA (Luijsterburg *et al.*, 2006). A role for Lrp of *E. coli* and *Salmonella* in DNA compaction has yet to be shown.

What is the physiological relevance of Lrp as a virulence gene regulator, and why is Lrp particularly suited to this role? It has been suggested that virulence gene expression is regulated by combinations of specific environmental conditions, to promote expression in specific sites in the host, and it has been shown that inappropriate expression of virulence genes reduces the virulence of *Salmonella* in the mouse model (Heithoff *et al.*, 1999; Lucas and Lee, 2000). *Salmonella* experience a wide range of environmental

conditions in the niches they inhabit, from the acidic pH of the stomach, to the abundant nutrients of the intestine, to the relatively nutrient poor spleen and liver (Slauch *et al.*, 1997). Lrp has been well characterised as a regulator of metabolic genes in nutrient-limiting conditions. Lrp may additionally act to aid *Salmonella* in distinguishing its unique environments, to allow appropriate expression of virulence factors. The expression of the *lrp* gene responds to nutrient availability, and acidic pH, as shown in Chapters 3 and 6 of this work. Lrp is not only regulated at the transcriptional level by environmental signals, but its regulatory activity is also modulated by the effects of leucine (Chen *et al.*, 2001a). This would ensure that Lrp is well placed to act as a sensor of environmental nutrient availability, and allowing it to regulate promoters, of both metabolic genes, and virulence genes. It is expected that Lrp would also act in concert with other regulatory proteins, as it is likely that an array of environmental conditions regulate virulence genes (Lucas and Lee, 2000). Guanosine tetraphosphate (ppGpp), which has been shown to regulate *lrp* expression, also strongly affects virulence gene expression. Recently, ppGpp has been shown to be required for the expression of both invasion and intracellular virulence genes in response to growth conditions that are relevant to host infection (Song *et al.*, 2004; Thompson *et al.*, 2006). ppGpp is synthesised at high levels by the *relA* product in response to amino acid starvation in the stringent response, and is degraded and synthesised by the *spoT* product in response to stress and nutrient limitation other than amino acid starvation (Cashel, 1996). It appears that the basal ppGpp levels synthesised by *spoT*, and not the stringent response, are responsible for the effect of ppGpp on SPI-1 gene expression (Pizarro-Cerdá and Tedin, 2004). Given that ppGpp and growth phase regulate *lrp* expression , it is tempting to suggest that Lrp may, in part, be responsible for indirect effects of ppGpp on virulence gene expression.

It is interesting to consider the properties of Lrp in relation to its regulation of horizontally-acquired virulence genes, such as those of SPI-2 and the *spv* operon, as well as those of the ancestral genome, such as metabolic genes. The consensus binding-site sequence for Lrp is relatively degenerate, and Lrp has recently been shown to bind non-specifically to DNA with a relatively high affinity. In addition, Lrp is a flexible regulator of gene expression: it can act as a dual regulator of transcription, and its effect can be modulated by leucine, or can be insensitive to leucine. This, coupled with the relatively

degenerate Lrp recognition sequence, may mean that horizontally-acquired genes are readily able to integrate into the Lrp regulon.

In conclusion, this work has examined, and confirmed, that the regulation of *lrp* in *S.* Typhimurium is affected by growth phase, nutrient availability, and negatively autoregulated in the same way as in *E. coli.* The key findings of this work are summarised in Figure 8.1. Additionally, it has identified further putative binding sites for Lrp on the *lrp* promoter, and has identified the transcriptional start site for the *lrp* gene in *S.* Typhimurium. The study elucidated genes that belong to the Lrp regulon in *S.* Typhimurium, including the *fim* operon for type 1 fimbriae, and genes of the SPI-2 intracellular virulence determinant. Lrp was shown to regulate *fimA* gene expression, and fimbriation in a novel way, by acting as a positive regulator of an essential regulator, FimZ. Lrp was also shown to influence the expression of *ssrA* of SPI-2 and *ompR* in response to acidic pH, and it was shown that the *lrp* gene itself is repressed by acidic pH. Finally, Lrp was shown to indirectly repress *ssrA* expression at neutral pH, and to interact directly with the *ompR* promoter. Together, these data point to a role for Lrp in virulence gene expression in *S.* Typhimurium, and suggest that Lrp could be an important regulator of genes that are expressed in the intracellular niche.

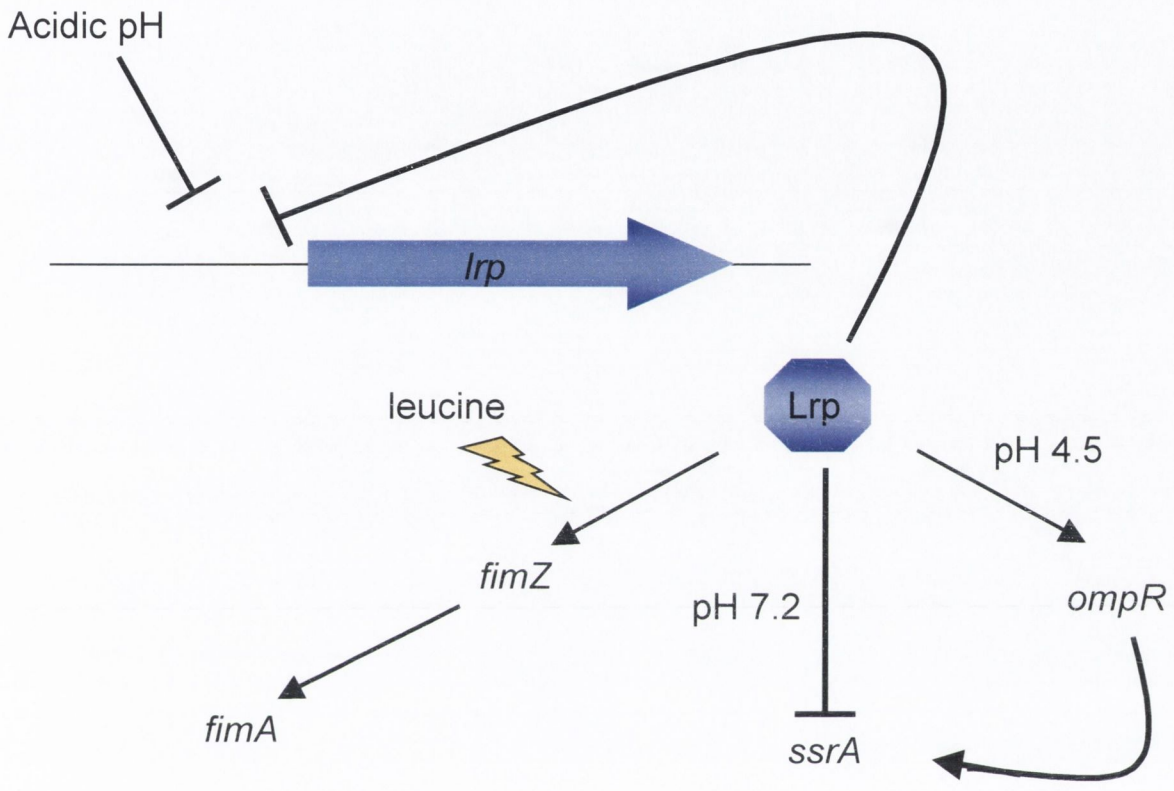


Fig. 8.1. Summary of key findings of this study. Acidic pH and Lrp were found to have negative effects on *lrp* gene expression. Lrp was shown to have a positive role in *ompR* expression at pH 4.5, and a negative role in *ssrA* expression at pH 7.2. Lrp was shown to positively regulate *fimZ*, and this regulation was enhanced by leucine. Lrp indirectly influenced *fimA* expression via its effect on *fimZ* expression.

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SUPPLEMENTARY DATA