The effect of glutathione on type 1 fimbriation in *Escherichia coli*

PhD Thesis for the degree of PhD in Microbiology

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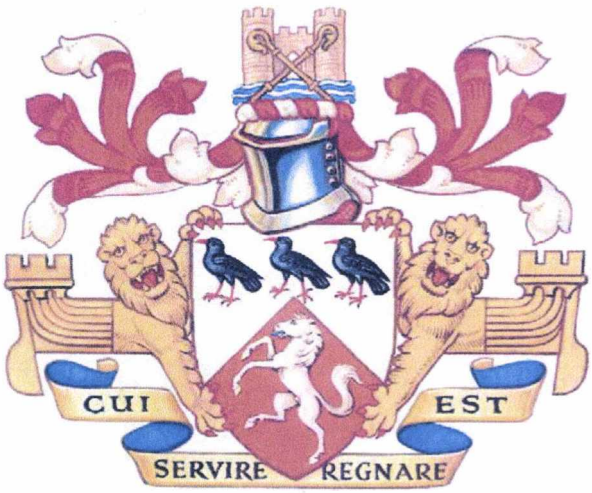

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent, or any other University or Institution of Learning.

Lynn Wales

20th July 2011

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Last, but far from least, I am enormously grateful to Chris, without whom (!) I would not be writing these words right now. Your never-ending support throughout my time in Canterbury was invaluable...

Abstract

A wide range of molecular studies are carried out on model organisms such as *Escherichia coli*. While most *E. coli* strains are commensal, some have evolved to produce toxins and other virulence factors and are pathogenic. Such a virulence factor are type 1 fimbriae, filamentous structures that are anchored in the outer membrane of the bacterial cell and enable the attachment of bacteria to host cells, as well as facilitating their invasion.

The production of type 1 fimbriae is regulated by phase variation. Inversion of *fimS*, a short DNA element that carries the promoter sequence for the *fim* gene operon, determines phase switching and requires the recombinases FimB and FimE. FimB catalyses OFF-to-ON switching, and levels of *fimB* expression regulate fimbrial production. The expression of *fimB* itself is regulated by many factors. This study demonstrates that the tripeptide glutathione is an activator of *fimB* expression. Glutathione is likely to have a periplasmic site of action and is suggested to target and inactivate the periplasmic enzyme SpeA. Since SpeA is the first enzyme in the polyamine biosynthetic pathway, levels of glutathione would determine levels of polyamines.

It is proposed that polyamines inhibit *fimB* expression at both the transcriptional and post-transcriptional level, an optimal level of polyamines allowing maximum *fimB* expression. The regulation of *fimB* expression at the transcriptional level is potentially mediated via elevated levels of the regulatory protein H-NS, a known inhibitor of *fimB* expression. However, the polyamines may have a direct effect on *fimB* translation as well. Type 1 fimbriae are proinflammatory and a virulence factor in pathogenic *E. coli*. The level of reduced GSH in the periplasm may thus provide the bacterium with a key indicator of host defence activation. Moreover, the fact that both GSH and the polyamines protect against oxidative stress suggests a *raison d'être* for their mutual regulation.

Abbreviations

Amp	ampicillin
(P)APS	(phosphor) adenosine 5'-phosphosulfate
bp	base pair
Cam	chloramphenicol
CTAB	hexadecyltrimethylammonium bromide
cys	cysteine
cys-gly	cysteinyl-glycine
Dam	deoxyadenosine methylase
ddH_2O	double-deionised water
DHL	dihydrolipoic acid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EHEC	enterohaemorrhagic *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ExPEC	extraintestinal pathogenic *Escherichia coli*
FAA	trifluoroacetylacetone
FID	flame ionization detection
fim	type 1 fimbriae (genes)
f_{met}	*N*-formylmethionine
Fur	ferric uptake regulator
Glc-6-P-DH	glucose-6-phosphate dehydrogenase
GC	gas chromatography
GlcNAc	*N*-acetylglucosamine
glu	glutamate
gly	glycine
GSH /GSSG	reduced/oxidised glutathione
GST	glutathione S-transferase
Gor	glutathione oxidoreductase
Gsp	glutathionylspermidine

H-NS	histone-like nucleoid-structuring protein
IHF	integration host factor
IBS	IHF binding site
IL	interleukin
IRL	left inverted repeat
IRR	right inverted repeat
IPTG	isopropyl β-D-1-thiogalactopyranoside
IVLA	isoleucine, valine, leucine and alanine
Kan	kanamycin
lac	lactose metabolism (genes)
LB	lysogeny broth
LA	lipoic acid
LOS	lipooligosaccharide
LPS	lipopolysaccharide
Lrp	leucine-responsive regulatory protein
MOPS	3-(*N*-morpholino)-propanesulphonic acid
M_{gly}	minimal glycerol MOPS medium
NaAc	sodium acetate
NADPH	nicotinamide adenine dinucleotide phosphate
Neu_5Ac	*N*-acetylneuraminic acid
OD	optical density
ONPG	ortho-nitrophenyl-β-galactoside
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
P-DHL	dihydrolipoamide
ppGpp	guanosine tetraphosphate
Put	putrescine
RBS	ribosome binding site
RD_{gly}	rich-defined glycerol MOPS medium
(m)RNA	(messenger) ribonucleic acid
RNR	ribonucleotide reductase
rpm	revolutions per minute
RT	room temperature

SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
Spe	spermidine
-SH	sulfhydryl
TAE	Tris/acetate/EDTA (buffer)
TCA	trichloroacetic acid
TCA cycle	tricarboxylic acid cyle
TE	Tris/EDTA (buffer)
TEMED	tetramethylethylenediamine
Tet	tetracycline
TLR	toll-like receptor
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection
UTR	untranslated region

Contents

Chapter 1

Introduction

1.1. *Escherichia coli*

Scientific progress on a wide range of molecular processes has been possible by studying simple organisms such as bacteria. *Escherichia coli*, in particular, has been widely researched. The gram-negative bacterium, commonly found in humans and mammals, belongs to the family of Enterobacteriaceae and is an intestinal facultative anaerobe with mostly commensal strains. However, some strains have evolved to produce toxins and hence to be pathogenic. While enteropathogenic and enterohaemorragic *E. coli* (EPEC and EHEC) may cause severe intestinal diseases, extraintestinal pathogenic *E. coli* (ExPEC) strains are able to infect other kinds of tissue. Hereby colonisation and adherence to the epithelia triggers the host cell's immune response to a disproportionate level and can lead to a wide range of diseases, a very common disease being urinary tract infection (UTI).

The host cell's immune system responds to pathogen-associated molecular patterns (PAMPs). These distinctive bacterial molecules, such as peptidoglycans, also include structures referred to as virulence factors that enable the bacteria to overcome host defences. These virulence factors include polysaccharide capsules, hemolysin, serum resistance as well as entero- and endotoxins (such as lipopolysaccharides LPS and lipooligosaccharides LOS) and adhesins such as fimbriae.
Despite being considered a commensal strain in humans, *E. coli* K-12 produces type 1 fimbriae, a virulence factor found in most *E. coli* strains, commensal as well as pathogenic. This adhesin has been shown to be involved in colonisation of the urinary tract by uropathogenic *E. coli* strains (UPEC) (Bahrani-Mougeot *et al.*, 2002). Besides additional virulence factors, the type 1 fimbriae enable UPEC strains to cause urinary tract infection.

1.1.1. Type 1 fimbriae and phase variation

Type 1 fimbriae are filamentous structures that are anchored in the outer membrane of the bacterial cell. They are a virulence factor in meningitis (Teng *et al.*, 2005) and urinary tract infection (Cornell *et al.*, 1996; Zhou *et al.*, 2001; Bahrani-Mougeot *et al.*, 2002). Besides mediating colonisation by bacteria (Wright *et al.*, 2007), the main role of type 1 fimbriae is to enable attachment of bacteria to host epithelial cells and promote invasion (Bower *et al.*, 2005). By interacting with host cells, the bacteria on the one hand have the benefit of gaining nutrients, but on the other hand activate the innate immune system of the host. The major component of the bacterial outer membrane, lipopolysaccharide (LPS), is hereby

recognized by the toll-like receptor 4 (TLR4) (Hedlund *et al.*, 2001), leading to the release of cytokines IL-6, IL-8 and TNF-α (Malaviya *et al.*, 1996; Godaly *et al.*, 1998; Samuelsson *et al.*, 2004). Besides LPS there are a range of other bacterial molecules that trigger the host's immune response. FimH, the protein that forms the tip of type 1 fimbriae, has recently been shown to be a novel TLR4 ligand (Ashkar *et al.*, 2008; Mossman *et al.*, 2008).

While FimA is the major subunit of type 1 fimbriae, the mannose-binding tip FimH (Klemm, 1984; Klemm *et al.*, 1987; Krogfelt *et al.*, 1990) recognises specific glycoproteins containing mannose on the host cell surfaces (Ofek *et al.*, 1977; Babu *et al.*, 1986). This enables bacteria to adhere to and colonise mammalian epithelial cells (Giampapa *et al.*, 1988; Cornell *et al.*, 1996; Zhou *et al.*, 2001; Bahrani-Mougeot *et al.*, 2002). Additional chaperones, adaptor and structural proteins (FimICDFG) are necessary for correct assembly (Figure 1.1). The subunits FimF and FimG link FimA to the adhesin FimH. Hereby FimF regulates assembly, while FimG controls length of the fimbriae by inhibiting polymerisation (Russell *et al.*, 1992). In addition to these structural proteins, the periplasmic chaperone FimC interacts with other fimbrial subunits (Jones *et al.*, 1993) and is itself recognised by the outer membrane protein FimD (Nishiyama *et al.*, 2003) for assembly outside the cell. The precise function of FimI is still unknown, although it has been shown to be essential for correct pilus biosynthesis (Valenski *et al.*, 2003).

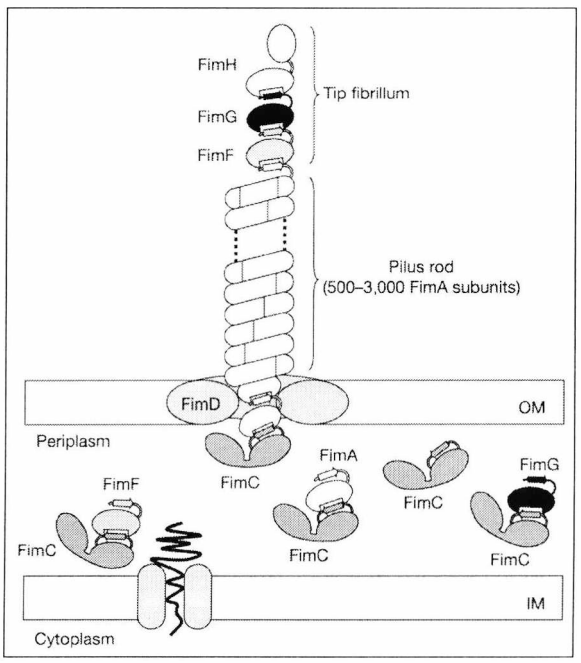


Figure 1.1: Assembly and structure of type 1 fimbriae (Vetsch *et al.*, 2004)

The genes encoding for all fimbrial proteins are located in the *fimAICDFGH* operon which is located adjacent to *fimS*, a 314 bp invertible element referred to as the *fim* switch. As this element contains the promoter of *fimA*, the adjacent gene operon can only be transcribed when *fimS* is in the ON orientation; inversion of *fimS* hence provides an ON and OFF switch for gene expression and fimbriae production.

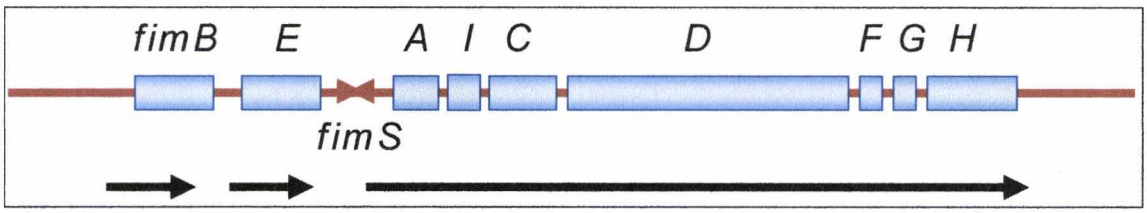


Figure 1.2: Organisation of the *fim* recombinase genes and the structural operon.

Inversion (i.e. switching) of *fimS* is carried out by the two recombinases FimB and FimE, the genes of which are located upstream of *fimS* and transcribed seperately from the other *fim* genes (Figure 1.2). These recombinases belong to the lambda integrase family of site-specific recombinases (Argos *et al.*, 1986; Dorman *et al.*, 1987; Eisenstein *et al.*, 1987) and catalyse the inversion of *fimS* by recognizing and binding to sequences that include 9bp inverted repeats (IRL and IRR) that flank the *fim* switch (Abraham *et al.*, 1985; Klemm, 1986; McClain *et al.*, 1991; Gally *et al.*, 1996). FimB is able to drive inversion in both directions (McClain *et al.*, 1991) at a relatively low frequency of approximately $2x10^{-3}$ per cell per generation (Blomfield *et al.*, 1993). However, while the activity of FimE is much higher (up to 0.7 per cell per generation) (Gally *et al.*, 1993), it catalyses switching from ON to OFF orientation selectively (McClain *et al.*, 1991).

The *fim* switch is an example for phase variation, a bacterial regulatory mechanism of gene expression. Phase variation is the reversible switching between expression ("ON") and non-expression ("OFF") of a gene and a means of regulating expression of a wide range of genes. This ultimately leads to a range of different phenotypes that, for example, enable the organism to adapt to changes in environmental conditions.

Phase variation can occur via distinct mechanisms. The *fim* switch, as described above, is an example for the genetic mechanism, as inversion of *fimS* alters the nucleotide sequence of the *fim* gene cluster. In contrast to this, phase variation of Ag43 occurs via an epigenetic mechanism. Ag43 is an outer membrane protein of *E. coli*, its variable roles include

autoaggregation, adherence and biofilm formation. Phase variation of the respective gene *agn43* involves deoxyadenosine methyltransferase (Dam) and the DNA binding protein OxyR (reviewed by van der Woude *et al.*, 2008). OxyR is a global regulator involved in protection against oxidative stress (Christman *et al.*, 1989) and changes its structure by oxidation and reduction (Tao *et al.*, 1991; Lee *et al.*, 2004). By binding to its specific binding site in the *agn43* promoter region, OxyR is able to repress and switch off *agn43* expression. As the OxyR binding site covers three Dam methylation sites (5'-GATC-3'), DNA methylation by Dam inhibits OxyR binding and repression. In contrast to the genetic mechanism of the *fim* switch, Ag43 switching follows an epigenetic mechanism of phase variation as it involves chemical modification of a nucleotide.

1.1.2. Regulation of the *fim* switch

The extent of interaction of bacteria with their environment and thus the extent of host response determines the extent of environmental stresses the bacteria have to endure.

1.1.2.1. Inversion of *fimS*

Phase variation, in general, and the *fim* switch, in particular, enables organisms like *E. coli* to avoid or regulate the extent of host defence. As the host defence jeopardises cell survival, it is inevitable that the production of type 1 fimbriae and hence the switching of *fimS* is closely monitored. This is accomplished at every level of fimbriae production, not only directly by regulating the inversion of *fimS*, but also indirectly by altering the level of *fimB* and *fimE* expression and their respective recombinase activity.

Inversion of *fimS* (switching) is carried out by cleavage of the DNA, strand exchange and subsequent re-ligation (Sadowski, 1993). For this process, physical proximity of the 9bp inverted repeats flanking *fimS* (IRL and IRR) is essential, as these regions represent the binding and cleavage sites for FimB and FimE (Gally *et al.*, 1996). The required structure is achieved by bending the DNA, whereby topological interference and steric hindrance is diminished by the integration host factor (IHF) (Thompson *et al.*, 1988; Kosturko *et al.*, 1989) and leucine-responsive regulatory protein (Lrp) (Wang *et al.*, 1993).

Lrp promotes inversion by binding to operator sites within the invertible element *fimS*, and its binding affinity depends on the levels of the branched-chain amino acids leucine (L), isoleucine (I) and valine (V) as well as alanine (A) (referred to as IVLA). Binding of Lrp

bends the DNA and hence facilitates *fimS* inversion. Three operator sites have been identified within the *fimS* region. Control by Lrp means that switching is inhibited when levels of leucine are low, but while binding to site 1 and 2 stimulates *fimS* inversion, binding to site 3 produces an inhibitory complex (Gally *et al.*, 1994; Roesch *et al.*, 1998). It transpires that Lrp binds with low affinity to the third site and the complex at this site is more responsive to IVLA. Elevated levels of leucine promote the selective loss of Lrp binding to site 3 (while not affecting binding to sites 1 and 2) and hence stimulate *fimS* inversion (Roesch *et al.*, 1998; Lahooti *et al.*, 2005). Besides Lrp, many other factors have been shown to directly affect inversion. Two binding sites for integration host factor (IHF) have been identified in the invertible element *fimS* (Blomfield *et al.*, 1997). Binding of IHF to site II (within the invertible element) produces sharp bends in the DNA, IHF hence enhancing and facilitating the effect of Lrp binding to its sites 1 and 2 (Figure 1.3). The function of IHF binding site I (adjacent to the invertible element) remains unclear, but is thought to involve steric hindrance of another protein that blocks FimB from binding to IRL, but not FimE. Studies on *fimS* inversion also revealed the histone-like nucleoid-structuring protein H-NS to have a repressing effect (Kawula *et al.*, 1991; Schembri *et al.*, 1998; O'gara *et al.*, 2000). This is supported by data showing that H-NS leads to promoter-specific repression of the *fimB* gene, hence repression of the OFF-to-ON inversion of *fimS* (Donato *et al.*, 1997).

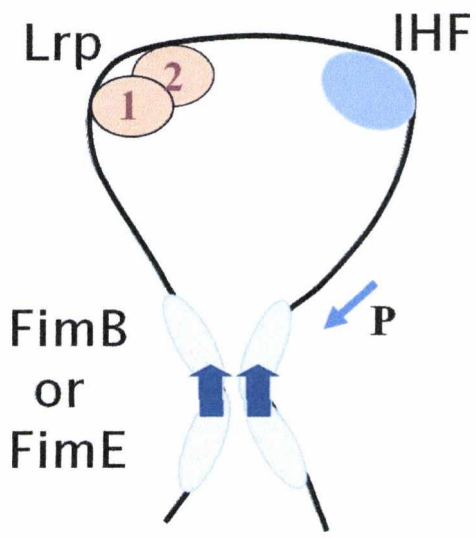


Figure 1.3: Binding of Lrp and IHF in the invertible element *fimS* facilitates bending of the DNA and thus promotes the inversion of *fimS*.

By regulating expression of the recombinase genes, the cells are able to specifically control ON-to-OFF (FimE) and OFF-to-ON (FimB) switching. It has been shown that orientation of *fimS* plays an important role in *fimE* expression, as the *fimE* transcript overlaps with the invertible element. The mRNA of *fimE* that is generated when *fimS* is in the OFF orientation is inactivated by 3' to 5' degradation (Sohanpal *et al.*, 2001). Hence FimE is produced at higher levels when in the ON orientation and its activity is restricted to switching from ON-to-OFF. Being able to switch from OFF-to-ON, the levels of *fimB* expression correlate directly to the rate of fimbriae production. Therefore the regulation of *fimB* has been well studied and is the focus of this study.

1.1.2.2 Regulation of *fimB* expression

An unusually large (1.4 kbp) intergenic region separates the *fimB* gene from the divergently transcribed *nanC* gene, which is involved in metabolism of *N*-acetylneuraminic acid (Neu_5Ac, sialic acid). This intergenic region provides binding sites for many regulatory factors that co-ordinately control expression of the two genes *nanC* and *fimB* (Figure 1.4).

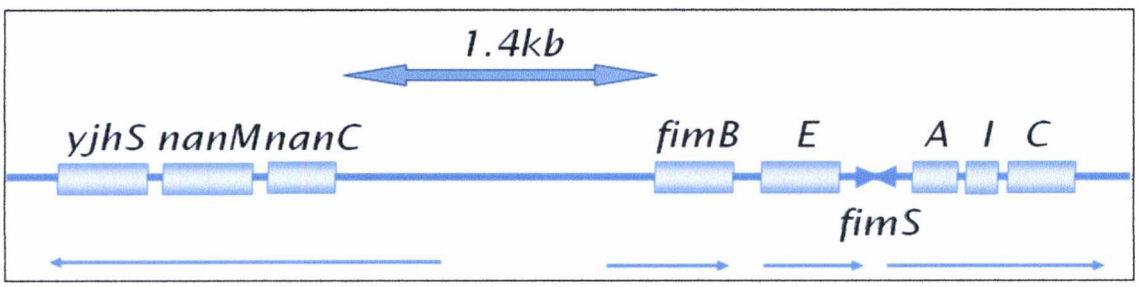


Figure 1.4: Organisation of the divergently transcribed *nanMC yjhS* and the *fim* operons

The regulatory proteins NanR (Neu_5Ac-responsive), NagC (GlcNAc-6P-responsive) and IHF are able to activate *fimB* and thus repress *nanC* expression. The operator site for NanR (O_{NR}) overlaps the -10 region of the *nanC* promoter (Condemine *et al.*, 2005) and binding of NanR is disrupted in the presence of *N*-acetylneuraminic acid (sialic acid, Neu_5Ac) (El-Labany *et al.*, 2003; Kalivoda *et al.*, 2003). NagC binds to two operator sites (O_{NC1} and O_{NC2}), that are located closer to the *fimB* promoter, and is responsive to the cell wall component *N*-acetylglucosamine (GlcNAc) (Sohanpal *et al.*, 2004; Sohanpal *et al.*, 2007). Binding of IHF to a site midway between O_{NC1} and O_{NC2} facilitates interaction between

these two sites and NagC binding, leading to the formation of a nucleoprotein complex that activates *fimB* expression (Sohanpal *et al.*, 2007) (Figure 1.5). The operator sites of NagC and NanR lie close to methylation sites recognised by deoxyadenosine methylase (Dam). Binding of NanR and NagC to their repective sites prevents Dam-methylation (El-Labany *et al.*, 2003). It has been shown that methylation protection only occurs at one site at a time, suggesting that NanR and NagC binding alternates and that *fimB* expression is activated alternatively by NanR and NagC (Sohanpal *et al.*, 2004; Sohanpal *et al.*, 2007). However, details on how these two regulators are actually able to activate *fimB* expression still remain to be clarified.

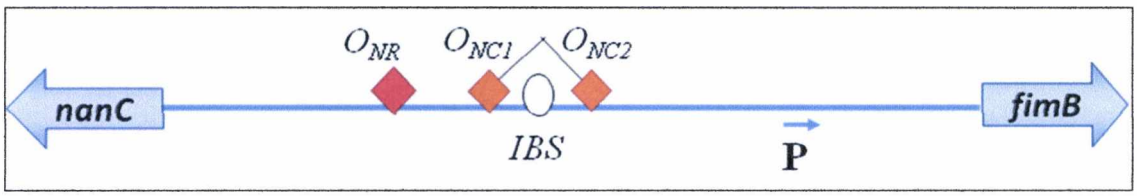


Figure 1.5: Binding sites for NanR (O_{NR}), NagC (O_{NC1}/O_{NC2}) and IHF (*IBS*) in the *nanC-fimB* intergenic region

Another protein that regulates *fimB* expression is H-NS. This global regulator has been shown to usually repress about 5% of all genes in *E. coli* (Hommais *et al.*, 2001) in response to environmental conditions (Schroder *et al.*, 2002). H-NS consists of an oligomerization domain and a nucleic acid binding domain, which preferentially binds to curved double-stranded DNA and thereby creates DNA-protein-DNA bridges (Dame *et al.*, 2001; Dame *et al.*, 2006). By inhibiting sigma factor σ^S at a post-transcriptional level (Barth *et al.*, 1995; Yamashino *et al.*, 1995), H-NS especially targets genes related to environmental stress. Sigma factor σ^S synthesis is positively regulated by guanosine 3',5'-bispyrophosphate (ppGpp) (Gentry *et al.*, 1993) and H-NS possibly disrupts this mechanism of activation. More importantly for this project, H-NS has been shown to be involved in the regulation of fimbriae production. H-NS inhibits *fimS* inversion by directly binding to the *fim* invertible element (Kawula *et al.*, 1991; Schembri *et al.*, 1998; O'gara *et al.*, 2000) and also specifically represses *fimB* transcription. Studies have not only revealed that H-NS represses *fimB* expression by binding tightly to the *fimB* promoter, but also that there may be two active *fimB* promoters (named P1 and P2) which are both repressed by

H-NS (Donato *et al.*, 1997). However work in our laboratory shows that only one of these promoters (P2) is active in the conditions used in this study (Sohanpal *et al.*, 2007).

Many regulatory proteins activate gene expression by antagonizing a repressor. In the case of H-NS and its inhibiting effect on *fimB* expression, one such activator is the protein SlyA. SlyA is a transcriptional regulator involved in the regulation of a wide range of cellular processes, including the resistance to host defences and other virulence factors. The protein belongs to the MarR family of transcriptional regulators, which primarily activate bacterial virulence and resistance mechanisms (reviewed by Ellison *et al.*, 2006). By associating with DNA in form of homodimers (Stapleton *et al.*, 2002), SlyA is able to disrupt and antagonise binding of other regulatory proteins. In *Salmonella typhimurium*, SlyA regulates a variety of extracellular, exposed virulence factors and is thought to protect from host defences by altering the bacterial cell surface (Navarre *et al.*, 2005; Corbett *et al.*, 2007). The SlyA mRNA features the poorly-translated UUG initiation codon (Kawakami *et al.*, 1999); production and hence levels of proteins with such poor translation initiation is enhanced during slow growth and by elevated levels of ppGpp (Liang *et al.*, 2000). In the case of SlyA, it is suggested that ppGpp not only stimulates translation, but also interacts directly with SlyA and enhances SlyA binding to target promoters by facilitating dimerisation (Zhao *et al.*, 2008).

Investigations on the interaction between SlyA and H-NS, i.e. antagonsim of H-NS repression by SlyA (reviewed by Stoebel *et al.*, 2008), suggest that SlyA can compete with H-NS for DNA binding sites and thus directly inhibit H-NS binding and repression as has been shown for *hlyE* activation (Wyborn *et al.*, 2004; Lithgow *et al.*, 2007). In this model of "mutual antagonsim" SlyA and H-NS can displace each other depending on the relative excess of these proteins. This model also provides a mechanism of how H-NS is able to re-associate and re-form an inhibitory complex (Lithgow *et al.*, 2007). In an alternative mechanism of SlyA activation, SlyA and H-NS bind simultaneously and form a nucleoprotein complex, a process that is dependent on the ratio of H-NS and SlyA in the cell. This mechanism (as decribed for the promoter of the K5 capsule gene cluster) requires the presence of H-NS for maximal SlyA stimulation of transcription (Corbett *et al.*, 2007; Xue *et al.*, 2009). Activation by SlyA hence not only occurs by disrupting H-NS binding, but also by functional interaction between SlyA and H-NS. Despite an apparent close link between SlyA and H-NS regulation, expression and auto-regulation of SlyA is independent of H-NS (Corbett *et al.*, 2007).

The involvement of H-NS in fimbriation and *fimB* regulation has been well-studied (Kawula *et al.*, 1991; Donato *et al.*, 1997; Schembri *et al.*, 1998; O'gara *et al.*, 2000), which does not apply to SlyA. In recent work, a member of our group (McVicker *et al.*, 2011) has identified SlyA as a novel activator of *fimB* expression. SlyA has multiple operator sites in the intergenic region upstream of the promoter *fimB*. However, there are subtle differences between these sites, and binding of SlyA to its individual sites depends on growth rate, growth conditions and other factors. SlyA binds to these sites to interact with H-NS in a mechanism that is consistent with the regulatory mechanisms described previously for other systems under SlyA control (Corbett *et al.*, 2007; Lithgow *et al.*, 2007; Xue *et al.*, 2009). SlyA interacts with H-NS, possibly not only by antagonising H-NS and preventing binding, but also by forming a complex with H-NS and modifying the position of H-NS. The basic model for SlyA activation suggests that SlyA activates *fimB* expression by binding to two operator sites O_{SA1} and O_{SA2} upstream of the *fimB* promoter. H-NS binds to overlapping operator sites and, upon H-NS binding, bridges between these and other sites both upstream and downstream are thought to cause repression (Figure 1.6). SlyA, in turn, antagonises H-NS binding to these sites and hence prevents H-NS repression.

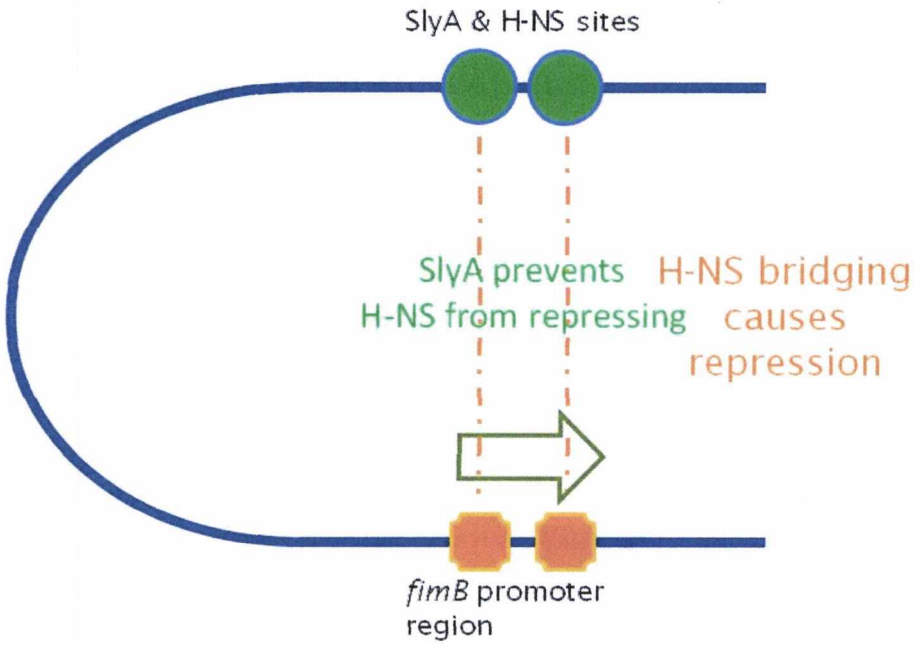


Figure 1.6: Schematic model for the activation of *fimB* expression by SlyA. SlyA antagonises H-NS repression by competing for binding sites in the *fimB* promoter region.

As type 1 fimbriae enable bacteria to interact with their environment, their production is bound to be subject to environmental control. Hence, research on the regulation of type 1 fimbriae, in general, and *fimB* expression, in particular, also covers a broader scope of regulatory factors such as temperature, media, pH, osmolarity and growth rate. Activation of the host immune system in response to bacterial infection generally entails a rise in temperature. The expression of *fimB* increases about two-fold from 30°C to 37°C, whereas the expression of *fimE* decreases about four-fold (Olsen *et al.*, 1998). In line with this, Gally et al. (1993) demonstrated that while switching by FimB increases with temperature, FimE-promoted switching is faster at lower temperatures than at higher temperatures. The number of fimbriate cells hence is proportional to the temperature, reaching optimal levels at mammalian body temperature between 37°C and 41°C. Furthermore, repression of *fimB* and *fimE* by H-NS differs between 30°C and 37°C, yet H-NS is not directly involved in the inversion of *fimS* (Olsen *et al.*, 1998). This suggests that H-NS might favour expression of type 1 fimbriae at body temperature by indirectly regulating *fimS* inversion via the transcription of the *fimB* and *fimE* genes.

Besides temperature, both *fimB* and *fimE* are also controlled by media, suggesting that expression of type 1 fimbriae could vary in different host compartments. The *fim* switch is controlled by levels of the aliphatic amino acids alanine, isoleucine, leucine, and valine (IVLA) and requires the protein Lrp (Gally *et al.*, 1993). As described above, Lrp promotes inversion by binding to operator sites within the invertible element *fimS*. While binding to sites 1 and 2 is stimulatory, binding to site 3 inhibits recombination, and the aliphatic amino acids (especially leucine) promote the selective loss of Lrp from site 3 (Gally *et al.*, 1994; Roesch *et al.*, 1998; Lahooti *et al.*, 2005). In conjuction with this regulatory complex, IHF is thought to assist the topological effects of Lrp and facilitate switching (Blomfield *et al.*, 1997).

fimB expression is also susceptible to pH and osmolarity. Low pH, as well as high osmolarity, reduce *fimB* (and *fimA*) expression, thus leads to the invertible element being in the phase-OFF orientation and to a loss of fimbriae expression (Schwan *et al.*, 2002). This may enable *E. coli* to regulate expression of type 1 fimbriation in order to adapt to specific host compartments like the bladder or kidney. However, details on the mechanism of this regulation remain to be elucidated. The same applies to the finding that transcription of *fimA* and *fimB* is repressed when cells enter the stationary phase. This repression is dependent on the stationary phase-specific sigma factor RpoS, but the mechanism by

which RpoS achieves negative control remains unknown (Dove *et al.*, 1997). Other environmental factors that control *fimB* expression include ppGpp, sialic acid and GlcNAc. While the effect of ppGpp was shown to be independent of the ppGpp accessory factor DksA (Åberg *et al.*, 2006; Åberg *et al.*, 2008), it is possibly mediated via SlyA, enhancing *fimB* activation by increasing levels of SlyA and/or SlyA dimerisation and binding (Zhao *et al.*, 2008). As part of host defence systems, sialic acid is essential for TLR function of host cells (Amith *et al.*, 2009) and *E. coli* does not produce sialic acid itself. Therefore it presents a key signal for activated host immune response. In the presence of sialic acid, NanR binding to its operator site on the *nanC* promoter is disrupted, which activates the *nanC* operon (for sialic acid catabolism) and inhibits *fimB* expression. Sialic acid catabolism, in turn, generates GlcNAc-6-P, a bacterial cell wall component which disrupts NagC binding to its operator sites (El-Labany *et al.*, 2003; Kalivoda *et al.*, 2003; Sohanpal *et al.*, 2004; Sohanpal *et al.*, 2007). As NanR and NagC are both activators of *fimB*, NanR and NagC work in conjunction to decrease type 1 fimbriation in response to potential threats such as host defence activation and cell wall damage, respectively.

Overall, type 1 fimbriae are required to be tightly regulated, as the extent of interaction of the cell with its environment inevitably relates to the extent of exposure to stress factors. In addition to the regulation of fimbriae production, the bacterial cell has evolved other sytems to protect against and counteract various environmental stress factors.

1.1.3. Environmental stress factors for *E. coli*

Interaction with the environment entails the exposure to a range of potentially harmful factors. As a consequence of aerobic metabolism, all aerobic organisms are subject to physiological oxidative stress in form of reactive oxygen species ROS such as superoxide ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•). Furthermore, ROS are generated as part of the host immune response, as is nitric oxide (NO) (Nathan *et al.*, 2000). In reaction with superoxide ($\bullet O_2^-$), NO generates reactive nitrogen species (RNS) such as peroxynitrite (ONOO-) (Wink *et al.*, 1998; Pacher *et al.*, 2007). Both ROS and RNS cause damage to a range of cell components including proteins, lipids and DNA.

An important reaction of nitric oxide is S-nitrosylation - the conversion of thiol groups to S-nitrosothiols. But it also specifically targets iron-containing proteins by degrading their Fe-S centers (Hibbs *et al.*, 1988), as does superoxide (Flint *et al.*, 1993). In response, the

cell activates numerous genes involved in protection, such as the genes for superoxide dismutases *sodA* (via SoxR and SoxS) (Wu *et al.*, 1991) and *sodB* (via NsrR) (Bodenmiller *et al.*, 2006; Filenko *et al.*, 2007). In scavenging the superoxide radical, superoxide dismutase enzymes generate hydrogen peroxide (Fridovich, 1975), which induces the OxyR regulon and thus activates the expression of many antioxidant genes (Zheng *et al.*, 2001). Iron (Fe) and Fe-S clusters play a major role in response to oxidative and nitrosative stress. NsrR contains an NO-sensitive Fe-S cluster that is required for DNA binding activity (Tucker *et al.*, 2008) and SodB contains Fe in its active center (Stallings *et al.*, 1983); furthermore, Fe-S clusters are a primary target for reactive oxygen species (Keyer *et al.*, 1996; Keyer *et al.*, 1997; reviewed by Imlay, 2003). Upon oxidation, Fe-S clusters release Fe^{2+} (Flint *et al.*, 1993). Free Fe^{2+} can reduce H_2O_2 in the Fenton Reaction, and thus generate the extremely reactive hydroxyl radical (OH•) (Imlay *et al.*, 1988). Hydroxyl radicals are able to oxidise most organic molecules and since it cannot be eliminated by an enzymatic reaction, the cell requires antioxidants (such as glutathione) and effective repair systems for protection.

Iron (Fe) plays an important role in the cell. It not only forms Fe-S clusters, the assembly of which is encoded by genes in the *isc* and the *suf* operon (Outten *et al.*, 2004), Fe^{2+} is also present in active sites of numerous enzymes and acts as a cofactor for cytochromes and other haem-containing enzymes. The uptake and homeostasis of Fe^{2+} is regulated by the ferric uptake regulator Fur (Hantke, 2001), a global regulator of many genes involved in a wide range of cellular functions. When activated in complex with Fe^{2+}, Fur regulates gene transcription by inhibiting the small regulatory RNA RyhB (Bagg *et al.*, 1987; Massé *et al.*, 2002; Massé *et al.*, 2003; Massé *et al.*, 2005) and is directly inhibited by NO (D'Autreaux *et al.*, 2002). Fe^{2+} can also be directly linked to a range of other metal ions. For example, Mn^{2+} uptake via the metal ion transporter MntH is repressed by Fur. Mn^{2+} can help protect against oxidative stress by substituting for Fe^{2+} in proteins and hence making them less vulnerable to oxidation and the Fenton Reaction (Patzer *et al.*, 2001; Anjem *et al.*, 2009). Furthermore, copper toxicity is primarily based on the fact that Cu(I) specifically targets Fe-S clusters by displacing the iron atoms and binding to the sulfur atoms (Macomber *et al.*, 2009). Fur and copper also suppress the expression of the metal binding protein ZinT, which binds other divalent metal ions like zinc, cadmium and nickel and is thus proposed to be part of a more general stress response (David *et al.*, 2003; Kershaw *et al.*, 2007).

Another environmental factor that affects homeostasis of the bacterial cell is the pH value. Acid stress resistance has been shown to be under the control of the global regulator H-NS and involves, amongst other systems, the amino-acid decarboxylases GadA and AdiA (Hommais *et al.*, 2001; Richard *et al.*, 2003; Kannan *et al.*, 2008). In response to low pH, GadA degrades glutamate to γ-aminobutyrate (GABA), while AdiA degrades arginine to agmatine, in both cases with consumption of a proton H^+ and release of CO_2. Both systems require a transmembrane antiporter (GadC and AdiC) that exchanges the extracellular substrate with the intracellular product (De Biase *et al.*, 1999; Gong *et al.*, 2003). An additional type of arginine decarboxylase (SpeA) is expressed regardless of pH variations, as it is ordinarily involved in the synthesis of polyamines. This protein is located in the cell envelope and, as AdiA, generates agmatine from extracellular arginine (Tabor *et al.*, 1969; Buch *et al.*, 1985) for subsequent polyamine biosynthesis. As AdiA is pH sensitive, AdiA and SpeA activity is probably coordinately controlled, complementing each other.

Most of these stress factors can be associated with the tripeptide glutathione. This antioxidant plays a key role in protecting the cells against a range of environmental stress factors. By oxidation and nitrosylation of its thiol group, it can counteract the effects of oxidative and nitrosative stress (Dalle-Donne *et al.*, 2009b) and as an efficient metal chelator (Ballatori, 1994), glutathione can prevent damage caused by free metal ions. Futhermore, it has been shown to be involved in pH-regulation (Ferguson *et al.*, 1998).

1.2. Glutathione – an abundant key molecule

Glutathione (GSH) is a globally active molecule involved in numerous cellular processes in almost all organisms. By serving as a reductant, it counteracts oxidative stress and protects the thiol groups of intracellular proteins, whereby its own thiol group has a key function.

1.2.1. The tripeptide – synthesis and metabolism

Glutathione (GSH) is the most abundant nonprotein thiol (1-10mM) in almost all aerobic organisms. The tripeptide consists of the amino acids glutamate, cysteine and glycine. It is an unconventional peptide due to an unusual link between the amino group of cysteine and the carboxyl group of the glutamate side chain (Figure 1.7).

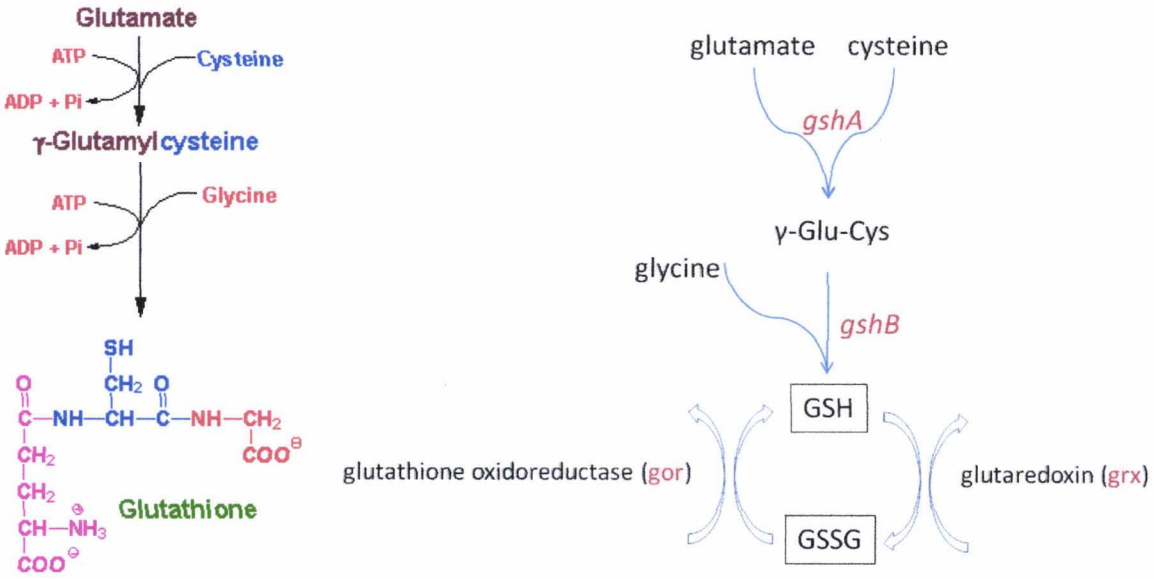


Figure 1.7: The biosynthesis of glutathione and the redox cycle between GSH and GSSG.

GSH synthesis and its metabolism have been described in detail by A. Meister and M.E. Anderson (Meister, 1988; Anderson, 1998) and has been reviewed by W. Wang (Wang *et al.*, 1998). GSH is synthesised in the cytoplasm in two consecutive, ATP-dependent steps. In the first step, γ-glutamylcysteine synthetase (GshA) catalyses the formation of a peptide bond between L-glutamate and L-cysteine. The limited availability of cysteine determines the rate of this reaction. Hence this reaction represents the rate-limiting step in glutathione biosynthesis and is controlled by negative feedback from the final end product GSH (Apontoweil *et al.*, 1975a; Richman *et al.*, 1975; Wang *et al.*, 1998). In the subsequent reaction, glycine is added to the C-terminal end of γ-glutamylcysteine by the enzyme glutathione synthetase (GshB) to yield γ-glutamylcysteinylglycine (GSH) (Apontoweil *et al.*, 1975a). Cysteine plays an important role in both GSH biosynthesis and activity. Not only is this amino acid the rate-limiting factor in glutathione synthesis, but the cysteine residue also provides the peptide with a free sulfhydryl (thiol) group (-SH). By serving as an electron donor, this thiol group gives GSH high electron-donating capacity and hence important reducing power (reviewed by Kidd, 1997).

Although GSH is synthesized inside the cell, it is transported out of the cell for degradation. The export of reduced glutathione from the cytoplasm to the periplasm is mediated by the heterodimeric ABC transporter CydDC (Pittman *et al.*, 2005). However,

GSH is not only degraded in the periplasm, but it also has a physiological periplasmic function. The periplasm of *E. coli* features a finely balanced redox control in which GSH acts as an additional reductant and supports disulfide bonding in proteins. Re-uptake of excreted GSH is possible via GsiABCD. This import system represents a salvage pathway for excreted GSH that works in conjunction with the periplasmic, membrane-bound cleavage enzyme γ-glutamyltransferase (Ggt). Ggt initiates breakdown of GSH by cleaving the γ-glutamyl linkage and transfers the γ-glutamyl group to acceptors like amino acids. The generated dipeptide cysteinylglycine (Cys-Gly) residue is taken up into the cytoplasm, cleaved by cysteinylglycinases (Aminopeptidases A, B, and N and dipeptidase D) and utilized as both cysteine and glycine sources (Suzuki *et al.*, 1986a; Suzuki *et al.*, 1986b; Suzuki *et al.*, 2001). This is particularly important in the case of cysteine, since the availability of this amino acid is limited in the cell. Potential import systems for Cys-Gly are the ABC transporters Dpp (for dipeptides) or Opp (for oligopeptides). The substrate-binding subunit DppA controls the specificity of Dpp (Olson *et al.*, 1991; Wu *et al.*, 1995; Smith *et al.*, 1999; Letoffe *et al.*, 2006). However, the binding site of DppA only recognises the peptide backbone and hence non-specifically binds a range of peptides for Dpp transport (Dunten *et al.*, 1995). DppA expression increases significantly in minimal medium in response to a greater need for amino acids (Olson *et al.*, 1991). For Opp-mediated transport of oligopeptides, the substrate-binding protein OppA recognises oligopeptides of up to a length of five amino acids (Gilvarg *et al.*, 1965; Gilvarg *et al.*, 1965; Payne, 1968; Guyer *et al.*, 1986). However, OppA shows no or only low affinity for free amino acids and dipeptides, which favours the Dpp-transporter as the main system for import of cysteinylglycine.

Another gene potentially involved in glutathione uptake and metabolism is *iaaA* (*ybiK*). According to Parry and Clark (2002), IaaA is required for GSH utilization as a sulfur source by playing a role in transport. The *iaaA* gene is located directly upstream of the *gsiABCD* operon, which Suzuki (2005) reports to be required for GSH uptake. However, other research reports IaaA to have L-asparaginase activity (Borek *et al.*, 2004) and hence may have a function in GSH degradation.

1.2.2. Glutathione – the redox cycle

Highly reactive intermediates such as superoxide and hydrogen peroxide are formed in aerobic metabolism. A dramatic increase in the levels of these reactive oxygen species

(ROS) may result in significant damage to DNA, lipids and proteins and thus cause metabolic disruptions. This damage commonly involves the generation of disulfide bonds by oxidation of thiol (-SH) groups. Being an abundant reductant and antioxidant, GSH has a key function in restoring thiol groups to their reduced state. As a consequence, reduced GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by glutathione reductase (Gor) (Figure 1.7). This reaction consumes reduced nicotinamide adenine dinucleotide phosphate (NADPH) which is generated in the pentose phosphate shunt by glucose-6-phosphate dehydrogenase (Glc-6-P-DH) (Kidd, 1997; Wang *et al.*, 1998), as well as in the TCA cycle (Sauer *et al.*, 2004; Csonka *et al.*, 1977). As Gor is constitutively active and is induced upon oxidative stress, this closed redox cycle ensures a stable reducing environment under physiological conditions. The majority (>90%) of the cellular glutathione pool is maintained in the reduced thiol form (GSH), the rest being present within the cell as mixed disulfides (mainly GS-S-protein), as the disulfide (GSSG) and as thioethers (R-S-R') (Kosower *et al.*, 1978). The GSH/GSSG ratio hence is an indicator of cellular stress level; in order to sustain a healthy ratio, glutathione homeostasis is tightly controlled by balancing its synthesis, redox-recycling and consumption.

Together with glutaredoxins, glutathione and Gor form the glutathione/glutaredoxin system. *E. coli* contains four glutaredoxins. Grx1 and Grx3 are classical dithiols and while Grx1 has been shown to be an electron donor for metabolic enyzmes like ribonucleotide reductase (RNR) and phosphoadenylsulfate (PAPS) reductase, the function of Grx3 remains unclear. Grx2 is a larger dithiol involved in monothiol-type reactions similarly to other GSH-dependent oxidoreductases. Grx4 is an unusual monothiol, as it can be reduced by thioredoxin reductase. It does not have classical Grx activity and is likely to be involved in iron homeostasis (Fernandes *et al.*, 2004; Lillig *et al.*, 2008). Like the thioredoxin system, glutaredoxins are induced by oxidative stress in order to maintain and regulate the cellular redox state (Tao, 1997; Carmel-Harel *et al.*, 2000; Song *et al.*, 2002). As opposed to the thioredoxins that are reduced by thioredoxin reductase (Arner *et al.*, 2000; Mustacich *et al.*, 2000), glutaredoxins require the reducing power of glutathione. Glutaredoxins reduce disulfide bonds of proteins through their redox-active thiol groups, but rely on glutathione for their own reduction. Oxidized glutathione is then regenerated by glutathione reductase (Holmgren, 1989; Carmel-Harel *et al.*, 2000; Fernandes *et al.*, 2004; Lillig *et al.*, 2008). Glutaredoxins reduce protein disulfide bonds by using both their cysteine residues, but also uniquely reduce mixed disulfides (glutathionylated proteins) by using only a single cysteine. The formation of mixed disulfides (glutathionylation),

catalysed by glutaredoxins themselves, is an important mechanism to sense the cellular redox potential (Fernandes *et al.*, 2004).

1.2.3. The thiol group – glutathionylation, metal chelation and pH

As described above, thiols groups are oxidised in response to oxidative stress and nitrosylated in response to nitrosative stress. GSH protects the cell from reactive species by oxidation or nitrosylation of its own thiol group and thus forming GSSG or GSNO, respectively. GSNO is considered to be a major reservoir of NO in cells, but remains reactive and has impact on metabolism by modifying proteins. GSNO primarily targets cysteine and homocysteine residues of regulators of the methionine pathway (MetJ, MetR, CysB; see more details below) (Jarboe *et al.*, 2008). However, a GSNO reductase is able to metabolise GSNO and hence control intracellular levels of both GSNO and *S*-nitrosylated proteins (Liu *et al.*, 2001). Both oxidative and nitrosative stress promote *S*-glutathionylation of proteins, i.e. the generation of a mixed disulfide bond between the cysteine residue of a protein and GSH. This provides a mechanism for post-translational modification of proteins in order to regulate cellular processes or to prevent oxidation of protein thiols (Dalle-Donne *et al.*, 2009a; Dalle-Donne *et al.*, 2009b). Thiol groups are involved in protein structure and are often present in the active site of a protein, thus modification of the thiol group affects structure and catalytic function of the protein. Although *S*-glutathionylation is usually considered to help protect against damage caused by oxidative or nitrosative stress, it also occurs under basal physiological conditions. Glutaredoxins and thioredoxins regulate deglutathionylation reactions and reverse related changes in biological activity. This redox regulation thus provides a specific control of many cellular processes. One example for redox regulation is OxyR, the activity of which is dependent on the formation of a disulfide bond and reversed by reduction with Grx1 (Zheng *et al.*, 1998). Furthermore, 3′-phosphoadenylylsulfate (PAPS) reductase is subject to redox regulation via reversible glutathionylation (Lillig *et al.*, 2003). This enzyme is involved in the sulfur assimilation pathway and the biosynthesis of cysteine (see below for details) and is regulated by oxidized glutathione (GSSG) and glutaredoxins. The formation of a mixed disulfide between GSSG and the active site Cys^{239} inhibits the activity of PAPS reductase and can be reduced by all glutaredoxins. In a physiological context, this regulation can be considered as an adaptation to oxidative stress, since oxidative stress elevates levels of oxidised GSSG. By inhibiting PAPS reductase, electrons are not used for

reduction of PAPS and sulfur assimilation, but can instead be targeted to reduce and repair oxidised proteins. Furthermore, the reversible inactivation of PAPS reductase by GSSG restricts cysteine biosynthesis and consequently glutathione biosynthesis. High levels of GSSG may indicate a sufficiency in glutathione and therefore have a negative feedback control on glutathione biosynthesis.

Conjugation with GSH does not necessarily involve the formation of a bond via the thiol group. In the case of the conjugate glutathionylspermidine (Gsp), an amide bond is formed between the glycine carboxyl group of GSH and the first amino group of spermidine (Tabor *et al.*, 1975). Gsp synthetase/amidase (GspSA) catalyzes both the synthesis and hydrolysis of Gsp (Bollinger *et al.*, 1995). Details on the physiological role of Gsp are still to be clarified, however it was recently discovered that Gsp is able to form mixed sulfides with protein thiols (GspSSPs) and thus provides an additional type of post-translational modification (Chiang *et al.*, 2010). Formation of these mixed thiols most likely occurs in order to prevent oxidation of protein thiols, as the level of GspSSPs is increased by oxidative stress. This increase is suggested to be caused by the selective inactivation of the GspSA amidase domain by H_2O_2, while the GspSA synthetase domain of the enzyme remains active. Elimination of the oxidative threat restores GspSA amidase activity which, together with GSH reductase (Gor) and glutaredoxin, leads to the recovery of basal levels of Gsp and GspSSPs (Chiang *et al.*, 2010). Gsp synthesis presents an intersection of GSH and spermidine metabolism and can account for up to 80% of the total glutathione content (Smith *et al.*, 1995). Hence Gsp might provide a way to control and balance cellular levels of the individual components GSH and spermidine.

Gsp is not the only connection between GSH and polyamines. As described below in more detail, polyamine biosynthesis can be linked to the metabolism of the individual amino acids that constitute the tripeptide GSH. Furthermore, polyamines are involved in translation initiation, especially of poorly translated genes due to their start codon UUG (Yoshida *et al.*, 2001). As a matter of fact, this also applies to *gshA*, the gene for GSH synthetase.

The protective and regulatory functions of GSH are also related to its ability to chelate reactive metals and thus coordinate metal transport, storage and metabolism. Again, it is the thiol group of GSH that plays a central role, as it has a high affinity for metals like mercury, cadmium, copper and zinc. Five other potential coordination sites (the glutamyl amino group, the glycyl and glutamyl carboxyl groups, and the two peptide linkages) have

lower affinity, but can help stabilise the coordination of a metal bound to the thiol group (Ballatori, 1994; Wang *et al.*, 1998). Since metal thiolates are generally kinetically labile, the metal ion is able to rapidly exchange between thiol ligands, a property that gives metal ions their toxicity. GSH can contain metal toxicity not only by regulating their transfer between ligands and their transport across cell membranes, but also as a source of cysteine for metal binding and as a reductant or cofactor in redox reactions involving metals. In connection with investigations into the mechanism of copper toxicity, Macomber and Imlay (2009) report that excess copper Cu(I) damages Fe-S proteins by displacing the iron atom and liganding to the coordinating sulfur atom. They conclude that chelation of copper by glutathione can enhance resistance to copper (Macomber *et al.*, 2009).

Lastly, GSH can be connected to the regulation of the cytoplasmic pH. GSH has been shown to protect against the electrophile methylglyoxal (Apontoweil *et al.*, 1975b). At the same time it is a negative regulator of the KefB and KefC potassium channels, whereby the absence of glutathione causes K^+ to leak out of these channels. These two systems correlate with each other in that the glutathione-dependent detoxification of methylglyoxal leads to the formation of *S*-lactoylglutathione which activates the KefB and KefC systems. Activation of the K^+ channels leads to a loss of K^+ and thus decreases the intracellular pH, which in turn protects against the toxic effects of methylglyoxal (Ferguson *et al.*, 1998). Hence GSH provides a link between detoxification and acidification, such that GSH is necessary for detoxification, but in doing so causes a decrease in pH.

GSH is associated with the protection against many stress factors. Since the extent of exposure to these stress factors inevitably relates to the extent of interaction of the cell with its environment, it is unsurprising that glutathione has been found to have a regulatory effect on *fimB* expression (as described below in further detail).

1.2.4. The individual amino acids – cellular function and significance

As outlined above, the importance of the tripeptide GSH is undisputed. However, the individual amino acids that form the tripeptide (glu, cys and gly) themselves play a major role in cellular processes such as replication, growth, metabolism and survival. GSH is considered to be a major "sink" for these amino acids and thereby potentially regulates and balances cellular levels of the free amino acids.

Glutamate is a non-essential amino acid found in many proteins and a key molecule in cellular metabolism. For glutamate generation, glutamate synthase (an aminotransferase) produces L-glutamate in a cyclic pathway. It first amidates L-glutamate to form L-glutamine. The following step involves the reductive transfer of the amide group to α-ketoglutarate to produce two molecules of L-glutamate. Alternatively, glutamate dehydrogenase (GdhA) uses ammonia itself as a nitrogen source and aminates α-ketoglutarate to yield L-glutamate (Miller *et al.*, 1972; Sakamoto *et al.*, 1975; Veronese *et al.*, 1975). This key role of glutamate in nitrogen assimilation and as an intermediate in nitrogen flux emphasises the importance of glutamate, as it makes glutamate a precursor for the biosynthesis of all other amino acids. Moreover, glutamate's precursor α-ketoglutarate directly links glutamate to the tricarboxylic acid (TCA) cycle, in which α-ketoglutarate is generated in successive reactions from citrate via isocitrate. In the steps preceding α-ketoglutarate synthesis, citrate is converted into D-*threo*-isocitrate (via cis-aconitase) in reactions catalysed by Aconitases A and B (AcnAB) and D-*threo*-isocitrate, in turn, is converted into α-ketoglutarate by isocitrate dehydrogenase (Icd). Glutamate is also linked to many other metabolic pathways by being converted into L-ornithine (Vogel, 1953). Ornithine is an important intermediate in the biosynthesis of a range of compounds, including the polyamine putrescine. Putrescine leads to the biosynthesis of spermidine, another common polyamine. Polyamines are involved in many biological processes and thus are essential for normal cell growth (see below). The direct pathway of putrescine biosynthesis from ornithine is the decarboxylation of L-ornithine (catalysed by ornithine decarboxylase, SpeC), while the indirect route involves the generation of L-arginine from L-ornithine (Vogel, 1953; Cunin *et al.*, 1986), its subsequent decarboxylation to agmatine (by SpeA) and hydrolysis to putrescine (by SpeB) (Morris *et al.*, 1966; Tabor *et al.*, 1985). Putrescine itself can be converted into spermidine (by SpeE) by transfer of a propylamine residue from decarboxylated S-adenosyl-L-methioninamine (dcSAM) (Bowman *et al.*, 1973). Its integration in a range of fundamental cellular processes clearly demonstrates the importance of glutamate and explains why its levels need to be tightly monitored.

Cysteine is a non-essential amino acid with a thiol side chain, which gives cysteine an important role in cellular processes. By participating in enzymatic reactions, the thiol side chain can be oxidised and form disulfide bonds, which have an important structural role in many proteins. L-cysteine is synthesised from L-serine in two steps via the intermediate *O*-acetyl-L-serine (Kredich *et al.*, 1966). The first step is carried out by serine acetylase

(CysE) and is inhibited by the final end product L-cysteine. The second step is catalysed by two isoforms of cysteine synthase (CysK and CysM) and combines sulfur in form of hydrogen sulfide with *O*-acetyl-L-serine to generate L-cysteine. For sulfur to be incorporated into cellular compounds, extracellular sulfate must first be reduced to sulfide. This occurs in the initial steps of the sulfur assimilation pathway via the intermediates adenosine 5'-phosphosulfate (APS), phosphoadenosine-5'-phosphosulfate (PAPS) and sulfite (Sekowska *et al.*, 2000). In reducing APS to PAPS, the cysteine residues of PAPS reductase (CysH) are oxidised and form a disulfide. Reduction and reactivation of the enzyme occurs via thioredoxins (Trx1 and Trx2) and the glutaredoxin Grx1 (Krone *et al.*, 1991; Berendt *et al.*, 1995; Lillig *et al.*, 1999). In response to oxidative stress, PAPS reductase can also be inactivated by reduced glutathione, forming a mixed disulfide with one if the cysteine residues. This inactivation can be reversed by all glutaredoxins and is intended to suppress electron flow through the sulfur assimilation pathway for the cell to be able to selectively use electrons to reverse undesired oxidation of proteins that are vital for cell survival (Lillig *et al.*, 2003).

Regulation of the second step of cysteine biosynthesis occurs via the activating complex of the cysteine-responsive transcription factor CysB and the intermediate *O*-acetyl-L-serine (Jones-Mortimer, 1968; Kredich, 1971; Kredich, 1992; van der Ploeg *et al.*, 1997). It is worth noting that CysB also regulates the expression of the *cysB*-like (*cbl*) gene, which encodes another transcription factor, Cbl. Cbl is inactivated by APS and is involved in an accessory regulatory circuit within the *cys* regulon. This could include genes required for sulfonate-sulfur utilization and genes that are induced by sulfate starvation and, in particular, the regulation of *cysM* (Iwanicka-Nowicka *et al.*, 1995; van der Ploeg *et al.*, 1997; van der Ploeg *et al.*, 2001; Bykowski *et al.*, 2002). Under conditions of sulfur starvation, i.e. the absence of sulphate or cysteine, CysB and Cbl hence induce the utilisation of alternative sulfur sources, such as aliphatic sulfonates or glutathione itself (Suzuki *et al.*, 1993; Iwanicka-Nowicka *et al.*, 1995; van der Ploeg *et al.*, 1997; van der Ploeg *et al.*, 2001; Bykowski *et al.*, 2002). Sulfur limitation stimulates the expression of the *cys* gene regulon: these conditions decrease the levels of L-cysteine and hence de-repress CysE, which generates *O*-acetyl-L-serine necessary for activation of CysB. CysB, in turn, activates expression of *cysK* and *cysM* and thus the production of L-cysteine (Jones-Mortimer, 1968; Kredich, 1971) (Figure 1.8).

Cysteine is a precursor for methionine (Soda, 1987), which is involved in the generation of the universal co-factor *S*-adenosyl-L-methionine (SAM) (Markham *et al.*, 1980). Thus, cysteine can be linked to many important cellular functions such as translation initiation, DNA methylation and polyamine biosynthesis (Lu, 2000). Almost all genes involved in methionine (*metB*, *metC*, *metE*) and SAM biosynthesis (*metK*) are repressed by MetJ. The affinity of MetJ to its target sites is enhanced when bound to SAM as co-repressor, giving SAM and MetJ negative feedback control on the synthesis of methionine as well as its own synthesis (Su *et al.*, 1971; Weissbach *et al.*, 1991; Gyaneshwar *et al.*, 2005; LaMonte *et al.*, 2006).

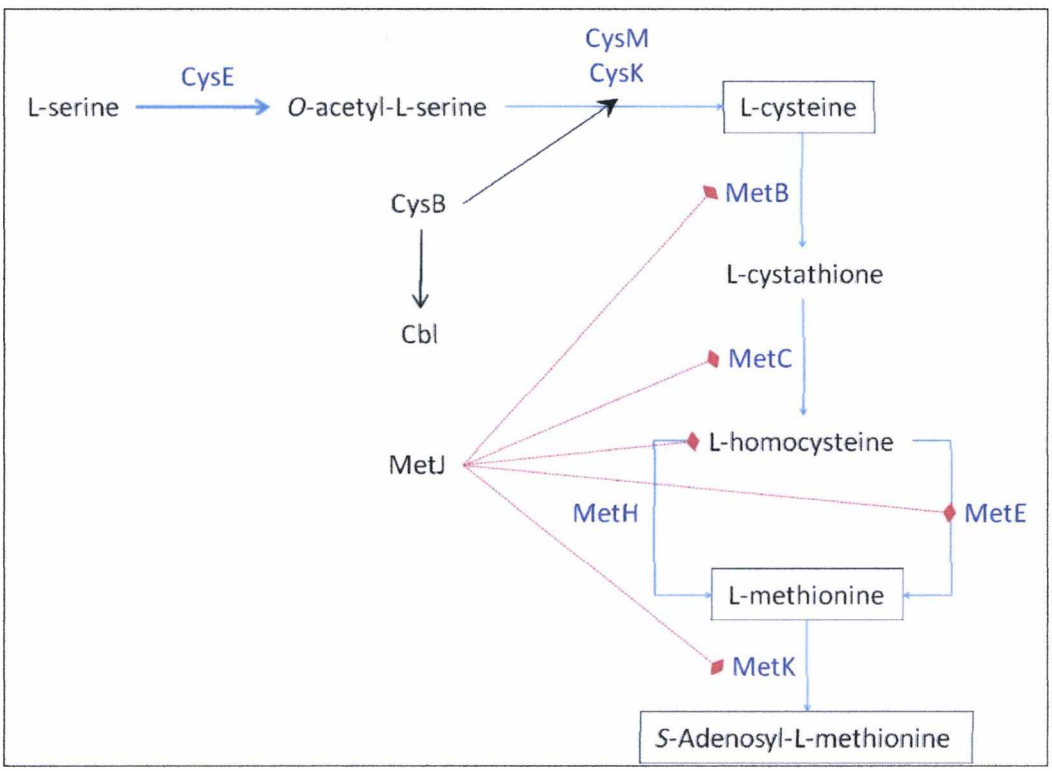


Figure 1.8: Pathway of cysteine and methionine biosynthesis

Due to its thiol group, cysteine and other compounds that incorporate cysteine are prone to oxidation of these groups. In the case of glutathione (GSH), the cysteine residue provides antioxidant properties, as well as the ability to glutathionylate proteins, i.e. form mixed disulfides (see above). This ultimately allows protection of proteins and/or modification of their biological activity (as described above). Oxidation of protein thiols may lead to disulfide bonds and thus affect protein folding and stability (via their tertiary structure or protein crosslinks) as well as activity (via cysteine residues in active sites of enzymes)

(Sevier *et al.*, 2002). Furthermore, cysteine is also an important source of sulfide. Cysteine desulfurase (IscS) converts cysteine into alanine and thereby releases sulfur (Schwartz *et al.*, 2000). Sulfur can then be used for the assembly and repair of iron-sulfur (Fe-S) clusters and other molecules. In fact, IscR, a sensor and regulator of iron-sulfur cluster assembly, has been shown to regulate expression of type 1 fimbriae via FimE (Schwartz *et al.*, 2001; Wu *et al.*, 2009). In addition to catalytic activity and redox regulation, the thiol group of cysteine also permits metal binding of proteins and hence the ability to sense and control levels of metal ions (Giles *et al.*, 2003). Overall, this clearly demonstrates the significance and importance of cysteine in the cell.

Glycine is the smallest proteinogenic amino acid with only two hydrogen atoms as its side chain. It is derived from L-serine by transfer of a methyl group to tetrahydrofolate. This reaction, catalysed by serine hydroxymethyltransferase (GlyA), is the primary source of glycine and one carbon (1C) units. Additionally, 1C units can be generated by the cleavage of glycine. The four proteins of glycine cleavage (Gcv) system (GcvT, GcvH, GcvP and GcvL) generate CO_2, NH_3 and a C1 unit from glycine (Kikuchi, 1973) and thereby balance the metabolic requirements for glycine and C1 units. While glycine is used in protein synthesis and in the biosynthesis of purines and cell walls, the C1 units are used in many processes, such as the biosynthesis of purines, thymidine, methionine, histidine, choline and lipids (Blakley, 1955; Pizer *et al.*, 1964; Mansouri *et al.*, 1972; Schirch *et al.*, 1985; Heil *et al.*, 2002). The genes for the Gcv proteins form the *gcv* operon which is induced in the presence of glycine and repressed in the presence of purines. This regulation is under the control of two *gcv*-specific transcriptional regulatory proteins (GcvA and GcvR) (Wilson *et al.*, 1994; Ghrist *et al.*, 1995). GcvA activates the *gcv* operon in the presence of glycine, while repression occurs in the absence of glycine and is dependent on GcvR. This can be explained by the formation of a repression complex between GcvR, GcvA and the promoter region of the *gcv* operon. Glycine disrupts this complex by directly binding to GcvR (Wilson *et al.*, 1993ab; Wilson *et al.*, 1993ba; Ghrist *et al.*, 1995; Ghrist *et al.*, 2001; Heil *et al.*, 2002).

Interestingly, glycine biosynthesis is potentially linked to methionine biosynthesis via the transcription regulator MetR, which controls several genes involved in methionine biosynthesis (Weissbach *et al.*, 1991) and activates *glyA* (Lorenz *et al.*, 1996). This suggests that cells coordinately control glycine and cysteine biosynthesis and thus balance levels of these (and other derivative) amino acids. Additional factors that link cysteine and

glycine are serine (as a common precursor) and 1C units (derived from glycine cleavage), as these are required for methionine biosynthesis from cysteine. Methionine is vital for the generation of SAM (for methylation of biological compounds) as well as for translation initiation (as *N*-formylmethionine f_{met}). This is significant with regard to glycine levels, as the *gcvR* gene has a poorly translated UUG start codon that benefits from increased levels of f_{met}.

Glutamate, cysteine and glycine, the three amino acids that form the tripeptide GSH, clearly are important in terms of regulating and maintaining cell homeostasis. Low GSH levels are bound to affect the cellular levels of these amino acids and hence their mutual balance. As illustrated above, there are a range of connections between cysteine and glycine. Regulation of glutamate levels, on the other hand, cannot be linked as easily to the levels of the other two amino acids. Apart from GSH itself, the polyamines could be claimed to be a secondary link. Cysteine and glycine both affect SAM metabolism and SAM, in turn, acts as a co-factor for SpeE in polyamine synthesis, i.e. the conversion of putrescine to spermidine (described in detail below). Glutamate, on the other hand, can be converted into the polyamine putrescine via ornithine, even though this is only a secondary pathway for putrescine biosynthesis.

1.3. Polyamines

1.3.1. Biosynthesis and Metabolism

E. coli produces four polyamines, putrescine, spermidine, cadaverine, and aminopropylcadaverine. While putrescine and spermidine are the primary polyamines, cadaverine and aminopropylcadaverine are alternative polyamines that partially substitute for putrescine and spermidine. Due to their positive charge, polyamines can bind to macromolecules such as DNA, RNA and proteins. Polyamines are essential for normal cell growth, since they are involved in a wide variety of biological processes, including regulation of gene expression, translation, cell proliferation, cell signalling and membrane stabilization (Tabor *et al.*, 1985; Igarashi *et al.*, 2000; Kusano *et al.*, 2008).

All polyamines contain putrescine as a backbone structure, and putrescine is the precursor of all polyamines. Putrescine, the direct precursor for spermidine, is synthesised via two pathways (Figure 1.9). In the direct pathway, L-ornithine (generated from L-glutamate) is decarboxylated by ornithine decarboxylase (SpeC) to form putrescine (Tabor *et al.*, 1985).

A second, inducible enzyme for this reaction (SpeF) is produced by some *E. coli* strains. This enzyme is induced at low pH, and as the reaction consumes a proton H^+, it is considered to be a defence mechanism against low environmental pH (Kashiwagi *et al.*, 1991). The indirect pathway of putrescine biosynthesis starts with the conversion of L-arginine to L-agmatine by arginine decarboxylase (SpeA) (Morris *et al.*, 1966; Wu *et al.*, 1973). L-agmatine is subsequently hydrolysed by agmatinase (SpeB) to yield putrescine. Urea is generated as a byproduct and is probably excreted due to lack of urea degradation enzymes in *E. coli* (Morris *et al.*, 1967). Although both pathways of putrescine biosynthesis are common in bacteria and are often found side by side in the same organism, the SpeA pathway is preferred over SpeC in the presence of exogenous arginine. This may be due to the fact that ornithine biosynthesis from glutamate is subject to feed-back inhibition by arginine, on the other hand SpeA is localised in the cell envelope and can therefore access exogenous arginine more readily than the cell can generate ornithine (Tabor *et al.*, 1969; Buch *et al.*, 1985).

Spermidine synthesis follows on from the formation of putrescine in that SpeE transfers a propylamine moiety to putrescine. This propylamine group derives from *S*-adenosyl-L-methionine (SAM), which is previously decarboxylated to *S*-adenosyl-L-methioninamine by SpeD (Bowman *et al.*, 1973). This step is inhibited by spermidine (Kashiwagi *et al.*, 1988) and thus is considered to be the rate-limiting step in spermidine biosynthesis. SAM is a universal compound in the cell that is generated from L-methionine by SAM synthase (MetK). This reaction is under negative feedback control with SAM as a corepressor for MetJ, a regulatory protein of genes involved in biosynthesis and transport of methionine (Weissbach *et al.*, 1991; Lu, 2000; LaMonte *et al.*, 2006). SpeE is also linked to the alternative polyamines cadaverine and aminopropylcadaverine, as it catalyses the conversion of cadaverine to aminopropylcadaverine. Cadaverine is formed by the decarboxylation of L-lysine (CadA), which itself is derived from L-aspartate. An intermediate of the pathway from aspartate to lysine is also a precursor for cell wall components.

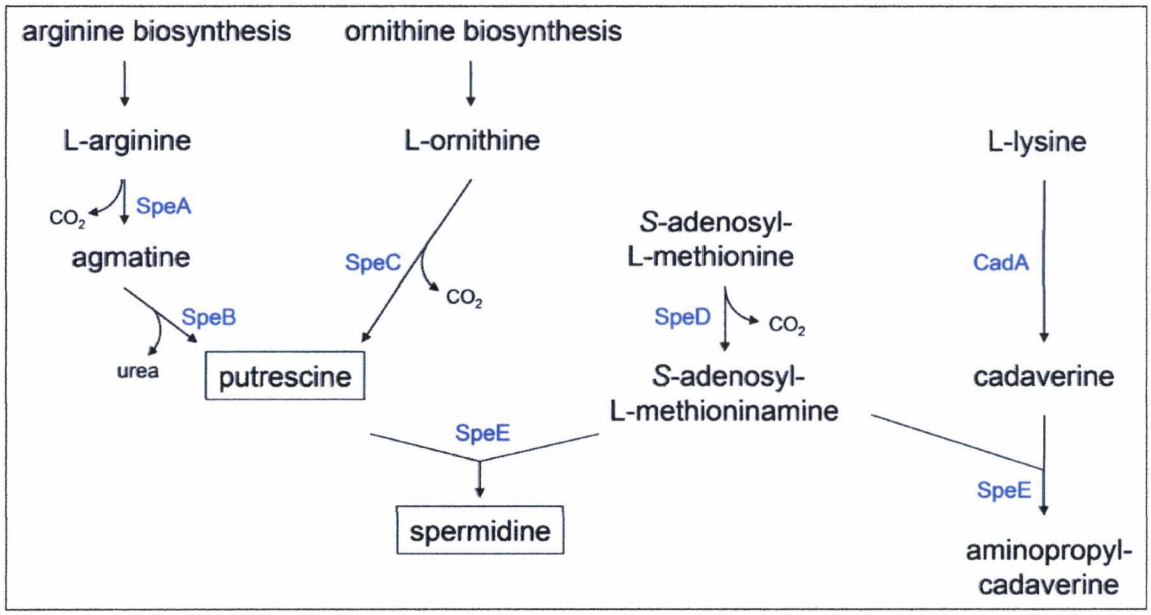


Figure 1.9: Pathways of polyamine biosynthesis

Synthesis, transport and degradation of polyamines are closely regulated. High levels of polyamines are toxic to cells and can lead to cell death, as excessive cross-linking of proteins forms cytoxic derivates (Kusano *et al.*, 2008). A main modulator of polyamine levels is the antizyme AtoC. An antizyme is a protein molecule that is induced by the end product of the enzymatic reaction that it inhibits. AtoC regulates putrescine biosynthesis by inhibiting ornithine decarboxylase (SpeC) and is induced by polyamines (Kyriakidis *et al.*, 1978; Canellakis *et al.*, 1993; Lioliou *et al.*, 2004). Transport of spermidine and putrescine across the inner cell membrane occurs via a range of transport systems. The PotABCD system preferentially binds spermidine, but also binds putrescine in support of the putrescine-specific PotFGHI system. Another putrescine transporter (PotE) not only mediates uptake of putrescine (in symport with a proton H^+), but also its excretion (in exchange for ornithine) (Kashiwagi *et al.*, 1991; Igarashi *et al.*, 1999). Furthermore, another importer for putrescine (PuuP) was discovered recently as part of the putrescine utilisation pathway (Puu) which specifically catabolises extracellular putrescine (Kurihara *et al.*, 2005; Kurihara *et al.*, 2008). After import, putrescine is degraded to succinate semialdehyde in five sequential steps. The initial step of degradation involves γ-glutamylation of putrescine by PuuA (Kurihara *et al.*, 2008), followed by subsequent steps catalysed by PuuB, C, D and E. The final step of this pathway is common to a second degradation pathway, in which putrescine is decarboxylated by YgjG (producing L-

glutamate) (Prieto-Santos *et al.*, 1986). The final product of these two pathways, succinate semialdehyde, is the precursor of succinate. Hence, succinate not only has negative feedback control on putrescine degradation, but it also links putrescine to the TCA cycle. One way of metabolising spermidine is its acetylation by SpeG in response to a variety of stresses (Carper *et al.*, 1991). Furthermore, spermidine is also present in conjugation with GSH to form glutathionylspermidine (Gsp), as decribed in more detail above. The response of Gsp synthetase/amidase (GspSA) to oxidative stress regulates levels of Gsp, which can form mixed disulfides with protein thiols (Bollinger *et al.*, 1995; Chiang *et al.*, 2010).

1.3.2. Molecular effects

Although the regulation of intracellular levels of polyamines is well characterised, this does not apply to the understanding of the molecular effects of polyamines. Studies have shown that polyamines are important in protecting cells from the toxic effects of oxygen (Ha *et al.*, 1998), but details remain to be elucidated. However, protection against paraquat-induced toxicity possibly involves downregulation of *soxS* expression by polyamines (Jung *et al.*, 2003). Furthermore, spermidine appears to be more protective against this kind of stress than putrescine (Chattopadhyay *et al.*, 2003) and the role of spermidine hereby is probably distinct from that played by glutathione (Rider *et al.*, 2007). It is also proposed that spermidine protects replicating DNA against damage by scavenging singlet oxygen (Khan *et al.*, 1992). With regard to DNA replication, polyamines affect the movement of the DNA replication fork rather than the initiation of DNA synthesis (Geiger *et al.*, 1980). Overall, polyamines increase the rate of cell growth and these proliferative effects are thought to be due to the stimulation of nucleic acid and protein synthesis (Tabor *et al.*, 1985). Since a large fraction of cellular polyamines exists in a polyamine-RNA complex (Igarashi *et al.*, 2000), the cellular function of polyamines may be primarily explained by a structural change of the RNA. The "polyamine modulon" (Igarashi *et al.*, 2006) refers to a group of about 300 genes that are stimulated by polyamines at the translational level and that are also important for cell growth. Many of these up-regulated genes are thereby under the control of transcription factors which themselves are part of the "polyamine modulon", namely Cya, RpoS, FecI, and Fis. Igarashi *et al.* (2006) describe three mechanisms by which polyamines stimulate protein synthesis. They report an enhancement of ribosome activity, which can be explained by enhanced assembly of the 30S ribosomal subunits as well as by a structural change of the Shine Dalgarno (SD)

sequence and the translation initiation codon (Yoshida *et al.*, 1999). This facilitates the interaction of mRNA with 30S ribosomal subunits. Furthermore, polyamines were found to stimulate translation initiation at UUG start codons, which are normally only poorly translated (Yoshida *et al.*, 2001). However, this effect is dependent on the secondary structure of the initiation region of the mRNA and requires exposure of the SD sequence. In *E. coli*, 34 genes have been identified with a UUG initiation codon (Blattner *et al.*, 1997). These genes include *gshA*, *gsiA*, *gcvR*, *speD*, *slyA* and *ygjG*, genes that are relevant within the scope of this project. The stimulatory effect of polyamines is also attributed to an increase in readthrough of the amber codon UAG. This is due to increased levels of both the suppressor $tRNA^{supE}$ and the binding affinity of Gln-$tRNA^{supE}$ for ribosomes (Yoshida *et al.*, 2001; Yoshida *et al.*, 2002). Thus, polyamines modulate protein synthesis not only at the level of translation initiation but also at the level of elongation.

Polyamines also affect cell proliferation through phosphorylation and dephosphorylation reactions involving ATP. Spermidine (and putrescine to a lesser extent) can form a ternary complex with ATP and Mg^{2+} and thus modulate reactions of ATPases and protein kinases (Igarashi *et al.*, 2000). Since the majority of cellular reactions involve ATP, this could account for the importance of polyamines for normal cell growth.

There appears to be a range of connections between the antioxidant glutathione and polyamines. The most direct link is the conjugation of these compounds to form glutathionylspermidine, but the fact that polyamines stimulate the translation of genes with a UUG initiation codon and that *gshA* is one of the few genes in *E. coli* that features this start codon, surely reinforces this link. A negative correlation between GSH and polyamines has furthermore been shown in other organisms. In the rat liver, the depletion of GSH induces SpeD and SpeC and therefore increases polyamine levels (Oguro *et al.*, 1990). In spinach, increased levels of polyamines reduce Gor activity (Erat *et al.*, 2008). This could be explained by polyamines decreasing levels of glutathione (and hence GSSG) and thus less requirement for Gor. Ultimately, negatively correlating levels of GSH and polyamines might even account for the activating effect of GSH on *fimB* expression and the inhibition of *fimB* in the absence of GSH, respectively.

1.4. Project aims

Prior to starting this project, data was obtained showing that glutathione has a very significant activating effect on *fimB* expression. Therefore the primary aim of this project was to investigate the mechanism behind this regulation. As glutathione is a universal molecule that is involved in a wide range of cellular processes, this was always considered to be a very complex and challenging task. The first approach was to determine the regulating molecule, i.e. whether glutathione itself or derivatives of glutathione are the activating factor, and the location of action, i.e. does glutathione act in the periplasm or the cytoplasm. This information could then lead on to investigate further details on the mechanism of regulation and the identification of a specific secondary factor that is responsible for the inhibition of *fimB* expression in the absence of glutathione. Moreover, this could help elucidate whether regulation occurs on the transcriptional or the translational level. Yet glutathione potentially not only has an effect on the expression of *fimB*, but also on the activity of the encoded recombinase FimB and hence on switching of the *fimS* invertible element. This project aimed to provide insight into the mechanism of *fimB* activation by glutathione, to identify intermediate factors in this regulatory pathway and their mode of action.

Chapter 2

Materials and Methods

2.1. Bacterial strains, plasmids and oligonucleotides

All bacterial strains used in this project are either obtained from the Keio collection (*Baba et al.*, 2006) or derivatives of *Escherichia coli* K-12 MG1655 (Guyer *et al.* 1981). Keio collection strains listed in table 2.1 are derived from strain BW25113 and were purchased from The Coli Genetic Stock Center (CGSC) at Yale. Most used derivatives of the *Escherichia coli* K-12 strain MG1655 are derived from strain BGEC905 and are MG1655 Δ*lacZYA* FimB-lacZ (*fimB* reporter fusion) in addition to the listed genotype. Strains with different backgrounds are described with their complete genotype.

All plasmids used for the construction of strains are listed in table 2.2. Plasmid pCP20 was used to cure Keio collection strains from their kan^R-cassette for the combination of multiple mutations; curing was carried out according to the standard procedure (Datsenko *et al.*, 2000) originally described by Cherepanov *et al.* (1995).

Oligonucleotide primers are presented in table 2.3 with their designated purpose of use.

Table 2.1: Bacterial strains used in this study. All strains except the marked (*) strains are derived from BGEC905 and are Δ*lacZYA* FimB-LacZ (*fimB* reporter fusions) in addition to their respective genotype. Strains marked with * are described individually.

Strain Number	Genetic Background	Source of Origin
AAEC072A	* MG1655 Δ*fimB-H*	Laboratory collection
AAEC090	* MG1655 Δ*lacZYA*::*sacB*-kan^R	Laboratory collection
AAEC100	* MG1655 Δ*lacZYA*	Laboratory collection
AAEC189A	* Λ- F- *endA1 Thi-1 hsdR17 supE44 ΔlacU169 recA mcrA mcrB ΔfimB-H*	Laboratory collection
AAEC261A	* MG1655 Δ*lacZYA*, *fimB'-'lacZYA*	Laboratory collection
AAEC370A	* MG1655 ΔlacZYA *fimA'-'lacZYA ΔfimE*	Laboratory collection
AAEC554	* MG1655 (*fimS* locked ON)	Laboratory collection
BGEC088	* MG1655 Δ*lacZYA* FimE'-'LacZ	Laboratory collection
BGEC905	* MG1655 Δ*lacZYA* FimB'-'LacZ	Laboratory collection
JW0117	* BW25113 *speE*::kan^R	Keio collection (Baba et al., 2006)
JW0669	* BW25113 *fur*::kan^R	Keio collection (Baba et al., 2006)
JW0801	* BW25113 *mntR*::kan^R	Keio collection (Baba et al., 2006)
JW0870	* BW25113 *cydD*::kan^R	Keio collection (Baba et al., 2006)
JW1267	* BW25113 *cysB*::kan^R	Keio collection (Baba et al., 2006)
JW1267	* BW25113 *cysB*::kan^R	Keio collection (Baba et al., 2006)
JW1966	* BW25113 *cbl*::kan^R	Keio collection (Baba et al., 2006)
JW1966	* BW25113 *cbl*::kan^R	Keio collection (Baba et al., 2006)
JW2092	* BW25113 *rcnR*::kan^R	Keio collection (Baba et al., 2006)
JW2388	* BW25113 *mntH*::kan^R	Keio collection (Baba et al., 2006)
JW2663	* BW25113 *gshA*::kan^R	Keio collection (Baba et al., 2006)
JW2904	* BW25113 *speB*::kan^R	Keio collection (Baba et al., 2006)
JW2905	* BW25113 *speA*::kan^R	Keio collection (Baba et al., 2006)
JW2956	* BW25113 *gsp*::kan^R	Keio collection (Baba et al., 2006)
JW2956	* BW25113 *gsp*::kan^R	Keio collection (Baba et al., 2006)
JW3446	* BW25113 *nikR*::kan^R	Keio collection (Baba et al., 2006)
JW3467	* BW25113 *gor*::kan^R	Keio collection (Baba et al., 2006)
JW3909	* BW25113 *metJ*::kan^R	Keio collection (Baba et al., 2006)

JW3933	* BW25113 *oxyR*::kan^R	Keio collection (Baba et al., 2006)
JW4023	* BW25113 *soxS*::kan^R	Keio collection (Baba et al., 2006)
JW4131	* BW25113 *nsrR*::kan^R	Keio collection (Baba et al., 2006)
JW4359	* BW25113 *rob*::kan^R	Keio collection (Baba et al., 2006)
JW5249	* BW25113 *marA*::kan^R	Keio collection (Baba et al., 2006)
JW5267	* BW25113 *slyA*::kan^R	Keio collection (Baba et al., 2006)
JW5714	* BW25113 *zur*::kan^R	Keio collection (Baba et al., 2006)
JW5843	* BW25113 *norR*::kan^R	Keio collection (Baba et al., 2006)
JW5897	* BW25113 *gsiA*::kan^R	Keio collection (Baba et al., 2006)
KCEC1243	*slyA*::kan^R	Laboratory collection
KCEC1288	*fur*::kan^R	Bacteriophage transduction; JW0669 into BGEC905
KCEC1294	*marA*::kan^R	Bacteriophage transduction; JW5249 into BGEC905
KCEC1334	*slyA*	Laboratory collection
KCEC1368	*slyA gshA*::kan^R	Laboratory collection
KCEC1428	* MG1655 Δ*lacZYA*::*sacB*-kan^R FimB'-'LacZ (intermediate strain)	Laboratory collection
KCEC1627	*gshA*	Laboratory collection
KCEC1757	*gor*::kan^R	Laboratory collection
KCEC1759	*gsiA*::kan^R	Laboratory collection
KCEC1769	*gshA gsiA*::kan^R	Bacteriophage transduction; JW5897 into KCEC1627
KCEC1771	*speA*::kan^R	Laboratory collection
KCEC1777	*speE*::kan^R	Laboratory collection
KCEC1838	*gshA cydD*::kan^R	Bacteriophage transduction; JW0870 into KCEC1627
KCEC1856	*cydD*::kan^R	Bacteriophage transduction; JW0870 into BGEC905
KCEC1904	*fur*	kan^R cassette curing; pCP20 into KCEC1288
KCEC1908	*gshA fur*::kan^R	Bacteriophage transduction; JW0669 into KCEC1627
KCEC1954	*mntR*::kan^R	Bacteriophage transduction; JW0801 into BGEC905
KCEC1956	*gshA mntR*::kan^R	Bacteriophage transduction; JW0801 into KCEC1627
KCEC1958	*mntH*::kan^R	Bacteriophage transduction; JW2388 into BGEC905
KCEC1960	*gshA mntH*::kan^R	Bacteriophage transduction; JW2388 into KCEC1627
KCEC1978	*ryhB*::cam^R	Bacteriophage transduction; EM1238 into BGEC905
KCEC1980	*gshA ryhB*::cam^R	Bacteriophage transduction; EM1238 into KCEC1627

KCEC2008	*oxyR*::kan^R	Bacteriophage transduction; JW3933 into BGEC905
KCEC2010	*gshA oxyR*::kan^R	Bacteriophage transduction; JW3933 into KCEC1627
KCEC2012	*fur ryhB*::cam^R	Bacteriophage transduction; EM1238 into KCEC1904
KCEC2134	*gshA ryhB*::cam^R *fur*::kan^R	Bacteriophage transduction; JW0669 into KCEC1980
KCEC2170	* MG1655 *gshA*::kan^R	Bacteriophage transduction; JW2663 into MG1655
KCEC2172	* MG1655 Δ*lacZYA fimA'-'lacZYA fimE gshA*::kan^R	Bacteriophage transduction; JW2663 into AAEC370A
KCEC2174	* MG1655 *gshA*::kan^R (*fimS* locked ON)	Bacteriophage transduction; JW2663 into AAEC554
KCEC2176	* MG1655 Δ*lacZYA* FimE'-'LacZ *gshA*::kan^R	Bacteriophage transduction; JW2663 into BGEC088
KCEC2206	*rob*::kan^R	Bacteriophage transduction; JW4359 into BGEC905
KCEC2228	*soxS*	kan^R cassette curing; pCP20 into KCEC932
KCEC2230	*soxS marA*::kan^R	Bacteriophage transduction; JW5249 into KCEC2228
KCEC2232	*rob marA*::kan^R	Bacteriophage transduction; JW5249 into KCEC2224
KCEC2234	*rob soxS*::kan^R	Bacteriophage transduction; JW4023 into KCEC2224
KCEC2371	*rob marA*	kan^R cassette curing; pCP20 into KCEC2232
KCEC2269	*soxS marA*	kan^R cassette curing; pCP20 into KCEC2230
KCEC2273	*rob soxS*	kan^R cassette curing; pCP20 into KCEC2234
KCEC2283	*soxS marA gshA*::kan^R	Bacteriophage transduction; JW2663 into KCEC2269
KCEC2287	*rob marA gshA*::kan^R	Bacteriophage transduction; JW2663 into KCEC2271
KCEC2289	*rob soxS marA*::kan^R	Bacteriophage transduction; JW5249 into KCEC2273
KCEC2291	*rob soxS gshA*::kan^R	Bacteriophage transduction; JW2663 into KCEC2273
KCEC2295	*soxR*::kan^R	Bacteriophage transduction; JW4024 into BGEC905
KCEC2297	*gshA soxR*::kan^R	Bacteriophage transduction; JW4024 into KCEC1627
KCEC2311	*rob soxS marA*	kan^R cassette curing; pCP20 into KCEC2289
KCEC2313	*soxS rob marA gshA*::kan^R	Bacteriophage transduction; JW2663 into KCEC2311
KCEC2353	*gshA hns*::tet^R	Bacteriophage transduction; KCEC755 into KCEC1627
KCEC2451	* Δ*soxS*, Δ*lacZYA*::*sacB*-Kan^R	Bacteriophage transduction; AAEC090 into KCEC2228
KCEC2465	* Δ*soxS*, *lac*UV5-*soxS*	Allelic exchange; pLW011 into KCEC2451
KCEC2484	*zur*::kan^R	Bacteriophage transduction; JW5714 into BGEC905
KCEC2486	*gshA zur*::kan^R	Bacteriophage transduction; JW5714 into KCEC1627
KCEC2492	* MG1655 Δ*lacZYA fimA'-'lacZYA fimE gor*::kan^R	Bacteriophage transduction; JW3467 into AAEC370A
KCEC2550	* MG1655 *gor*::kan^R	Bacteriophage transduction; JW3467 into MG1655

KCEC2554	* MG1655 *gor*::kan^R (*fimS* locked ON)	Bacteriophage transduction; JW3467 into AAEC554
KCEC2576	*nikR*::kan^R	Bacteriophage transduction; JW3446 into BGEC905
KCEC2578	*gshA nikR*::kan^R	Bacteriophage transduction; JW3446 into KCEC1627
KCEC2586	*rcnR*::kan^R	Bacteriophage transduction; JW2092 into BGEC905
KCEC2588	*gshA rcnR*::kan^R	Bacteriophage transduction; JW2092 into KCEC1627
KCEC2600	*cbl*::kan^R	Bacteriophage transduction; JW1966 into BGEC905
KCEC2602	*gshA cbl*::kan^R	Bacteriophage transduction; JW1966 into KCEC1627
KCEC2638	*nsrR*::kan^R	Bacteriophage transduction; JW4136 into BGEC905
KCEC2640	*gshA nsrR*::kan^R	Bacteriophage transduction; JW4136 into KCEC1627
KCEC2642	*norR*::kan^R	Bacteriophage transduction; JW5843into BGEC905
KCEC2644	*gshA norR*::kan^R	Bacteriophage transduction; JW5843 into KCEC1627
KCEC2679	*metJ*::kan^R	Bacteriophage transduction; JW3909 into BGEC905
KCEC2679	*gshA metJ*::kan^R	Bacteriophage transduction; JW3909 into KCEC1627
KCEC2712	*gshA speA*::kan^R	Bacteriophage transduction; JW2905 into KCEC1627
KCEC2714	*gshA speE*::kan^R	Bacteriophage transduction; JW2904 into KCEC1627
KCEC2734	*gss*::kan^R	Bacteriophage transduction; JW2956 into BGEC905
KCEC2746	*gshA gss*::kan^R	Bacteriophage transduction; JW2956 into KCEC1627
KCEC2760	*gor*	kan^R cassette curing; pCP20 into KCEC1757
KCEC2790	*speA*	kan^R cassette curing; pCP20 into KCEC1771
KCEC2860	* MG1655 Δ*lacZYA*, *fimB*'-'*lacZYA gshA*::kan^R	Bacteriophage transduction; JW2663 into BGEC261A
KCEC2862	* MG1655 Δ*lacZYA*, *fimB*'-'*lacZYA slyA*::kan^R	Bacteriophage transduction; JW5267 into BGEC261A
KCEC2973	*cysB*	kan^R cassette curing; pCP20 into KCEC920
KCEC2982	*cysB cbl*::kan^R	Bacteriophage transduction; JW1966 into KCEC2973
KCEC2993	*speB*::kan^R	Bacteriophage transduction; JW2904 into BGEC9054
KCEC2995	*gshA speB*::kan^R	Bacteriophage transduction; JW2904 into KCEC1627
KCEC3052	*speA cbl*::kan^R	Bacteriophage transduction; JW1966 into KCEC2790
KCEC3052	*speA cbl*::kan^R	Bacteriophage transduction; JW1966 into KCEC2760
KCEC3100	*cydD*	kan^R cassette curing; pCP20 into KCEC1856
KCEC3112	*cydD cbl*::kan^R	Bacteriophage transduction; JW1966 into KCEC3100
KCEC755	*hns*::mTn*10*	Laboratory collection
KCEC920	*cysB*::kan^R	Laboratory collection

KCEC928	*gshA*::kan^R	Laboratory collection
KCEC932	*soxS*::kan^R	Laboratory collection
MG1655	* Λ- F- rph-1	*E. coli* Genetic Stock Centre (Guyer, 1981)

Table 2.2: Plasmids used in this study.

Plasmid	Characteristics	Source
pCP20	FLP$^+$ Λcl857$^+$ ΛP_R ts amp^R cam^R	Cherepanov *et al.*, 1995
pIB462	ts, cam^R; *lac*UV5 region; *Bam*HI and *Hind*III restriction sites	Laboratory collection
pLW01	pIB462 *soxS* (*lac*UV5 fusion with *soxS* gene)	This study

Table 2.3: Oligonucleotides used in this study

Oligo ID	Sequence	Purpose
soxS:lac_fw	5'- GCGCGGATCCCCAACAGATGAATTAACG -3'	Construction of pLW011
soxS:lac_rv	5'- GCGCAAGCTTTGCGAAGGCGATGCCGCCGC -3'	Construction of pLW011
lacSeqF	5'- CCCGACTGGAAAGCGGGCAG -3'	Sequencing *lacUV5* construct
lacSeqR2	5'- CGGCTAATCTAGATCGCTGAAC -3'	Sequencing *lacUV5* construct

2.2. Media, growth conditions and storage

Sterilisation of solutions was carried out by autoclaving at 121°C for 15 minutes or by filtration with sterile 0.2μm pore filters (Nalgene).

Double-deionized water (ddH_2O) used for solutions was purified through multiple de-ionising and filtration cartridges (Millipore MilliQ®) and further sterilized by autoclaving.

All chemical and media reagents were standard laboratory grade or higher and obtained from Sigma-Aldrich UK unless otherwise stated.

Concentrations of buffer components indicated in percentages refer to % (w/v) (weight:volume ratio) for solids and % (v/v) (volume:volume ratio) for liquids, both at room temperature (RT).

Liquid bacterial cultures were grown in conical flasks, aerated at 37°C and 180rpm in a waterbath (New Brunswick Scientific, Innova 3100) unless otherwise noted. If required, a static waterbath (Grant Instruments) was used. Bacterial cultures on solid media were grown in a static incubator at the temperature stated in the relevant protocol. For long-term storage, bacterial strains were grown in LB and stored in 15% glycerol (Fisher Scientific) at -70°C. For inoculation, a sample of the freezer stock was transferred to the appropriate medium and grown overnight under the required conditions before further use.

2.2.1. Liquid Media

The liquid media used in this study included lysogeny Luria-Bertani (LB) broth as well as a medium based on minimal 3-(N-morpholino)-propanesulphonic acid (MOPS) as described by Neidhardt *et al.* (1974). The original composition of both minimal and rich-defined MOPS medium was modified in terms of their supplemental carbon source, replacing glucose with glycerol (M_{gly} and RD_{gly}).

MOPS was further supplemented with defined ingredients such as nucleotide bases, specific amino acids and vitamins as described below. This ensured reproducibility of its composition and hence of results obtained with this medium.

The detailed composition of MOPS M_{gly} and RD_{gly} is described in tables 2.4 and 2.5.

Luria-Bertani (LB) broth and LB-agar plates

The preparation of LB broth complied with the description by Sambrook *et al.* (1989): 10g/l tryptone (Becton-Dickinson & Co.), 5g/l sodium chloride NaCl (Fisher Scientific UK) and 5g/l yeast extract (Oxoid) were dissolved in ddH_2O before autoclaving and stored at room temperature.

MOPS medium

Minimal MOPS medium (M_{gly}) and rich-defined medium (RD_{gly}) were made up of the components listed in tables 2.4 and 2.5, respectively. The MOPS medium was then filter sterilized, covered with foil and stored at 4°C. Tables 2.6 to 2.10 describe the composition of the stock solutions for M_{gly} and RD_{gly}.

Table 2.4: Components required for minimal MOPS medium (M_{gly})

Component	**to make 1 litre M_{gly}**
x10 MOPS	100ml
40% glycerol	10ml
132mM $K_2HPO_4 \cdot 3H_2O$	10ml
20mM thiamine	0.5ml
ddH_2O	make up to 1 litre

Table 2.5: Components required for rich defined MOPS (RD_{gly}) medium

Component	**to make 1 litre RD_{gly}**
x10 MOPS	100ml
40% glycerol	10ml
132mM $K_2HPO_4 \cdot 3H_2O$	10ml
x10 Amino Acid solution	100ml
x10 G solution	100ml
x10 CAU solution	100ml
x100 vitamin B supplement	10ml
Cysteine (0.0369g in 5ml ddH_2O)	2.38ml
Methionine (0.0448g in 5ml ddH_2O)	3.33ml
ddH_2O	make up to 1 litre

Stock solutions of all components were prepared in advance, except for Cysteine and Methionine, which were made up fresh before use and added at a final concentration of 0.1mM and 0.2mM, respectively.

Stock solutions for RD glycerol

Table 2.6: Components required for x10 MOPS

Component	**to make 500ml 10x MOPS**
MOPS (Melford Laboratories Ltd)	41.86g in 200ml dH2O
Tricine	3.58g to MOPS solution, pH7.4 with 10M KOH (Fisher Scientific UK)
$FeSO_4 \cdot 7H_2O$	0.274g in 100ml ddH_2O. Add 5ml to MOPS solution
NH_4Cl	10.163g in 100ml ddH_2O. Add 25ml to MOPS solution
K_2SO_4	4.809g in 100ml ddH_2O. Add 5ml to MOPS solution
$CaCl_2$	0.735g in 100ml ddH_2O. Dilute 1:100 and add 5ml to MOPS solution
$MgCl_2 \cdot 6H_2O$	5.027g in 100ml ddH_2O. Add 5ml to MOPS solution
NaCl	29.22g in 100ml ddH_2O. Add 50ml to MOPS solution
Micronutrients solution	add 5ml of stock solution to MOPS solution
ddH_2O	make up to 500ml, filter sterilize and store at -20°C

Table 2.7: Components required for 10x Amino Acid solution

Component	**to make 500ml 10x Amino Acid solution**	**concentration**
Alanine	0.356g in 5ml ddH_2O	8mM

Arginine	0.422g in 5ml ddH_2O	52mM
Asparagine	0.3008g in 40ml ddH_2O	4mM
Aspartic acid	0.342g +335µl 10M KOH in 5ml ddH_2O	4mM
Glutamic acid	0.56g +430µl 10M KOH in 5ml ddH_2O	6mM
Glutamine	0.439g in 25ml ddH_2O	6mM
Glycine	0.3g in 5ml ddH_2O	8mM
Histidine	0.21g in 5ml ddH_2O	2mM
Isoleucine	0.262g in 10ml ddH_2O	4mM
Leucine	0.5248g in 80ml ddH_2O	8mM
Lysine	0.366g in 5ml ddH_2O	4mM
Phenylalanine	0.3304g in 40ml ddH_2O	4mM
Proline	0.23g in 5ml ddH_2O	4mM
Serine	5.25g in 125ml ddH_2O	100mM
Threonine	0.238g in 5ml ddH_2O	4mM
Tryptophan	0.1022g in 10ml ddH_2O	1mM
Tyrosine	0.1812g in 100ml ddH_2O	2mM
Valine	0.352g in 10ml ddH_2O	6mM
ddH_2O	make up to 500ml, filter sterilize and store at 4°C	

Table 2.8: Components required for stock solutions of the bases, 10x G and 10x CAU

Component	**to make 500ml 10x G solution**	**concentration**
Guanine	0.15g	2mM
0.015M KOH (Fisher Scientific)	make up to 500ml, filter sterilize and store at 4°C	
	to make 500ml 10x CAU solution	**concentration**
Adenine	0.135g	2mM
Cytosine	0.11g	2mM
Uracil	0.1126g	2mM
0.015M KOH (Fisher Scientific)	make up to 500ml, filter sterilize and store at 4°C	

Table 2.9: Components required for micronutrient solution

Component	**to make 100ml micronutrient solution**
$(NH_4)_6MO_7O_{24}$	1.236g in 100ml ddH_2O, add 30µl to final stock solution
H_3BO_3	0.6183g in 100ml ddH_2O, add 400µl to final stock solution
$CoCl_2$	0.238g in 100ml ddH_2O, add 300µl to final stock solution
$CuSO_4 \cdot 6H_2O$	0.25g in 100ml ddH_2O, add 100µl to final stock solution
$MnCl_2$	0.1979g in 100ml ddH_2O, add 800µl to final stock solution
$ZnSO_4 \cdot 7H_2O$	0.2875 in 100ml ddH_2O, add 100µl to final stock solution
ddH_2O	make up to 100ml, filter sterilize and store at 4°C

Table 2.10: Components required for x100 Vitamin B supplements (500ml)

Component	**to make 500ml 100x vitamin B supplement**
Thiamine HCl	0.169g
Pantothenate	0.238g
ρ-aminobenzoic acid	0.069g
ρ-hydroxybenzoic acid	0.069g
2,3-dihydroxybenzoic acid	0.077g in 0.02M KOH (Fisher Scientific)
ddH_2O	make up to 500ml, filter sterilize and store at 4°C

2.2.2. Solid Media

For preparation of solid L-agar plates 15g/l agar (Difco) was added to the LB solution before autoclaving. For *fimS* recombination experiments special Indicator medium plates were made up of 50g/l premixed MacConkey powder (Difco). L-agar sucrose medium for the allelic exchange procedure (Blomfield *et al.*, 1991) was prepared without NaCl in the LB mixture and by adding sterile sucrose to a final concentration of 6% after autoclaving.

2.2.3. Supplements

For selection purposes, antimicrobial agents were added to the media after autoclaving. Stock solutions of antibiotics were made up as listed in table 2.11, filter sterilised and kept at -20°C.

Isopropyl β-D-1-thiogalactopyranoside (IPTG; Melford) was used for complementation experiments at a final concentration of 0.1mM. The stock solution was made at a concentration of 100mM in ddH_2O, filter sterilised and stored at -20°C.

Table 2.11: Antibiotic stock solutions and working concentrations

Component	Stock solution	working concentration
Ampicillin	125mg/ml in ddH_2O	125µg/ml
Chloramphenicol	34mg/ml in ethanol (Fisher Scientific)	25µg/ml
Kanamycin	kanamycinA: 25mg/ml in ddH_2O	25µg/ml
Tetracycline	Tetracycline hydrochlorine: 10mg/ml in 50% ethanol	10µg/ml

2.3. Centrifugation

This project made use of following centrifuges:
Centrifugation of 1.5ml and 2ml Eppendorf tubes was carried out in a benchtop microcentrifuge (Eppendorf MiniSpin, rotor F-45-12-11). Larger volumes were centrifuged in 15ml and 50ml Falcon tubes by use of a Sigma 2K15 centrifuge (rotor Sigma 256/97 Nr.12149). Unless otherwise stated, the Sigma centrifuge was precooled and used at 4°C.

2.4. Transformation of DNA

Transformation is an established method to introduce foreign (mainly plasmid) DNA into bacterial cells. Transformation of plasmids into bacterial cells requires the target cells to be made competent to enable them to take up DNA from the environment.
Production of competent cells and their subsequent transformation were based on Sambrook *et al.* (1989), and carried out as described below.

2.4.1. Production of competent cells

In the calcium chloride procedure described by Sambrook *et al.* (1989), competent cells are made from an overnight culture, grown in 10ml LB broth at 37°C in a shaking waterbath (180rpm). 28µl of overnight culture was sub-cultured into 28ml fresh pre-warmed LB

broth and grown to an OD_{600nm} of 0.5. Five minutes before reaching this density, 3.75ml of warmed, sterile 100% glycerol was slowly added to the shaking flask. Upon reaching an OD_{600nm} of 0.5, the culture was transferred to a 50ml falcon tube and chilled on ice for 10 minutes. After centrifuging the culture at 4°C for 10 minutes at 4000rpm, the cell pellet was resuspended in 25ml ice-cold solution of 0.1M $MgCl_2$ with 15% glycerol. The cells were centrifuged again (8min, 3800rpm, 4°C) and the cell pellet was resuspended in 6.25ml ice-cold T-salt solution (75mM $CaCl_2$, 6mM $MgCl_2$, 15% glycerol). After incubating the cells on ice for 20 minutes with occasional gentle shaking, the cells were centrifuged (6min, 3600rpm, 4°C), the supernatant discarded and replaced with 1.25ml ice-cold T-salt solution. Aliquots of these cells (100µl) were then stored immediately in pre-cooled tubes at -70°C; competent cells were left at -70°C for at least 24 hours before the first use.

2.4.2. Transformation protocol

For each transformation, 100µl of competent cells were thawed on ice and mixed with DNA. For standard transformations, up to 10µl of plasmid DNA solution was used, while the cloning procedure required 20µl of overnight ligation mixture. The cell-DNA mixture was incubated on ice for 30 minutes and then heat-shocked at 42°C for 30 seconds. 1ml LB broth was added before incubating the cells for 1 hour in a static waterbath at the required temperature (37°C, or 28°C for temperature sensitive plasmids). 200µl aliquots of the cells sample were then spread onto L-agar plates containing an appropriate antibiotic to select for cells that have successfully taken up the plasmid. After incubation overnight at the same temperature as before, colonies of transformants were picked and grown in LB broth plus antibiotic before further use or stocking in 15% glycerol at -70°C.

2.5. Isolation and Analysis of DNA

In order to construct new strains, various methods were used to isolate and modify DNA. DNA was eluted in TE buffer (10mM Tris pH 8.0, 1mM EDTA; Fisher Scientific) during purification or isolation and either used immediately or kept at -20°C for long-term storage.

2.5.1. Isolation of plasmid DNA

Plasmid DNA was isolated with QIAprep Spin Miniprep Kit (Qiagen) and eluted in 50µl elution buffer.

For the plasmid isolation, the bacterial strain carrying the plasmid was grown overnight in LB broth supplemented with an appropriate antibiotic. The cells were grown at 37°C, while cells carrying temperature sensitive plasmids were grown at 28°C. The overnight culture was centrifuged at 4,500 x *g* for 10 minutes and the cell pellet was subsequently processed following the protocol of the QIAprep Spin Miniprep Kit. At the final step, the obtained plasmid DNA was eluted in TE buffer and stored at -20°C.

2.5.2. Isolation of genomic DNA

Isolation of chromosomal (genomic) DNA was carried out as described by Silhavy *et al.*, 1984).

567µl of an overnight cell culture of these cells were added directly to 30µl 10% SDS (BDH Laboratory Supplies) and 3µl Proteinase K (4mg/ml) (Fisher Scientific). The cells were incubated at 65°C for at least 2 hours before adding 100µl 5M NaCl and mixing gently. 80µl of warm CTAB solution (10% hexadecyl trimethyl ammonium bromide in 0.7M NaCl (Fisher Scientific)) was then added and repeated incubation at 65°C for 10 minutes was followed by adding 780µl chloroform (BDH Laboratory Supplies) and mixing gently. The sample was then centrifuged at 10,000 x *g* for 5 minutes and the obtained upper aqueous phase was transferred to a fresh tube. An equal volume of phenol/chloroform/isoamyl alcohol (24:25:1) was added and the mixture was centrifuged again at 10,000 x *g* for 5 minutes. Again, the upper aqueous phase was transferred into a fresh tube and 0.6 volumes of isopropanol added, leading to a high molecular weight precipitate. The sample was pelleted for 2 minutes at 10,000 x *g*, the supernatant removed by aspiration and the pellet washed with 1ml 70% ethanol (Fisher Scientific). A final centrifugation step at 10,000 x *g* for 1 minute and removal of the supernatant yielded a DNA pellet which was then air-dried briefly before resuspending in 10µl TE buffer and storing at 4°C. For successful use in PCR this DNA stock solution was required to be further diluted (see below).

2.5.3. Restriction endonuclease digestion of DNA

Restriction endonuclease enzymes were purchased from New England Biolabs, Promega and Roche Applied Science. Digests and reaction conditions followed the supplier's instructions, mainly for 2 hours at the temperature and in the buffer conditions recommended by the manufacturer. Double digests were carried out by adding both restriction endonuclease enzymes to a single reaction. In this case, the temperature and reaction buffer most compatible for both endonucleases was chosen according to the supplier's recommendation. The digested DNA was subsequently purified with a Promega Wizard® SV Gel and PCR Clean-up Kit either through a column or via gel-purification with an agarose gel. Gel-purification was required as part of the cloning procedure to isolate plasmid DNA inserts and is described in detail below.

2.5.4. Ligation of DNA

DNA fragments obtained by restriction enzyme digestion were ligated using T4 DNA ligase (Roche Applied Science). Ligation was typically carried out in a reaction volume of 20µl following the manufacturer's instructions and using the supplied reaction buffer solutions. Ligation of DNA fragments into plasmids for subsequent transformation was carried out overnight at 16°C, with a vector:insert DNA ratio of approximately 1:1.

2.5.5. PCR amplification of DNA

Amplification of specific DNA segments was achieved by polymerase chain reaction (PCR). This reaction involves oligonucleotides (primers) that are specifically designed to flank the target DNA region. In order to create DNA insertions, replacemants or deletions in specific DNA sequences (site-directed mutagenesis), these oligonucleotides feature mutagenic sequences. All oligonucleotides were synthesised by Eurofins MWG Operon (Germany), dissolved in sterile water and kept at 500µM in ddH_2O at -70°C for long-term storage. A sample of each primer was diluted to a working concentration of 50µM and kept at -20°C. PWO polymerase (Roche Applied Science) was used for cloning and strain construction.

The PCR reactions were set up as described in table 2.12 using filter pipette tips (Promega) to minimise contamination. A master mix containing buffer, dNTPs, primers and ddH_2O

was prepared to ensure uniformity between individual samples. The template DNA and PWO polymerase were added to this master mix in individual Eppendorf PCR tubes.

PCR was carried out in a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems), programmed with following settings for standard solutions:

1x (94°C for 30 seconds)

25x (94°C for 30 seconds, 55°C for 45 seconds, 72°C for 30 seconds)

1x (72°C for 7 minutes)

PCR reactions were then cooled down to storage temperature of 4°C. In case of nonspecific products forming under these conditions, the annealing temperature was increased gradually from 55°C to 65°C to increase the specificity of the primers.

DNA was subsequently purified through a column using a Promega Wizard® Gel and PCR Clean-Up kit according to manufacturer's instructions.

Table 2.12: PCR reaction (50µl final volume)

10x PCR buffer (with $MgSO_4$)	5µl
10x dNTP solution (2mM each, Roche Applied Science)	5µl
forward primer (50µM)	1µl
reverse primer (50µM)	1µl
DNA template (approx. 5ng/µl)	1µl
PWO polymerase	0.5µl
ddH_2O	36.5µl

The concentration of DNA after purification was determined in a spectrophotometer by measuring the absorbance at 260nm. At a concentration of 50µg/ml, DNA has an OD_{260nm} of 1, hence the concentration of a 100-fold dilution of DNA can be calculated as follows:

undiluted DNA conc. (µg/ml) = 100 x 50 x OD_{260nm}

2.5.6. Agarose gel electrophoresis

DNA fragments were separated by size via agarose gel electrophoresis for purification or analysis.

For DNA analysis, the agarose gel was made up of 1% agarose (Bio-Rad) in 30ml TAE buffer (1 litre made up of 242g Tris (Fisher Scientific), 57.1ml acetic acid (Fisher Scientific), 100ml 0.5M EDTA (Fisher Scientific), pH 8.0 in a total volume of 1 litre ddH_2O) and a 12-lane comb was used.

1µl 6x loading dye (50mM Tris pH 8.0 (Fisher Scientific), 50mM EDTA (Fisher Scientific), 40% sucrose (Fisher Scientific), 1% SDS (BDH Laboratory Supplies), 0.25% bromophenol blue in ddH_2O) was added to 5µl DNA solution and 5µl loaded into each lane. 5 µl DNA marker (100µl Promega 1kb DNA Ladder, 100µl loading dye and 400µl TE buffer) was applied to a separate lane for size determination. The gel was run at 120V and 150mA for 30 minutes and subsequently stained in 0.5µg/ml ethidium bromide for 30 minutes. The DNA lanes were then visualised on a UV transilluminator using Kodak 1D software.

Gel purification of DNA bands was carried out as described above with minor alterations. The agarose gel was made up with 0.8% agarose LE (Roche Applied Science) in 40ml TAE buffer and a 8-lane comb was used to increase the sample volume to 25 µl per well. After running the gel, DNA was only viewed under UV light to cut out required bands, but not imaged at this point to minimise exposure to UV light and the associated mutagenic impact on the sample. The DNA excised from the gel was then purified as described above, using the Promega Wizard® SV Gel and PCR Clean-up Kit.

2.5.7. DNA sequencing

Newly constructed plasmids were checked with regard to their correct nucleotide sequence before further use. For this purpose, a standard PCR was carried out on the isolated plasmid as described above using appropriate non-mutagenic primers that frame the mutated region. This ensured a higher and cleaner concentration of DNA target sequence than a plasmid Miniprep alone would produce. The obtained PCR product was purified and the concentration of the obtained solution was measured in a spectrophotometer as described above.

Sequencing was carried out by Beckman Coulter Ltd (UK) using a primer at 25μM that is designed to bind approximately 100bp upstream of the target sequence.

2.6. Genetic Manipulations and Strain construction

Genetic manipulations were obtained by use of various classical microbiological and molecular techniques. The following section covers the techniques used to create mutations on plasmids and the bacterial chromosome.

2.6.1. $P1_{vir}$ transduction

Bacteriophage $P1_{vir}$ is a phage specific to *E. coli* that enables general transduction of DNA and is therefore an established means in strain construction. By using $P1_{vir}$, specific mutations tagged with antibiotic resistance can be transduced into appropriate backgrounds and hence enable combination of various different mutations. In this study, $P1_{vir}$ transduction enabled the transfer the majority of gene knockout mutations between individual bacterial strains and hence the creation of a wide range of mutant strains. The standard procedure as described by Silhavy *et al.* (1984) requires the generation of a bacteriophage lysate as initial step before infection of bacterial cells, which was prepared as follows (Silhavy *et al.*, 1984).

The donor strain was grown overnight and 200μl of this culture was added to two flasks with 10ml of fresh pre-warmed LB broth containing 0.2% glucose and 5mM calcium chloride. After an incubation period of 30min at 37°C in a shaking waterbath (180rpm), 200μl of a previously prepared $P1_{vir}$ bacteriophage lysate was added to one of two the cultures, the second culture serving as control. This lysate should carry a different antibiotic resistance as the donor cell in order to avoid selection for the incorrect mutation after transduction. The added phage lysate leads to lysis of the donor cells and production of mature bacteriophages. Lysis occurs during an incubation period of 1-3 hours and leads to clarification of the bacterial culture compared to the control culture. The lysed culture was then transferred to a 50ml falcon tube, 200μl chloroform added and centrifuged for 10 minutes at 4500*g* at 4°C. 5ml of the obtained supernatant was then transferred to a sterile glass test tube, mixed well with 100μl chloroform, before sealing the tube with parafilm and storing the lysate at 4°C overnight before first use.

The prepared bacteriophage lysate contains a range of DNA fragments. The fragment of interest, carrying an antibiotic resistance marker in place of the deleted gene can be transduced into a recipient strain as described below.

200µl of an overnight culture of the recipient strain was added to 200µl filter sterilized bacteriophage buffer (20mM $MgSO_4$, 10mM $CaCl_2$) in a sterile 1.5ml tube. The obtained solution was mixed and 100µl aliquoted into three sterile 1.5ml tubes, two which then receiving 10 or 50µl $P1_{vir}$ lysate, the third tube serving as cell-only negative control. A fourth tube was prepared with only 50µl lysate as bacteriophage-only negative control. Each sample was gently mixed, incubated at 28°C for 30 minutes before adding 1ml of LB broth supplemented with 0.1M sodium citrate and incubating at 37°C for 2-3 hours. Following incubation, 100µl of each sample was plated out onto L-agar plates made up with the antibiotic matching resistance of the donor cell. The plates were incubated overnight at 37°C and obtained colonies of transductants were then re-streaked onto fresh L-agar plates (containing the same antibiotic). After another overnight incubation at 37°C, transductants were grown in LB for stocking at -70°C.

2.6.2. Curing of the kan^R cassette

Keio collection strains are mutant strains that carry a kan-resistance (kan^R) cassette in place of a deleted gene. In order to combine multiple gene deletions, the kan^R cassette needs to be cured, resulting in clean gene deletion. This procedure was carried out by use of the plasmid pCP20, which containes a gene for removal of the kan cassette, is temperature sensitive (28°C) and carries a resistance against chloramphenicol (cam).

For curing, competent target cells were transformed with pCP20 and grown overnight on cam plates at 28°C. Colonies of the obtained transformants were grown for at least 3 hours in 5ml LB broth at 42°C to ensure loss of any remaining free plasmids in the cells. The culture was then diluted 10^{-3} to 10^{-6}-fold and 100µl of each dilution was plated out onto L-agar plates pre-warmed to 42°C and incubated at 42°C overnight. The following day single colonies were restreaked using a 6x6 grid system onto plain L-agar plates as well plates supplemented with cam and kan. This enabled the screening of a high number of colonies for sensitivity to both cam and amp, as the kan^R cassette should be removed and the plasmid carrying cam resistance should have been lost due to excision of the plasmid from the bacterial chromosome at 42°C. The plates were grown overnight at 37°C and colonies showing sensitivity to the antibiotics were picked from the respective grid location on the

plain L-agar plate, restreaked onto fresh plain L-agar plates and incubated overnight at 37°C before stocking the cured strains the following day.

2.6.3. *lac*UV5 promoter fusion

The fusion of a target gene to the *lac*UV5 promoter provides a means for over-expressing genes. The target gene hereby is under the control of the *lac*UV5 promoter and transcription can be induced with the nonhydrolyzable lactose analog Isopropyl β-D-1-thiogalactopyranoside (IPTG).

The construction of these complementation strains was carried out by inserting the target gene into the vector plasmid pIB462. This plasmid (cam^R, temperature sensitive 28°C) contains the *lacZ* start codon just upstream of restriction endonuclease recognition sites for *Bam*HI and *Hind*III. Hence the amplification of the target gene was carried out with primers that carry restriction sites for *Bam*HI and *Hind*III, respectively. The forward primer, with the *Bam*HI restriction site at its 5'-end, was designed to anneal upstream of the ribosome binding site (RBS), but downstream of the target gene promoter which includes the transcription start site. The reverse primer was complementary to the region downstream of the transcription terminator and carried the *Hind*III restriction site at its 3'-end. PCR on wild type genomic DNA hence yielded a DNA fragment with a 5' *Bam*HI site and a 3' *Hind*III restriction site.

In order to insert this DNA fragment into the vector pIB462, both the fragment and the vector were digested with the same pair of restriction enzymes, i.e. *Bam*HI and *Hind*III. Subsequent ligation resulted in a plasmid containing the *lac*UV5-target gene fusion, the promoter of the target gene being replaced by the *lac*UV5 promoter.

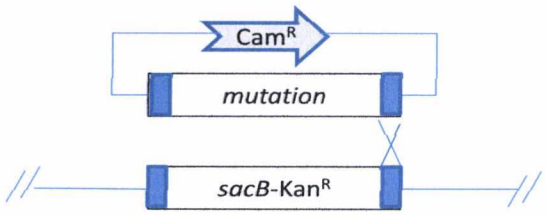
The plasmid was then transformed into competent AAEC189 cells. Due to a mutation in the *recA* gene, this strain is not able to integrate the plasmid into its chromosome and hence provides a means to amplify the free plasmid and thus check the plasmid nucleotide sequence for successful integration. For this, the plasmid was isolated with a Qiagen QIAprep Spin Miniprep Kit and re-digested with the previously used pair of restriction enzymes (*Bam*HI and *Hind*III). Plasmids containing the inserted DNA fragment were sequenced as described in Section 2.5.7 to confirm the correct fusion of *lac*UV5 promoter and target gene.

Plasmids with the correct DNA sequence were then transformed into an "intermediate strain", a strain in which the *lacZYA* genes have been replaced by a *sacB*-kan^R cassette. In

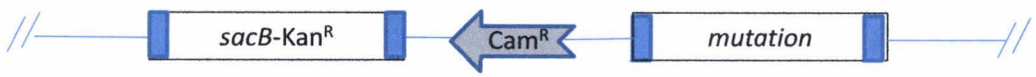
this study, strain KCEC1428 (MG1655 Δ*lacZYA*::*sacB*-kan^R, FimB'-'LacZ fusion; laboratory collection) was used as wildtype strain as it also carries the FimB-LacZ fusion necessary for determination of *fimB* expression. The exchange of the *sacB*-kan^R cassette with the designed *lac*UV5-target gene fusion was achieved by Allelic Exchange (described below) and produced a new strain that can overexpress the target gene by induction with IPTG.

2.6.4. Allelic exchange

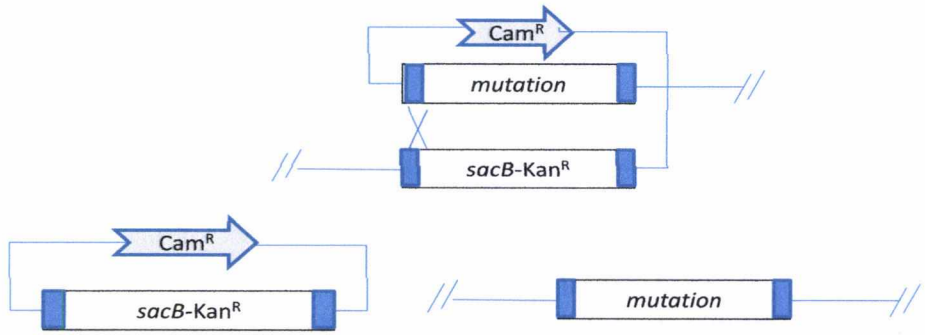
Allelic exchange is a method used to transfer mutations from a plasmid into non-essential regions of the *E. coli* chromosome. As described by Blomfield *et al.* (1991), this procedure makes use of a temperature sensitive vector as well as an "intermediate strain". This strain contains the *B. subtilis sacB* gene (*sacB*-kan^R cassette) as a selection marker in place of the target sequence for mutation (Figure 2.1).



Recombination between the intermediate strain (containing *sacB*-kan^R cassette in place of target sequence) and the vector (containing mutated sequence and marker)...



...leads to integration of the vector...



...while subsequent excision produces strain with mutation

Figure 2.1: Schematic diagram of Allelic exchange.

In this project, the strain KCEC1428 (laboratory collection) was used as intermediate strain, as it carries both a *sacB*-kan^R cassette in place of the *lacZYA* gene as well as the FimB-LacZ translational fusion. Intermediate strains must be recA positive for integration and excision of the plasmid, events that are rare and form the basis for selection in the Allelic Exchange procedure. Furthermore, temperature-sensitivity of the vector allows for loss of the vector during the exchange procedure. The plasmid for Allelic Exchange (pIB462 in this study) must also have regions that are homologous to the regions flanking the *sacB*-kan^R cassette of the intermediate strain and was created and verified by using the techniques described above.

The plasmid was transformed into competent cells of the relevant intermediate strain, plated out on cam plates and incubated overnight at 42°C. This step selected for integration of the plasmid, as any free plasmid is lost due to its temperature sensitivity and only cells carrying antibiotic resistance markers from the integrated plasmid are viable.

The following day, single colonies were grown in plain LB for 3 hours at 42 °C. A dilution series in LB from 10^{-1} to 10^{-4} was made up and 100µl of the 10^{-1}, 10^{-2} and 10^{-3} dilutions was plated out onto sucrose plates pre-warmed to 42°C. The 10^{-4} dilution was plated out onto a plain L-agar plate as control. The non-selective medium allowed excision of the plasmid while the high temperature ensured the curing of all plasmids. The excision can either lead to the *sacB*-kan^R cassette remaining in the bacterial chromosome or the desired mutation being exchanged for the *sacB*-kan^R cassette. Hence the subsequent step selected for loss of the *sacB* marker by growing the cells overnight on sucrose agar plates. The product of SacB is lethal to *E. coli* in the presence of sucrose as it produces levans which accumulate in the periplasmic space and causes cell lysis (Gay *et al.*, 1983; Gay *et al.*, 1985; Tang *et al.*, 1990). The plates were incubated at 28°C overnight, before transferring single colonies onto a selection of antibiotic plates. Colonies were picked from the sucrose plate containing roughly the same number of colonies as the control plate (10^{-4} dilution). Kanamycin sensitivity indicated the loss of the kan^R cassette, while chloramphenicol and/or ampicillin sensitivity (depending upon the resistance marker present on the plasmid) indicated the successful curing and removal of the plasmid. Clones that successfully exchange the *sacB*-kan^R cassette for the desired mutation only are able to form colonies on non-selective agar plates. After incubating overnight at 37°C, these colonies were grown in LB to be stocked.

2.7. Measuring gene expression by *β*-galactosidase assay

The β-galactosidase assay is a method to determine the level of expression of a specific gene. This method makes use of the cleaving activity of β-galactosidase (product of the *lacZ* gene). While it hydrolyses lactose to glucose and galactose in the cell, it also recognises the synthetic compound o-nitrophenyl-β-D-galactoside (ONPG) as a substrate. Cleavage of ONPG results in a yellow colour due to the generation of o-nitrophenol. The level of gene expression can thus be determined by means of a LacZ reporter fusion and relates to the intensity of colour.

Based on a protocol described by Miller (1972), expression of a LacZ reporter fusion is measured in Miller units according to the following procedure. Z-Buffer is composed of 60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $Na_2HPO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$ and 50 mM β-mercaptoethanol. Before adding the final component β-mercaptoethanol , the solution was adjusted to pH 7.0 with Na_2HPO_4 and made up to a final volume of 500ml with ddH_2O. Z-buffer was stored foilcovered and sealed with parafilm at 4°C.

2.7.1. *β*-galactosidase assay protocol

An overnight culture was grown in the required medium (either RD_{gly} or M_{gly}) and 15µl overnight culture (RD_{gly}) or 300 µl overnight culture (M_{gly}) was inoculated into 15ml fresh medium. The cultures were grown to an OD_{600nm} of 0.18 and placed on ice to reach an OD_{600nm} of 0.2, remaining in exponential phase. This ensured best reproducibility and reliability of results, as *fimB* expression varies as growth phase changes. The final OD_{600nm} was measured after 20 minutes incubation on ice and the assay was started by adding 250µl culture to four test tubes previously prepared with 250µl Z-buffer. 250µl sterile medium was added to two additional tubes as a negative control. 10µl chloroform and 5µl 0.1% SDS (BDH Laboratory Supplies) was added to all six tubes to lyse the cells. The tubes were then vortexed for 10 seconds and transferred to a static water bath at 28°C. After 5 minutes, the reaction was started by adding 100µl 4mg/ml ONPG. The samples remained at 28°C until a colour change became noticeable, generally for about 90 minutes. At this point, each reaction was stopped in the same order by addition of 250µl 1M Na_2CO_3 and the OD_{420nm} and OD_{550nm} were measured using the negative control tubes as blanks. These values were obtained were then entered into following equation to calculate Miller Units:

$$\text{Miller Units} = \frac{1000x\ (OD_{420} - 1.75\ x\ OD_{550})}{OD_{600}\ x\ Time\ of\ Assay\ (min)\ x\ 0.5}$$

2.7.2. Statistical analysis

The Miller Units for each strain in an experiment was calculated from 4 technical replicates. The obtained values were compared by Student's *t*-test to obtain the 95% confidence interval, i.e. the range of values within which the "true" value would fall in 95% of the cases. The difference between two values was considered statistically significant, if the mean of the values did not cover each other's range plus or minus its 95% confidence interval. The calculation is carried out as follows:

$$95\ \%\ \text{confidence interval for 4 replicates} = (3.182\sigma) / \sqrt{4}$$

with the letter σ representing the sample standard deviation, calculated by Microsoft Excel and the number 3.182 being the α/2 critical value for n-1 (i.e. 3) degrees of freedom.

2.8. Measuring fimbriation

2.8.1. Agglutination Assay

Type 1 fimbriae expression was observed qualitatively by yeast agglutination experiments. This assay was carried out by mixing equal volumes (5µl) of refrigerated *Saccharomyces cerevisiae* solution and *E. coli* culture at OD_{600nm}=2.0 on a glass microscope slide. After incubation at room temperature for 30 seconds, the samples were imaged with a Leica MZFLIII microscope, a binocular dissecting microscope with transmitted illumination, and pictures were captured with a Leica DC300F camera. The extent of type 1 fimbriation could be determined by the appearance of white precipitates which formed due to the agglutination of bacterial fimbriae with yeast cells.

Hereby, a *fimS* locked ON strain (AAEC356) was used as positive control, while a *fimS* locked OFF strain (AAEC358) served as negative control. Equal volumes (5µl) of refrigerated *Saccharomyces cerevisiae* solution and test strain were mixed on a glass microscope slide and then examined under the microscope.

2.8.2. Measuring *fimS* inversion in *fimB*$^{+}$*fimE*$^{-}$ strains

A *fimA-lacZ* reporter fusion was used to measure rate of OFF-to-ON inversion of the *fimS* invertible element, as described in previous publications (Gally et al., 1993; Lahooti et al., 2005). In this procedure, a single phase-OFF cell is grown for a known number of generations and plated out on MacConkey agar. As expression of LacZ causes a colour change, this allows the number of phase-ON colonies to be counted and thus the calculation of inversion events per generation.

To obtain a single phase-OFF colony, strains were streaked from frozen stocks onto MacConkey agar and grown overnight at 28°C. The following day, a phase-OFF colony was restreaked onto a fresh MacConkey plate and grown overnight again at 28°C. For the actual procedure, 25ml RD_{gly} medium was prewarmed to 37°C in a water bath shaking at 180rpm before inoculating a single phase-OFF colony. Upon reaching an OD_{420nm} of approximately 0.1, the OD_{420nm} was precisely measured and the culture diluted $x10^{-6}$. Per strain, 25 tubes containing 5 ml medium were prewarmed at 37°C and inoculated with the 10^{-6} diluted culture (xµl) according to the following equation:

$$x\ \mu l = 0.1 \times 18.2 / OD_{420nm}\ (\text{for } RD_{gly})$$

The tubes were then shaken at 37°C and 200rpm until bacterial growth became visible in about a third of all tubes (i.e. the medium started to become cloudy) and the OD_{420nm} was measured for each sample. Remaining below an OD_{420nm} of 0.2, a dilution was prepared according to table 2.13 and 100µl of this dilution was plated out onto MacConkey agar (5 plates per tube). The dilution is based on the measured OD_{420nm} and is achieved by diluting the cells by a factor D_1, mixing D_2 µl of this with D_3 µl of sterile medium. For example, if OD_{420nm} for a tube measured 0.17, the correct concentration of cells would be obtained by diluting the sample 10000-fold, then adding 925 µl of this mixture to 75 µl of sterile medium.

This procedure not only ensures a countable and comparable number of colonies between individual plates, but is also required for the final calculation of switching frequency.

OD	D_1	D_2	D_3
0.05	$1x10^3$	314	686
0.06	$1x10^3$	262	738
0.07	$1x10^3$	224	776
0.08	$1x10^3$	196	804
0.09	$1x10^3$	174	826
0.1	$1x10^3$	157	843
0.11	$1x10^3$	143	857
0.12	$1x10^3$	131	869
0.13	$1x10^3$	121	879
0.14	$1x10^3$	112	888
0.15	$1x10^3$	104	896
0.16	$1x10^4$	983	17
0.17	$1x10^4$	925	75
0.18	$1x10^4$	873	127
0.19	$1x10^4$	827	173
0.2	$1x10^4$	786	214
0.21	$1x10^4$	749	251
0.22	$1x10^4$	715	285
0.23	$1x10^4$	683	317
0.24	$1x10^4$	655	345
0.25	$1x10^4$	629	371
0.26	$1x10^4$	605	395
0.27	$1x10^4$	582	418
0.28	$1x10^4$	561	439
0.29	$1x10^4$	542	458
0.3	$1x10^4$	524	476

Table 2.13: Dilutions for FimB recombination experiment.

The MacConkey agar plates were incubated at 28°C for at least 24 hours, a longer period making it easier to distinguish phase-ON and phase-OFF colonies. The number of phase-ON and total colonies were counted and recorded by adding together the numbers from all

plates taken from one particular tube. The switching frequency was then calculated via the following equations:

$V = (1000 / D_2) \times D_1 \times (C_T / P)$

$G = (log(V) - log(0.2)) / log(2)$

Inversion frequency per cell per generation $= 1 - (1 - C_O / C_T)^{(1 / G)}$

where V is the viable count per ml, D_1 and D_2 are taken from Table 2.11, C_T is the total number of colonies, P is the number of plates per tube, G is the number of generations and C_O is the number of phase-ON colonies.

2.9. Determination of Polyamine levels

Levels of putrescine and spermidine levels were measured by Gas chromatography (GC) with trifluoroacetylacetone (FAA) as derivatising reagent. Separation and determination of the polyamines putrescine and spermidine in various mutant backgrounds was carried out as described by Khuhawar *et al.* (1999). Hereby, FAA reacts with the diamines to form H_2FAA_2Pu and H_2FAA_2Spe, respectively.

For the preparation of polyamine standards, 1ml of putrescine and spermidine solution (2mg/ml) was added to trifluoroacetyl acetone (FAA) (1.5ml, 3% v/v in methanol), sodium acetate (1M) buffer, pH 6.75 (2ml) and heated to 95°C for 15 minutes in a water bath. After cooling, 3ml chloroform was added and the layers were allowed to separate. The organic fraction was collected before repeating the extraction with 2ml chloroform and combining both extracts. The solvent chloroform was subsequently evaporated in a waterbath at 62°C and the residue was dissolved in 0.4ml ethanol.

The cell samples were prepared by growing up to 4 overnight cultures per strain to an OD_{600nm} of 0.2 and pooling these cultures. This increased the amount of cells present per sample. After pelleting the cells and removing the supernatant by aspiration, the cells were washed once with 2ml of a buffer containing 30mM Tris-HCl, pH7.5 and 10mM Magnesium acetate and then sonicated (8x 30s) in the same buffer. At this point, additional cell samples were spiked with polyamine standard solution for control purposes, to ensure that the subsequent treatment does not affect polyamine derivatisation and detection.

After sonication, extraction of the polyamines was carried out by TCA-diethyl ether extraction. 2ml of trichloroacetic acid TCA (10% w/v in water) was added to the sonicated

samples, the suspension mixed thoroughly and cooled on ice for 30 minutes. The samples were then centrifuged at 10,000rpm for 10 minutes and the supernatant was collected. The residue was resuspended in 2ml TCA (10%), mixed thoroughly and centrifuged for 10-15 minutes and the supernatant collected again. The combined supernatant was subsequently added to 5ml water-saturated diethyl ether, mixed well and after separation the upper organic layer was removed. The extraction of the remaining aqueous layer was repeated twice with 5ml diethyl ether. The final aqueous layer was collected to add 1.5ml trifluoroacetylacetone (FAA) (3% v/v in methanol) and 2ml sodium acetate (1M), and the samples were checked for a pH of 6.75. For the remaining procedure, the cell samples were treated in the same way as the polyamine standard solutions: incubation at 95°C for 15 minutes being followed by chloroform extraction. After evaporation of the chloroform, the residue was dissolved in 0.1ml ethanol to further increase the concentration of present polyamines.

1ul of the individual solutions was then injected onto a HP-5MS column (30mx0.25mm I.D.) at a column temperature of 240°C. The programmed heating rate of 2°C/min continued up to 260°C, before holding the temperature at 260°C for 10 minutes. The flow-rate of the carrier gas helium was set to 4.5ml/min.

Chapter 3

Glutathione

an activator of *fimB* expression

3.1. Summary

In *E. coli* MG1655, *fimB* expression is regulated by a range of factors and pathways and glutathione was identified as another regulatory factor. Initial research focussed on the oxidation state and site of action of glutathione before investigating its mode of action. Glutathione was shown to activate *fimB* expression in its reduced form (GSH) and exogenous GSH compensates for the loss of glutathione production in the Δ*gshA* strain even in the absence of its cytoplasmic membrane transporter. This suggests that GSH acts in the periplasmic space. Furthermore, Cbl was identified as a repressor of *fimB* expression, yet only in the presence of functional glutathione production. The effect of Cbl can possibly be linked to a limitation of the intracellular GSH pool by regulating GSH biosynthesis or degradation. While loss of GSH export across the inner bacterial membrane by CydDC does not affect the activating effect of GSH in the wild type, the stimulatory effect in the absence of *cbl* is dependent upon this GSH transporter. This provides further evidence for the hypothesis that GSH acts on a periplasmic target, but does not rule out the involvement of additional cytoplasmic factors.

3.2. Reduced GSH activates *fimB* expression

Glutathione biosynthesis requires the enzymes γ-glutamylcysteine synthetase (GshA) and glutathione synthetase (GshB), which form peptide bonds between the individual amino acids in two consecutive steps (Meister, 1988). Therefore the effect of glutathione on *fimB* expression was examined by transducing Δ*gshA*::kan^R and Δ*gshB*::kan^R mutations into *E. coli* MG1655 strains containing a FimB-LacZ translational fusion. This translational fusion carries the *lacZ* reporter gene within the *fimB* gene, downstream of the *fimB* promoter and ribosome binding site (RBS). This produces a hybrid protein of FimB and LacZ. Therefore the level of FimB production can be directly related to the levels of LacZ, which is measured by β-galactosidase activity. A transcriptional fusion, on the other hand, would transcribe both genes under the control of one promoter and translate these genes as two distinct proteins via a different RBS. Moreover, if expression of FimB is controlled at the level of translation, not transcription, this effect will only be identified by the translational fusion.

The absence of glutathione biosynthesis (Δ*gshA* and Δ*gshB*) resulted in a 4-fold decrease in *fimB* expression compared to the wild type (Figure 3.1) and the phenotype of both the Δ*gshA* and Δ*gshB* mutants were identical. This rules out the possibility that the intermediate in glutathione biosynthesis, the dipeptide γ-glutamylcysteine generated by *gshA*, is the activating factor.

In the cell, glutathione is present in two oxidative states, as reduced GSH and oxidised GSSG, with glutathione oxidoreductase (Gor) ensuring a high GSH:GSSG ratio by reducing GSSG to GSH (Kidd, 1997; Wang *et al.*, 1998). A Δ*gor* mutant thus contains mainly oxidised glutathione (GSSG) and could give an indication whether it is reduced or oxidised glutathione that activates *fimB* expression. Deletion of *gor* significantly decreased *fimB* expression to levels just above those observed for Δ*gshA* and Δ*gshB* mutants (Figure 3.1). This data provides evidence that the tripeptide glutathione activates *fimB* expression and moreover suggests that this effect is mainly attributed to reduced GSH, as opposed to oxidised GSSG.

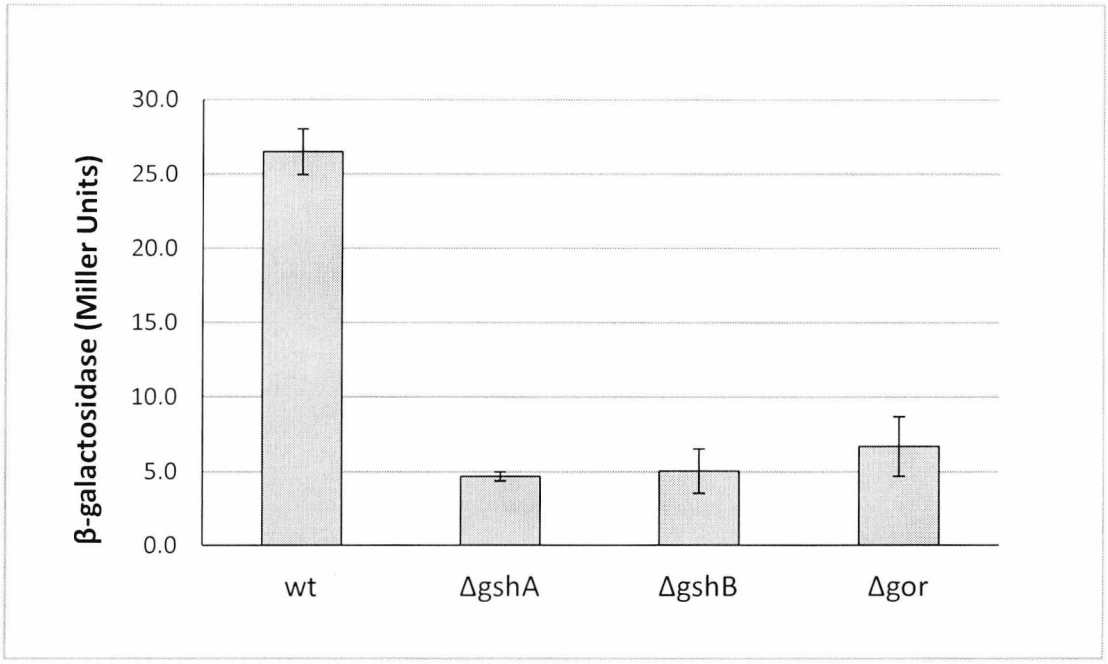


Figure 3.1: β-galactosidase assay showing the effect of the absence of glutathione biosynthesis (Δ*gshA*, Δ*gshB*) and glutathione reduction (Δ*gor*) on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC1611 and KCEC1757.

3.3. Localisation of activating GSH

3.3.1. Addition of exogenous glutathione

The activating effect of glutathione was further investigated by treating the wild type and Δ*gshA* cells with exogenous glutathione to determine whether it could compensate for the loss of glutathione production. At concentrations corresponding to cytoplasmic physiological levels (0.25mM - 4mM) (Meister, 1988), reduced glutathione GSH complemented the Δ*gshA* mutant and also stimulated *fimB* expression in the wild type (Figure 3.2). An initial response was observed at a concentration of 0.25mM and the level of complementation increased with the concentration of GSH (Figure 3.3). Addition of oxidised glutathione GSSG, in contrast, did not complement *fimB* expression (Figure 3.2). Along with the result obtained for the Δ*gor* mutant, this indicates that glutathione activates *fimB* expression in its reduced state.

The levels of GSH required for complete complementation were beyond the physiological levels of total glutathione that are expected to be in periplasm. However, under the growth conditions, GSH is readily converted to GSSG by air oxidation (Eser *et al.*, 2009), which decreases the amount of reduced GSH that is actually added to the cells. The high levels of GSH for complementation might also be explained by its transport into the cytoplasm via an uptake system located in the inner membrane. To test this hypothesis, complementation was studied in cells lacking the glutathione transport system GsiABCD (Suzuki *et al.*, 2005). Exogenous GSH proved to stimulate *fimB* expression even in the absence of glutathione uptake (Figure 3.3), supporting the hypothesis that GSH regulates *fimB* expression via a periplasmic target. Furthermore, the result that exogenous GSH stimulates in the wild type as well as the Δ*gshA* mutant implies that the level of GSH in the wild type is sub-optimal for *fimB* expression and that GSH activates *fimB* expression more efficiently at elevated levels.

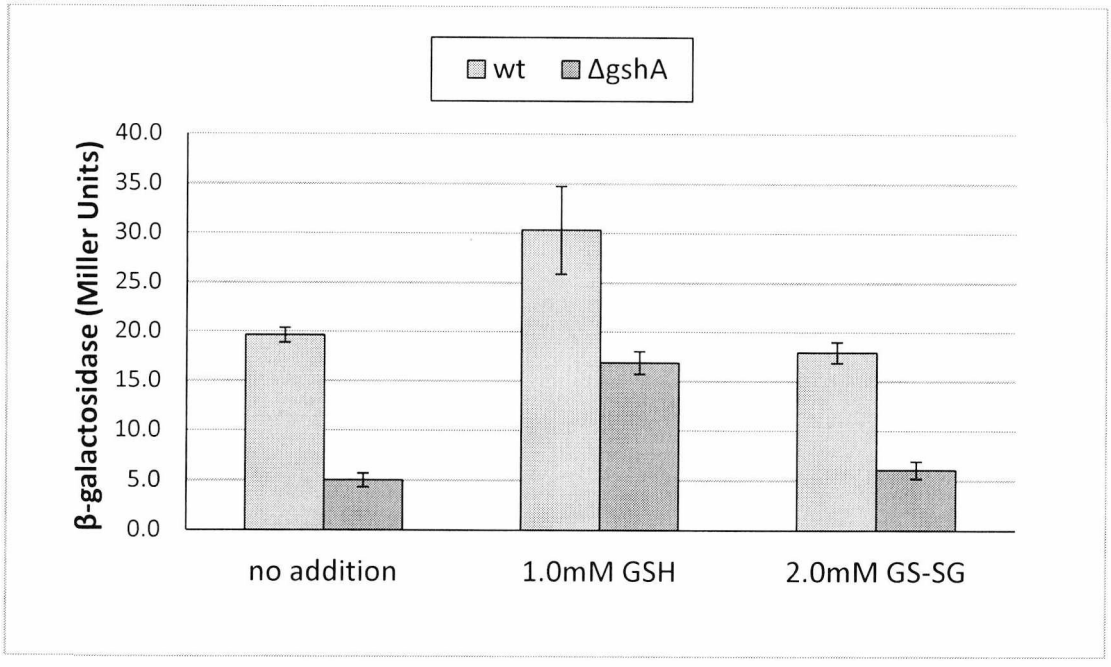


Figure 3.2: β-galactosidase assay showing the effect of exogenous GSH and GSSG on *fimB* expression. Strains were grown in RD_{gly} with addition of 1mM GSH and 2mM GSSG. Strains used were BGEC905 and KCEC1627.

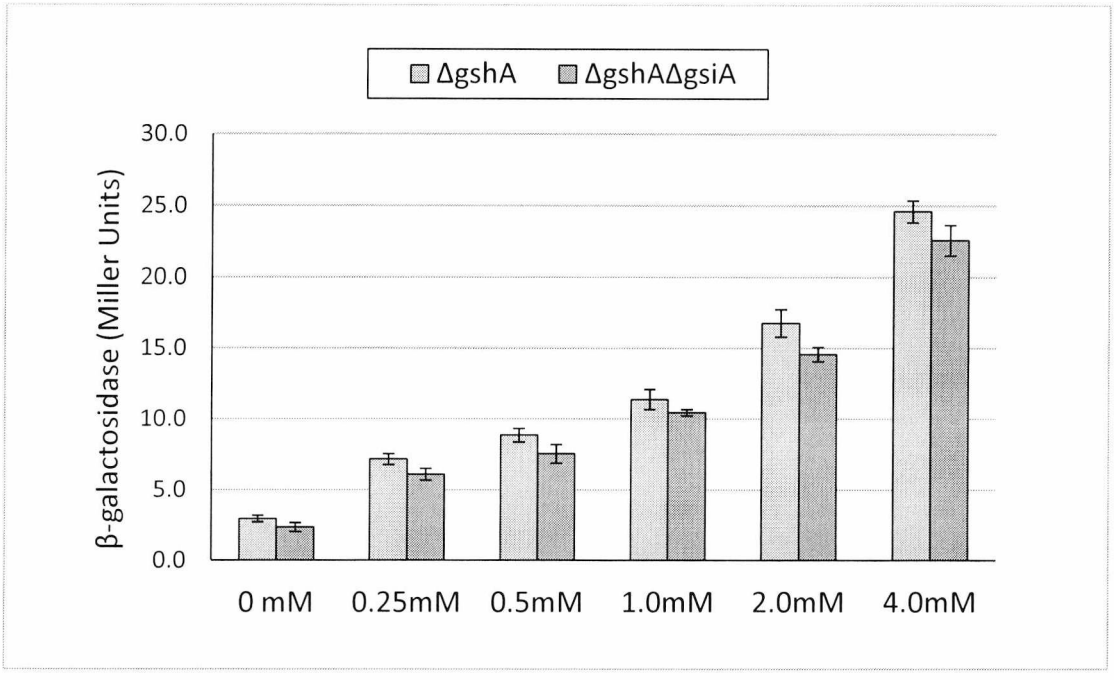


Figure 3.3: β-galactosidase assay showing the effect of exogenous GSH on *fimB* expression in the absence of glutathione uptake (Δ*gsiA*). Strains were grown in RD_{gly} with addition of 0.25, 0.5, 1.0, 2.0 and 4.0mM GSH. Strains used were KCEC1627 and KCEC1769.

3.3.2. Mutation of the GSH export protein CydD

Data obtained so far suggests a periplasmic site of action for GSH. Supposing this to be the case, GSH export would be required for the activating effect of GSH on *fimB* expression. The glutathione export protein CydDC mediates transport of GSH across the cytoplasmic membrane into the periplasm to maintain an optimal redox balance in the periplasm (Pittman *et al.*, 2005). *cydD* was deleted in both the wild type and Δ*gshA* background, but did not show any effect on *fimB* expression (Figure 3.4). While this suggests that CydDC is not essential for the activating effect in the wild type, this does not refute the hypothesis that GSH has a periplasmic site of action. CydDC is the only identified GSH exporter so far, but work by other groups (Eser *et al.*, 2009; Smirnova *et al.*, 2011) shows that export of GSH occurs even in the absence of CydD. CydDC may not be induced in the respective genetic backgrounds and under the experimental conditions and GSH export is carried out by an alternative transport system. Alternatively, GSH might not only be acting in the periplasm, but also on an additional cytoplasmic target.

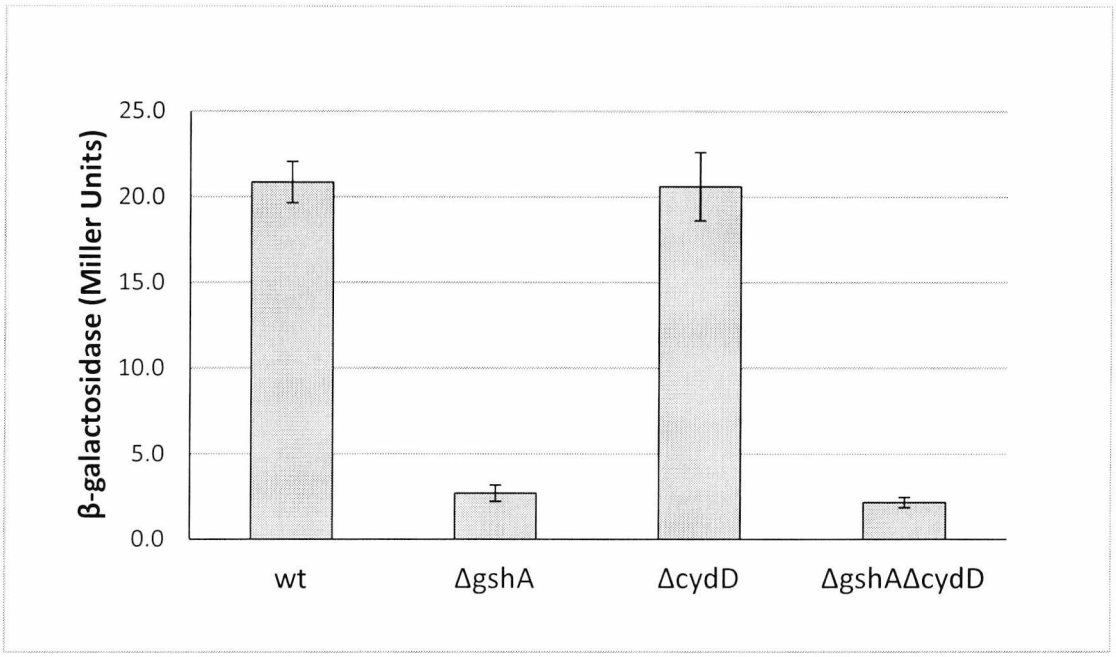


Figure 3.4: β-galactosidase assay showing the effect of the absence of GSH export (Δ*cydD*) on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC1856 and KCEC1838.

3.4. Cbl: a potential suppressor?

The transcription factor Cbl was considered to be a potential cytoplasmic regulator of the GSH pool. This regulatory protein is required for the use of alternative sulfur sources (van der Ploeg *et al.*, 1997). Cbl is activated via CysB under sulfur-deficient conditions and at low levels of cysteine (Iwanicka-Nowicka *et al.*, 1995). Cysteine, in turn, is a component of GSH, providing a direct link between Cbl and GSH biosynthesis. It seems unlikely that conditions that induce the activity of Cbl would also induce additional consumption of the scarce cysteine by producing more GSH than is necessary. Therefore Cbl was thought likely to be limiting GSH levels.

It was found that mutation of *cbl* had a significant effect on *fimB* expression, with levels increasing 3-4 fold in the wild type background. This effect was suppressed by mutation of *gshA* (Figure 3.5), indicating that the ability to make GSH is required for Cbl to affect *fimB* expression. The effect of *cbl* mutation was dependent upon CysB (Figure 3.6). This was expected, as *cbl* expression is activated by CysB (Iwanicka-Nowicka *et al.*, 1995). This is also consistent with the hypothesis that Cbl regulates GSH levels in response to sulfur deficiency and low levels of cysteine. It further suggests that the absence of Cbl leads to an increase in GSH, which in turn stimulates *fimB* expression.

In contrast to the situation in the wild type background, the stimulating effect of *cbl* mutation is dependent upon the export protein CydD (Figure 3.5). This suggests that loss of Cbl either induces *cydD* expression or that it raises GSH levels beyond a threshold needed to become a substrate for CydDC mediated export.

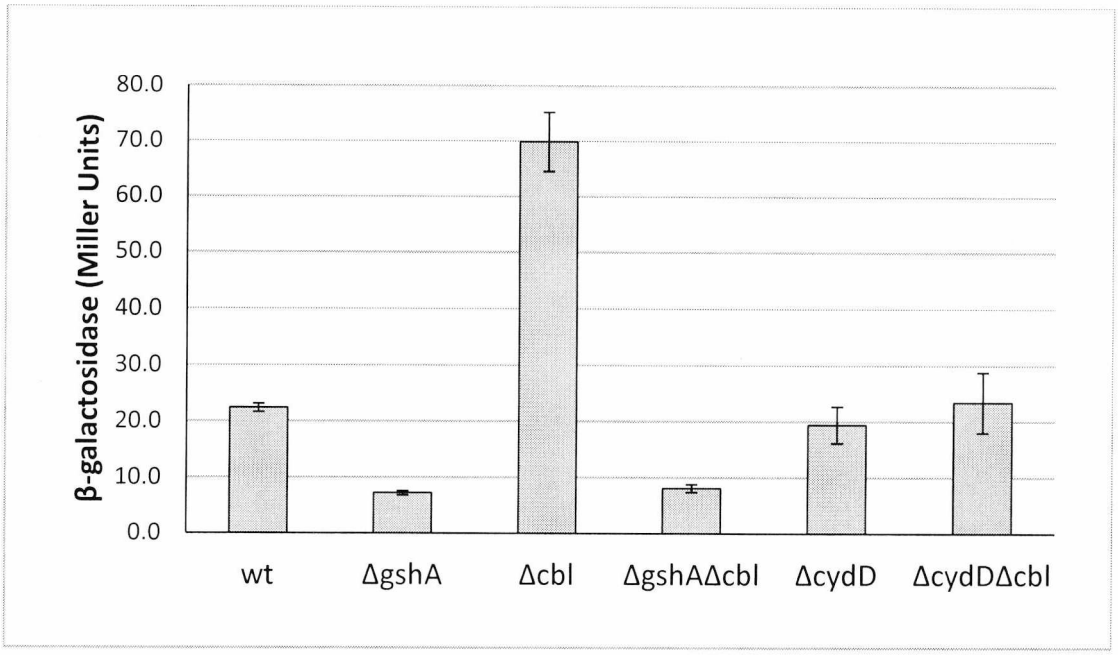


Figure 3.5: β-galactosidase assay showing the effect of *cbl* mutation on *fimB* expression in the presence and absence of GSH export (Δ*cydD*). Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC2600, KCEC2602, KCEC3100 and KCEC3112.

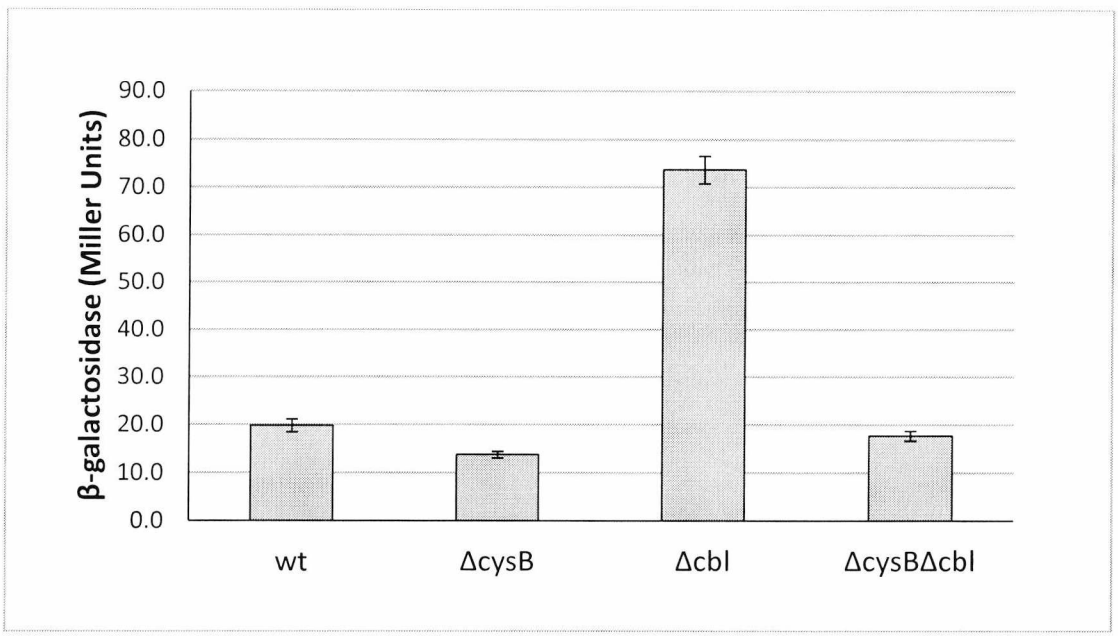


Figure 3.6: β-galactosidase assay showing the effect of *cbl* mutation on *fimB* expression in the absence of CysB activation (Δ*cysB*). Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC2973, KCEC2600 and KCEC2982.

3.5. RD +/- cys/met: deprivation of sulfur source

In the sulfur assimilation pathway, extracellular sulfate is reduced to sulphide, generating the intermediate product adenosine 5'-phosphosulfate (APS) (Sekowska *et al.*, 2000). APS, in turn, inactivates Cbl (Bykowski *et al.*, 2002) to prevent sulfur assimilation via alternative sulfur sources. The RD_{gly} medium used in our experiments is supplemented with cysteine and methionine, for which reason the cells can reduce biosynthesis of these amino acids. Consequently, the sulfur assimilation pathway is less active, leading to less APS to inactivate Cbl. In RD_{gly} medium without cysteine and methionine, increased sulfur assimilation will elevate APS levels and render Cbl less active. The effect of *cbl* mutation was therefore expected to be diminished in the absence of cysteine and methionine.

Indeed, *cbl* mutation had a much less pronounced effect on *fimB* expression in these media (Figure 3.7). Both a decreased activity of Cbl and the low level of cysteine may account for this suppression. With less active Cbl enzyme in the cell, mutation of *cbl* has less impact. Moreover, the stimulatory effect of *cbl* mutation is dependent upon GSH biosynthesis (Figure 3.5). Supposing that loss of Cbl increases the intracellular GSH pool, low levels of cysteine limit GSH production and thus the effect of *cbl* mutation on *fimB* expression.

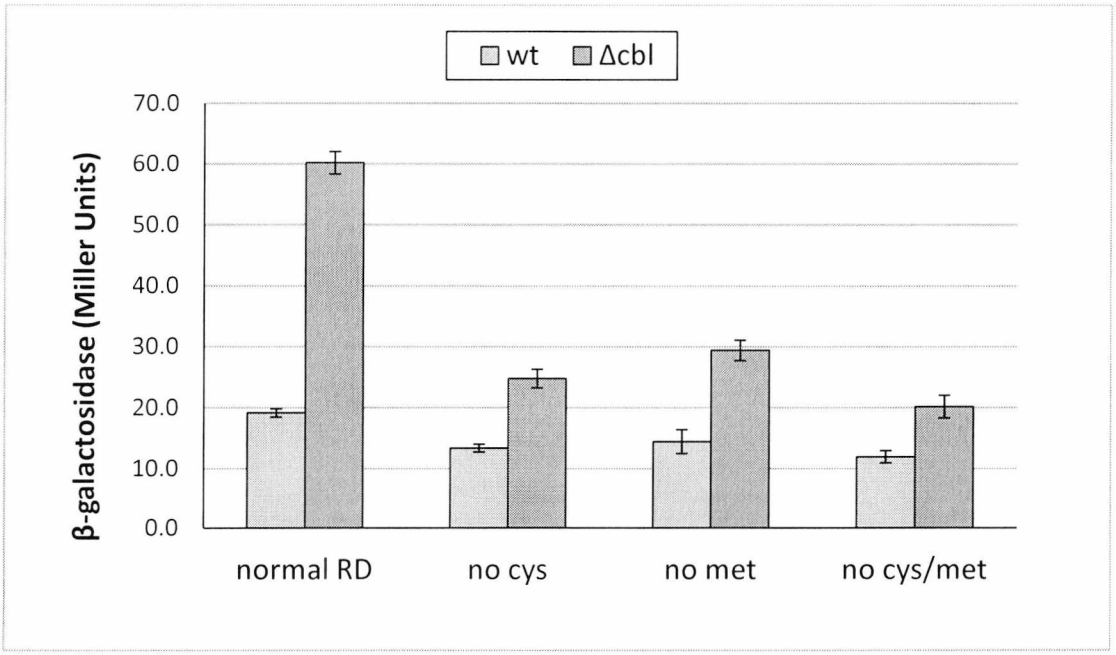


Figure 3.7: β-galactosidase assay showing the effect of *cbl* mutation on *fimB* expression in the absence of cysteine and methionine. Strains were grown in normal RD_{gly} RD_{gly} without cysteine, RD_{gly} without methionine and RD_{gly} without cysteine and methionine. Strains used were BGEC905 and KCEC2600.

3.6. Discussion

A range of mutations were transduced into *E. coli* MG1655 strains in order to delete genes involved in glutathione biosynthesis and metabolism. The mutations were obtained from the Keio collection (Baba *et al.*, 2006), a library of gene deletions that are tagged with a kan^R-cassette, and moved into strains containing a FimB-LacZ fusion. In this translational fusion strain, the level of FimB expression is directly related to the level of LacZ expression, and therefore can be measured via β-galactosidase activity.

Initially it was confirmed that glutathione activates *fimB* expression. The loss of both genes required for its synthesis (Δ*gshA* and Δ*gshB*) show the same decrease in *fimB* expression. This indicated that the intact tripeptide stimulates *fimB* expression, as opposed to the dipeptide γ-glutamylcysteine, the intermediate in glutathione biosynthesis. Levels of *fimB* expression in glutathione deficient mutants (Δ*gshA* and Δ*gshB*) were comparable to the level obtained for mutants lacking glutathione oxidoreductase (Δ*gor*). These mutants produce glutathione, but are not able to reduce oxidised GSSG back to reduced GSH. Loss of Gor not only decreases the GSH:GSSG ratio in the cell, but is also known to diminish the overall glutathione pool (Kunert *et al.*, 1990). This suggested that glutathione is required to be in its reduced form to activate *fimB* expression. In this regard, the result that only exogenous GSH, and not GSSG, complements in the Δ*gshA* mutant, provides a weight of evidence that glutathione activates *fimB* expression in its reduced state GSH.

To be able to complement in the Δ*gshA* mutant, exogenous was required to be added GSH at physiological intracellular levels (0.1-10mM) (Meister, 1988). Approximately 30% of the entire glutathione pool is found outside the cell in the medium (Owens *et al.*, 1986) and the level of added GSH exceeded the physiological levels that are expected to be found in the periplasm. However, under the growth conditions, GSH is probably largely present in the oxidised form (Eser *et al.*, 2009), which decreases the amount of reduced GSH that is effectively added to the cultures. As only reduced GSH appears to activate *fimB* expression, the amount of GSH required for complementation has to take this oxidation into account.

As an alternative explanation, exogenous GSH might be imported into the cytoplasm. However, complementation with exogenous GSH was obtained even in the absence of the functional GSH import system GsiABCD (Suzuki *et al.*, 2005). This suggests that GSH

stimulates *fimB* expression via a target that is located in the periplasmic space of the bacterial cell. It therefore seemed likely that the GSH transporter CydCD (Pittman *et al.*, 2005) would be involved in the stimulatory effect of endogenous GSH by enabling its export to the periplasm. Although the mutation of *cydD* did not confirm this, this does not rule out a periplasmic site of action of endogenous GSH as GSH is exported into the periplasm even in the absence of *cydD* (Eser *et al.*, 2009). The CydDC transporter may not be induced under the conditions used. Alternatively, an additional cytoplasmic factor may be involved in the activating effect of GSH on *fimB* expression.

The addition of GSH not only stimulated *fimB* expression in the Δ*gshA* mutant, but also in the wild type. This suggests that levels of GSH are sub-optimal for *fimB* expression in the wild type. By increasing levels of GSH, the responsive factor is able to stimulate *fimB* expression even more efficiently.

The transcription factor Cbl was considered to be a potential regulator. This regulator is required for the use of alternative sulfur sources (van der Ploeg *et al.*, 1997) and is activated via CysB under sulfur-deficient conditions and hence low levels of cysteine (Iwanicka-Nowicka *et al.*, 1995). Cbl can therefore be linked to glutathione biosynthesis via the amino acid cysteine, a component of glutathione. Another potential link between GSH and Cbl is provided by the fact that *E. coli* is also able to use glutathione as an alternative sulfur source (Suzuki *et al.*, 1993), although there is no evidence so far that Cbl is required for GSH utilisation.

The mutation of *cbl* significantly increased *fimB* expression in the wild type background, but not in Δ*gshA* strains. This result shows that Cbl is involved in *fimB* regulation, although this requires the cells to be able to synthesise GSH. The response to *cbl* mutation was furthermore shown to be dependent upon the transcription factor CysB, which is consistent with previous reports that CysB activates *cbl* expression (Iwanicka-Nowicka *et al.*, 1995). Cbl activity is inhibited by APS, an intermediate in the sulfur assimilation pathway (Bykowski *et al.*, 2002). Under conditions of sulfur starvation, a low rate of flux through this pathway will decrease the accumulation of APS and thus increase levels of active Cbl. The sulfur-rich medium used for the experiments, should cause a high rate of sulfur assimilation, bringing about high levels of APS that inactivates the majority of Cbl. This would mean that the mutation of *cbl* would not be expected to produce as strong a phenotype as it does. Yet, the presence of cysteine and methionine in the RD_{gly} medium

reduces the requirement for the cells to synthesise these amino acids themselves. Cysteine and methionine have negative feedback control on sulfur assimilation (Jones-Mortimer, 1968) and lower the flux through the assimilation pathway. This leads to lower levels of APS and consequently more active Cbl. Leaving out cysteine and methionine in the RD_{gly} medium hence induces sulfur assimilation, which should lower the activity of Cbl. Experiments carried out in RD_{gly} medium without cysteine and/or methionine were consistent with this expectation, as the effect of *cbl* mutation was decreased under these conditions.

The decreased impact of *cbl* mutation on *fimB* expression in the absence of cysteine and methionine can be explained via the GSH pool as well as the net activity of Cbl. On the one hand, a low level of cysteine limits GSH biosynthesis and the GSH pool. This in turn limits the effect that the absence of Cbl can have on GSH levels. On the other hand, the cells are required to take up more sulfur for cysteine and methionine biosynthesis. This increases the flow through the sulfur assimilation pathway, increases levels of APS and thus inactivates Cbl. Although the absence of cysteine also induces CysB to activate cysteine biosynthesis – and thus antagonises the inactivation of Cbl by stimulating *cbl* expression - the increase in APS would cause a net decrease in active Cbl. In the absence of the sulfur amino acids, limited resources for GSH biosynthesis as well as diminished Cbl activity hence both reduce the potential to increase the GSH pool with loss of Cbl.

As mentioned above, Cbl is activated by CysB (activator of cysteine biosynthesis) under conditions of sulfur starvation (Iwanicka-Nowicka *et al.*, 1995). Thus Cbl possibly limits GSH levels as a way of conserving cysteine with GSH serving as reservoir for cysteine. This would be consistent with the result that Δ*cbl* only affects *fimB* expression in the presence of functional GSH biosynthesis. Cbl might limit the GSH pool by activating its degradation or by inhibiting its biosynthesis. Along with the observation that exogenous GSH stimulates *fimB* expression in the wild type, the substantial stimulatory effect of Δ*cbl* further suggests that the level of GSH in the wild type is sub-optimal for *fimB* expression.

The stimulation observed in the *cbl* mutant is furthermore dependent upon the GSH export protein CydD (Figure 3.5). In response to *cbl* mutation, raised levels of intracellular GSH are possibly exported to the periplasm in order to stimulate *fimB* expression. Loss of Cbl may induce *cydD* expression to allow GSH to be exported for subsequent activation of *fimB* expression via a periplasmic target. Alternatively, levels of GSH may rise beyond a

threshold level needed for CydDC export. Both explanations account for the lack of response to Δ*cydD* in the wild type background (Figure 3.4).

As mutation of *cydD* also blocks cysteine export and thus leads to accumulation of cysteine in the cell. Elevated levels of cysteine, in turn, will signal sulfur sufficiency and hence inactivate Cbl. However, while this could explain the lack of response to mutation of *cbl* in the Δ*cydD* mutant background, the inactivation of Cbl should increase *fimB* expression as observed in the Δ*cbl* single mutant. The result obtained for the Δ*cbl*Δ*cydD* double mutants hence strongly suggests the involvement of CydD in effect of *cbl* mutation.

In a physiological context, the presence of sufficient sulfur sources (and hence cysteine levels) will lead to inactivation of Cbl, which, in turn, increases the intracellular GSH pool and its export via CydDC. This allows GSH to act on its periplasmic target to activate *fimB* expression. High levels of cysteine – and hence GSH – would therefore be a signal for low stress levels and represent good conditions to increase the production of type 1 fimbriae.

In order to confirm that Cbl alters GSH levels and to link *fimB* expression to optimal levels of GSH levels, the measurement of intracellular levels of GSH was attempted. This was carried out with a Glutathione Assay Kit purchased from Abnova, which is designed to detect GSH, GSSG and total glutathione individually. Unfortunately this was unsuccessful, as high background readings in the GSH deficient Δ*gshA* strain showed that the assay was not specific for GSH and GSSG.

While it clearly would be of interest to measure GSH and GSSG levels to confirm the hypothesis described above, subsequent work focussed on the identification of the periplasmic target of GSH and of cytoplasmic factors that affect *fimB* expression in response to GSH instead.

Chapter 4

The level of regulation

4.1. Summary

Investigations so far suggest that GSH activates *fimB* expression by acting on a periplasmic target. To investigate the mechanism of regulation further, the initial approach was to study if GSH affects *fimB* transcription or translation. It is shown that mutation of *gshA* affects *fimB* expression at both levels. While GSH forms a covalent adduct with spermidine (Tabor *et al.*, 1985; Smith *et al.*, 1995), polyamines have an effect on the translation of many genes (Tabor *et al.*, 1985; Igarashi *et al.*, 2006). This suggests that this polyamine could have a selective affect on *fimB* translation that contributes to the Δ*gshA* mutant phenotype. Results show that the *fimB* promoter is responsive to exogenous spermidine. There is a greater response to spermidine in the translational fusion than in the transcriptional fusion, which indicates that spermidine affects *fimB* expression at both the transcriptional and post-transcriptional level.

4.2. LacZ fusion strains

The level of *fimB* expression in the experiments described in earlier chapters was estimated using a FimB-LacZ translational fusion. This fusion carries the *lacZ* reporter gene within the *fimB* gene. The fusion contains the 5' end of the *fimB* orf fused inframe with the 3' end of the *lacZ* orf to generate a hybrid FimB-LacZ protein that has β-galactosidase activity. The level of LacZ therefore directly relates to the level of *fimB* expression and takes into consideration regulatory effects at the level of transcription as well as translation.

However, the activating effect of GSH may be taking place at the level of *fimB* transcription, but not translation, or vice versa. In contrast to the translational fusion, a *fimB-lacZ* transcriptional fusion only detects regulatory effect at the level of transcription. In this strain, the inserted *lacZ* gene contains its own ribosome binding site. Therefore *lacZ* is transcribed under the control of the *fimB* promoter, but translation produces only the LacZ protein via its own RBS. Any post-transcriptional modification and regulatory effect on *fimB* translation is hence unlikely to be detected. In order to determine at which level GSH regulates *fimB* expression, the effect of *gshA* mutation was compared between a transcriptional and a translational fusion strain.

4.3. Regulation of *fimB* expression at transcriptional and translational level

Transcriptional and translational fusion strains where used to investigate whether the *gshA* mutation affects *fimB* expression at the transcriptional or the translational levels selectively. In order to distinguish an effect on transcription from an effect on translation, respective Δ*slyA* mutant strains were used as a control. SlyA activates *fimB* transcription, by preventing H-NS repression (McVicker *et al.*, 2011). Assuming that SlyA and GSH both activate *fimB* transcription alone, it is possible to predict the relative effects of the Δ*gshA* mutant on expression of the transcriptional and translational fusions. An unexpectedly large effect of the Δ*gshA* mutant on the translational fusion relative to the transcriptional fusion would be interpreted as evidence for post-transcriptional control.

The experimental value obtained for the Δ*gshA* transcriptional fusion was significantly higher than predicted (Figure 4.1) given its effect on the translational fusion. While the levels of *fimB* expression in the translational fusion of Δ*slyA* and Δ*gshA* are decreased to a comparably low level, the respective values obtained for the transcriptional fusion show a big discrepancy. As the transcriptional fusion should not detect regulation at the post-transcriptional level, this difference implies that the Δ*gshA* mutant has a specific effect on translation. Considering that both fusion strains show a decrease in *fimB* expression, it seems reasonable to propose that GSH activates *fimB* expression at both the transcriptional and translational level.

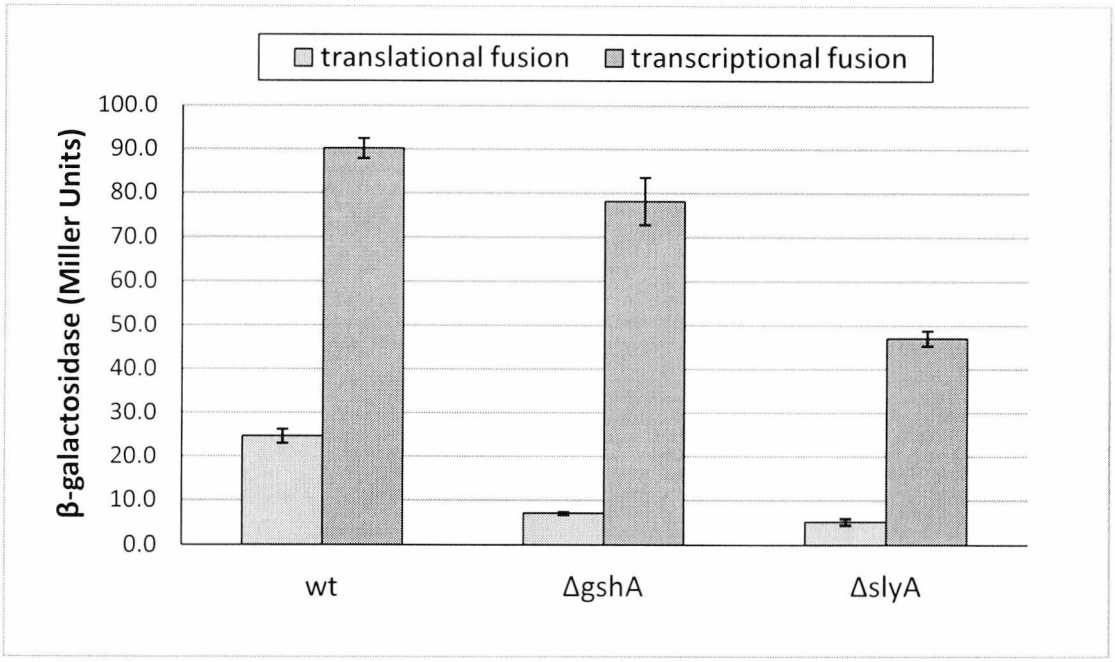


Figure 4.1: β-galactosidase assay showing the effect of *gshA* mutation and *slyA* mutation on *fimB* expression in a translational and transcriptional FimB-LacZ fusion strain. Strains were grown in RD_{gly}. Strains used were BGEC905, AAEC261A, KCEC1627, KCEC2860, KCEC1243 and KCEC2862.

4.4. Spermidine inhibits *fimB* expression

Polyamines are a global regulator in the cell and are involved in many cellular processes. This includes protein synthesis, since polyamines have been shown to stimulate a wide range of genes at the translational level (Tabor *et al.*, 1985; Igarashi *et al.*, 2006). The majority of the cellular GSH pool is present in complex with the polyamine spermidine, forming glutathionylspermidine (Gsp). In this regard, it was considered that polyamines might be involved in the translational effect on *fimB* expression in the Δ*gshA* mutant. To test this hypothesis, exogenous spermidine was added to the translational and transcriptional fusion strains. Spermidine was shown to affect both fusions, however the response to spermidine was greater in the translational fusion than in the transcriptional fusion (Figure 4.2). This leads to the conclusion that spermidine affects *fimB* expression at both the transcriptional and post-transcriptional levels.

The formation of Gsp provides a direct link between GSH and spermidine. Cellular levels of glutathione hence are likely to affect the levels of free spermidine. In the absence of

GSH, the loss of Gsp formation should cause an increase in levels of free spermidine, which may account for the inhibitory effect of *gshA* mutation on *fimB* expression. The enzyme glutathionylspermidine synthase (Gss) catalyses the formation of Gsp. Supposing that mutation of *gss* increases levels of free spermidine, it was considered that this should have the same effect as the *gshA* mutation. However, mutation of *gss* did not affect *fimB* expression (Figure 4.3). This discrepancy may indicate that GSH controls spermidine biosynthesis by negative feedback inhibition. Supposing this to be the case, loss of Gsp formation would not be expected to affect spermidine levels and hence *fimB* expression.

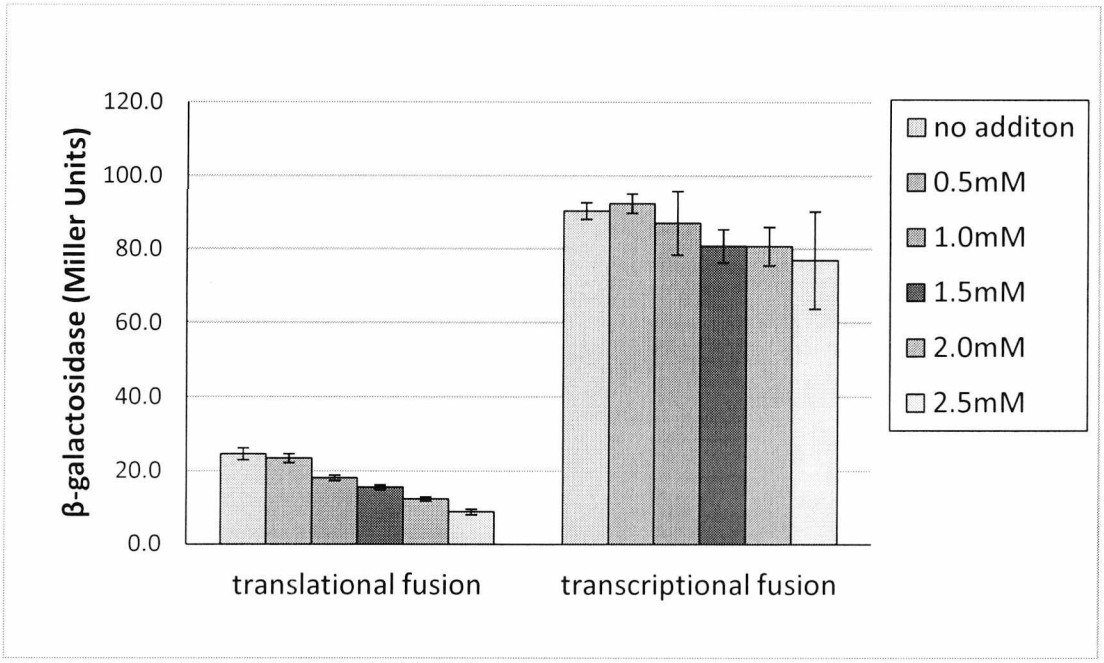


Figure 4.2: β-galactosidase assay showing the effect of exogenous spermidine on *fimB* expression in a translational and transcriptional FimB-LacZ fusion strain. Strains were grown in RD_{gly} with addition of 0-2.5mM spermidine. Strains used were BGEC905 and AAEC261A.

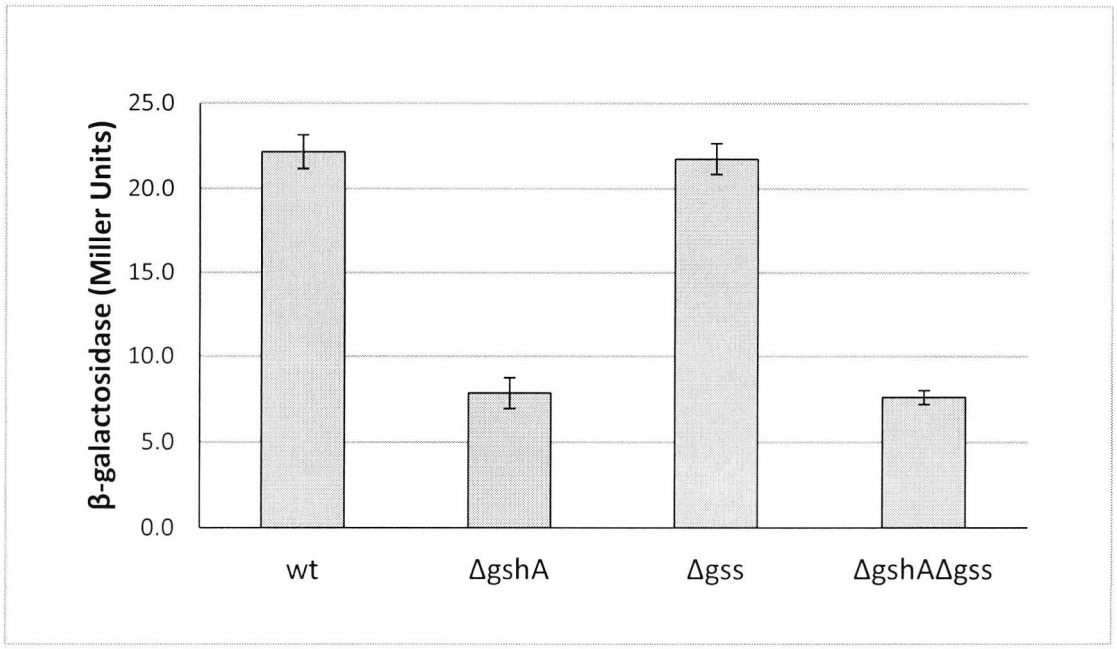


Figure 4.3: β-galactosidase assay showing the effect of *gsp* mutation on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC2734 and KCEC2746.

4.5. Discussion

Having shown that GSH has an activating effect on *fimB* expression, the mechanism of regulation was further studied in regard to the level of regulation. To investigate whether GSH has an effect on transcription or translation of *fimB*, the effect of *gshA* mutation was compared in a translational and a transcriptional fusion strain. The translational fusion detects regulatory effects at the level of transcription and translation, while the transcriptional fusion is unlikely to detect any post-transcriptional regulatory effects on *fimB* translation. It was shown that the Δ*gshA* mutant has a specific effect on translation and results further show that mutation of *gshA* affects *fimB* expression at both the transcriptional and translational level.

Polyamines are known to regulate protein synthesis (Tabor *et al.*, 1985; Igarashi *et al.*, 2006; Igarashi *et al.*, 2010). Moreover, GSH and the polyamine spermidine form glutathionylspermidine Gsp (Smith *et al.*, 1995). Therefore it was considered that polyamines could be involved in the translational effect of the *gshA* mutation on *fimB* expression. The addition of exogenous spermidine had an inhibitory effect in both the translational and transcriptional fusion. However, the translational fusion was much more

sensitive to spermidine than the transcriptional fusion, which indicated that spermidine affects *fimB* expression at both the transcriptional and post-transcriptional level. This is consistent with the result that GSH affects *fimB* expression at the level of transcription and translation. Taken together, it seems reasonable to propose that low levels of GSH lead to an elevation of spermidine levels. An excess of spermidine, in turn, inhibits transcription as well as translation of *fimB*.

GSH forms a complex with spermidine, which is catalysed by glutathionylspermidine synthase (Gss). It was considered that loss of GSH elevates levels of free spermidine, which in turn inhibits *fimB* expression. Mutation of *gss* had no effect on *fimB* expression and did therefore not support this hypothesis. However, this might be explained by negative feedback control of GSH on spermidine biosynthesis, whereby elevated levels of free GSH diminish spermidine biosynthesis and thus prevent any considerable rise in spermidine levels.

Subsequent studies first focused on the regulation of *fimB* at the transcriptional level, while the potential involvement of spermidine in the Δ*gshA* mutant phenotype will be further pursued in Chapter 6.

Chapter 5

Potential regulators of *fimB* expression

5.1. Summary

As a potent reductant, GSH protects the cell against oxidative and nitrosative stress (Dalle-Donne *et al.*, 2009b). OxyR and SoxRS are key regulators induced in response to oxidative stress and NorR and NsrR are activated under nitrosative stress. These regulators therefore had potential to be involved in the regulatory effect of GSH on *fimB* expression, though this could not be confirmed. GSH also acts as an important metal chelator (Ballatori, 1994). High levels of free metal ions present a risk to the cells. While excess copper damages protein structure, free ferrous iron produces harmful oxygen radicals. It was found that the addition of the metal chelator EDTA suppressed the effect of *gshA* mutation, suggesting that a metal ion-responsive regulator might be involved in the phenotype. While the ferric uptake regulator Fur activates *fimB* expression in the wild type, it is a repressor in the Δ*gshA* mutant. Further work shows that the Fur-repressed regulatory RNA RyhB is required for suppression of the Δ*gshA* mutant phenotype, suggesting that a RyhB-repressed target could inhibit *fimB* expression selectively in the absence of GSH. Furthermore, mutation of *hns*, which encodes the histone-like nucleoid-structuring protein H-NS, a known inhibitor of *fimB* expression, partially suppressed the Δ*gshA* mutant phenotype. Thus it is proposed that elevated *hns* expression, or an increase in the activity of H-NS, is required for the Δ*gshA* mutant phenotype.

5.2. Oxidative and nitrosative stress

The reductant GSH protects the bacterial cell against oxidative and nitrosative stress by activating a range of regulatory proteins. In the absence of GSH, elevated stress levels will activate these regulators. It was considered that one of these regulators might have a direct regulatory effect on *fimB* expression and that this contributes to the Δ*gshA* mutant phenotype.

The initial approach focused on the key regulators OxyR and SoxRS. While OxyR responds to elevated levels of hydrogen peroxide (Storz *et al.*, 1999; Zheng *et al.*, 2001), SoxR activates the transcriptional activator SoxS, to protect cells from superoxide and nitric oxide stress (Demple, 1996; Demple, 1999; Manchado *et al.*, 2000; Imlay, 2008). However, the deletion of *oxyR* and *soxR* did not suppress the Δ*gshA* phenotype (Figure 5.1), indicating that these regulators are not involved in the regulation of *fimB* expression. As SoxS is induced in the absence of GSH (Ding *et al.*, 1996), increased levels of SoxS

may inhibit *fimB* expression. By creating a fusion between the *lac*UV5 promoter and the *soxS* gene, it was possible to induce *soxS* expression by addition of IPTG. However, this had no effect (Figure 5.2), indicating that SoxS, too, does not regulate *fimB* expression. The structure of SoxS is very similar to the regulatory proteins MarA and Rob (Cohen *et al.*, 1993). All three proteins bind to the "Marbox" sequence in the promoter region to activate transcription of a range of genes, including genes involved in protection against superoxide stress (Martin *et al.*, 1999; Martin *et al.*, 2002). It was considered that the loss of one of these regulators can be compensated for by the other two proteins, for which reason all three genes were deleted in various combinations. However, this range of mutants did not have an effect on *fimB* expression (Figure 5.3). It should be noted that combined deletion of *soxS*, *marA* and *rob* in the Δ*gshA* background proved to be growth inhibitory, which was possibly due to highly elevated levels of oxidative stress.

The transcriptional regulators NorR and NsrR are involved in the response to nitric oxide (Gardner *et al.*, 2002; Bodenmiller *et al.*, 2006). In the absence of GSH, an increase in nitric oxide stress will activate these regulators. Yet again, mutation of *norR* and *nsrR* did not suppress the Δ*gshA* mutant phenotype (Figure 5.4). However, the levels of nitrosative stress in the experimental conditions may be too low for NorR and NsrR to be activated.

However, raised levels of oxidative and nitrosative stress can also be linked to metal ions. While nitric oxide has been shown to inhibit the ferric uptake regulator Fur (D'Autreaux *et al.*, 2002; Mukhopadhyay *et al.*, 2004), OxyR or SoxS are both activators of *fur* transcription (Zheng *et al.*, 1999). Furthermore, OxyR induces Mn^{2+} uptake via MntH in response to hydrogen peroxide (Anjem *et al.*, 2009). It was therefore considered that metal ion-responsive regulators may be involved in the regulatory effect of GSH on *fimB* expression.

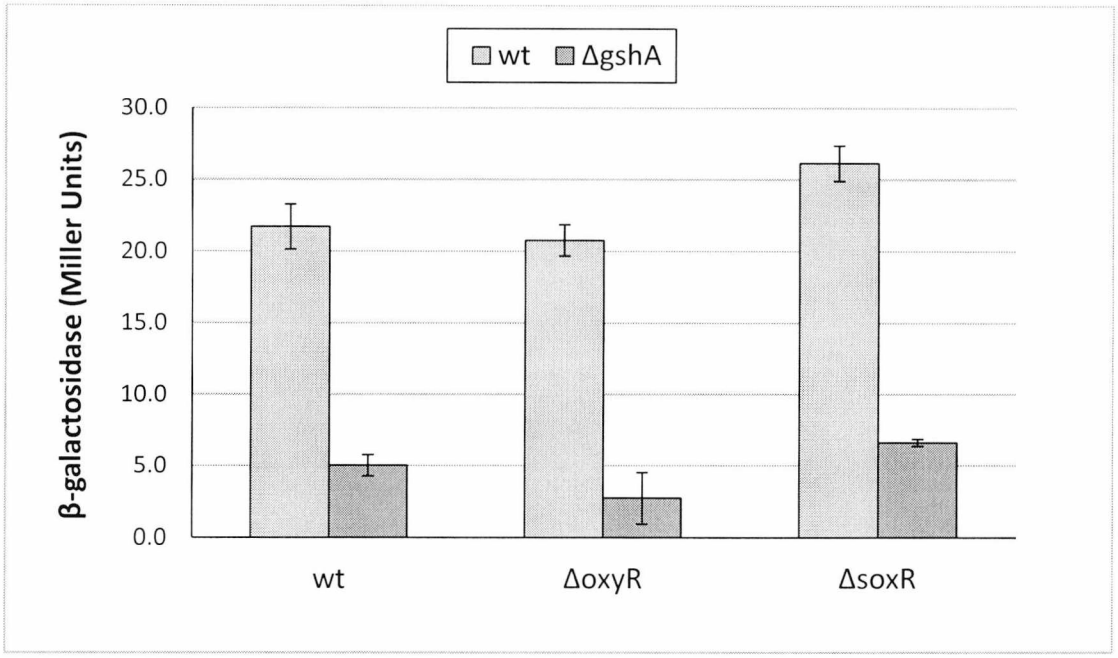


Figure 5.1: β-galactosidase assay showing the effect of the absence of oxidative stress-responsive regulators OxyR and SoxR on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC2008, KCEC2010, KCEC2295 and KCEC2297.

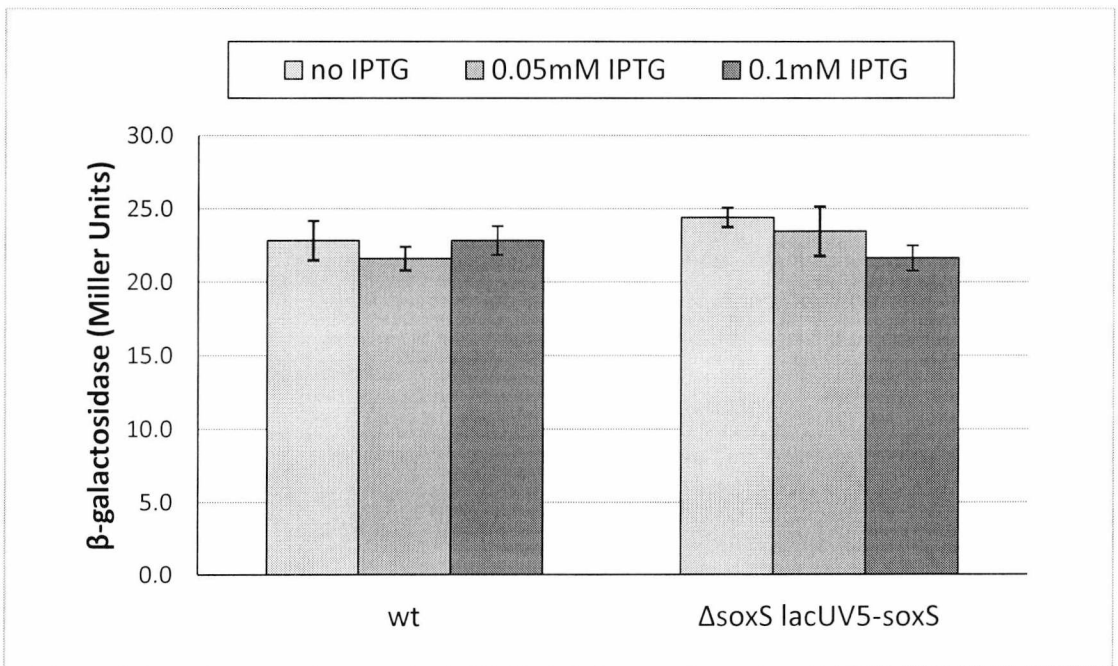


Figure 5.2: β-galactosidase assay showing the effect of *lac*UV5-induced over-expression of SoxS on *fimB* expression. Strains were grown in RD_{gly} and *soxS* expression was induced by addition of 0.05mM and 0.1mM IPTG. Strains used were BGEC905 and KCEC2465.

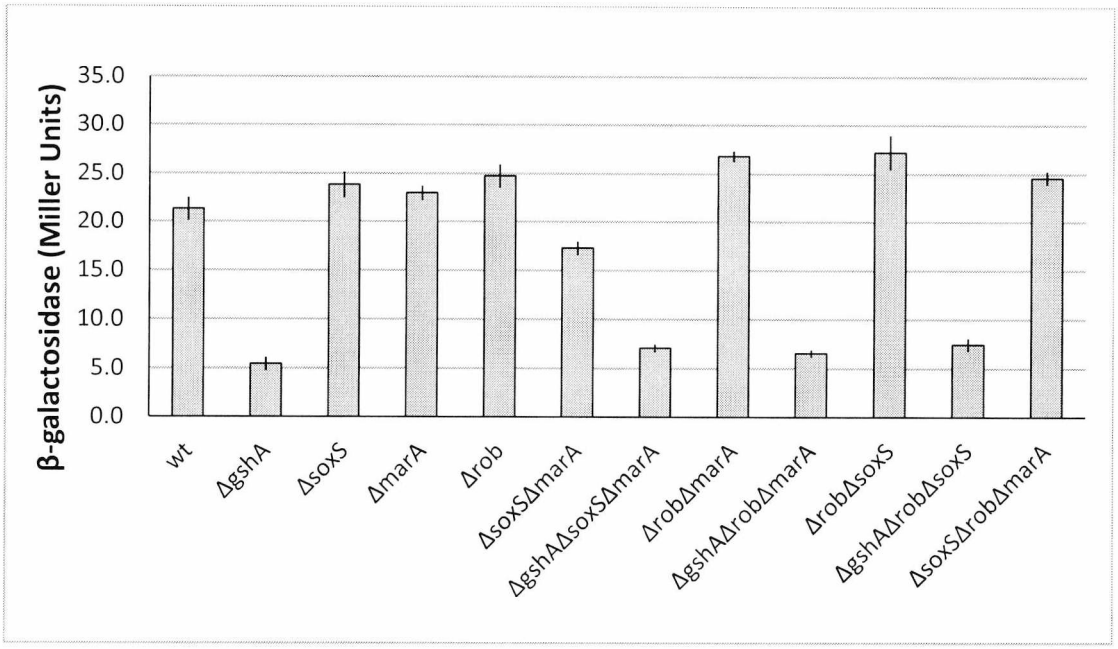


Figure 5.3: β-galactosidase assay showing the effect of the absence the Marbox regulatory proteins SoxS, MarA and Rob on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC932, KCEC1294, KCEC2206, KCEC2230, KCEC2232, KCEC2234, KCEC2283, KCEC2287, KCEC2291 and KCEC2289.

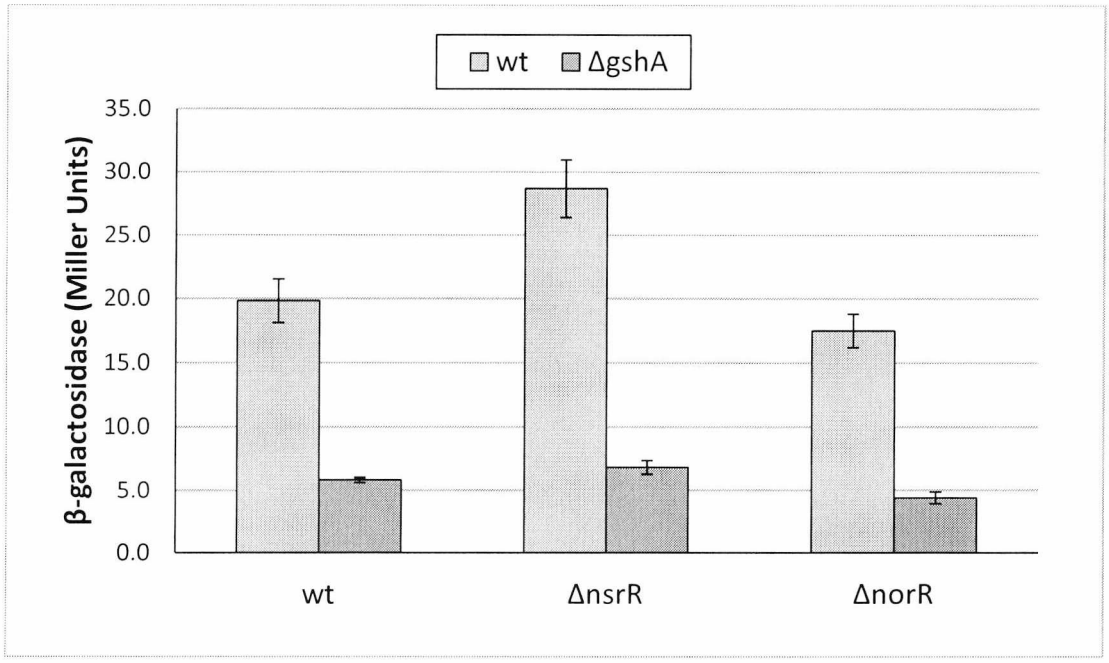


Figure 5.4: β-galactosidase assay showing the effect of the absence of nitrosative stress-responsive regulators NorR and NsrR on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC2638, KCEC2640, KCEC2642 and KCEC2644.

5.3. Metal ion regulators

As an efficient metal chelator (Ballatori, 1994), GSH regulates metal toxicity via the levels of free metal ions. Elevated levels of free metal ions are highly reactive and can thus cause damage to the cells. Excess copper Cu^{2+} targets iron-sulfur clusters in proteins (Macomber *et al.*, 2009), while ferrous iron Fe^{2+} drives the generation of reactive oxygen species via the Fenton reaction (Imlay *et al.*, 1988). In the absence of the metal chelator GSH, levels of free metal ions will rise and with this the activity of their respective regulators. This, in turn, may inhibit *fimB* expression. To test this hypothesis, the non-specific metal chelator EDTA was added to the culture medium to determine if it could suppress the Δ*gshA* mutant phenotype. As shown in Figure 5.5, the addition of EDTA suppressed the Δ*gshA* mutant phenotype successfully, suggesting that an exess of one or more metal ions could be involved.

Copper Cu^{2+} could be involved in the Δ*gshA* mutant phenotype, as chelation by glutathione can prevent copper toxicity (Macomber *et al.*, 2009). Cu^{2+} affects the activity of proteins by damaging Fe-S clusters as well as by competing with other metal ions for their binding sites. Furthermore, copper can generate toxic hydroxyl radicals (Kershaw *et al.*, 2005; Macomber *et al.*, 2009). However, diminishing Cu^{2+} levels in the growth medium and subsequently adding it back to the medium did not have any effect on *fimB* expression (Figure 5.6). Cu^{2+} suppresses the expression of *zinT*, which encodes a metal binding protein and general stress factor (Kershaw *et al.*, 2007; Graham *et al.*, 2009). More importantly, excess copper also downregulates *fimA* expression (Kershaw *et al.*, 2005). An inverted repeat overlapping the -10 promoter region of *fimB* could be matched to the same region in the *zinT* promoter (Figure 5.7), which suggested that a common transcription factor inhibits the expression of *zinT* and *fimB* at elevated levels of copper. The zinc uptake regulator Zur inhibits *zinT* expression (Panina *et al.*, 2003). However, mutation of *zur* did not affect *fimB* expression, either in the presence or absence of GSH (Figure 5.8).

Ferrous-iron-loaded enzymes are vulnerable to damage from the Fenton reaction and manganese protects these enzymes from oxidation by substituting for iron (Anjem *et al.*, 2009). In the absence of GSH, a rise in labile manganese levels activates the Mn^{2+}-responsive transcriptional regulator MntR to repress further Mn^{2+} uptake via *mntH*. Interestingly, the MntR binding site in the *mntH* promoter region could be mapped to a close consensus sequence in the *fimB* promoter region (Figure 5.9). However, like *zur*,

mutation of *mntR* did not affect *fimB* expression (Figure 5.10). Futhermore, the investigation of alternative metal ions and their respective regulators – NikR (Ni^{2+}) and RcnR (Co^{2+}) – did not reveal any regulatory effect on *fimB* expression either (Figure 5.8).

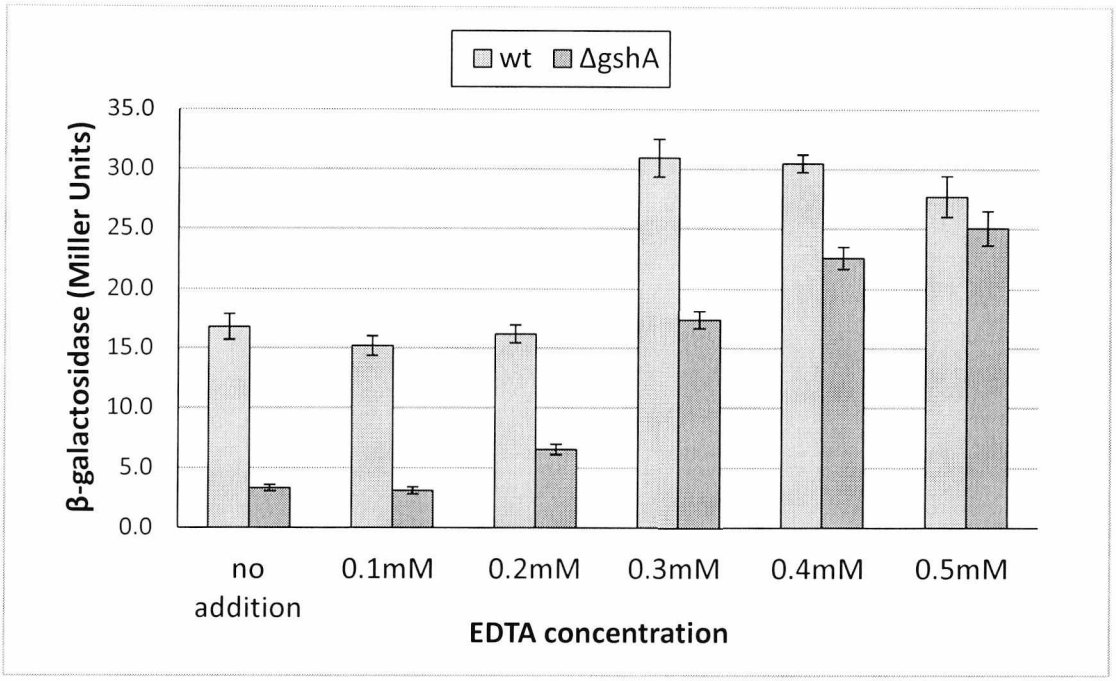


Figure 5.5: β-galactosidase assay showing the effect of metal chelation by EDTA on *fimB* expression. Strains were grown in RD_{gly} with addition of 0-0.5mM EDTA. Strains used were BGEC905 and KCEC1627.

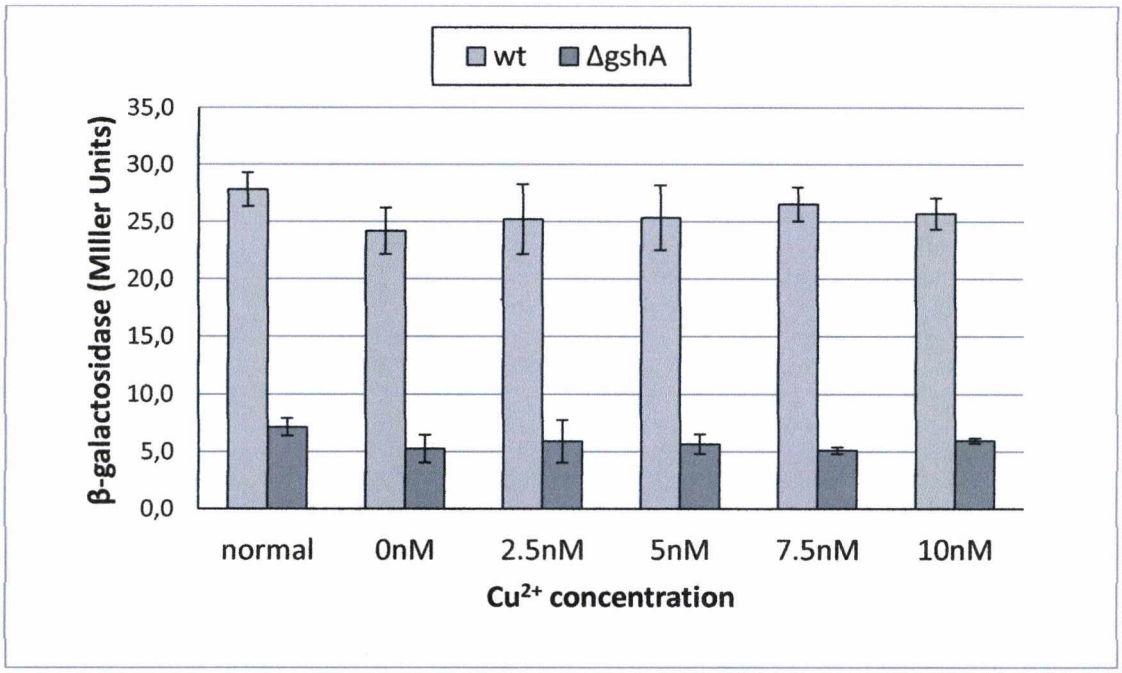


Figure 5.6: β-galactosidase assay showing the effect of the absence of Cu^{2+} and its subsequent addition to the medium on *fimB* expression. Strains were grown in RD_{gly} with 0-10nM Cu^{2+}. Strains used were BGEC905 and KCEC1627.

TTGCTATATGTTACAATATAACATTACACATCATATACATTAACTCTGG *zinT*

ACAGAACATATCACATGATATATA *fimB*

Figure 5.7: Comparison of the promoter regions of *zinT* and *fimB*. The transcriptional start site for *zinT* is highlighted in red, the -10 promoter region of *fimB* is highlighted in blue.

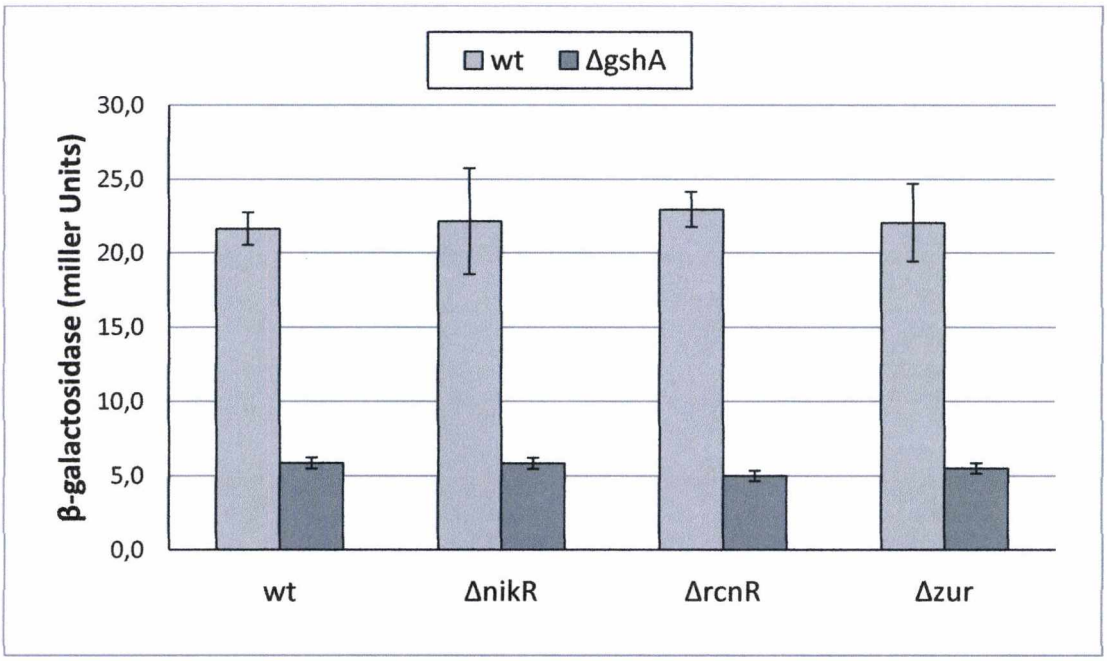


Figure 5.8: β-galactosidase assay showing the effect of the absence of metal ion regulators (Δ*nikR*, Δ*rcnR*, Δ*zur*) on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC2576, KCEC2578, KCEC2586, KCEC2588, KCEC2516 and KCEC2486.

ATCTTGTGCCTCTA**AAACATAGCCTTTGCTATGTTT**CATGCTATGCCAA *mntH*

TTTTACTTGTTACA**GAACATATCACATGATATATAG**ATAAGATTAGTTG *fimB*

-10

Figure 5.9: Comparison of the DNA region encoding for the binding site of MntR in the *mntH* gene with the *fimB* promoter region. Underlined are the nucleotides that match the sequence of the MntR binding site at *mntH*.

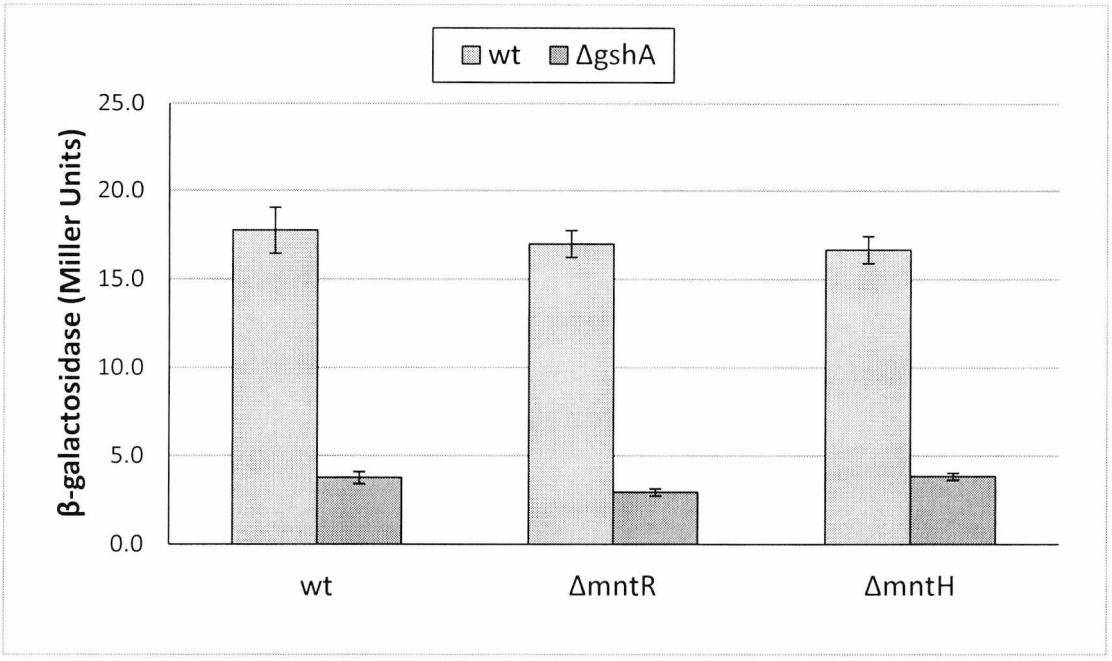


Figure 5.10: β-galactosidase assay showing the effect of the absence of the Mn^{2+}-responsive transcriptional regulator MntR and the Mn^{2+}-uptake protein MntH (Δ*mntR* and Δ*mntH*) on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC1954, KCEC1956, KCEC1958 and KCEC1960.

5.4. The ferric uptake regulator Fur

Intracellular iron levels and iron uptake are controlled by the ferric uptake regulator Fur. Fur is activated in complex with free Fe^{2+} to repress iron acquisition genes, as well as its own synthesis (Bagg *et al.*, 1987; De Lorenzo *et al.*, 1988; Hantke, 2001; Andrews *et al.*, 2003). Free Fe^{2+} reacts with hydrogen peroxide in the Fenton reaction to generate reactive oxygen species (Imlay *et al.*, 1988). To decrease the labile iron pool and prevent further damage under stress conditions, OxyR and SoxS activate *fur* transcription (Zheng *et al.*, 1999). Furthermore, the labile iron pool is closely controlled by GSH (Thorgersen *et al.*, 2008). In the absence of GSH, an increase of free Fe^{2+} is likely to increase levels of Fur-Fe^{2+}, which might in turn have an inhibitory effect on *fimB* expression. To test this hypothesis, the effect of Δ*fur* on *fimB* expression was investigated in both the wild type and Δ*gshA* mutant background. While mutation of *fur* decreased *fimB* expression in the wild type, it suppressed the effect of the *gshA* mutation and generated *fimB* expression levels similar to that seen in the single Δ*fur* mutant background (Figure 5.11). Fur therefore appears to be a net activator of *fimB* expression in the wild type, yet a net repressor in the

Δ*gshA* mutant background. This suggests that Fur controls a regulatory protein that selectively suppresses *fimB* expression in the Δ*gshA* mutant background.

Fur activates the expression of several genes by inhibiting the expression of the regulator RNA RyhB (Massé *et al.*, 2002). Therefore the possibility that mutation of *fur* affects *fimB* expression by inducing *ryhB* expression was considered. To test this hypothesis, *ryhB* was deleted in the Δ*fur* mutant background in the presence of either the wild type or the *gshA* mutant allele. While *fimB* expression was restored to wild type levels in the Δ*fur*Δ*ryhB* mutant, the effect of *gshA* mutation was no longer suppressed in the Δ*gshA*Δ*fur*Δ*ryhB* mutant (Figure 5.11). This showed that the effect of *fur* mutation is dependent upon RyhB and suggests that Fur regulates *fimB* expression indirectly by inhibiting *ryhB* expression. RyhB, in turn, must be acting as a dual regulator. It represses *fimB* expression in the wild type, but has a net activating effect in the Δ*gshA* mutant. It was proposed that RyhB represses an additional unknown repressor that is only activated in the absence of GSH. Taken together with the previous result obtained for *fur* mutants, this suggests that Fur regulates a repressor of *fimB* expression that is under the control of RyhB. RyhB regulates the expression of an extensive collection of genes involved in iron metabolism (Massé *et al.*, 2002). However, inspection of this collection of genes did not reveal any known regulators of *fimB* expression and nor did it suggest any likely candidate regulators. Thus, how Fur and RyhB control *fimB* expression, and how they participate in the Δ*gshA* mutant phenotype, was not investigated further at this stage of the project.

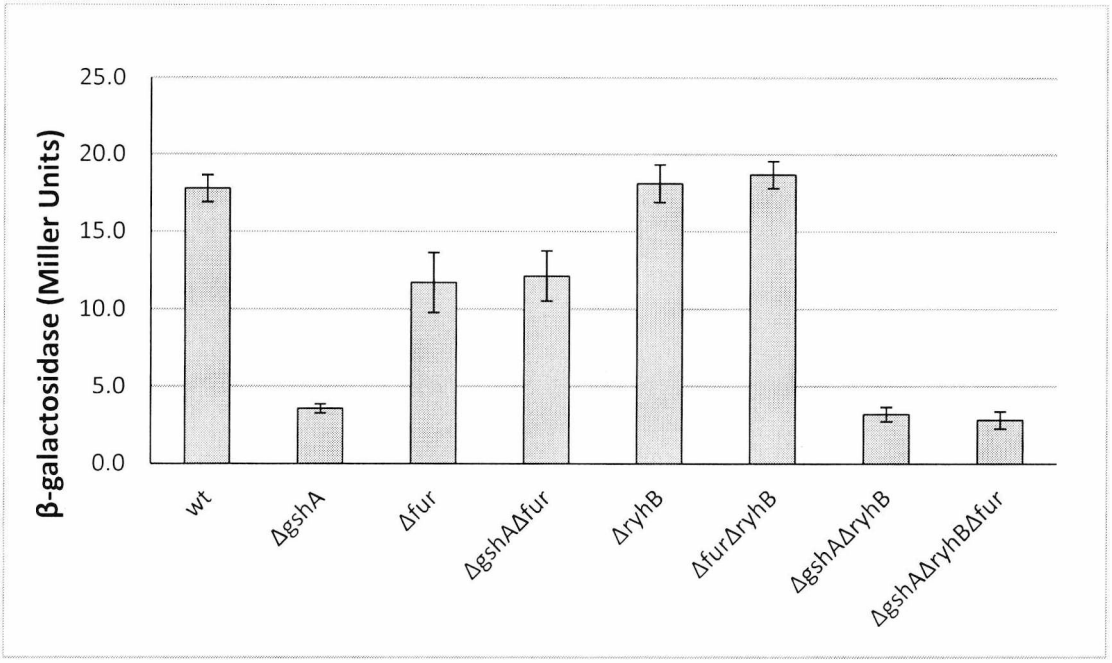


Figure 5.11: β-galactosidase assay showing the effect of the absence of the ferric uptake regulator Fur and the regulator RNA RyhB (Δ*fur*, Δ*ryhB*) on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC1288, KCEC1908, KCEC1978, KCEC2012, KCEC1980 and KCEC2134.

5.5. Histone-like nucleoid-structuring protein H-NS

The histone-like nucleoid-structuring protein H-NS is a global regulator that mainly represses gene expression. H-NS especially targets genes that are related to environmental stress conditions (Schroder *et al.*, 2002) and thus the possibility was considered that H-NS might be involved in the effect of *gshA* mutation on *fimB* expression.

The mutation of *hns* in the wild type significantly increased *fimB* expression, which can be explained by the direct interaction of H-NS with the *fimB* promoter (Donato *et al.*, 1997). Furthermore, mutation of *hns* partially suppressed the effect of *gshA* mutation on *fimB* expression (Figure 5.12). This suggests that H-NS is involved in the activating effect of GSH and that GSH may affect either the levels or the activity of H-NS.

The transcriptional regulator SlyA competes with H-NS for DNA binding sites (reviewed by Stoebel *et al.*, 2008), and work by others in the laboratory confirms that SlyA activates *fimB* expression by this mechanism (McVicker *et al.*, 2011). Combined mutation of Δ*gshA* and Δ*slyA* had a profound compound effect on *fimB* expression (Figure 5.13). This is

consistent with the conclusion that GSH somehow prevents H-NS from inhibiting *fimB* expression.

While the results described above provide support for the hypothesis that the *gshA* mutant phenotype arises in part as a result of enhanced H-NS repression of *fimB* transcription, the regulatory effect of GSH on translation of *fimB* still remained to be clarified. The synthesis of H-NS is stimulated by polyamines (Terui *et al.*, 2007), but polyamines also directly regulate protein synthesis (Tabor *et al.*, 1985; Igarashi *et al.*, 2000; Igarashi *et al.*, 2006; Igarashi *et al.*, 2010). The possibility that the polyamines link GSH, H-NS and *fimB* expression was therefore next considered and is described in Chapter 4.

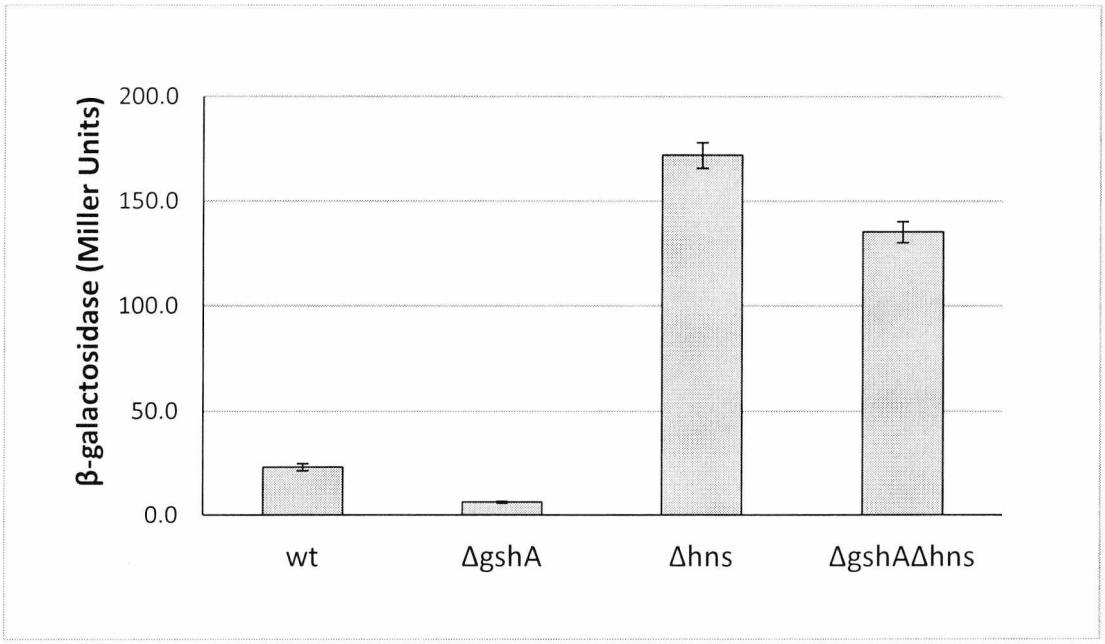


Figure 5.12: β-galactosidase assay showing the effect of *hns* mutation on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC1631 and KCEC2353.

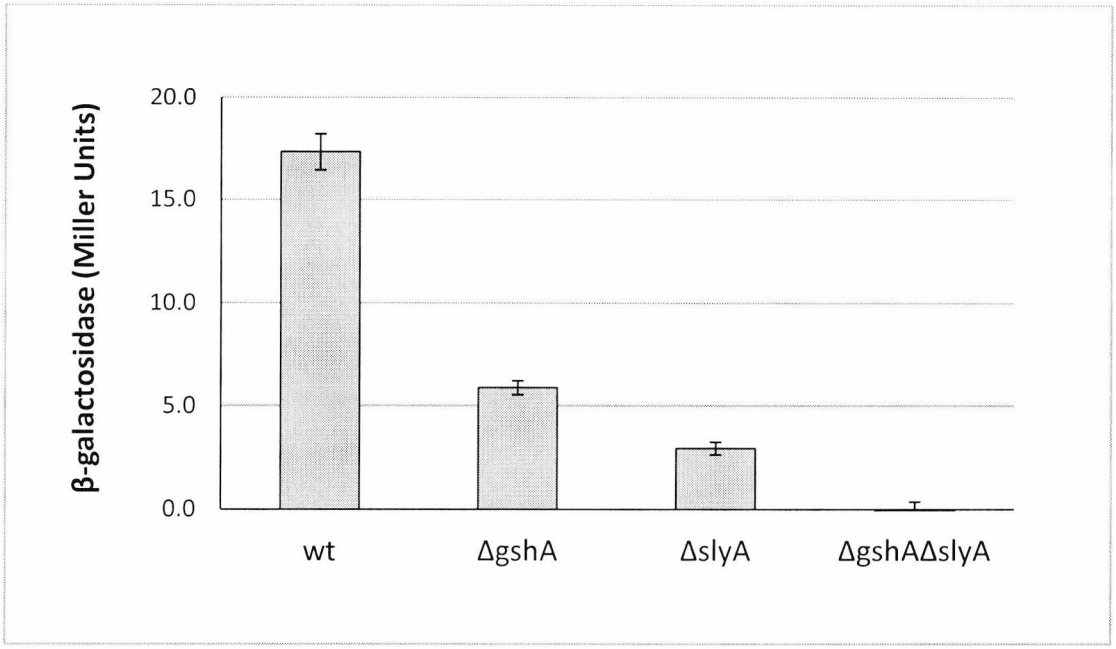


Figure 5.13: β-galactosidase assay showing the effect of *gshA* and *slyA* mutation on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC1243 and KCEC1368.

5.6. Discussion

GSH activates *fimB* expression and results suggest a periplasmic target for GSH. However, the regulation of *fimB* will also involve one or more cytoplasmic factors in response to a signal from the periplasmic target, or even by direct interaction with GSH in the cytoplasm. The cellular functions of GSH were used as the basis to identify this cytoplasmic factor. By use of transcriptional and translational fusion strains, it was possible to determine that the mutation of *gshA* affects *fimB* expression at both the transcriptional and the translational level. Initial investigations focused on transcriptional regulators that are involved in the response to oxidative and nitrosative stress, as well as metal toxicity.

OxyR and SoxRS respond to hydrogen peroxide and superoxide-generating compounds, respectively. By activating the OxyR and SoxRS regulons, they help protect the cells from oxidative damage, as these regulons include a wide range of genes that are involved in oxygen scavenging (Christman *et al.*, 1989; Storz *et al.*, 1999; Zheng *et al.*, 2001). In the absence of GSH, elevated oxidative stress levels will activate OxyR and SoxR. Therefore a direct inhibitory effect of OxyR and SoxS on *fimB* expression was considered. However,

the deletion of *oxyR* and *soxR* did not have any effect on *fimB* expression, nor did the induction of *soxS* expression. SoxS has been shown to be induced in the absence of GSH (Ding *et al.*, 1996), for which reason it was considered to be involved in the *gshA* mutant phenotype. The investigation of the nitric oxide-responsive regulators NorR and NsrR (Gardner *et al.*, 2002; Bodenmiller *et al.*, 2006) did also not show any connection to *fimB* expression.

While OxyR and SoxS are also involved in metal ion regulation (Zheng *et al.*, 1999; Anjem *et al.*, 2009), GSH is an efficient metal chelator (Ballatori, 1994). It is therefore possible that an excess of one or more metal ions is involved in the inhibition of *fimB* expression. This was further supported by the addition of the non-specific metal chelator EDTA, which suppressed the *gshA* mutant phenotype. However, the deletion of a range of metal-ion responsive regulators as well as diminishing Cu^{2+} levels in the medium did not reveal any connection to the regulation of *fimB* expression. Especially copper and manganese had high potential to be involved. While an excess in copper damages Fe-S clusters in proteins and generates harmful oxygen radicals, GSH can prevent this by chelation of free Cu^{2+} (Macomber *et al.*, 2009). Manganese, on the other hand, has been shown to protect proteins from oxidative damage by substituting for Fe^{2+} as cofactor for some metalloenzymes (Anjem *et al.*, 2009). Iron plays a central role in the cell. While Fe-S proteins require Fe^{2+} for their activity, Fe^{2+} also leads to the production of highly damaging hydroxyl radicals (OH•) in the Fenton reaction (Imlay *et al.*, 1988). Chelation by GSH helps controls the labile Fe^{2+} pool to prevent this reaction. Furthermore, GSH also acts as a "sink" for cysteine, which drives the Fenton reaction by reducing ferric iron Fe^{3+} back to ferrous iron Fe^{2+} (Park *et al.*, 2003). The ferric uptake regulator Fur forms a complex with free Fe^{2+} to repress further iron uptake and thus regulates intracellular iron levels. In the absence of GSH, increased levels of free Fe^{2+} will activate Fur. Therefore the possibility was considered that elevated levels of Fur-Fe^{2+} inhibits *fimB* expression.

While mutation of *fur* suppressed the Δ*gshA* mutant phenotype, it decreased *fimB* expression in wild type. This effect was shown to be dependent upon the regulator RNA RyhB (Figure 5.11). It was therefore proposed that RyhB acts as a dual regulator. While it only inhibits *fimB* expression in the wild type, RyhB may additionally suppress the expression of a repressor that is activated in the Δ*gshA* mutant, but not the wild type.

According to this model, mutation of *fur* would activate RyhB and thus suppress the Δ*gshA* mutant phenotype – but only to the Δ*fur* single mutant level.

RyhB plays a major role in the regulation of iron metabolism (Massé *et al.*, 2002). Further research in this regard did not reveal any known or potential regulator of *fimB* expression. However, the histone-like nucleoid-structuring protein H-NS was considered to be involved in the effect of *gshA* mutation on *fimB* expression. H-NS is a global regulator that targets genes related to environmental stress and also a known inhibitor of *fimB* expression (Donato *et al.*, 1997; Schroder *et al.*, 2002). Mutation of *hns* partially suppressed the Δ*gshA* mutant phenotype. Furthermore, mutation of *gshA* enhanced the effect of *slyA* mutation. The transcriptional regulator SlyA is an antagonist for H-NS and activates *fimB* expression by competing with H-NS for its DNA binding site in the *fimB* promoter. This compound effect of combining Δ*gshA* and Δ*slyA* mutation suggests that GSH prevents H-NS from inhibiting *fimB* expression, possibly by affecting the levels or the activity of H-NS.

As described in Chapter 2, the *gshA* mutant phenotype is most likely caused by an effect on *fimB* expression at the transcriptional as well as the translational level. Since polyamines stimulate H-NS synthesis (Terui *et al.*, 2007), but also directly regulate protein synthesis (Tabor *et al.*, 1985; Igarashi *et al.*, 2006; Igarashi *et al.*, 2010), it was considered that polyamines are involved in the *gshA* mutant phenotype. While polyamines may indirectly regulate *fimB* transcription via H-NS, they may be having a direct effect on *fimB* translation.

Chapter 6

Polyamines regulate *fimB* expression

6.1. Summary

The addition of exogenous spermidine was found to inhibit *fimB* expression. Since the majority of the intracellular GSH pool is present in a complex with the polyamine spermidine (Smith *et al.*, 1995), it was proposed that elevated levels of spermidine contribute to the Δ*gshA* mutant phenotype.

However, while H-NS was shown to be involved in the inhibitory effect of exogenous spermidine, it was not possible to provide evidence that an excess of endogenous spermidine is involved in the Δ*gshA* mutant phenotype. On the other hand, the disruption of the initial steps in the polyamine biosynthesis pathway indicated that an excess of the polyamine putrescine inhibits *fimB* expression in the absence of GSH. Futhermore, stimulation of spermidine biosynthesis enhanced the inhibitory effect in the Δ*gshA* mutant. It was therefore suggested that an excess of both putrescine and spermidine inhibit *fimB* expression, yet physiological conditions limit the levels of spermidine. Further investigations led to the hypothesis that GSH limits polyamine biosynthesis by inactivating the periplasmic enzyme SpeA, the first enzyme in polyamine biosynthesis.

6.2. The effect of exogenous spermidine

The regulatory effect of GSH on *fimB* expression occurs at both the transcriptional and translational level. While exogenous spermidine was shown to have an inhibitory effect on *fimB* expression (Chapter 4), GSH possibly activates *fimB* transcription by inhibiting the repressor protein H-NS. Taking into consideration that polyamines regulate translation of a range of genes and, more specifically, stimulate the synthesis of H-NS, it was thought likely that polyamines provide a link between GSH, H-NS and *fimB* expression. Moreover, this hypothesis is supported by the fact that the majority of the intracellular GSH pool is present in a complex with the polyamine spermidine (Smith *et al.*, 1995).

In support of the previous result, the effect of exogenous spermidine was investigated in the wild type and the Δ*gshA* mutant. Spermidine was shown to be inhibitory in the wild type and Δ*gshA* mutant background (Figure 6.1), which confirmed that spermidine somehow inhibits *fimB* expression.

As shown previously, the effect of *gshA* mutation was suppressed by mutation of *hns*. Since polyamines stimulate H-NS synthesis (Terui *et al.*, 2007) and H-NS inhibits *fimB* expression by binding to the *fimB* promoter (Donato *et al.*, 1997), it was proposed that

polyamines regulate *fimB* transcription indirectly via H-NS. The inhibitory effect of exogenous spermidine was thus expected to be partially dependent upon H-NS. To test this, exogenous spermidine was added to the *hns* mutant. Results show that the *hns* mutant is less responsive to exogenous spermidine (Figure 6.2), indicating that H-NS is involved in the inhibitory effect of spermidine.

While these results relate to the effect of exogenous spermidine, it remained to be clarified whether the Δ*gshA* mutant phenotype is actually caused by an excess of spermidine.

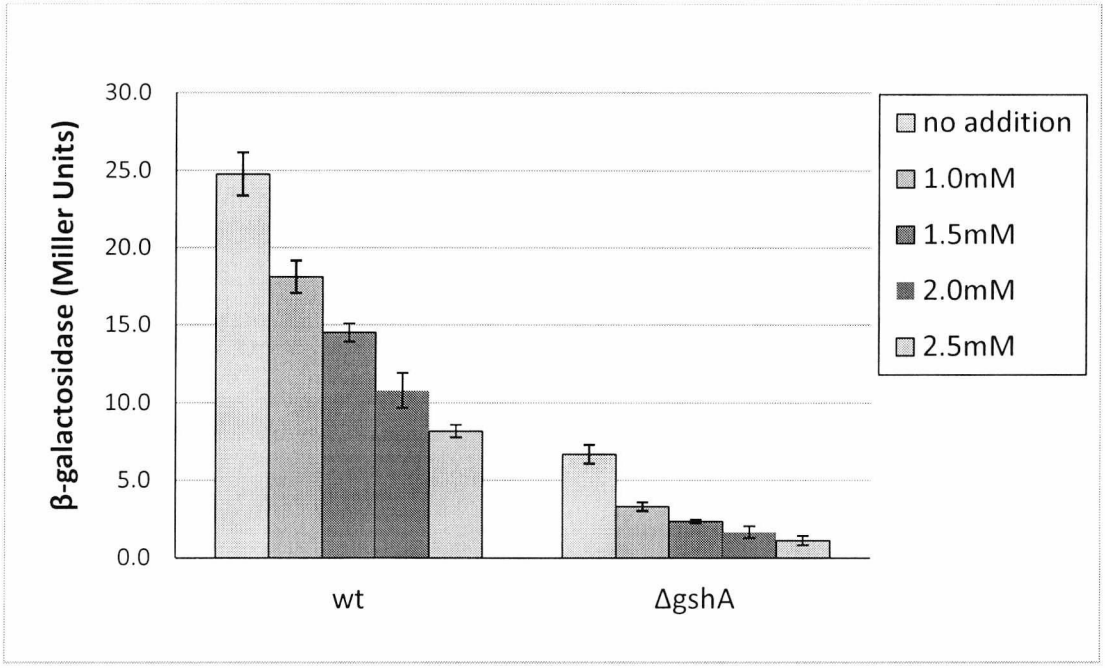


Figure 6.1: β-galactosidase assay showing the effect of exogenous spermidine on *fimB* expression. Strains were grown in RD_{gly} with addition of 0-2.5mM spermidine. Strains used were BGEC905 and KCEC1627.

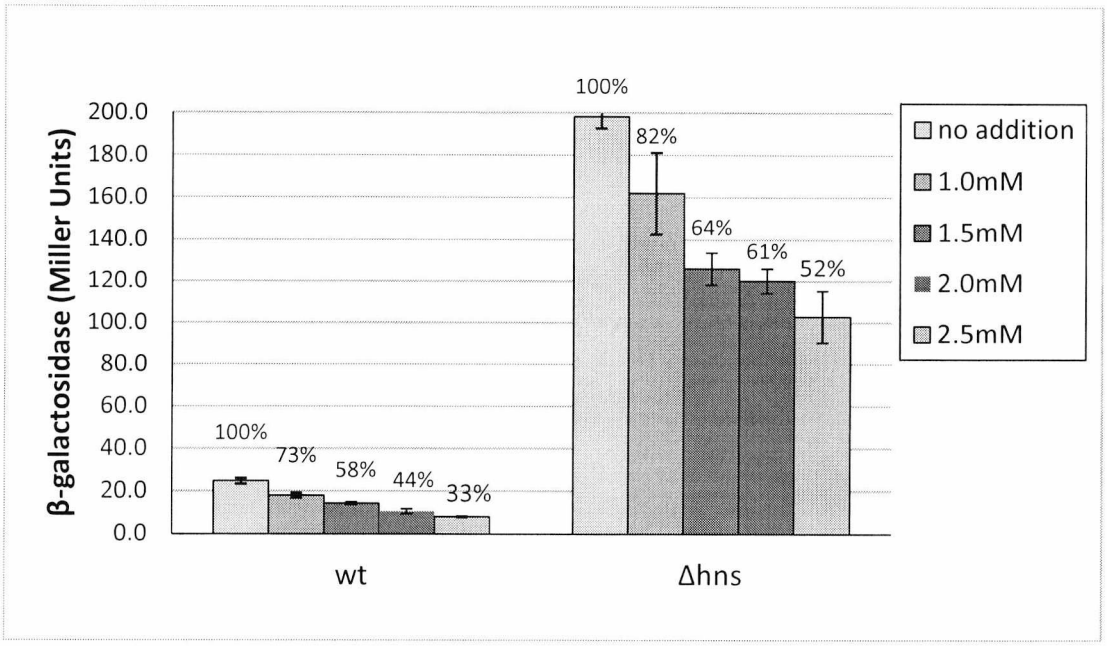


Figure 6.2: β-galactosidase assay showing the effect of exogenous spermidine on *fimB* expression in *hns* mutants. Strains were grown in RD_{gly} with addition of 0-2.5mM spermidine. Strains used were BGEC905 and KCEC1631.

6.3. GSH regulates *fimB* expression via polyamines

The results obtained with exogenous spermidine suggest that an excess of spermidine accounts for the Δ*gshA* mutant phenotype. To verify this, levels of endogenous spermidine were manipulated by deletion of *speE*. SpeE catalyses the final step in spermidine biosynthesis by converting putrescine to spermidine (Bowman *et al.*, 1973; Tabor *et al.*, 1985). Mutation of *speE* was thus expected to suppress the Δ*gshA* mutant phenotype by diminishing any excess of spermidine.

However, mutation of *speE* decreased *fimB* expression in the wild type, and had a weak compound effect in the Δ*gshA* mutant background (Figure 6.3). This indicated that the Δ*gshA* mutant phenotype is not caused by an excess of spermidine. Since the absence of SpeE blocks the conversion of putrescine to spermidine and had a compound effect on *fimB* expression, it was considered that the accumulation of a precursor of spermidine accounts for the Δ*gshA* mutant phenotype. Thus it is possible that loss of GSH elevates polyamine biosynthesis up to the level of putrescine, but does not affect the final step, the generation of spermidine.

To test whether the Δ*gshA* mutant phenotype is caused by a precursor of spermidine, the polyamine biosynthesis pathway was disrupted by mutation of *speA*. SpeA is the first enzyme in the polyamine biosynthetic pathway. Moreover, SpeA is located in the periplasm (Buch *et al.*, 1985) and therefore could be the so far unidentified periplasmic target of GSH. Mutation of *speA* was found to decrease *fimB* expression in the wild type background, but also suppressed the Δ*gshA* mutant phenotype (Figure 6.3). Taken together with the result obtained for the Δ*speE* mutant, this suggests that mutation of *gshA* leads to an excess in putrescine biosynthesis, which inhibits *fimB* expression. Furthermore, it was considered that GSH limits polyamine biosynthesis by inhibiting SpeA activity in the periplasm. It remained to be clarified whether the inhibitory effect is caused by putrescine, an intermediate product of putrescine biosynthesis or a breakdown product of putrescine.

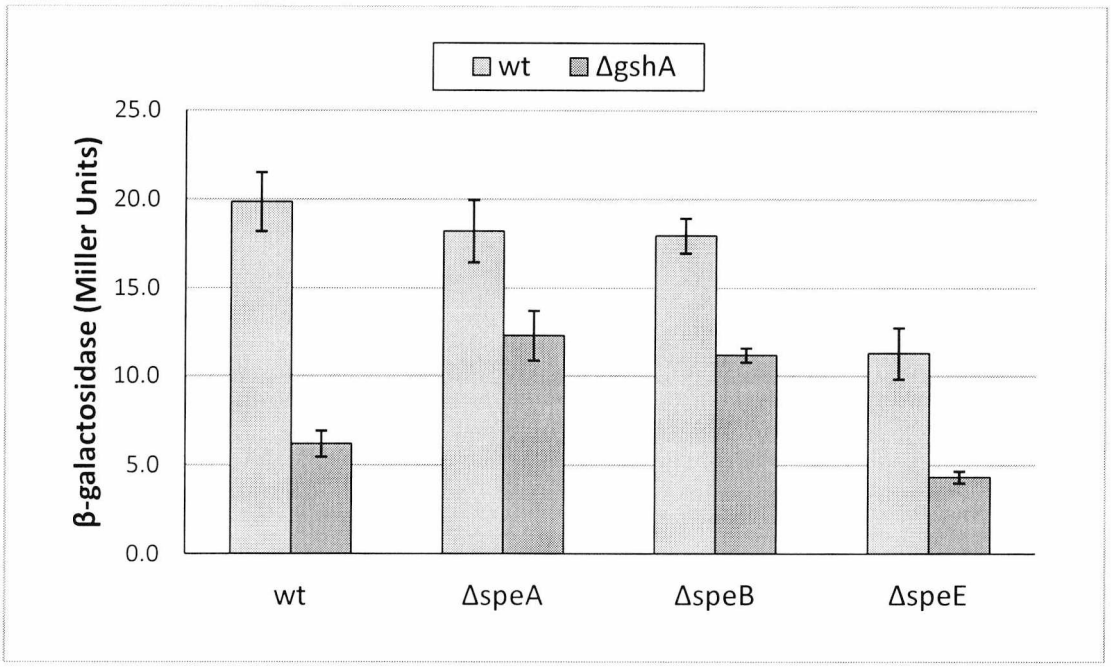


Figure 6.3: β-galactosidase assay showing the effect of disrupted polyamine biosynthesis (Δ*speA*, Δ*speB*, Δ*speE*) on *fimB* expression. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC1771, KCEC2712, KCEC2993, KCEC2995, KCEC1777 and KCEC2714.

6.4. Putrescine inhibits *fimB* expression in the Δ*gshA* mutant

The suppression of the Δ*gshA* mutant phenotype by mutation of *speA* suggests that GSH controls polyamine biosynthesis by inactivating SpeA in the periplasm. Since mutation of *speE* did not suppress the effect of *gshA* mutation, the inhibitory effect of exogenous spermidine (Figure 6.1) may be explained by negative feedback control of spermidine on SpeE. The consequent accumulation of putrescine or an intermediate product in putrescine biosynthesis then inhibits *fimB* expression in the Δ*gshA* mutant. To test this, exogenous putrescine was added to the wild type and the Δ*gshA* mutant. However, this did not have any effect on *fimB* expression, nor did the addition of its precursor agmatine (Figure 6.4 and 6.5).

The decrease of *fimB* expression in the Δ*speE* single mutant is possibly caused by an accumulation of the inhibitory factor in the polyamine biosynthesis pathway. Mutation of *speE* most likely has a feedback effect and thus increases the inhibitory effect of the respective factor. The same effect was to be expected by adding exogenous putrescine and agmatine. Thus, an explanation for the lack of response to exogenous putrescine and agmatine may be that these compounds are not taken up by the cell.

The formation of putrescine from agmatine is catalysed by SpeB. Mutation of *speB* will hence prevent the generation of putrescine and should help determine whether putrescine inhibits *fimB* expression in the absence of GSH. Results show that mutation of *speB* decreases *fimB* expression in the wild type, but suppresses the effect of *gshA* mutation (Figure 6.3), as was found previously by mutation of *speA*. Since *speB* (and *speA*) are required for normal levels of putrescine biosynthesis, this indicates that the generation of putrescine is required for the Δ*gshA* mutant phenotype. Thus it is proposed that while GSH limits the intracellular levels of putrescine, an excess of putrescine in the absence of GSH inhibits *fimB* expression. Taken together with the decrease in *fimB* expression in the wild type background, this further suggests that there is an optimal level of putrescine for maximal *fimB* expression. This optimal level is lower than Δ*gshA* mutant levels, but higher than levels in the Δ*speA* and Δ*speB* mutants.

The conversion of putrescine from agmatine generates urea as a unique side product in *E. coli* (Morris *et al.*, 1967). The loss of SpeA and SpeB hence also prevents the generation of urea. It was speculated whether urea has an inhibitory effect on *fimB* expression, which would provide an alternative explanation for the suppressing effect of *speA* and *speB*

mutation. However, this could not be confirmed by adding urea to the cells (Figures 6.6). As *E. coli* is not able to degrade urea, it is excreted into the medium without further processing. The lack of response to urea could therefore simply indicate that exogenous urea is not taken up by *E. coli*.

Results obtained so far suggest that GSH activates *fimB* expression by limiting the levels of putrescine, but not spermidine, biosynthesis. This suggests that, under physiological conditions, loss of GSH does not elevate polyamine synthesis beyond the level of putrescine. The conversion of putrescine to spermidine by SpeE requires a propylamine group from decarboxylated *S*-adenosyl-L-methionine (dcSAM). It is considered that dcSAM levels limit the rate of spermidine production (Kashiwagi *et al.*, 1988; Morgan, 1999). By increasing the SAM pool, it should therefore be possible to elevate levels of spermidine and thus test whether elevated levels of endogenously generated spermidine have the same effect as exogenous spermidine.

SAM is generated from L-methionine and is also a cofactor for MetJ, a repressor of genes involved in methionine biosynthesis (Weissbach *et al.*, 1991; LaMonte *et al.*, 2006). By growing the cells in medium without methionine as well as by mutating *metJ*, it is possible to induce both methionine and SAM biosynthesis. This, in turn, should increase spermidine levels. Mutation of *metJ* in the Δ*gshA* mutant produced a further decrease in *fimB* expression, as did growth in methionine-less medium (Figure 6.7). This additional inhibition suggests that endogenous spermidine does have an inhibitory effect on *fimB* expression. However, mutation of *speE* alone does not elicit a decrease in *fimB* expression (Figure 6.3), probably because SAM levels are likely to limit spermidine synthesis. Raised levels of putrescine would hence not raise levels of spermidine under physiological conditions. Taken together, these results indicate that the polyamines putrescine and spermidine inhibit *fimB* expression. However, due to low levels of SAM, the absence of GSH is predicted to cause putrescine levels, but not spermidine levels, to rise.

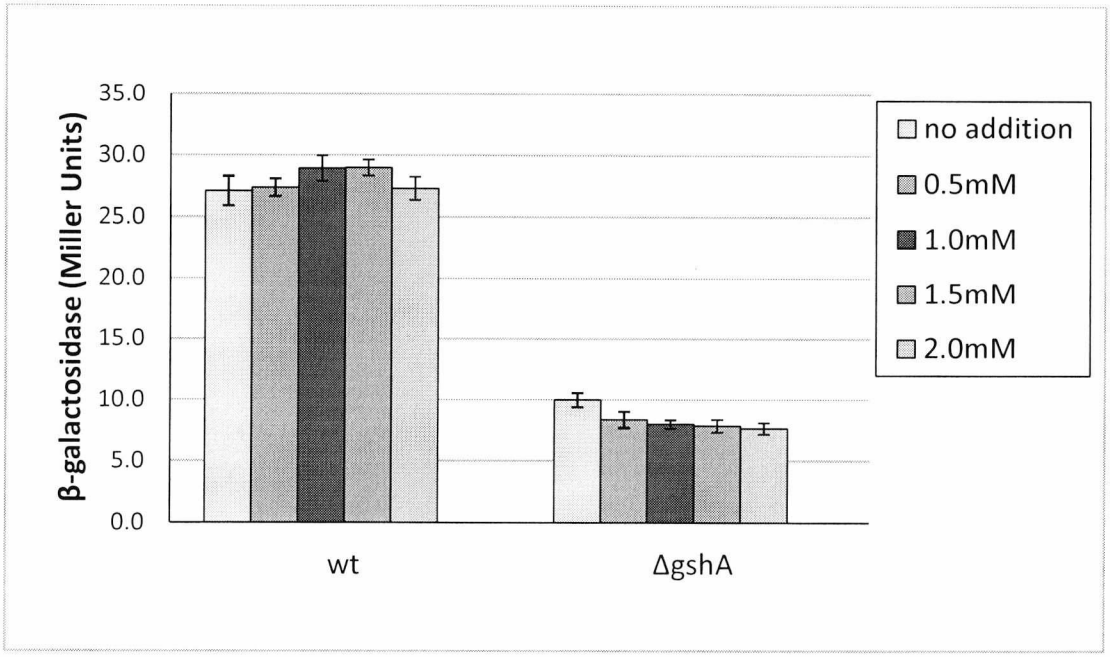


Figure 6.4: β-galactosidase assay showing the effect of exogenous putrescine on *fimB* expression. Strains were grown in RD_{gly} addition of 0-2.0mM putrescine. Strains used were BGEC905 and KCEC1627.

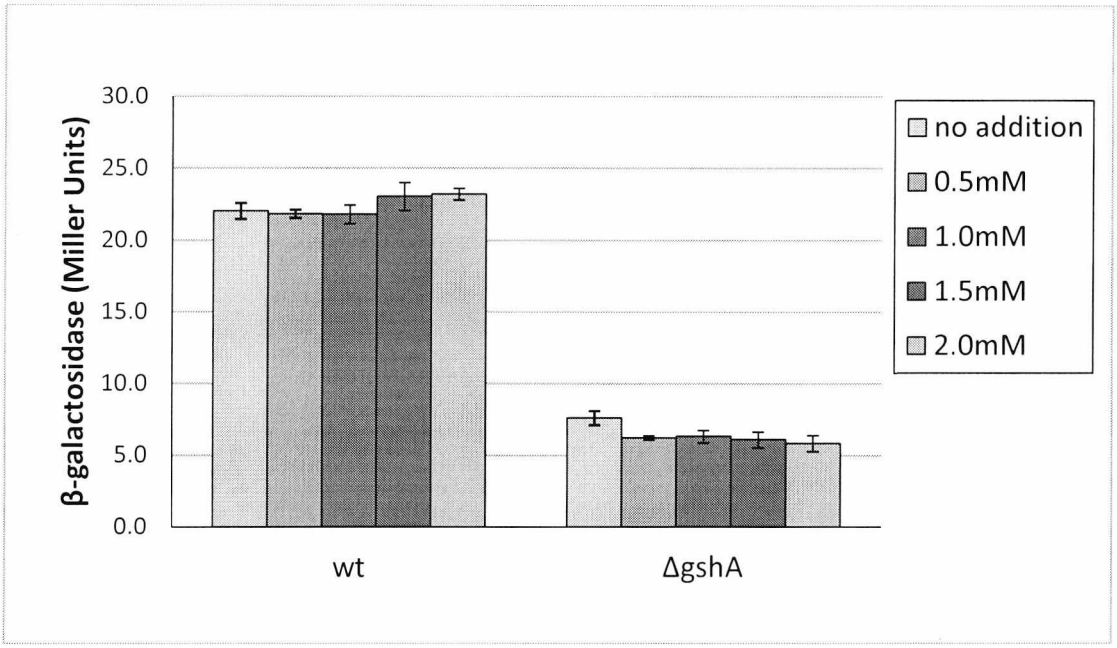


Figure 6.5: β-galactosidase assay showing the effect of exogenous agmatine on *fimB* expression. Strains were grown in RD_{gly} addition of 0-2.0mM agmatine. Strains used were BGEC905 and KCEC1627.

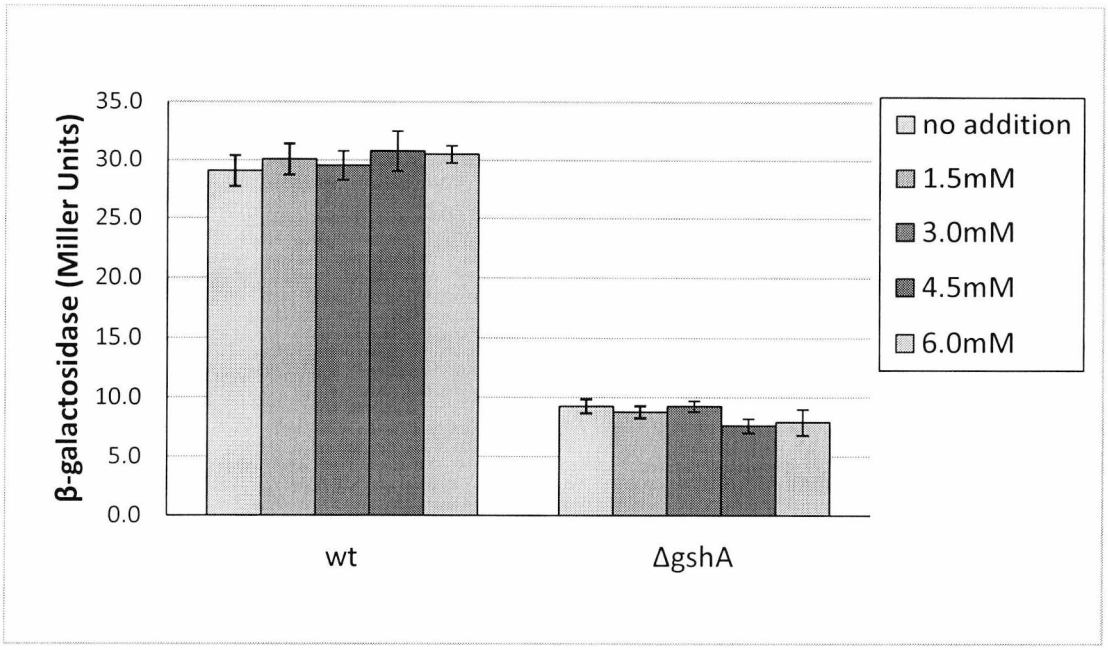


Figure 6.6: β-galactosidase assay showing the effect of exogenous urea on *fimB* expression. Strains were grown in RD_{gly} addition of 0-6.0mM urea. Strains used were BGEC905 and KCEC1627.

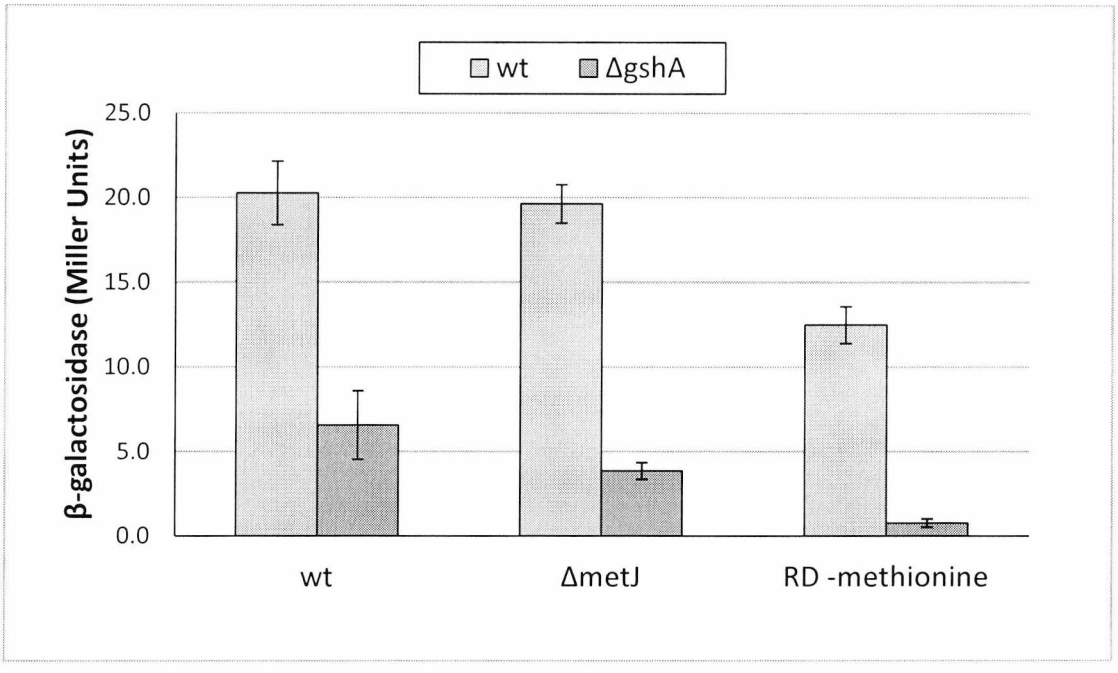


Figure 6.7: β-galactosidase assay showing the effect of induced SAM biosynthesis on *fimB* expression in Δ*metJ* mutants and in the absence of methionine. Strains were grown in RD_{gly} or RD_{gly} without methionine, respectively. Strains used were BGEC905, KCEC1627 and KCEC2679.

6.5. SpeA as periplasmic target for GSH

The results described above indicate that GSH regulates *fimB* expression by limiting cellular levels of putrescine. Furthermore, earlier results suggest a periplasmic target for GSH (Chapter 3). Since SpeA is located in the inner periplasmic space (Buch *et al.*, 1985) and the mutation of *speA* suppresses the Δ*gshA* mutant phenotype, SpeA seemed likely to be the target for GSH control.

It is proposed that the absence of GSH renders SpeA more active and hence leads to inhibitory levels of putrescine. Exogenous GSH, in turn, suppresses the Δ*gshA* phenotype by directly inhibiting SpeA in the periplasm. Supposing this to be the case, exogenous GSH should not be able to stimulate *fimB* expression in Δ*speA* mutants. To test this, exogenous GSH was added to strains carrying the *speA* mutation in both the wild type and Δ*gshA* mutant background. The response to exogenous GSH was diminished significantly in the absence of *speA* (Figure 6.8), indicating that the effect of exogenous GSH is largely dependent upon SpeA. This further supports the hypothesis that SpeA is the periplasmic target for GSH.

Previous data also showed that the absence of the transcriptional regulator Cbl stimulates *fimB* expression (Chapter 3.4). This effect is dependent upon GSH biosynthesis, as well as the GSH export system CydDC. Taking into account the latest results, it is proposed that mutation of *cbl* increases *fimB* expression by increasing levels of GSH in the periplasm.

Results show that the stimulatory effect of *cbl* mutation was lost in the absence of SpeA (Figure 6.9). This implies that Cbl affects *fimB* expression by somehow affecting *speA* expression or SpeA activity. Since previous results indicate that GSH inhibits SpeA activity, it is suggested that mutation of *cbl* stimulates *fimB* expression by somehow increasing the periplasmic reduced glutathione concentration to diminish SpeA activity. These observations also suggest that while the wild type has putrescine levels above the optimum for *fimB* expression, the Δ*cbl* mutant has levels closer to the optimum due to optimal control of SpeA activity by GSH.

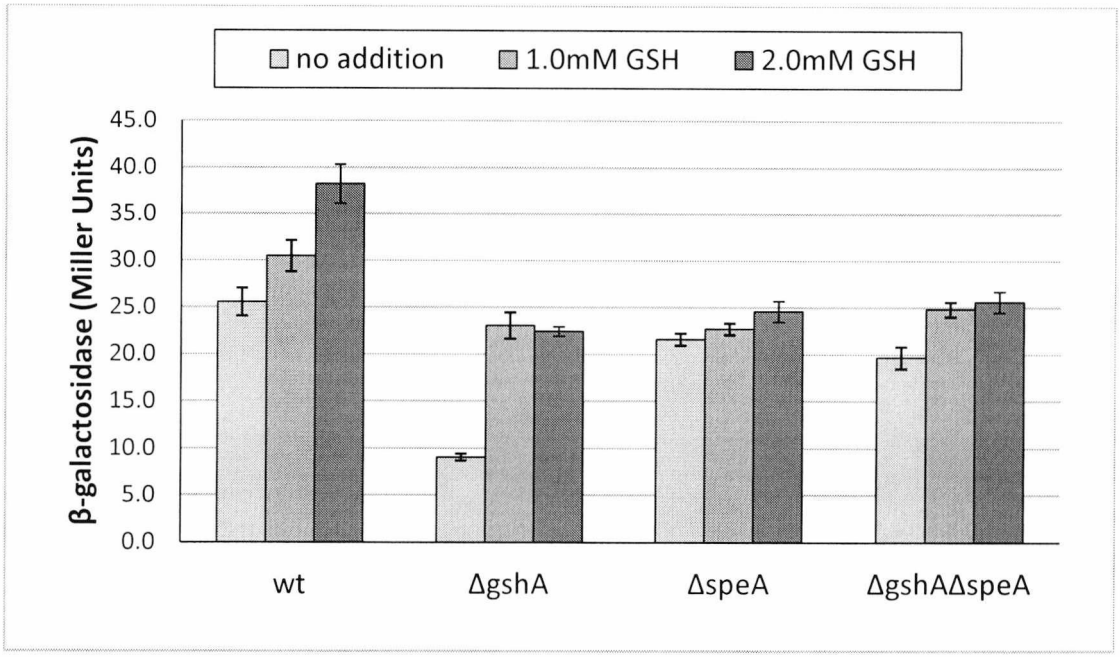


Figure 6.8: β-galactosidase assay showing the effect of exogenous GSH on *fimB* expression in *speA* mutants. Strains were grown in RD_{gly} with addition of 0-2.5mM spermidine. Strains used were BGEC905, KCEC1627, KCEC1771 and KCEC2712.

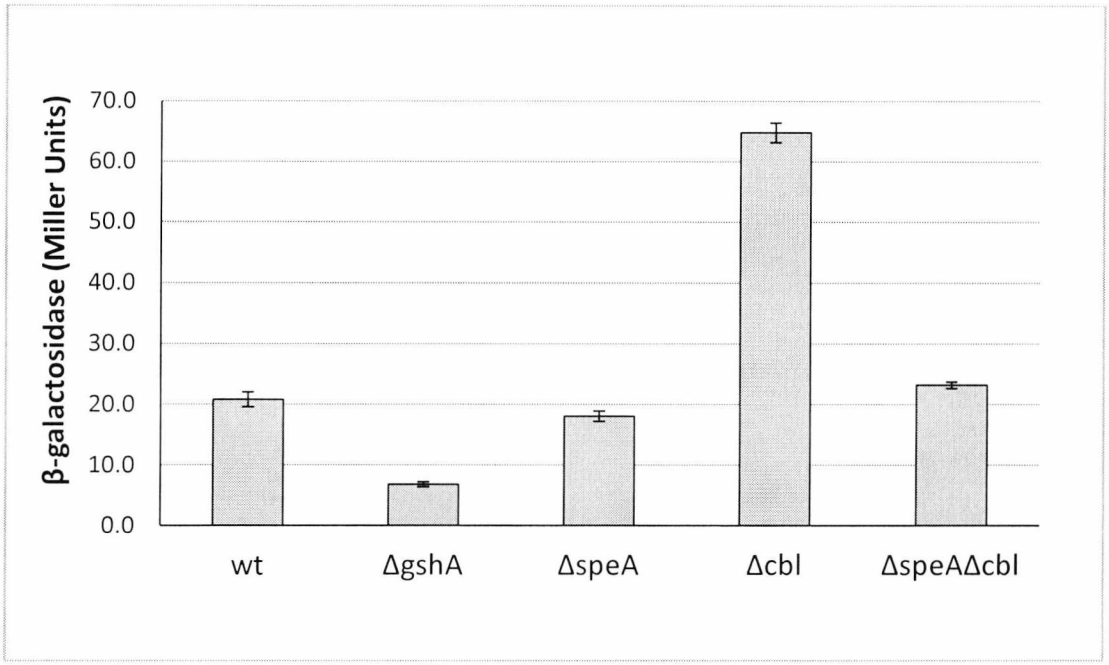


Figure 6.9: β-galactosidase assay showing the effect of *cbl* mutation on *fimB* expression in the absence of SpeA. Strains were grown in RD_{gly}. Strains used were BGEC905, KCEC1627, KCEC2760, KCEC2600 and KCEC3052.

6.6. Discussion

GSH was shown to activate *fimB* expression at the translational and the transcriptional level. Results furthermore suggest that the effect of *gshA* mutation on *fimB* expression involves an increase in the inhibitory effect of the repressor H-NS. Since polyamines regulate protein synthesis (Tabor *et al.*, 1985; Igarashi *et al.*, 2000; Igarashi *et al.*, 2006; Igarashi *et al.*, 2010) and also stimulate H-NS synthesis (Terui *et al.*, 2007), it was considered that polyamines are involved in the regulatory effect of GSH on *fimB* expression. This is supported by the result that exogenous spermidine inhibits *fimB* expression in the wild type and Δ*gshA* mutant, but that the response to spermidine is decreased in Δ*hns* mutants. Furthermore, the formation of glutathionylspermidine (Gsp) provides a direct link between GSH and spermidine.

Neither loss of Gsp biosynthesis, nor mutation of *speE* supported the hypothesis that an excess of spermidine accounts for the inhibitory effect of *gshA* mutation. However, exogenous spermidine may be causing product inhibition of SpeE activity, its inhibitory effect on *fimB* expression would thus be due to the accumulation of an intermediate product in the spermidine biosynthetic pathway. In support of this hypothesis, mutation of *speA*, which catalyses the initial step in the polyamine biosynthesis pathway, does suppress the Δ*gshA* mutant phenotype.

SpeB catalyses the generation of putrescine from agmatine. Mutation of *speB* suppressed the effect of *gshA* mutation to the same extent as mutation of *speA*. The common effect of *speA* and *speB* mutation is the lack of putrescine biosynthesis, which indicates that excess putrescine contributes to Δ*gshA* mutant phenotype. Mutation of *speB* not only decreases putrescine levels, but also causes the accumulation of agmatine. Since mutation of *speA* and *speB* has a comparable effect, this furthermore suggests that agmatine does not have an additional inhibitory effect on *fimB* expression. Thus, an excess of putrescine appears to be sufficient to inhibit *fimB* expression in the Δ*gshA* mutant.

However, addition of exogenous agmatine and putrescine did not have an effect on *fimB* expression. It has been reported that putrescine is normally synthesised in excess amounts, and the excess amount of putrescine is excreted to maintain levels (Kashiwagi *et al.*, 1988). The lack of response to putrescine and agmatine could thus be explained by a lack of uptake.

It is proposed that the absence of GSH causes a rise in putrescine levels, yet does not significantly affect spermidine levels. The conversion of putrescine to spermidine requires decarboxylated SAM, which is generated from SAM by SpeD (Bowman *et al.*, 1973). This step is an important regulator of polyamine biosynthesis (Kashiwagi *et al.*, 1988; Morgan, 1999) and probably limits spermidine biosynthesis when the intracellular spermidine concentration becomes excessive. It was considered that SAM levels limit the activity of SpeE and thus spermidine biosynthesis. By mutation of *metJ*, a repressor of methionine and SAM biosynthesis, and by growth in methionineless medium, it is possible to induce methionine biosynthesis and thus elevate SAM levels. This enhanced the effect of the Δ*gshA* mutation on *fimB* expression, which indicates that spermidine does have an inhibitory effect on *fimB* expression, along with putrescine. However, under physiological conditions low SAM levels limit the generation of spermidine (Kashiwagi *et al.*, 1988; Morgan, 1999). Elevated levels of putrescine in the Δ*gshA* mutant will hence probably not lead to a rise in spermidine levels. It is therefore suggested that mutation of *gshA* leads to an elevation of putrescine levels, which inhibits *fimB* expression.

The quantification of polyamine levels in the cell would provide direct evidence that an excess of putrescine is sufficient to account the Δ*gshA* mutant phenotype. However, the quantification by gas chromatography (GC) only detected putrescine and spermidine in the standard control samples, not the cell samples (see Appendix A). It is possible that the cellular levels of polyamines are below the detection limit or that the extraction of the polyamines from the cells was not efficient enough. An alternative approach to determine putrescine levels was based on the fact that SpeB produces urea as a side product (Morris *et al.*, 1967). The rate of urea production therefore is a measure of the flux through the arginine to putrescine pathway. This proved to be more complicated than expected, as conventional urea assay kits are based on the breakdown of urea to CO_2 and NH_3 and the presence of ammonia in the medium will affect this method. An alternative method has been described by Zawada *et al.* (2009) and used a modified Jung reagent containing *o*-phthalaldehyde and primaquine bisphosphate to detect urea. Yet this method did not produce data that allowed to quantify and compare putrescine levels (data not shown).
However, work by others in the laboratory did provide further support for the hypothesis that an excess of putrescine accounts for the Δ*gshA* mutant phenotype. An increase in osmolarity of the medium (by addition of 0-400mM NaCl) completely suppressed the Δ*gshA* mutant phenotype and increased *fimB* expression in the wild type (Dr Lei Sun,

unpublished data). In response to increased osmolarity, *E. coli* increases its internal solutes by taking up potassium K^+. To maintain charge balance, this occurs in exchange for positively charged putrescine (Munro *et al.*, 1972). Thus, at higher osmolarity, *E. coli* effluxes putrescine – but not spermidine – into the medium via the ornithine/putrescine antiporter PotE (Schiller *et al.*, 2000). As only putrescine is exported with an increase in osmolarity and the Δ*gshA* mutant phenotype was completely suppressed by inhibition of putrescine biosynthesis, this supports the hypothesis that excess putrescine inhibits *fimB* expression in the absence of GSH.

The results described above not only directly link putrescine to the regulatory effect of GSH on *fimB* expression, but are also consistent with the hypothesis that GSH acts on a periplasmic target. SpeA is located in the periplasm (Buch *et al.*, 1985) and while mutation of *speA* suppressed the effect of Δ*gshA* mutant phenotype, the effect of exogenous GSH is largely dependent upon SpeA. This suggests that GSH activates *fimB* expression by inactivating SpeA and thus limiting the levels of putrescine biosynthesis. Furthermore, the stimulatory effect of *cbl* mutation is lost in the absence of SpeA, which implies that Cbl regulates *fimB* expression via SpeA. While the effect of *cbl* mutation is dependent upon GSH biosynthesis and export, results suggest that GSH inhibits SpeA activity. This reinforces the hypothesis that mutation of *cbl* stimulates *fimB* expression by increasing levels of periplasmic GSH to inactivate SpeA.

It is possible that Cbl regulates GSH levels in the cytoplasm by regulating the biosynthesis or degradation of GSH. Alternatively, Cbl may control *cydDC* expression. Loss of Cbl would thus induce CydDC synthesis and GSH export to the periplasm.

Taken together, the results suggest that GSH is exported to the periplasm, where it inhibits SpeA activity to give an optimal level of putrescine for *fimB* expression. In the wild type background, *fimB* expression is stimulated by *cbl* mutation as well as by exogenous GSH. This indicates that periplasmic levels of GSH limit *fimB* expression in the wild type and that there is an optimal level of putrescine for maximal *fimB* expression. An increase of GSH in the Δ*cbl* mutant leads to optimal control of SpeA and thus decreases putrescine levels to a level closer to the optimum. Accordingly, putrescine levels are suggested to be above the optimum in the wild type, below the optimum in the Δ*speA* mutant, yet closer to the optimum in the Δ*cbl* mutant (Figure 6.11).

As discussed in Chapter 3, complementation requires high levels of exogenous GSH. It was proposed that the majority of GSH will be readily oxidised under the growth conditions. However, assuming that SpeA is the periplasmic target for exogenous GSH, this may provide another explanation for the high levels of exogenous GSH. The localisation of SpeA in the cell envelope (Buch *et al.*, 1985) could make it poorly accessible to GSH. Alternatively, SpeA might have low affinity for GSH, which means that the threshold level for GSH binding is beyond normal physiological levels.

Furthermore, there is a potential link between the role of SpeA in the regulation of *fimB* expression by GSH and the suppressing effect of *fur* mutation. As described in Chapter 5, Fur regulates *fimB* expression indirectly by inhibiting *ryhB* expression. It transpires that the *speA* gene has a potential binding site for RyhB (Figure 6.10). *sodB* is a known target for RyhB hybridisation and has a perfect antisense homology to RyhB. Comparison with *sodB* shows a homologous sequence in *speA* with only two mismatches. *speA* could hence be a target for RyhB inhibition. This suggests that mutation of *fur* at least partially suppresses the *gshA* mutation by induction of RyhB (as shown above), which then inhibits *speA* translation. It is reported that Fur is partially inactivated in the absence of GSH (Thorgersen *et al.*, 2008). This activates RyhB, which should partially help to counteract any excess in putrescine levels by inhibiting *speA*.

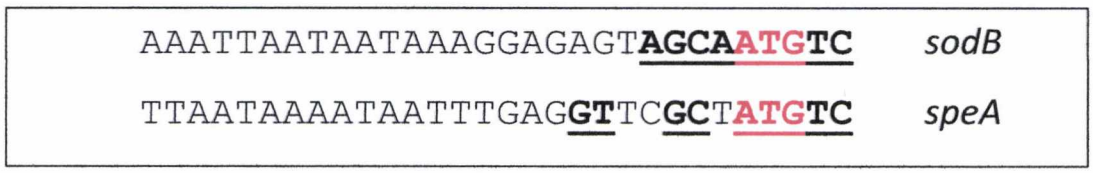


Figure 6.10: Comparison of the DNA region encoding for the binding site of RyhB in the *sodB* gene. The translational start site is highlighted in red. Underlined is the known target for RyhB hybridisation.

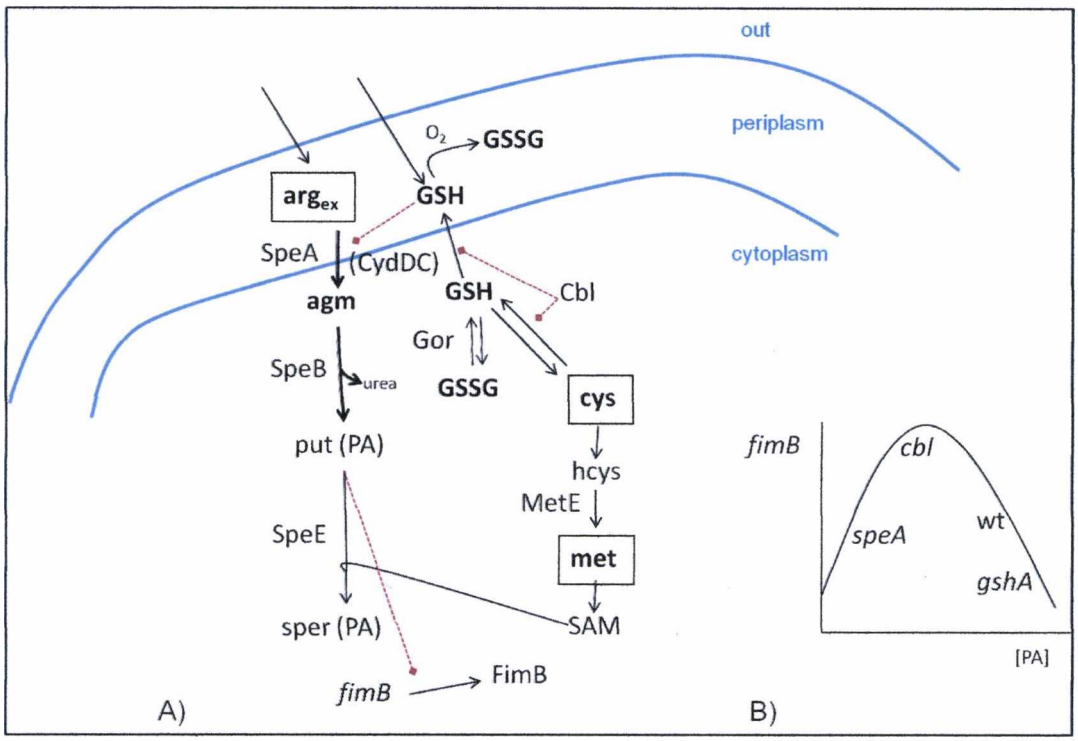


Figure 6.11: A) Model for the control of *fimB* expression in the Δ*gshA* mutant. (⟶) symbolises an activating effect, (----◆) symbolises an inhibitory effect. B) Graph illustrates the hypothesised relationship between polyamine [PA] levels and *fimB* expression. While PA levels are beyond the optimal level in the wild type and Δ*gshA* mutant and below the optimal level in the Δ*speA* mutant, PA levels in the Δ*cbl* mutant are close to the optimum for maximal *fimB* expression.

Chapter 7

GSH and FimB recombination

7.1. Summary

The production of type 1 fimbriae is regulated by phase variation. This involves the inversion of the *fim* switch (*fimS*), a 314 bp invertible element which contains the *fimA* promoter. Inversion is controlled by the recombinases FimB and FimE. Hereby FimE switches the system from ON-to-OFF orientation, while FimB is able to catalyse inversion in both directions. Having shown that GSH activates *fimB* expression, this chapter investigates the effect of GSH on FimB-mediated recombination as well as *fimE* expression. While GSH does not affect *fimE* expression, results show that despite the significant decrease in *fimB* expression, mutation of *gshA* only has a relatively weak inhibitory effect on FimB recombination. The Δ*gor* mutant, on the other hand, does not show this discrepancy. This suggests that the Δ*gshA* mutant has a way to compensate for low levels of *fimB* expression by somehow affecting the FimB protein or DNA to stimulate the recombinase activity of FimB. The Δ*gor* mutant, however, shows a level of FimB recombination that is consistent with its level of *fimB* expression. Hence the effect of *gor* mutation is explained adequately by the change in the *fimB* expression alone. It is further shown that GSH does not affect fimbriation beyond the level of *fimS* inversion.

7.2. Effect of GSH on *fimE* expression

FimB and FimE catalyse the inversion of the *fim* switch *fimS*. While FimB switches the system in both directions, FimE only acts in the ON-to-OFF direction. Since *fimB* and *fimE* are regulated and transcribed independently from one another, it was supposed that mutation of *gshA* not only affects *fimB* expression, but also *fimE* expression. By using a FimE-LacZ fusion, it was found that the absence of GSH only causes a minor change in *fimE* expression compared to the wild type (Figure 7.1). This indicates that GSH selectively activates *fimB* expression. Although *fimB* switches in both direction, this occurs at a relatively low frequency compared to *fimE*. It can therefore be proposed that GSH stimulates OFF-to-ON switching via *fimB*, but has negligible effect on the reverse inversion from ON-to-OFF.

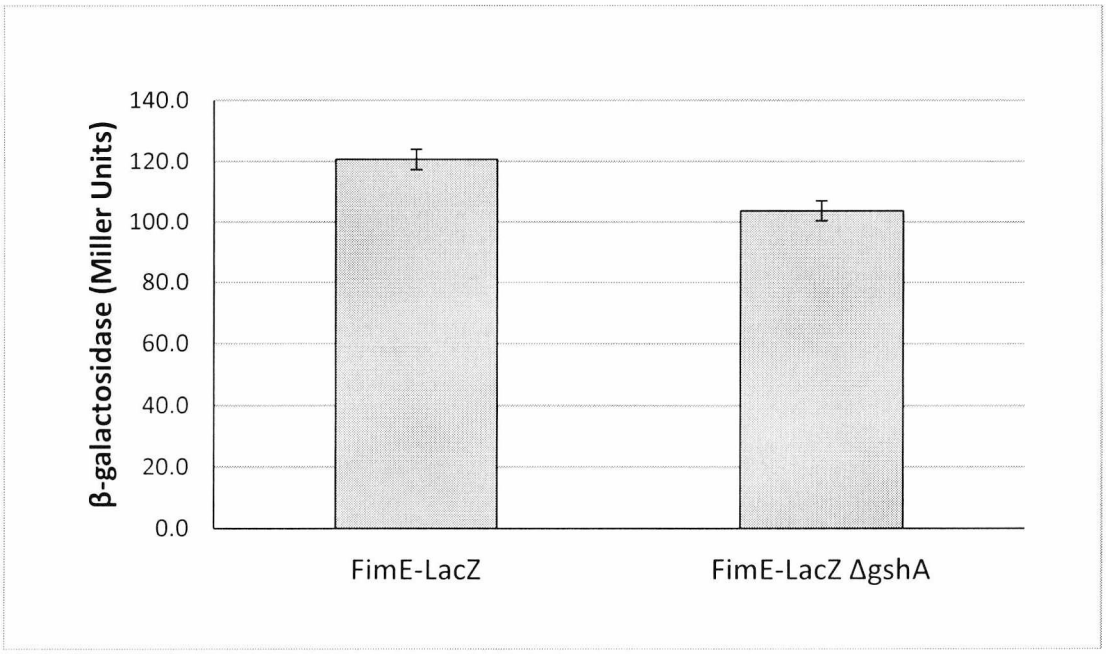


Figure 7.1: β-galactosidase assay showing the effect of *gshA* mutation on *fimE* expression. Strains were grown in RD_{gly}. Strains used were BGEC088 and KCEC2176.

7.3. Effect of GSH on FimB recombination

As shown in Chapter 3, the absence of GSH significantly decreases the level of *fimB* expression. While both FimE and FimB catalyse the inversion of the *fim* switch, only FimB switches *fimS* from the OFF-to-ON orientation (McClain *et al.*, 1991; Gally *et al.*, 1996). It has been shown that the transcription factors NanR and NagC affect fimbriation simply by regulating *fimB* trancription (El-Labany *et al.*, 2003; Sohanpal *et al.*, 2004). This may also apply to GSH, in which case FimB levels would be the limiting factor for OFF-to-ON recombination. Accordingly, low levels of *fimB* expression in the Δ*gshA* mutant would be reflected in a low rate of OFF-to-ON recombination.

The rate of FimB-mediated phase switching was investigated in a Δ*fimE* mutant that carries a *fimA-lacZ* reporter fusion. This fusion allows "OFF" and "ON" colonies to be distinguished. These colonies contain a majority of "OFF" and "ON" cells, respectively. Provided that the rate of phase switching is slow enough, when plated out onto MacConkey agar, phase OFF cells produce pale colonies, whereas phase ON cells produce dark red colonies. Starting with a single "OFF" cell, bacteria are grown for a known number of generations, before plating out. By comparing the number of phase ON colonies

to the total number of colonies, it is possible to determine the frequency of OFF-to-ON inversion per cell per generation.

Mutation of *gshA* in the respective Δ*fimE* background reduced the frequency of FimB-mediated inversion only slightly (Figure 7.2). The effect of the *gshA* mutation on FimB recombinase activity was thus surprisingly small given its effect on *fimB* expression (Figure 7.3). Mutation of *gor*, on the other hand, significantly decreased the frequency of FimB recombination to levels that are more consistent with the respective low level of *fimB* expression (Figure 7.2 and 7.3). This shows that while mutation of *gshA* and *gor* have a comparable effect on *fimB* expression, the effect on FimB recombination is much greater in the Δ*gor* mutant. Furthermore, the disproportionate effect of *gshA* mutation on *fimB* expression and FimB recombination suggests that the activity of FimB is enhanced in the Δ*gshA* mutant, but not the Δ*gor* mutant.

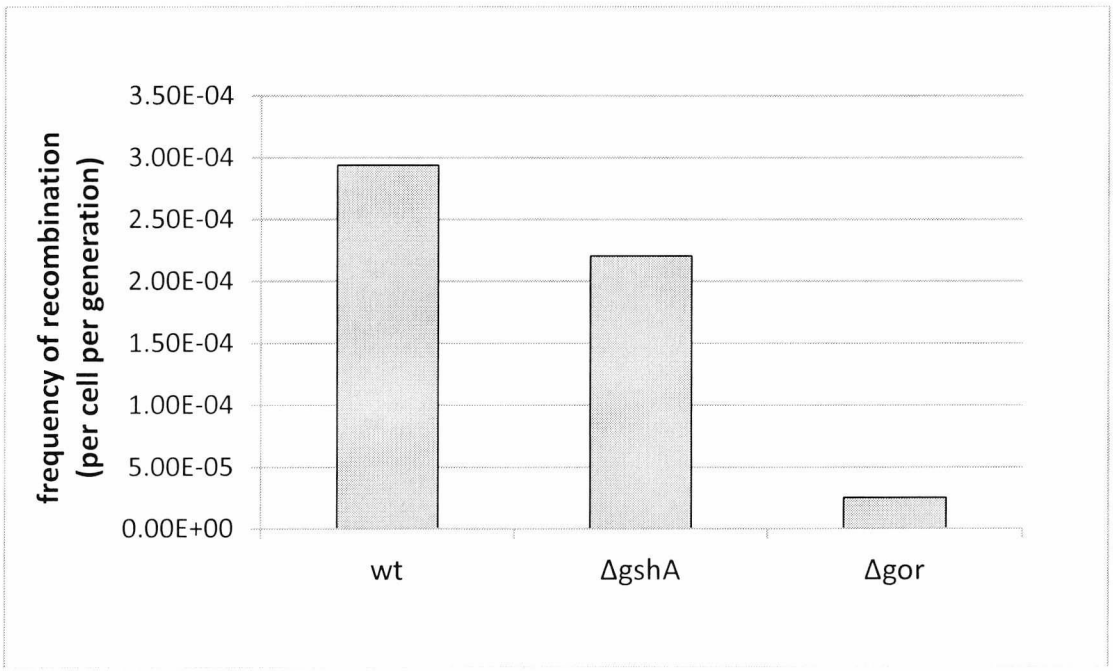


Figure 7.2: The effect of Δ*gshA* and Δ*gor* mutation on FimB-mediated recombination. Strains used were AAEC370A, KCEC2172 and KCEC2492.

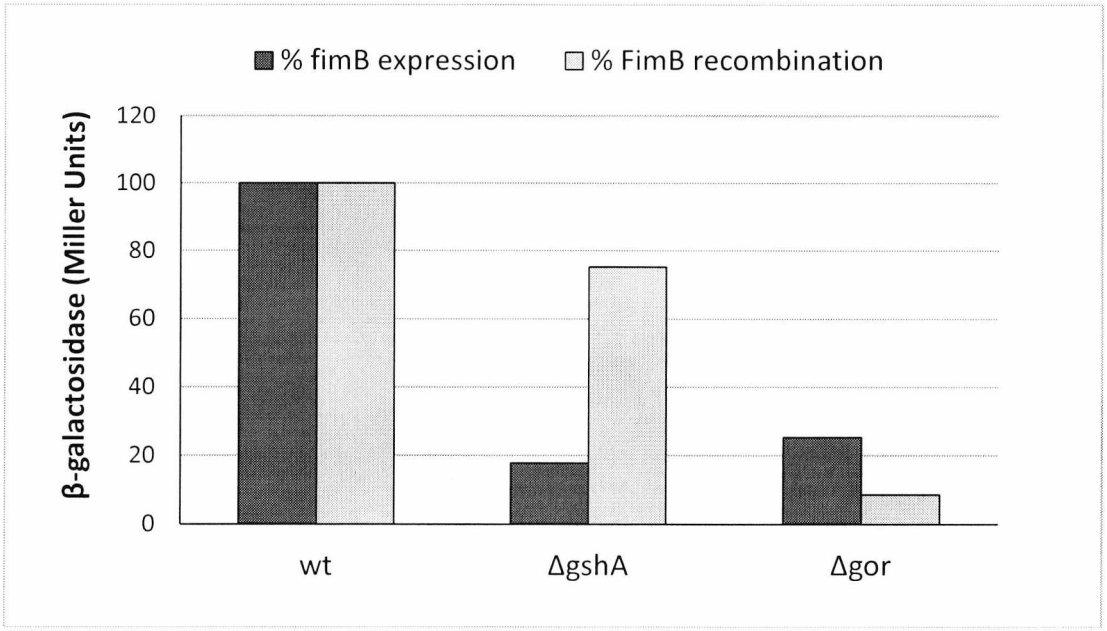


Figure 7.3: Comparison of the effect of Δ*gshA* and Δ*gor* mutation on *fimB* expression and FimB-mediated recombination. Percentages are calculated in relation to wild type values. Strains used were BGEC905, KCEC1627, KCEC1757, AAEC370A, KCEC2172 and KCEC2492.

7.4. Effect of GSH on *fimA* expression

Type 1 fimbriae consist of a mannose-binding tip FimH that enables interaction with mannose receptors on eukaryotic cell surfaces. This provides the basis for the yeast agglutination assay, in which type 1 fimbriae cause *Saccharomyces cerevisiae* cells to visibly clump together. This enables the assessment of the production of type 1 fimbriae in a physiologically relevant context. Yeast agglutination experiments were carried out on wild type and mutant strains, using *fimS* locked ON (constitutively expressing type 1 fimbriae) and afimbriate (Δ*fimB-H*) strains as positive and negative controls respectively. The relative amount of visible clumping is directly proportional to the amount of type 1 fimbriae on the bacterial cell surface. As seen in Figure 7.4, the agglutination is in line with the effect on FimB recombination, showing a further decrease in the Δ*gor* mutant. It seemed possible that GSH or GSSG may have an effect on fimbriation by regulating transcription, translation, export or assembly of the adhesin per se. To test this, *gshA* and *gor* were deleted in the *fimS* locked ON background. However, these mutants did not differ from its parent strain, implying that glutathione has no effect on *fimA* expression beyond the point of *fimS* inversion.

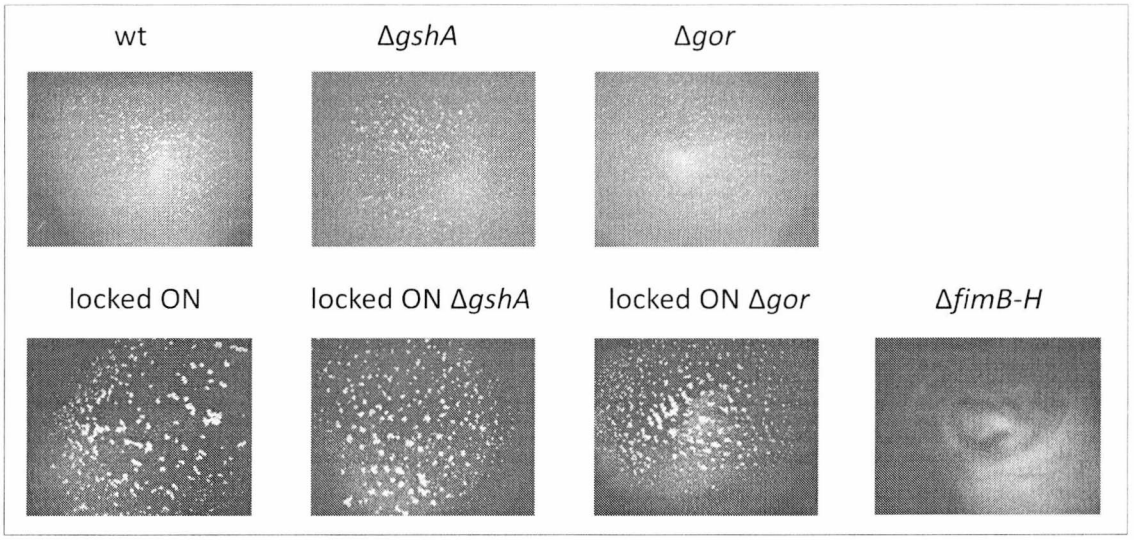


Figure 7.4: Yeast agglutination experiment showing relative decrease in visible cell clumping in the wild type, Δ*gshA* and Δ*gor* mutant compared to the positive (locked ON) and negative (Δ*fimB-H*) control. Strains MG1655, KCEC2170, KCEC2550, AAEC554, KCEC930, KCEC2554 and AAEC072A. Images are shown to identical scale (x1.25).

7.5. Discussion

The production of type 1 fimbriae is controlled in many ways. Phase variation is regulated by two recombinases, FimB switching the system in both directions, FimE only switching from ON-to-OFF. The ratio of FimB and FimE is thus critical for type 1 fimbriation. While it has been shown that GSH regulates the expression of *fimB*, it was supposed that this may be associated to a change in *fimE* expression to either enhance or diminish the effect of GSH on the production of type 1 fimbriae. However, mutation of *gshA* was not found to affect *fimE* expression, which suggests that GSH enhances type 1 fimbriation by selectively activating the OFF-to-ON inversion of the *fim* switch. So far, an effect on *fimB* expression was always found to correlate to the respective effect on FimB recombination. It was therefore expected that low levels of *fimB* expression in the Δ*gshA* mutant cause a decrease in FimB recombination. However, the absence of GSH only had a weak effect on FimB-mediated switching compared to the effect on *fimB* expression. Mutation of *gor*, on the other hand, decreased FimB recombination to levels that correspond to the levels of *fimB* expression. The yeast agglutination assay confirmed that this effect on FimB

recombination does not extend to altering *fimA* expression or fimbriae assembly and thus is likely to be an effect on FimB activity.

Previous studies show a correlation between levels of *fimB* expression and the frequency of FimB switching (El-Labany *et al.*, 2003; Sohanpal *et al.*, 2004), indicating that FimB levels limit switching. In this regard, the effect of *gshA* mutation on switching is not as expected. It was assumed that mutation of *gshA* would have a similar effect to *gor* mutation, i.e. a significant decrease in the rate of FimB recombination.

The absence of *gor* leads to high levels of oxidised GSSG, which are not present in the Δ*gshA* mutant. GSSG could hence be involved in the differential between the Δ*gshA* and the Δ*gor* mutant. It was supposed that GSSG could have a specific inhibitory effect on FimB recombination. This effect could possibly involve glutathionylation of a regulator of FimB recombination.

Chapter 8

Discussion

8.1. Background

Type 1 fimbriae are filamentous adhesins that allow bacterial attachment to host cell surfaces, a key step in pathogenesis. The production of type 1 fimbriae is controlled by phase variation, which is determined by the inversion of the *fim* switch (*fimS*). *fimS* is a 314bp invertible element that contains the promoter for the *fim* gene operon encoding for the structural proteins of fimbriae. While two recombinases, FimB and FimE, are able to catalyse inversion of *fimS*, only FimB is capable of turning the system from OFF-to-ON (McClain *et al.*, 1991). The expression of *fimB* therefore correlates to the level of fimbriae production. Since type 1 fimbriae are a virulence factor, *fimB* expression is required to be tightly controlled to avoid or minimise the extent of host defence. Several factors have been shown to be involved in this control.

These factors include the leucine-responsive regulatory protein (Lrp), which responds to the branched-chain amino acids and alanine (Roesch *et al.*, 1998; Lahooti *et al.*, 2005). Other environmental conditions such as growth medium and temperature (Gally *et al.*, 1993) also affect *fimB* expression. The regulatory proteins NanR and NagC respond to sialic acid and the cell wall component *N*-acetylglucosamine (GlcNAc), respectively, and bind to the large 1.4kbp *nanC-fimB* intergenic region to activate *fimB* expression (Sohanpal *et al.*, 2004; Condemine *et al.*, 2005; Sohanpal *et al.*, 2007). The integration host factor IHF acts as an accessory factor to facilitate Lrp binding as well as NagC activation (Blomfield *et al.*, 1997; Sohanpal *et al.*, 2007). Furthermore, repressors such as the global regulatory protein H-NS inhibit *fimB* expression by binding to the *fimB* promoter (Donato *et al.*, 1997; Olsen *et al.*, 1998).

By producing type 1 fimbriae and hence activating the host defence system, the bacteria are exposed to a range of stress factors such as oxidative and nitrosative stress. The tripeptide glutathione can protect against these stress factors via its thiol group. Prior to this project, it was found that GSH activates *fimB* expression. This project focuses on the effect of glutathione on fimbriation and investigates the mechanism of *fimB* activation by glutathione.

8.2. The importance of glutathione

Glutathione is a tripeptide that is comprised of glutamate, cysteine and glycine. It is ubiquitous in animals, plants and microorganisms and has a function in many cellular processes, such as protein synthesis, enzyme catalysis, metabolism, and transmembrane transport (Meister, 1988; Kidd, 1997). The cysteine residue provides glutathione with a thiol (-SH) group, which enables it to reduce the disulfide linkages of proteins to maintain their structure and function (Wang *et al.*, 1998). By glutathionylation, the formation of mixed disulfides between glutathione and the thiol group of proteins, GSH can regulate the activity of proteins (Akerboom *et al.*, 1982; Ondarza, 1989). Protein thiols are susceptible to oxidation and thus are affected by changes in the redox balance. Reduced glutathione (GSH), on the other hand, can act as an electron-donor and reductant to protect from oxidative damage, for which reason it is present in almost all aerobic species (reviewed by Kidd, 1997).

Aerobic metabolism leads to the formation of highly reactive oxygen species (ROS) that can damage DNA, lipids and proteins (Bandyopadhyay *et al.*, 1999; Valko *et al.*, 2007). These ROS include superoxide and hydroxyl radicals, as well as hydrogen peroxide. While glutathione is able to protect against oxidative stress by scavenging hydroxyl radicals and singlet oxygen directly, it can also detoxify hydrogen peroxide as a cofactor for antioxidant enzymes such as glutathione peroxidase (Kidd, 1997; Masella *et al.*, 2005; Valko *et al.*, 2007). Besides peroxidases, glutathione S-transferases (GSTs) carry out detoxification reactions (Jakoby, 1978; 2006; Kaplowitz, 1980). This is especially important for those organs most directly exposed to exogenous toxins, particularly the liver in mammals (Kaplowitz, 1981). In addition to the protection against oxidative stress and detoxification, GSH also plays an important role in metal ion homeostasis (Ballatori, 1994). The thiol group of the cysteine moiety of GSH has a high affinity for metal ions such as copper, zinc or cadmium (Ballatori, 1994; Wang *et al.*, 1998) and thus enables GSH to chelate free metal ions. In this context, the regulation of free iron Fe^{2+} levels is of great importance. Fe^{2+} generates the reactive hydroxyl radical (OH•) in the Fenton reaction (Imlay *et al.*, 1988). Glutathione, on the other hand, can help prevent this reaction not only by chelating free Fe^{2+}, but also by acting as a storage for cysteine (Meister, 1988), since free cysteine drives the Fenton reaction by reducing Fe^{3+} back to Fe^{2+} (Park *et al.*, 2003).

The formation of glutathionylspermidine (Gsp) appears to be another physiologically important function of glutathione, since most of the cellular GSH pool is present as this conjugate (Smith *et al.*, 1995). Like GSH, Gsp forms mixed disulfides with protein thiols (Chiang *et al.*, 2010) and thus regulates enzyme activity and helps to protect against oxidative damage.

In mammals, glutathione has also been shown to have an effect on the immune system to control inflammation (reviewed by Perricone *et al.*, 2009). The activation of T-lymphocytes is regulated by the redox state of glutathione (reviewed by Valko *et al.*, 2007), while bacteria like *E. coli*, on the other hand, activate the host immune system. Glutathione hence could be involved in the maintenance of a healthy host-parasite balance.

Since glutathione is not produced by all bacterial strains (Fahey *et al.*, 1978), it is not required for essential cellular processes, but probably helps protect against harmful agents. The main function will thereby be the maintenance of a healthy redox environment to ensure a thiol-disulfide balance and thus regulate the activity of key enzymes.

8.3. Possible effects of glutathione on *fimB* expression

There are many ways in which glutathione could be affecting the fimbrial system in *E. coli*. As a potent reductant, glutathione is involved in maintaining the redox state of the cell. Moreover, γ-glutamylcysteine (γ-GC), the precursor of glutathione, can substitute for the redox properties of GSH to a significant extent (Faulkner *et al.*, 2008). The activating effect of glutathione on *fimB* expression may hence be down to its precursor γ-GC.

The cytoplasm provides a reducing environment, which is maintained by the thioredoxin and glutaredoxin systems (Holmgren, 1989; Carmel-Harel *et al.*, 2000). It is therefore possible that glutathione regulates the activity of a regulatory protein that is responsive to oxidative stress and to changes in the redox balance of the cell. While OxyR and SoxRS play a key role in the response to oxidative and nitrosative stress (Demple, 1996; Demple, 1999; Storz *et al.*, 1999), SoxS has been shown to be induced in the absence of GSH (Ding *et al.*, 1996).

Furthermore, these regulators are also linked to levels of free metal ions (Zheng *et al.*, 1999; Anjem *et al.*, 2009). Metal toxicity is a major threat to many cellular processes. Excess Fe^{2+} can produce harmful oxygen radicals in the Fenton reaction (Imlay *et al.*,

1988), and while excess copper causes damage to iron-sulfur clusters in proteins (Macomber *et al.*, 2009), manganese is able to prevent this to an extent by substituting for iron (Anjem *et al.*, 2009). Glutathione, on the other hand, is a potent metal chelator (Ballatori, 1994) and can also act as a "sink" for cysteine, which drives the Fenton reaction by reducing ferric iron Fe^{3+} back to ferrous iron Fe^{2+} (Park *et al.*, 2003). It is therefore possible that glutathione affects *fimB* expression by controlling the levels of free metal ions and thus metal toxicity.

The translational regulator AconitaseB (AcnB) contains a 4Fe-4S cluster and is thus very sensitive to oxidative stress and low iron levels. Furthermore, aconitase activity has been shown to be decreased in the absence of GSH (Gardner *et al.*, 1993). It was thus thought possible that glutathione activates *fimB* by enhancing aconitase activity.

AcnB catalyzes the isomerisation of citrate to isocitrate as part of the TCA cycle. The subsequent conversion of isocitrate to α-ketoglutarate by isocitrate dehydrogenase generates NADPH, which is required as a reductant for correct formation of periplasmic disulfide bonds. Although the mutation of *acnB* had a small suppressive effect in the Δ*gshA* mutant, this line of enquiry did not lead to any conclusive results. However, the involvement of NADPH seems relevant in terms of the formation of disulfide bonds. FimH, a structural component of type 1 fimbriae, contains cysteine (-SH) residues and requires disulfide bond formation for fimbrial assembly in the periplasm (Lowe *et al.*, 1987; Soto *et al.*, 1999), as well as for binding to mannose (Nilsson *et al.*, 2007). The formation of disulfide bonds requires an oxidative environment (reviewed by Raina *et al.*, 1997), yet the thioredoxin and glutaredoxin systems (Holmgren, 1989; Carmel-Harel *et al.*, 2000), together with GSH and NADPH, provide a reducing environment in the cytoplasm. However, periplasmic redox proteins such as DsbA, DsbC and the membrane protein DsbB (Bardwell *et al.*, 1991; Bardwell *et al.*, 1993; Ito, 2010), as well as a low GSH:GSSG ratio in the periplasm, facilitate the formation and correct folding of disulfide-containing proteins (reviewed by Raina *et al.*, 1997; Ito, 2010). For this reason, most disulfide-bond-containing proteins are periplasmic proteins that are secreted prior to being folded in the periplasm. This further suggests that glutathione may have a periplasmic role in regulating *fimB* expression by ensuring correct disulfide bond formation.

Furthermore, transmembrane proteins as part of 2-component regulatory systems may sense changes in the redox state and transmit this signal to its cytoplasmic regulator to

control *fimB* expression. In this context, the ArcA/B system may be involved in the regulation of *fimB* expression. Under reducing conditions, the transmembrane sensor kinase ArcB is reduced and hence activated to phosphorylate the cytoplasmic response regulator ArcA (Georgellis *et al.*, 1997; Malpica *et al.*, 2004). Phosphorylated ArcA (ArcA-P), in turn, regulates gene expression (Iuchi *et al.*, 1988) and thus could be controlling *fimB* expression in response to redox changes. It has been proposed that high levels of ArcA-P inhibit *fimB* expression under anaerobic conditions (Liu *et al.*, 2004). Our data show that mutation of *arcA* decreases *fimB* expression only in the presence of *gshA* (data not shown). It therefore seemed possible that GSH is required for ArcB activity, with reduced GSH activating ArcB to increase levels of ArcA-P. However, further investigations did not further support this hypothesis.

ArcA also regulates the expression of *lpdA*, the gene encoding lipoamide dehydrogenase. This enzyme is a component of the α-ketoglutarate dehydrogenase and glycine cleavage multi-enzyme complexes (Pettit *et al.*, 1967; Steiert *et al.*, 1990), and hence provides a link to the reductants NAPDH and GSH. Lipoamide refers to the protein-bound form of lipoic acid, which transpires to be a biological antioxidant (Packer *et al.*, 1995). Low levels of LpdA will cause the accumulation of dihydrolipoamide (P-DHL) (Feeney *et al.*, 2011), and hence a decrease of free lipoic acid. Not only has it been shown that P-DHL can replace redox function of GSH (Feeney *et al.*, 2011), but also that Lpd is repressed by spermidine (Yohannes *et al.*, 2005). Since the majority of the GSH pool is present in complex with spermidine (Smith *et al.*, 1995), this presents a further potential mechanism for the regulation of *fimB* expression by GSH.

GSH and polyamines have common functions in the cell by protecting against oxidative stress as well as copper toxicity (Ha *et al.*, 1998; Wang *et al.*, 2007). Polyamine biosynthesis involves cysteine and glutamate, two of the three amino acids that GSH is comprised of (Bowman *et al.*, 1973; Tabor *et al.*, 1985). Furthermore, polyamines enhance translation of genes with the poorly transcribed UUG start codon (Yoshida *et al.*, 1999), which applies to the *gshA* gene. Polyamines also stimulate the synthesis of a range of proteins, one of which is the transcriptional regulator H-NS, a known repressor of *fimB* expression. The regulation of *fimB* expression may thus occur via an effect on translation, or at the level of transcription. The investigation of these possible ways for glutathione to affect *fimB* expression ultimately led to the model described in the following subchapters.

8.4. Model for the regulation of *fimB* expression by glutathione

8.4.1. Periplasmic GSH activates *fimB* expression

Research in the laboratory is focused on investigating the regulation of fimbrial production. Preliminary work to this project showed that *fimB* expression is significantly decreased in strains that do not produce glutathione (Δ*gshA*). Glutathione is an important molecule in the cell. Via its thiol group, it is able to protect against a range of environmental stress factors, such as oxidative and nitrosative stress (Dalle-Donne *et al.*, 2009b), as well as metal toxicity (Ballatori, 1994). It is this thiol group of glutathione that also makes it a key reductant in the cell. Next to the total glutathione pool, the oxidation state of glutathione is hence likely to be an indicator for the physiological state of the cell. The production of type 1 fimbriae, on the other hand, can generate stress conditions by activating the host defence system. Type 1 fimbriae not only enable attachment to epithelial cells, but also promote their invasion. In this process, lipopolysaccharide (LPS), the major component of the bacterial outer membrane, is presented to host cell receptors. LPS is recognised by the toll-like receptor 4 (TLR4) (Schilling *et al.*, 2001), which leads to the release of cytokines IL-6, IL-8 and TNF-α (Malaviya *et al.*, 1996; Godaly *et al.*, 1998; Schilling *et al.*, 2001; Samuelsson *et al.*, 2004). Moreover, FimH itself is a ligand for TLR4 (Ashkar *et al.*, 2008), while type 1 fimbriae have been shown to be specifically required for the late IL-8 production (Semiramoth *et al.*, 2009).

It is hence reasonable to suggest that glutathione not only protects against damage caused by the host defence system, but that glutathione also plays a role in regulating the production of type 1 fimbriae to minimise host defence in the first case.

Initial investigations into this regulatory effect showed that glutathione has an activating effect on *fimB* expression (Chapter 3). Mutation of *gshA* and *gshB*, the genes that encode the enzymes required for glutathione biosynthesis (Meister, 1988), caused a significant decrease in *fimB* expression. This indicates that the intact tripeptide stimulates *fimB* expression, as opposed to the dipeptide γ-glutamylcysteine, which is the intermediate in glutathione biosynthesis.

Glutathione is present in a reduced (GSH) as well as an oxidised state (GSSG), with glutathione oxidoreductase (Gor) ensuring a constant ratio between GSH and GSSG (Kunert *et al.*, 1990). Since mutation of *gor* had a comparable effect on *fimB* expression as

observed in the Δ*gshA* and Δ*gshB* mutants, glutathione is probably required to be in its reduced state GSH to activate *fimB* expression. This hypothesis was supported by complementation assays. While exogenous reduced GSH completely suppressed the Δ*gshA* mutant phenotype, this effect was not shown for oxidised GSSG. Furthermore, complementation was not dependent upon the GSH import system GsiABCD, which suggests that GSH acts on a periplasmic target.

The bacterial cell produces CydDC, an export system for GSH and cysteine (Pittman *et al.*, 2005). Since it was proposed that GSH acts in the periplasm to regulate *fimB* expression, this effect was expected to be influenced by CydDC. However, mutation of *cydD* did not affect the Δ*gshA* mutant phenotype. An explanation for this result may be that CydDC is not induced under the present growth conditions and that GSH is exported by other transport systems, as has been suggested previously (Eser *et al.*, 2009; Smirnova *et al.*, 2011). An alternative explanation for the lack of response to *cydD* mutation is that GSH not only acts on a periplasmic target, but has a regulatory effect on an additional cytoplasmic regulatory protein.

Initial investigations to identify a cytoplasmic regulatory protein that is involved in the regulation of *fimB* expression were based on the key function of glutathione as reductant and metal chelator. In this regard, the oxidative stress-responsive regulators OxyR and SoxRS seemed to be promising candidates. However, the effect of GSH on *fimB* expression could not be linked to OxyR and SoxRS, nor to the nitrosative stress-responsive regulators NsrR and NorR. It was also not possible to show that the Δ*gshA* mutant phenotype is caused by metal toxicity. Although the ferric uptake regulator Fur was shown to be involved in the Δ*gshA* mutant phenotype - possibly by regulating a repressor of *fimB* expression via the regulator RNA RyhB - we were not able to elucidate more details on this regulatory effect.

However, further investigations of regulatory proteins that might potentially respond to, or control the levels of, GSH revealed that the transcription factor Cbl regulates *fimB* expression. This regulator is required for the use of alternative sulfur sources (van der Ploeg *et al.*, 1997). This means that Cbl is activated under sulfur-deficient conditions and hence low levels of cysteine (Iwanicka-Nowicka *et al.*, 1995). Since cysteine is a component of GSH, this provides a direct link between Cbl and GSH biosynthesis.

The absence of Cbl had a stimulatory effect on *fimB* expression, though this effect proved to be dependent upon GSH biosynthesis, as well as the GSH export system CydDC. This leads to the hypothesis that Cbl limits GSH biosynthesis and thus regulates the GSH pool in the cell. Since GSH can be considered as a "sink" for cysteine, this would ensure that, despite sulphur deficiency, there is sufficient cysteine for other cellular processes. These processes include methionine biosynthesis and thus translation initiation, as well as the generation of *S*-adenosyl-L-methionine (SAM), the major methyl donor in the cell (Lu, 2000).

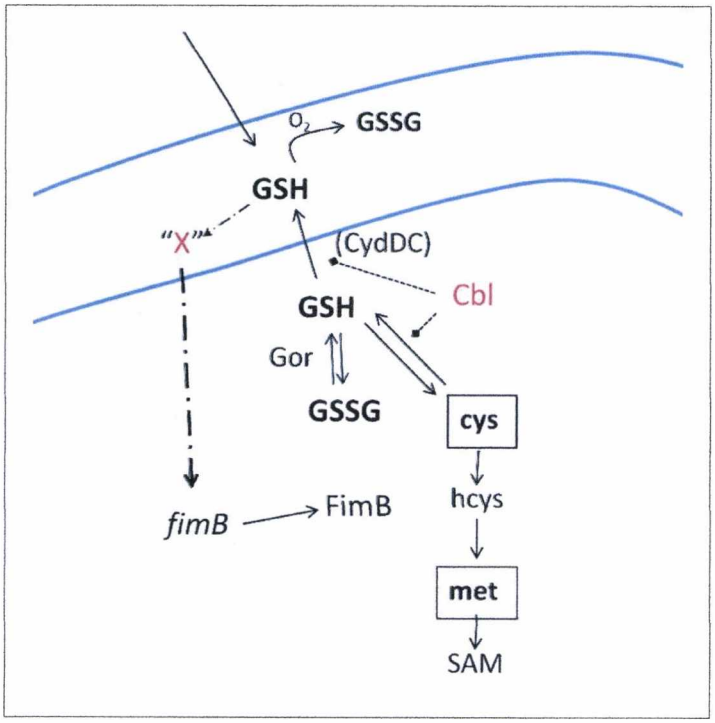


Figure 8.1: Model for the interrelation between glutathione and Cbl and the subsequent effect of periplasmic GSH on *fimB* expression. (⟶) symbolises an activating effect, (- - -◆) symbolises an inhibitory effect.

In the absence of Cbl, GSH levels will rise and GSH will subsequently be exported to the periplasm via CydDC and/or an alternative export system, where it can access its periplasmic target to regulate *fimB* expression (Figure 8.1). While mutation of *cydD* did not affect *fimB* expression in the wild type background, it was shown to be involved in the Δ*cbl* mutant background. It is possible that levels of GSH are below the threshold for CydDC export in the wild type, but rise above the threshold in the Δ*cbl* mutant. Another

explanation would be that *cydD* expression is only induced in the absence of Cbl, which signals sufficient sulfur levels. This would provide another way for the cell to prevent sulfur and cysteine insufficiency.

8.4.2. GSH regulates polyamine levels

The majority of the cellular GSH pool is present as glutathionylspermidine (Gsp), a covalent adduct with the polyamine spermidine (Smith *et al.*, 1995). Polyamines have many functions in the cell. Being positively charged, polyamines are able to bind to DNA, RNA and proteins and thus regulate cell growth, gene expression and protein synthesis, among other things (Tabor *et al.*, 1985; Igarashi *et al.*, 2000; Kusano *et al.*, 2008). While polyamines have been shown to protect against oxidative stress (Ha *et al.*, 1998), toxic levels of copper induce putrescine biosynthesis in plants (Wang *et al.*, 2007), which also suggests a role in protection against metal toxicity. Along with the formation of glutathionylspermidine, this provides a direct link between glutathione and polyamines. Moreover, SpeA does not only initiate the polyamine biosynthesis pathway, but is also located in the inner periplasmic space (Buch *et al.*, 1985). It was therefore proposed that the regulatory effect of GSH is linked to polyamines.

Addition of exogenous spermidine was shown to have an inhibitory effect on *fimB* expression, at both the translational and the transcriptional level (Chapter 4). The analysis of a range of mutations that disrupt the polyamine biosynthetic pathway at various stages led to the hypothesis that the Δ*gshA* mutant phenotype is caused by elevated levels of putrescine, not spermidine. However, further results indicate that endogenous spermidine does inhibit *fimB* expression, though the levels of *S*-adenosyl-L-methionine (SAM) limit spermidine production under our physiological conditions (Chapter 6.4). SAM is required for the conversion of putrescine to spermidine by SpeE (Bowman *et al.*, 1973; Tabor *et al.*, 1985). By mutation of *metJ*, as well as by growing the cells in medium without methionine, it was possible to further decrease *fimB* expression, presumably by increasing the SAM pool and hence enhancing spermidine biosynthesis.

Mutation of *speA* suppressed the effect of *gshA* mutation. Furthermore, Δ*speA* mutants were less responsive to exogenous GSH, which indicates that the effect of exogenous GSH is dependent upon SpeA. Considering that SpeA is located in the periplasmic space and that previous results suggest a periplasmic role of GSH in *fimB* regulation, it was proposed

that GSH regulates *fimB* expression by limiting polyamine biosynthesis via SpeA. Furthermore, the stimulatory effect previously observed in the Δ*cbl* mutant was lost in the absence of SpeA. Since it was proposed that loss of Cbl elevates GSH levels and that periplasmic GSH stimulates *fimB* expression by inactivating SpeA, this suggests that mutation of *cbl* increases the levels of periplasmic GSH to diminish SpeA activity. It is possible that Cbl regulates GSH levels in the cytoplasm via its degradation or its biosynthesis pathways. Alternatively, Cbl may repress *cydD* expression and thus the rate of GSH export to the periplasm. In either case, loss of Cbl would increase periplasmic GSH levels, which then inactivates SpeA, diminishes putrescine biosynthesis and hence stimulates *fimB* expression (Figure 8.2).

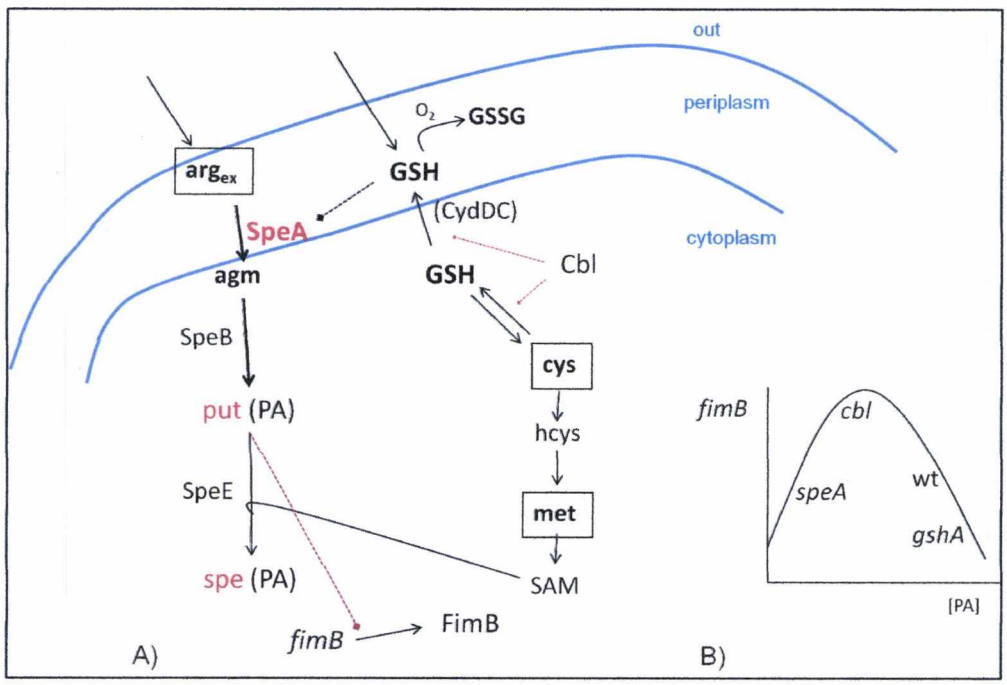


Figure 8.2: A) Model for the regulation of polyamine (PA) levels by GSH. (⟶) symbolises an activating effect, (----◆) symbolises an inhibitory effect. B) The graph describes the correlation between polyamine (PA) levels and *fimB* expression.

These observations further suggest that there is an optimal level of putrescine for maximal *fimB* expression. In the wild type, putrescine levels will be above the optimum. However, increased levels of GSH allow optimal control of SpeA in the Δ*cbl* mutant and lead to putrescine levels closer to the optimum. In the Δ*gshA* mutant, on the other hand, putrescine levels will be far beyond the optimum (Figure 8.2).

8.4.3. A potential role for H-NS

The level of *fimB* expression was measured by using translational FimB-LacZ fusion strains in β-galactosidase assays. However, by comparing the effect of *gshA* mutation in both a translational and a transcriptional *fimB-lacZ* fusion strain, it was possible to determine that GSH regulates *fimB* expression at both the transcriptional and the translational level.

Polyamines regulate protein translation (Tabor *et al.*, 1985; Igarashi *et al.*, 2010), but also specifically stimulate H-NS synthesis (Terui *et al.*, 2007). H-NS is a global regulatory protein that represses the expression of genes that are related to environmental conditions (Schroder *et al.*, 2002). Furthermore, H-NS has been shown to repress *fimB* transcription by binding to the *fimB* promoter (Donato *et al.*, 1997). Results show that mutation of *hns* suppresses the Δ*gshA* mutant phenotype. Δ*hns* mutants also showed to be less responsive to exogenous spermidine, suggesting that H-NS is involved in the inhibitory effect of spermidine. Moreover, combining a mutation in *gshA* with a mutation in *slyA* had a strong compound effect. The transcriptional regulator SlyA is an antagonist for H-NS and competes with H-NS for its DNA binding site in the *fimB* promoter (McVicker *et al.*, 2011). Loss of SlyA hence decreases *fimB* expression by enhancing the activity of H-NS. Taken together, this suggests that the activating effect of GSH on *fimB* expression is mediated via polyamines and H-NS, whereby GSH might have a related function to SlyA, in that GSH prevents H-NS from inhibiting *fimB* expression. While excess polyamines inhibit *fimB* translation directly, the effect on *fimB* transcription is suggested to be indirect via H-NS. Elevated levels of polyamines in the absence of GSH stimulate H-NS synthesis, which in turn represses the *fimB* promoter.

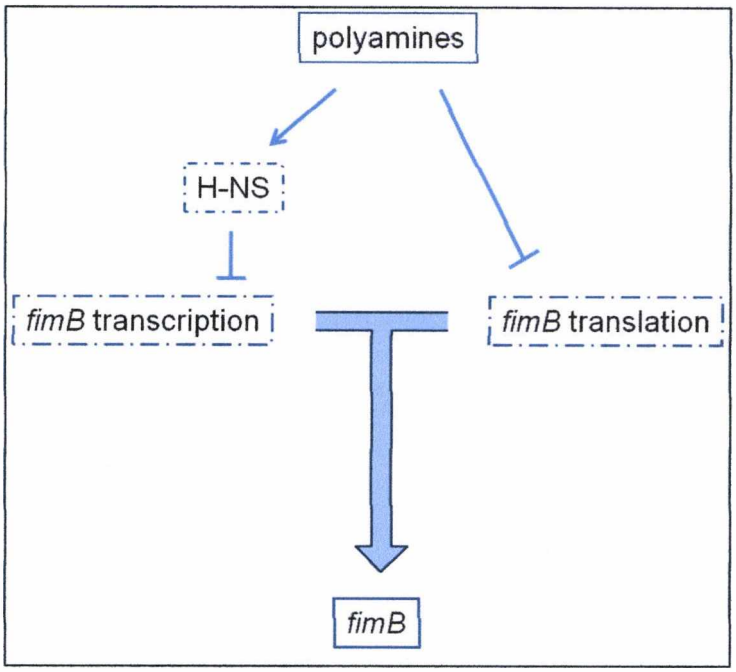


Figure 8.3: Model for the effect of polyamines on *fimB* transcription and translation.
(⟶) symbolises an activating effect, (⟞) symbolises an inhibitory effect.

Supposing this to be the case, *fimB* regulation by GSH and polyamines would be under dual control (Figure 8.3). The direct inhibition of *fimB* translation by polyamines is complemented by the indirect inhibitory effect on *fimB* transcription via H-NS. While this indirect effect can be controlled via levels of H-NS and its direct antagonists such as SlyA, the direct effect can be regulated by balancing GSH and polyamine levels. The results described above suggest that GSH and polyamines are able to mutually regulate their respective cellular levels by feedback control (also see page 133).

8.4.4. In a physiological context...

Type 1 fimbriae can activate host defence systems and thus expose the bacteria to a range of harmful stress factors. Glutathione is able to protect the cells from damage caused by this stress, and has also been shown to regulate fimbriation to avoid the exposure to stress in the first case. The results and regulatory effects described above hence provide a model for *fimB* regulation, and are also of physiological relevance.

Under conditions of stress, GSH will help protect the cell from damage, which will consequently diminish the cellular pool of reduced GSH. The GSH:GSSG ratio can

therefore be considered as an indicator for cellular stress levels. Low levels of GSH will be a signal for the cell to decrease the production of type 1 fimbriae in order to avoid further exposure to environmental stress factors. Vice versa, high levels of GSH will activate *fimB* expression, as was shown in the results described in Chapter 3.

The stimulatory effect on *fimB* expression in the Δ*cbl* mutant was explained by an increase in the level of periplasmic GSH. While Cbl is activated under conditions of sulfur starvation, GSH is a major reservoir for cysteine (Tateishi *et al.*, 1977; Suzuki *et al.*, 1993). It is therefore likely that Cbl limits GSH levels, as well as GSH export, to help maintain cysteine levels in the cell. Under sufficient sulfur availability, the inactivation of Cbl would therefore increase GSH production to conserve cysteine. In this regard, the inactivation of Cbl would be a signal for conditions good enough to allow an increase in GSH levels and in fimbrial production.

The assembly of type 1 fimbriae involves the formation of disulfide bonds between its structural components (Jacob-Dubuisson *et al.*, 1994; Soto *et al.*, 1999). These components contain multiple cysteine residues, and thus provide the thiol groups for disulfide bonds. Disulfide bonds are generated by coupling of reduced thiol residues (-SH) (Sevier *et al.*, 2002). The formation of type 1 fimbriae will thus require a reducing agent to ensure the thiol groups of its components to be in the reduced state. While GSH probably will not play a direct role in this, its levels nevertheless will certainly reflect the cell's ability to carry out correct disulfide bond formation necessary for fimbrial biosynthesis. Since fimbriae assembly occurs in the periplasmic space (Dodd *et al.*, 1984; Lowe *et al.*, 1987; Klemm, 1992; Jones *et al.*, 1993), this further supports the importance of periplasmic GSH in the regulation of fimbrial production.

Our results propose that periplasmic GSH activates *fimB* expression by diminishing polyamine biosynthesis. The mutual control of GSH and polyamine biosynthesis seems reasonable in a physiological context. High levels of GSH reflect low stress levels and thus represent conditions that do not necessarily require polyamines to additionally stimulate cell growth and protein synthesis. One way for the cell to diminish free polyamine levels is the formation of Gsp (Tabor *et al.*, 1975). However, this is not favourable from a metabolic point of view, as Gsp formation requires ATP, as does GSH biosynthesis. It is thus less

energetically costly to coordinate the cellular levels of GSH and polyamines to prevent excess biosynthesis.

The model described above describes a mechanism by which GSH regulates polyamine levels, suggesting that periplasmic GSH diminishes polyamine biosynthesis by inactivating SpeA. While stress conditions will diminish the cytoplasmic GSH pool, this will also affect GSH levels in the periplasm. Consequently, SpeA will be rendered more active to increase polyamine biosynthesis. Polyamines will not only help counteract oxidative damage (Ha *et al.*, 1998), but simultaneously increase GSH levels by stimulating its translation initiation at the poorly translated UUG start codon (Yoshida *et al.*, 2001). High levels of GSH, in turn, will reflect good growth conditions and low stress levels and thus diminish polyamine biosynthesis, as well as stimulate *fimB* expression. If the expression of *gshA* is stimulated by polyamines via translation initiation as this model suggests, this will consequently have negative feedback on GSH production and thus increase polyamine biosynthesis. According to this model, GSH and polyamines can mutually regulate their levels by negative feedback in a closed circuit system (Figure 8.4).

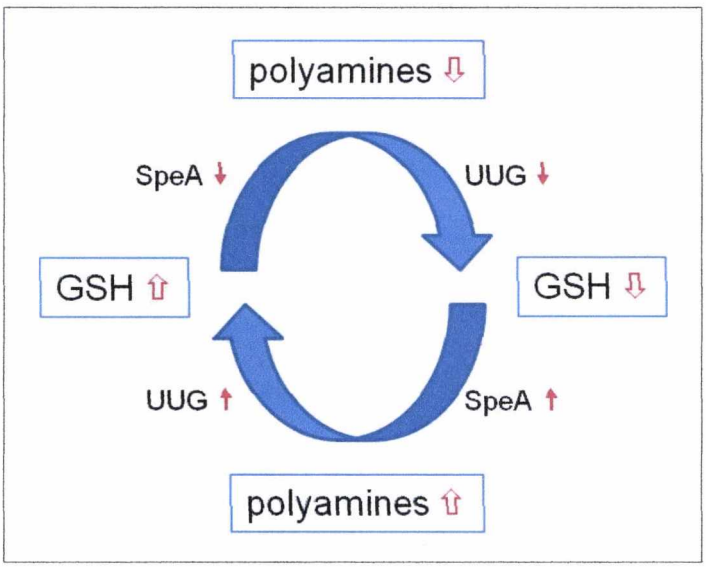


Figure 8.4: Model for the mutual regulation of GSH and polyamine levels by feedback control. GSH regulates polyamine levels via inactivation of SpeA, while polyamines increase translation initiation at the poorly translated UUG start codon of the *gshA* gene.

There are a range of mechanisms by which the cell can regulate polyamine levels. While the activity of arginine decarboxylase (SpeA) and ornithine decarboxylase (SpeC) is controlled by the amount of polyamines in the cell (Kyriakidis *et al.*, 1978; Tabor *et al.*, 1985), adenosylmethionine decarboxylase (SpeD) is strongly inhibited by spermidine (Kashiwagi *et al.*, 1988). Furthermore, it has been shown that excess putrescine is exported into the medium via the putrescine-ornithine antiporter PotE (Kashiwagi *et al.*, 1988; Kashiwagi *et al.*, 1991). While these mechanisms are mostly linked to feedback control of intermediates or products in the biosynthetic pathway, the regulation by GSH would provide a means for the cells to adapt polyamine levels in response to physiological conditions.

8.5. Balancing GSH and polyamines: lipoic acid

As mentioned previously, GSH and polyamines appear to be linked in many different ways, ranging from glutathionylspermidine Gsp and gene expression (UUG translation initiation) to biosynthesis (inactivation of SpeA), amino acids (cysteine and glutamate) and protection against oxidative and metal ion stress.

The results described above have led to the conclusion that GSH regulates *fimB* expression via polyamines. It was hypothesised that GSH regulates polyamine biosynthesis by inactivating SpeA, while polyamines, in turn, inhibit *fimB* expression directly as well as indirectly via H-NS. However, an alternative explanation for these results obtained within the scope of this project could be the induction of a factor that compensates for GSH, but is normally repressed by putrescine (or another polyamine). The common role of both GSH and polyamines in oxidative stress suggests that polyamines might suppress the synthesis of an alternative reductant that can replace GSH.

In this regard, lipoic acid is a potential factor to be involved in the regulation of *fimB* expression. Lipoic acid and its reduced form dihydrolipoic acid act as an antioxidant by reacting with reactive oxygen species (such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen) (reviewed by Packer *et al.*, 1995; Shay *et al.*, 2009). Furthermore, lipoic acid can reduce GSSG (Packer *et al.*, 1995) and has been shown to induce de novo GSH synthesis in humans and rats by reducing cystine to cysteine (Han *et al.*, 1997; Sen *et al.*, 2000; Suh *et al.*, 2004). It is therefore possible that

lipoic acid can reduce a target that is normally reduced by GSH and that it is not the loss of redox function of GSH that is key to the Δ*gshA* mutant phenotype.

The protein-bound form of lipoic acid, lipoamide, undergoes redox cycling by lipoamide dehydrogenase (Lpd), which reoxidises reduced dihydrolipoamide (P-DHL). It has been reported that glutaredoxins are able to catalyse the reduction of GSSG by P-DHL (Porras *et al.*, 2002), which suggests that P-DHL reduces GSSG in place of Gor to some extent. Furthermore, Feeney et al. (2011) report that P-DHL can replace the redox function of GSH. It has also been shown that *lpdA*, the gene encoding for lipoamide dehydrogenase, is repressed by spermidine (Yohannes *et al.*, 2005). Thus it is possible that endogenous putrescine also has this effect on *lpdA*. In the absence of GSH, a rise in putrescine levels will thus decrease *lpdA* expression and lead to the accumulation of P-DHL. This ultimately decreases the levels of free lipoic acid. Since lipoic acid may replace GSH as a reductant, this will consequently mean that neither GSH nor lipoic acid are able to stimulate *fimB* expression, which causes the observed low levels of *fimB* expression.

Mutation of *speA*, on the other hand, may suppress the Δ*gshA* mutant phenotype by decreasing levels of putrescine and spermidine and thus increasing levels of *lpdA* expression and free lipoic acid. Lipoic acid, in turn, is able to replace GSH by reducing a common target.

This suggests two possible mechanisms for GSH to regulate *fimB* expression. The first possibility is based on the assumption that GSH has no direct effect on polyamine levels per se. While spermidine inhibits lipoic acid biosynthesis via *lpdA*, mutation of *speA* induces the generation of lipoic acid. The *gshA* mutant phenotype is thus repressed by mutation of *speA* due to the generation of lipoic acid that can replace GSH.

In the second possible mechanism, GSH does control polyamine levels. Elevated polyamine levels caused by mutation of *gshA* diminish lipoic acid levels, which in turn leads to a lower level of *fimB* expression. According to this, lipoic acid would be an activator of *fimB* expression, but is suppressed in the absence of GSH by an excess of putrescine.

Results show that the ability of exogenous GSH to stimulate *fimB* expression is dependent upon SpeA. Taking this into account, one is able to distinguish between these possibilities, in favour of the second which suggests GSH to control polyamine levels.

Supposing that GSH has no effect on polyamine levels, exogenous GSH would not affect spermidine levels and thus not be able to overcome the constitutive inhibition of lipoic acid biosynthesis by spermidine. Furthermore, GSH would be affecting *fimB* expression by a mechanism other than spermidine and lipoic acid, for which reason complementation should be independent of SpeA. It therefore seems reasonable to assume that GSH has a direct effect on polyamine levels and controls polyamine biosynthesis by inactivating SpeA. Exogenous GSH thus inactivates SpeA and stimulate *fimB* expression by elevating levels of lipoic acid, yet complementation will only occur in the presence of SpeA.

8.6. Effect of GSH on *fimB* expression and FimB recombination

8.6.1. Differential between Δ*gshA* and Δ*gor* mutants

The absence of GSH significantly decreases *fimB* expression. Since levels of *fimB* expression have been shown to correlate to the frequency of FimB switching (El-Labany *et al.*, 2003; Sohanpal *et al.*, 2004), the Δ*gshA* mutant was expected to have low FimB activity. However, results show that FimB-mediated switching is hardly affected by mutation of *gshA*. Mutation of *gor*, on the other hand, had the expected effect on FimB activity, as low levels of *fimB* expression led to low switching activity of FimB. Despite a comparable effect on *fimB* expression, there is thus a differential effect on FimB recombination in the *gshA* and *gor* mutant. It was therefore suspected that glutathione exerts additional unexpected effects on FimB recombination, which would lead to enhanced activity of FimB in the Δ*gshA* mutant, but not the Δ*gor* mutant. By loss of Gor, oxidised GSSG will not be reduced back to GSH, which hence leads to an accumulation of GSSG in the cell. The Δ*gshA* mutant, on the other hand, will not produce any GSSG. It was therefore supposed that GSSG may have a specific inhibitory effect on FimB recombination. Possible alternative inhibitory factors may be the redox state of the cytoplasm, which could affect FimB recombinase activity, or glutathionylation of FimB.

GSSG is able to glutathionylate proteins by forming mixed disulfide bonds between the cysteine residues of a protein. Thiol groups support the protein structure and are also often part of the active site of a protein (Dalle-Donne *et al.*, 2009a). Post-translational modification by glutathionylation hence is a mechanism to control the activity of a protein. In the case of FimB recombination, it is possible that high levels of GSSG in the Δ*gor*

mutant lead to glutathionylation of a protein that directly or indirectly alters the activity of FimB. Consequently, FimB activity would be enhanced in the Δ*gshA* mutant.

8.6.2. Possible explanations

8.6.2.1. MetE, SAM and spermidine

An alternative explanation for the differential between the *gshA* and *gor* mutant was considered to be a difference in spermidine levels. As described above, the absence of GSH is suggested to elevate levels of polyamines (Chapter 6). Moreover, it has been shown that spermidine stimulates FimB recombination in vitro (Gally *et al.*, 1996). Since the generation of spermidine appears to be limited by SAM levels, it was proposed that excess cysteine in the Δ*gshA* mutant is converted to SAM, which enhances spermidine production. Spermidine, in turn, will stimulate FimB recombination. In the Δ*gor* mutant, however, GSSG could be inhibiting SAM biosynthesis by inactivating MetE. MetE is required for the terminal step in methionine biosynthesis and is reversibly inactivated by GSSG (Hondorp *et al.*, 2004). It therefore seems possible that inactivation of MetE by glutathionylation prevents the stimulatory effect of spermidine in the Δ*gor* mutant. Thus, the hypothesis is that Δ*gshA* and Δ*gor* mutants have a different effect on spermidine levels, which then affect the activity of FimB. For this hypothesis to apply, it is presumed that spermidine and putrescine have different effects on FimB recombination. As has been shown for the lambda integrase recombination system, spermidine stimulates recombination far more efficiently than putrescine (Boldt *et al.*, 2007). Since FimB and FimE are also tyrosine recombinases like the lambda integrase protein, this is likely to be directly relevant to FimB recombination.

However, it should be taken into consideration that the cells are grown in methionine-containing medium. This may mean that there are only low levels of active MetE in the cell, in which case glutathionylation of MetE is not expected to have much of an effect on switching to account for the difference observed between the Δ*gshA* and the Δ*gor* mutants.

8.6.2.2. Aeration

Glutathione is a key reductant in the cell. Any effect of GSH on cellular processes would hence be dependent on the availability of oxygen in the cell growth conditions. In the presence of less oxygen, there would be less requirement for a reducing agent such as

GSH. Any regulatory effect of GSH may therefore be less pronounced under less aerobic conditions.

The β-galactosidase assays were carried out in well-aerated flasks, while the switching experiments were carried out in less well aerated test tubes. Supposing that GSH activates *fimB* expression by acting as a reductant, it is possible that loss of GSH has less effect in the less aerated tubes. Δ*gshA* mutants would hence have higher levels of *fimB* expression in tubes than in flasks. Moreover, this would be consistent with the relatively high frequency of FimB recombination compared to the low level of *fimB* expression measured by β-galactosidase assay in flasks.

With regard to the Δ*gor* mutant, accumulating GSSG could become an oxidant in the less aerated tubes. The requirement for GSH as a reductant will hence be greater in the Δ*gor* mutant background to activate *fimB* expression. In this case, mutation of *gor* would have more impact on *fimB* expression than mutation of *gshA*. This ultimately leads to lower levels of *fimB* expression and thus FimB recombination in the Δ*gor* mutant, as was demonstrated in the switching experiment.

For consistent and comparable results, it would thus be necessary to measure *fimB* expression and FimB recombination under identical conditions. This would eliminate any discrepancy in the activity of GSH and thus allow to directly compare any effect on *fimB* expression to effects on FimB recombination.

8.7. Further experiments to verify the model

There are many experiments that could and should be carried out to clarify and verify the results of this study. Most importantly, it is necessary to measure the polyamine levels in various mutant backgrounds. While the measurement of polyamine levels by GC-MS was not successful (Appendix A), it has in the meantime been possible to arrange for a collaborator to take over this measurement. However, the results were not obtained at the time of submission of this thesis.

The determination of glutathione levels with a commercially available kit was not successful (see Chapter 3). A range of factors were proposed to affect glutathione levels in the cell – such as mutation of *gor* and *cbl*, polyamines or the availability of the individual amino acids cysteine, glutamate and glycine. This could be confirmed by measuring of cellular glutathione levels. Changes in the total glutathione content of the cell, as well as in

the levels of reduced and oxidised glutathione could potentially be determined by HPLC, as described by Cereser *et al.* (2001) or Hiraku *et al.* (2002). An alternative method has been developed with *o*-phthalaldehyde as a fluorescent reagent (Hissin *et al.*, 1976).

In Chapter 3 it was proposed that Cbl regulates levels of periplasmic GSH. This may occur by a regulatory effect on glutathione biosynthesis (via *gshA* and/or *gshB*) or degradation or by repression of *cydD* expression. It would therefore be helpful to carry out RT-PCR to see whether Cbl controls *gshA*, *gshB* or *cydD* expression. Moreover, RT-PCR would also be a useful method to test whether there is a change in either *speA* or *speB* expression in the Δ*gshA* and Δ*gor* mutant backgrounds.

A key hypothesis in this project is the mutual regulation of GSH and polyamine levels. In this context it was proposed that polyamines enhance *gshA* expression by enhancing translation initiation at its weak start codon UUG. This can be tested by mutating the UUG start to a AUG start by site-directed mutagenesis, since the AUG start codon is less sensitive to changes in polyamine levels and thus stimulation by polyamines.

An alternative approach to determine the level of regulation of GSH and the polyamines would be to measure and compare the levels of *fimB* mRNA by quantitative RT-PCR. An effect on transcription would alter the levels of mRNA present in the cell, while levels would remain unaltered in the case of a regulatory effect on *fimB* translation.

Furthermore, results in Chapter 7 show that the low level of *fimB* expression in the Δ*gshA* mutant does not correlate to the relatively high frequency of FimB recombination. As discussed above, it is possible that the availability of oxygen may be a key factor causing this effect. Since the main function of GSH is to act as a reductant, the oxidative state of the cell is likely to play a crucial role in a regulatory effect of GSH. It is possible that the level of *fimB* expression differs between the growth conditions for the β-galactosidase assay and the switching assay, for which reason it would be important to measure the level of *fimB* expression under the same growth conditions as FimB recombination. This would eliminate any potential discrepancy between levels of *fimB* expression and thus allow direct comparison of *fimB* expression and FimB recombination.

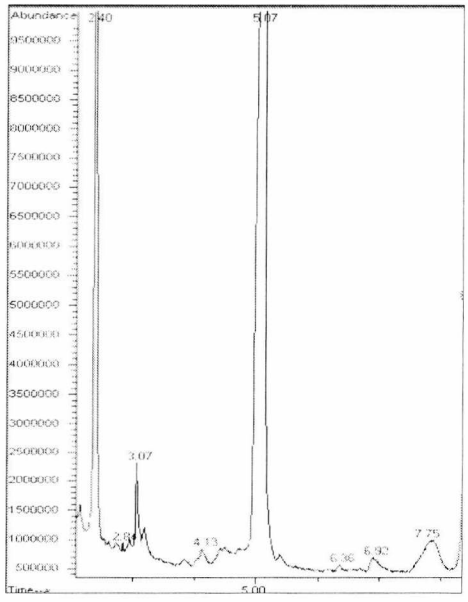
It is possible that exposed cysteine residues in the FimB protein structure are susceptible to oxidation, which may alter the activity of FimB under conditions of oxidative stress caused by the absence of GSH. It would therefore be interesting to investigate the activity of FimB in both reduced and oxidised conditions.

Appendix A

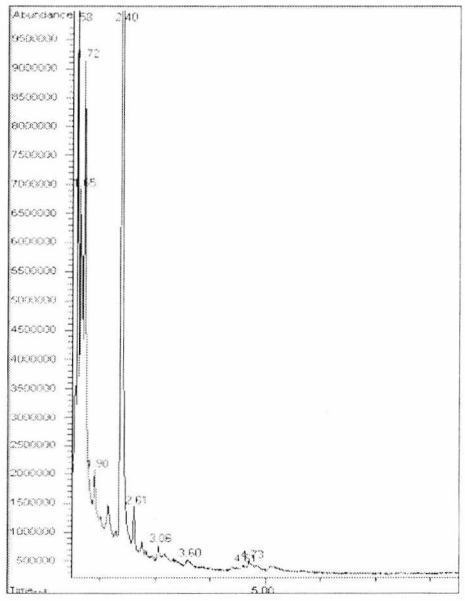
Polyamine levels

A.1 Measurement of polyamine levels by GC-MS

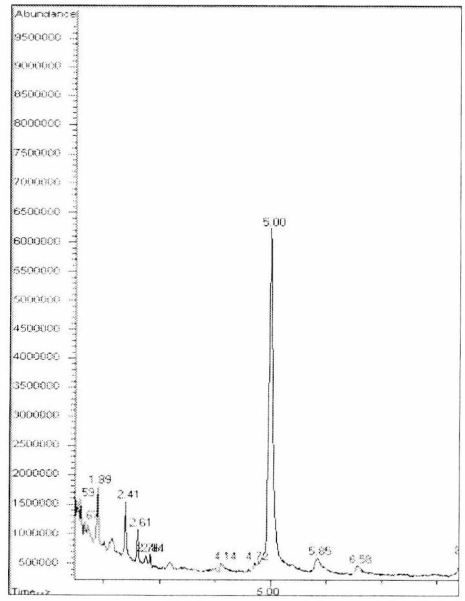
Results obtained so far provide strong evidence that GSH limits levels of polyamines levels. In the absence of GSH, a rise in putrescine levels inhibits *fimB* expression. By measuring the intracellular levels of polyamines in different mutant backgrounds, it should be possible to verify this hypothesis. Moreover, this would also help determine the specific polyamine (putrescine or spermidine) that is associated with the respective response on *fimB* expression. The quantification of polyamines was approached by gas chromatography (GC). According to the protocol described by Khuhawar et al. (1999), putrescine and spermidine were derivatised with trifluoroacetylacetone (FAA) for their subsequent separation and detection. While derivatised putrescine and spermidine was detected in standard solutions (Figure A.1A) this was not successful in the cell samples (Figure A.1D). However, when adding the standard solution of putrescine and spermidine to the cell samples, it was possible to detect a signal for the respective polyamine (Figure A.1B and C). This showed that the method of derivatisation and detection was effective. However, intracellular polyamines were possibly not extracted sufficiently from the cells or the cellular levels of polyamines are below the detection limit, which would explain the failure to detect a signal in cell samples.



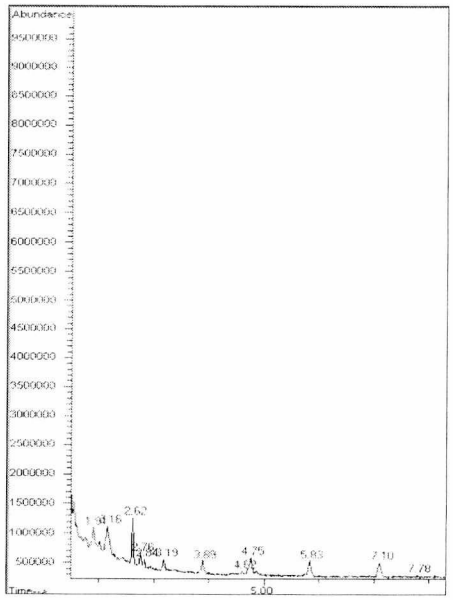
A



B



C



D

Figure A.1: Gas chromatographic separation of A) standard solutions; B) and C) wild type cell sample treated with standard solutions of putrescine and spermidine; D) wild type cell sample.

Appendix B

Thermoregulation

B.1 Temperature Control by GSH

Recombination by FimB and FimE is thermoregulated, presumably at the level of expression of *fimE* and *fimB* (Gally *et al.*, 1993). Moreover, the *fim* switch is affected by H-NS and H-NS repression is responsive to temperature (Olsen *et al.*, 1998). However, it has not been proven that H-NS accounts for the thermoregulation of *fimB* expression and FimB recombination. Since the activating effect of GSH on *fimB* expression is suggested to be linked to H-NS, it was investigated whether GSH is involved in temperature control of *fimB* expression.

In the wild type, *fimB* expression is reduced going from 37°C to 28°C. However, this effect was found to be reversed in the Δ*gshA* mutant background, showing an increase in *fimB* expression at 28°C (Figure B.1). Taken together, these results show that the net stimulatory effect of GSH is diminished at 28°C.

It has been shown that H-NS represses *fimB* expression in a temperature-dependent manner (Olsen *et al.*, 1998). It was therefore proposed that H-NS accounts for this temperature effect by having a greater inhibitory effect on *fimB* expression at reduced temperatures. Preliminary results suggest that the absence of GSH elevates levels of polyamines, which stimulate H-NS synthesis (Terui *et al.*, 2007) and thus increases H-NS activity. In this regard, it may be the case that the change in temperature affects polyamine levels and thus the effect of *gshA* mutation.

However, the mechanism behind this temperature effect was not found. As described in Chapter 3, deletion of the ferric uptake regulator Fur suppressed the Δ*gshA* mutant phenotype. Moreover, the effect of *speA* mutation mirrors the effects of Δ*fur* and temperature. It was therefore proposed that these effects are mediated via polyamine levels. Ferrous iron (Fe^{2+}) is less soluble at lower temperatures. A decreased availability of Fe^{2+} will inactivate Fur and hence induce *ryhB* expression. While the effect of *fur* mutation was shown to be dependent upon RyhB (Chapter 3), it was proposed that RyhB may have a binding site in the *speA* promoter (Chapter 4). It is therefore possible that RyhB decreases putrescine biosynthesis at lower temperatures to give suppression of the Δ*gshA* effect.

Supposing this to be the case, the increased activity of RyhB will cause sub-optimal levels of putrescine in the wild type and hence decrease *fimB* expression. In the Δ*gshA* mutant, however, RyhB will diminish the excess of putrescine. This not only suppresses, but actually reverses the effect of *gshA* mutation.

This hypothesis is consistent with the diminished net stimulatory effect of GSH at 28°C. With diminished putrescine biosynthesis at lower temperatures, there will be less excess putrescine in the absence of GSH. Thus, loss of GSH will not have as much of an inhibitory effect.

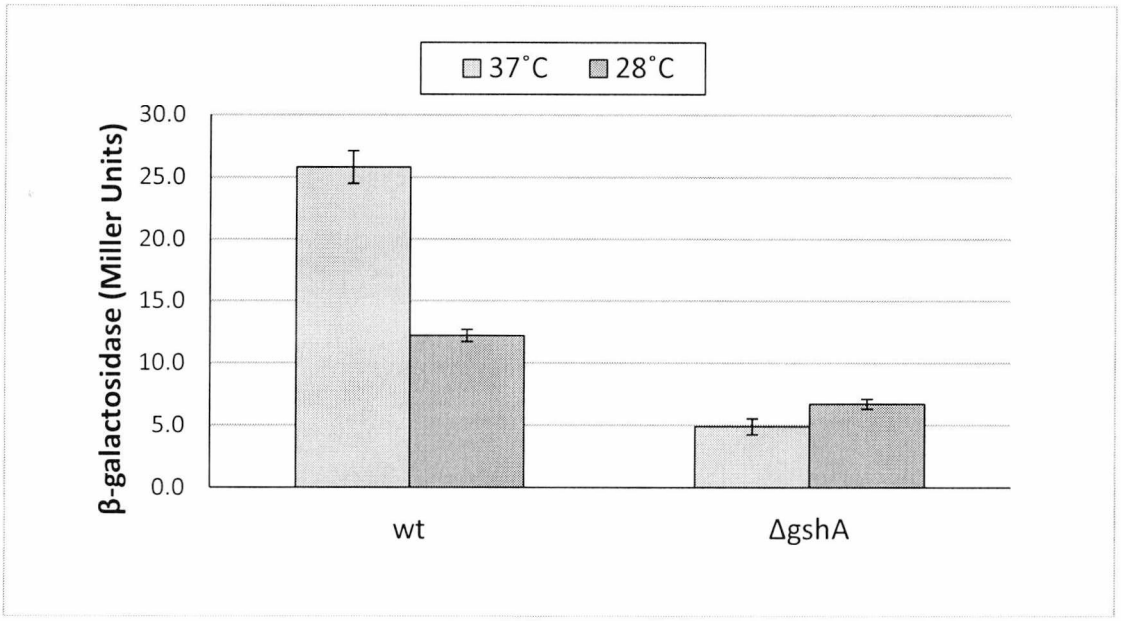


Figure B.1: β-galactosidase assay showing the effect of *gshA* mutation on the temperature response of *fimB* expression at 28°C vs. 37°C. Strains were grown in RD_{gly}. Strains used were BGEC905 and KCEC1627.

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