Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.
The Dps protein of *Salmonella enterica* serovar Typhimurium

by

Jennifer T. Sankey

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

Moyne Institute of Preventive Medicine
School of Genetics and Microbiology
Trinity College Dublin

March 2008
DECLARATION

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

I agree that the Librarian, Trinity College Dublin, may lend or copy this thesis on request.

[Signature]

Jennifer T. Sankey
Summary

As a nucleoid-associated protein, Dps has long been thought to influence gene expression at a global level. In this study the role of Dps in genetic regulation in *Salmonella enterica* serovar Typhimurium was investigated.

Microarray analysis revealed that Dps had little effect on the transcriptional profile of SL1344, few genes responded to its absence. Of the Dps-regulated genes identified one group was involved in amino acid transport and metabolism. Subsequent investigations revealed that Dps has a role to play in the metabolism of SL1344 particularly in stationary phase.

Proteomic analysis revealed that RpoS and H-NS, two global regulators of *S. Typhimurium* were affected by the mutation in Dps. Transcriptional analysis revealed that the *rpoS* mRNA was expressed at a higher level in the *dps* mutant than in the wild-type and that the stability of the mRNA was reduced in the absence of Dps. The RpoS protein was shown to be downregulated by the absence of Dps. The H-NS protein was also shown to be downregulated by the absence of Dps, however the *dps* mutation did not affect the *hns* mRNA. It is postulated that Dps may be affecting the production of a small non-coding RNA thus influencing global regulation in *S. Typhimurium*.

Evidence is presented here for the first time that seems to indicate that the influence Dps has on gene regulation in *S. Typhimurium* is primarily found at the post-transcriptional level, distinguishing it from other members of the nucleoid-associated family of proteins.
Acknowledgements

My sincere thanks to Charlie for giving me the opportunity to work in his lab and in particular for his advice throughout this project. I would also like to thank my thesis committee, Tim, Cyril and Stephen, for their helpful insights over the past four years. Thanks also to my collaborators Jay Hinton and Stephen Smith.

Thank you to all the prep room staff Paddy, Joe, Ronan, Fionnuala, Margaret, Dave and Henry for keeping me well stocked with unusual media and implements.

To Connie thank you so much for your help over the years and in particular for allowing me to read the Viking catalogue every so often. To everyone in the Dorman Lab who helped especially Dr Tadhg O’Croinin, Kirsty McFarland and Dr Dan Stoebel.

To my friends from home, Michelle, Lou, Michele and Louise thanks for keeping me sane.

To my brother, sister, sister-in-law and brother-in-law to be, thanks for bringing me back to normality when I need it. To my Goddaughter Eva thank you for brightening the world.

Finally a huge thank you to my parents for their love and support I could never have done it without you
For my parents
with thanks
Table of Contents

Title page 1
Declarations II
Summary III
Acknowledgments IV
Table of Contents VI
List of Figures XII
List of Tables XV

Chapter 1  General Introduction 1

1.1  \textit{Salmonella enterica} 2
1.2  Infection by \textit{S. enterica} 2
1.2.1  \textit{Salmonella} pathogenicity islands 3
1.2.2  \textit{Salmonella} in macrophage 4
1.3  Stationary Phase 4
1.4  Genetic regulation 6
1.4.1  Regulation of transcription 6
1.4.1.1  Promoter regions and $\sigma$ factors 6
1.4.1.2  Transcription factors 7
1.4.1.3  DNA Supercoiling 8
1.4.2  Posttranscriptional regulation 9
1.4.3  Posttranslational regulation by proteolysis 11
1.5  The bacterial nucleoid 11
1.5.1  Nucleoid-associated proteins 12
1.5.2  Fis 12
1.5.3  HU 13
1.5.4  IHF 13
1.5.5  H-NS 14
1.6  Dps 14
1.6.1  The structure of Dps 15
1.6.2  DNA binding by Dps 15
1.6.3  Ferritins and other iron storage proteins 16
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6.4</td>
<td>Protection from oxidative stress by Dps</td>
<td>17</td>
</tr>
<tr>
<td>1.6.5</td>
<td>Regulation of $dps$ gene expression</td>
<td>17</td>
</tr>
<tr>
<td>1.6.6</td>
<td>Regulation of Dps Protein</td>
<td>17</td>
</tr>
<tr>
<td>1.7</td>
<td>Aim of this study</td>
<td>18</td>
</tr>
</tbody>
</table>

**Chapter 2 Materials and methods**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Chemicals and reagents</td>
<td>20</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Growth media, Antibiotics, X-Gal and IPTG</td>
<td>20</td>
</tr>
<tr>
<td>2.2</td>
<td>Bacterial strains and culture conditions</td>
<td>23</td>
</tr>
<tr>
<td>2.3</td>
<td>Plasmids and oligonucleotides</td>
<td>23</td>
</tr>
<tr>
<td>2.4</td>
<td>Creation of the $dps$ mutant JTS004</td>
<td>24</td>
</tr>
<tr>
<td>2.5</td>
<td>Transformation of bacterial cells with plasmid DNA</td>
<td>24</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Preparation and transformation of calcium chloride-competent cells</td>
<td>24</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Preparation and transformation of electro-competent cells</td>
<td>25</td>
</tr>
<tr>
<td>2.6</td>
<td>Transduction with bacteriophage P22</td>
<td>26</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Preparation of P22 phage lysate</td>
<td>27</td>
</tr>
<tr>
<td>2.6.2</td>
<td>P22 phage transduction</td>
<td>27</td>
</tr>
<tr>
<td>2.7</td>
<td>Oxidative stress assay</td>
<td>27</td>
</tr>
<tr>
<td>2.8</td>
<td>Acid stress assay</td>
<td>28</td>
</tr>
<tr>
<td>2.9</td>
<td>Spectrophotometric assays</td>
<td>28</td>
</tr>
<tr>
<td>2.9.1</td>
<td>Monitoring bacterial growth</td>
<td>28</td>
</tr>
<tr>
<td>2.9.2</td>
<td>Determination of nucleic acid concentration</td>
<td>28</td>
</tr>
<tr>
<td>2.9.3</td>
<td>Determination of protein concentration by the Bradford assay</td>
<td>28</td>
</tr>
<tr>
<td>2.10</td>
<td>Assays of $gfp$ reporter gene expression by flow cytometry</td>
<td>29</td>
</tr>
<tr>
<td>2.11</td>
<td>Preparation of nucleic acids</td>
<td>29</td>
</tr>
<tr>
<td>2.11.1</td>
<td>Small-scale isolation of high purity plasmid DNA</td>
<td>29</td>
</tr>
<tr>
<td>2.11.2</td>
<td>Large-scale isolation of high purity plasmid DNA</td>
<td>29</td>
</tr>
<tr>
<td>2.11.3</td>
<td>Purification of chromosomal DNA</td>
<td>30</td>
</tr>
<tr>
<td>2.11.4</td>
<td>Isolation of RNA</td>
<td>30</td>
</tr>
<tr>
<td>2.12</td>
<td>Manipulation of DNA in vitro</td>
<td>31</td>
</tr>
<tr>
<td>2.12.1</td>
<td>Restriction endonuclease cleavage of DNA</td>
<td>31</td>
</tr>
</tbody>
</table>
2.12.2 Phosphatase treatment of restriction endonuclease-cleaved DNA
2.12.3 Purification of linear DNA
2.12.4 Ligation of DNA molecules
2.12.5 Ethanol precipitation of DNA/RNA
2.13 Polymerase Chain Reaction (PCR)
2.13.1 Amplification of DNA
2.13.2 Reverse Transcriptase-PCR (RT-PCR)
2.13.2.1 cDNA synthesis and amplification
2.14 RNA stability assay
2.15 Gel electrophoresis
2.15.1 Agarose gel electrophoresis
2.15.2 SDS-PAGE
2.15.3 Staining of proteins
2.16 Northern blotting
2.16.1 Denaturing electrophoresis of RNA
2.16.2 Transfer of the resolved RNA to nylon membrane
2.16.3 DIG (Digoxigenin) probe synthesis
2.16.4 Hybridisation and detection of DIG probe
2.17 Southern blotting
2.17.1 Electrophoresis, denaturation and transfer of DNA to nylon membrane
2.18 Western immunoblot analysis
2.18.1 Preparation of total cellular protein extracts
2.18.2 Transfer of proteins to nitrocellulose membrane
2.18.3 Detection of bound antigens
2.19 Autoradiography
2.20 DNA microarray analysis
2.20.1 RNA isolation
2.20.2 cDNA synthesis and Cy5-dye labeling
2.20.3 Cy3-labelling of genomic DNA
2.20.4 1,2-dichloroethane (DCE) blocking of microarray slides
2.20.5 Hybridisation of DNA to slides
2.20.6 Scanning of slides and microarray data handling 45
2.21 2-D PAGE 45
2.21.1 Protein sample preparation 45
2.21.2 Immobilized pH gradient (IPG) strip loading 46
2.21.3 First dimension isoelectric focusing 46
2.21.4 Equilibration and second dimension SDS-PAGE 47
2.21.5 Protein spot data handling 47
2.21.6 Spot Excision and identification 47
2.22 Tissue cell culture 48
2.22.1 Epithelial cell invasion assays 48
2.22.2 Macrophage survival assays 49
2.22.3 Intracellular expression profile of $dps$ 49
2.23 Phenotypic Array 50
2.23.1 Culture preparation 50
2.23.2 Phenotype microarray panel loading 51
2.23.3 Phenotype microarray panel reading 51
2.24 Relative fitness assay 51

Chapter 3 Characterisation of the role of the Dps protein of *Salmonella enterica* serovar Typhimurium 53

3.1 Introduction 54
3.2 Results 56
3.2.1 Examination of SL1344$dps$ 56
3.2.2 Construction of the $dps$ mutant JTS004 57
3.2.3 Genetic confirmation of the $dps$ mutation 57
3.2.4 The effect of the $dps$ mutation on growth of *S. Typhimurium* in batch culture 59
3.2.5 Phenotypic confirmation of the $dps$ mutation 59
3.2.6 Complementation of $dps$ in trans 60
3.2.7 Phenotypic confirmation of the complementation of $dps$ in trans 60
3.2.8 Construction of a $dps$ promoter fusion plasmid 61
3.2.9  
*dps* promoter activity is not subject to autoregulation  
61

3.2.10  
Intracellular characterisation of Dps  
62

3.2.10.1  
Effect of Dps on invasion/uptake  
62

3.2.10.2  
Effect of Dps on survival in macrophage  
63

3.2.10.3  
Intracellular expression of *dps*  
64

3.2.10.4  
Effect of Dps on SPI-2 regulators *ssrA* and *ssrB*  
65

3.3  
Discussion  
66

**Chapter 4**  
Microarray transcriptomic analysis of the Dps regulon of  
*Salmonella enterica* serovar Typhimurium  
72

4.1  
Introduction  
73

4.2  
Results  
75

4.2.1  
DNA microarray analysis to determine the Dps regulon  
75

4.2.2  
The global transcription profile  
76

4.2.3  
Amino acid transport and metabolism  
77

4.2.3.1  
Branched-chain amino acid transport  
77

4.2.4  
Motility gene expression  
79

4.2.5  
Regulator gene expression  
79

4.2.6  
Iron regulated genes  
79

4.2.7  
Virulence gene expression  
80

4.2.8  
Stress response gene expression  
80

4.3  
Discussion  
81

**Chapter 5**  
2-D PAGE proteomic analysis of the Dps regulon of *Salmonella enterica* serovar Typhimurium  
83

5.1  
Introduction  
84

5.2  
Results  
87

5.2.1  
2D-PAGE proteome analysis to determine the Dps regulon  
87

5.2.2  
The protein expression profiles of SL1344 and JTS004 in
stationary phase

5.2.3 Impact of the \textit{dps} mutation on carbon catabolism in \textit{S. Typhimurium} 88

5.2.4 Effect of the mutation of \textit{dps} on growth in low iron concentration 91

5.2.5 The protein expression profiles of SL1344 and JTS004 in exponential phase 93

5.2.6 The \textit{dps} mutation has no significant effect on morphology of \textit{S. Typhimurium} 93

5.2.7 The \textit{dps} mutant has lower levels of H-NS than wild-type SL1344 94

5.2.8 The \textit{dps} mutation does not display reduced motility 94

5.2.9 The effect of the \textit{dps} mutation on flagellin subunits 95

5.2.10 The \textit{dps} mutant has lower levels of RpoS than wild-type SL1344 96

5.2.11 The \textit{dps} mutant has higher levels of \textit{rpoS} transcript than wild-type SL1344 97

5.3 Discussion 98

Chapter 6 The effect of Dps on global regulators RpoS and H-NS in \textit{S. Typhimurium} 108

6.1 Introduction 109

6.2 Results 111

6.2.1 The \textit{dps} mutant has higher levels the major transcript of \textit{rpoS} 111

6.2.2 The effect of the \textit{dps} mutation on the stability of \textit{rpoS} mRNA is dependent on growth phase 111

6.2.3 The mutation in \textit{dps} has no effect on the level of expression of \textit{hns} 113

6.2.4 The mutation in \textit{dps} has no effect on the stability of \textit{hns} mRNA 113

6.3 Discussion 114

XI
Chapter 7  General discussion  117

Bibliography  125
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic model of <em>Salmonella</em> survival in macrophage and subsequent dissemination.</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Genetic structure and organization of <em>Salmonella</em> pathogenicity island-2 (SPI-2).</td>
<td>4</td>
</tr>
<tr>
<td>1.3</td>
<td>Small RNA regulators in global regulation cascades.</td>
<td>10</td>
</tr>
<tr>
<td>1.4</td>
<td>Translational regulation of rpoS by the sRNA DsrA</td>
<td>10</td>
</tr>
<tr>
<td>1.5</td>
<td>Crystal structure of (A) Dps (B) Ferritin</td>
<td>17</td>
</tr>
<tr>
<td>3.1</td>
<td>Investigation of SL1344<em>dps</em> from the IFR</td>
<td>56</td>
</tr>
<tr>
<td>3.2</td>
<td>Molecular examination of SL1344<em>dps</em></td>
<td>57</td>
</tr>
<tr>
<td>3.3</td>
<td>Generation of the <em>dps</em> mutant JTS004</td>
<td>57</td>
</tr>
<tr>
<td>3.4</td>
<td>Molecular examination of SL1344 and JTS004</td>
<td>58</td>
</tr>
<tr>
<td>3.5</td>
<td>Growth of SL1344 and JTS004 in batch culture</td>
<td>59</td>
</tr>
<tr>
<td>3.6</td>
<td>Phenotypic testing of JTS004</td>
<td>60</td>
</tr>
<tr>
<td>3.7</td>
<td>Phenotypic confirmation of the complementation of <em>dps</em> in <em>trans</em></td>
<td>60</td>
</tr>
<tr>
<td>3.8</td>
<td>Cloning of the <em>dps</em> promoter region into plasmid pZep08</td>
<td>61</td>
</tr>
<tr>
<td>3.9</td>
<td><em>dps</em> is not subject to autoregulation.</td>
<td>61</td>
</tr>
<tr>
<td>3.10</td>
<td>The effect of the <em>dps</em> mutation on S Typhimurium invasion of CHO-K1 epithelial cells</td>
<td>62</td>
</tr>
<tr>
<td>3.11</td>
<td>The effect of the <em>dps</em> mutation on S Typhimurium uptake by J774 macrophage-like cells</td>
<td>63</td>
</tr>
<tr>
<td>3.12</td>
<td>The effect of the <em>dps</em> mutation on intracellular survival</td>
<td>63</td>
</tr>
<tr>
<td>3.13</td>
<td>Intracellular expression of <em>dps</em></td>
<td>64</td>
</tr>
<tr>
<td>3.14</td>
<td>The intracellular levels of Fis do not change in the <em>dps</em> mutant.</td>
<td>65</td>
</tr>
<tr>
<td>3.15</td>
<td>Effect of the <em>dps</em> mutation on SPI-2 regulators <em>ssrA</em> &amp; <em>SsrB</em></td>
<td>65</td>
</tr>
<tr>
<td>4.1</td>
<td>Impact of <em>dps</em> mutation on the transcriptome of SL1344</td>
<td>76</td>
</tr>
<tr>
<td>4.2</td>
<td>Genetic organisation of <em>livFGMHK</em> and <em>livFGMHJ</em> operons.</td>
<td>77</td>
</tr>
<tr>
<td>4.3</td>
<td>Dps affects LS and LIVII</td>
<td>77</td>
</tr>
<tr>
<td>4.4</td>
<td>The flagellar regulatory network.</td>
<td>79</td>
</tr>
<tr>
<td>5.1</td>
<td>The protein expression profile of SL1344 and JTS004 in stationary phase</td>
<td>88</td>
</tr>
<tr>
<td>5.2</td>
<td>Outline diagram of the Tri-carboxylic acid (TCA) cycle</td>
<td>88</td>
</tr>
<tr>
<td>5.3</td>
<td>JTS004 has no growth defect associated when growing on acetate</td>
<td>88</td>
</tr>
</tbody>
</table>
as the sole carbon source

5.4 The effect of the *dps* mutation on the fitness of *S. Typhimurium* growing in defined carbon sources

5.5 The effect of the *dps* mutation on expression of selected genes of the TCA cycle and glyoxylate bypass

5.6 Effect of the *dps* mutation on the fitness of *S. Typhimurium* growing in the presence of the iron chealator 2,2-dipyridyl

5.7 Effect of the *dps* mutation on growth in low iron concentration

5.8 The protein expression profiles of SL1344 and JTS004 in exponential phase

5.9 The intracellular level of H-NS is reduced in JTS004

5.10 Effect of the *dps* mutation on *S. Typhimurium* motility

5.11 Effect of the *dps* mutation on the intracellular levels of flagella proteins and on flagella transcription

5.12 Effect of the *dps* mutation on the *hin* switch of *S. Typhimurium*

5.13 The intracellular level of RpoS is reduced in JTS004

5.14 The levels of *rpoS* transcript are increased in the *dps* mutant

6.1 Expression of *rpoS* from the major *rpoS* promoter is increased in *dps* mutant JTS004

6.2 Stability of *rpoS* mRNA is maintained in the *dps* mutant at exponential phase

6.3 Stability of *rpoS* mRNA is not maintained in the *dps* mutant at stationary phase.

6.4 The mutation in *dps* has no effect on the transcription of *hns*.

6.5 Stability of *hns* mRNA is not affect by the mutation in *dps* at exponential phase

6.6 Stability of *hns* mRNA is not affect by the mutation in *dps* at stationary phase.
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Strains used in this study</td>
<td>23</td>
</tr>
<tr>
<td>2.2</td>
<td>Plasmids used in this study</td>
<td>23</td>
</tr>
<tr>
<td>2.3</td>
<td>Oligonucleotides used in this study</td>
<td>24</td>
</tr>
<tr>
<td>4.1</td>
<td>12-h Gene Expression Difference 1.5-fold FDR 0.10</td>
<td>76</td>
</tr>
<tr>
<td>4.2</td>
<td>4-h Gene Expression Difference 1.5-fold FDR 0.10</td>
<td>76</td>
</tr>
<tr>
<td>4.3</td>
<td>22-h Gene Expression Difference 1.5-fold FDR 0.10</td>
<td>76</td>
</tr>
<tr>
<td>4.4</td>
<td>Genes involved in amino-acid transport and metabolism affected by the <em>dps</em> mutation</td>
<td>77</td>
</tr>
<tr>
<td>4.5</td>
<td>Fitness measurements</td>
<td>78</td>
</tr>
<tr>
<td>4.6</td>
<td>Motility genes affected by the <em>dps</em> mutation</td>
<td>79</td>
</tr>
<tr>
<td>4.7</td>
<td>Virulence genes affected by the <em>dps</em> mutation</td>
<td>79</td>
</tr>
<tr>
<td>5.1</td>
<td>Carbon sources contained in phenotype microarray</td>
<td>89</td>
</tr>
<tr>
<td>5.2</td>
<td>OD_{540nm} values for the carbon sources outlined in table 5.1</td>
<td>89</td>
</tr>
<tr>
<td>5.3</td>
<td>Carbon sources whose catabolism was affected in JTS004</td>
<td>89</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
1.1 *Salmonella enterica*

*Salmonella enterica* is a species of *Salmonella* associated with infection of a wide variety of animal hosts including humans, chickens, mice, pigs and cattle (Ohl and Miller, 2001). *Salmonella* infections are acquired through the ingestion of contaminated food or water, the symptoms of the disease caused by infection with *Salmonella*, in humans, range from a self-limiting gastroenteritis to typhoid fever and bacteraemia (Ohl and Miller, 2001). Serovars such as *S. enterica* serovar Typhi (*S. Typhi*) and *S. enterica* serovar Paratyphi (*S. Paratyphi*) can result in a systemic infection called typhoid fever (Ohl and Miller, 2001). The pathology associated with such infections is macrophage infiltration and hypertrophy of the reticuloendothelial system, including the intestinal Peyer’s patches, mesenteric lymph nodes, spleen and bone marrow. Mortality without treatment is 10-15% (Ohl and Miller, 2001). Infection with the non-typhoidal serovars of *S. enterica* such as *S. enterica* serovar Typhimurium (*S. Typhimurium*) and *S. enterica* serovar Enteriditis (*S. Enteriditis*) can result in a self-limiting gastroenteritis, characterized by diarrhoea in healthy individuals, however bacteraemia can occur in rare instances in the young and immunocompromised (Jones and Falko, 1996; Wallis and Galyov, 2000). *S. Typhimurium* is a particularly well-studied serovar of *S. enterica*, as in the mouse it invades epithelial cells and survives and proliferates in macrophages before entering the blood stream and causing a systemic infection similar to the pathology associated with *S. Typhi* in humans (Kingsley and Baumler, 2000).

1.2 *Infection by S. enterica*

As *Salmonella* infections are acquired through the ingestion of contaminated food or water the bacteria must, following oral ingestion, survive the acidic pH of the stomach before entering the small intestine. Once inside the small intestine the bacteria traverse the intestinal mucosal layer and adhere to intestinal epithelial cells, primarily specialized M cells of Peyers’s patches, by means of the fimbriae found on the surface of the bacteria (Clegg *et al.*, 1996; Darwin and Miller, 1999; Finlay and Brumell 2000; Jones *et al.*, 1994; Lucas and Lee, 2000). *Salmonella* promotes its own uptake into the epithelial cells by translocating a series of effector proteins into the host cell via an apparatus known as a bacterial type III secretion system (TTSS) (Finlay and Brumell, 2000; Galan, 1996). Once these effector proteins have been injected into the
host cell they induce it to rearrange its actin cytoskeleton in the vicinity of the adhering bacteria in a way that results in denuding of the microvilli brush border on the surface of the host cell and subsequent ruffling of the cell membrane around the bacteria (Finlay and Brumell, 2000; Galan, 2001). Once the membrane has surrounded the bacteria, they are engulfed by the host cell by a process know as bacterial-mediated endocytosis (Francis et al., 1992). Following internalisation into vesicles the bacteria can transcytose to the basolateral surface of the intestinal epithelia where they exit and encounter host macrophages (Alpuche-Aranda et al., 1994). The bacteria are phagocytosed by the macrophages. *Salmonella* can survive and replicate in macrophages despite the low pH and presence of reactive oxygen species, the bacteria are localized within a membrane compartment known as the *Salmonella*-containing vacuole (SCV) (Fig 1.1). A separate TTSS and related effector proteins are necessary for bacterial survival in macrophage (Ochman et al., 1996; Cirillo et al., 1998; Hensel et al., 1998). The presence of replicating *Salmonella* in the macrophage will eventually lead to apoptosis of the macrophage and release and proliferation of the bacteria through the lymphatics and bloodstream, giving rise to a systemic infection (Hueffer and Galan 2004; Ohl and Miller 2001).

1.2.1  *Salmonella* pathogenicity islands

The genes responsible for the production of the specific TTSS and effector proteins needed for invasion of epithelial cells are located in a region of the *Salmonella* genome that has a different GC content and codon usage to the rest of the genome as a whole. Such regions of the genome are known as pathogenicity islands, and are thought to have been acquired by horizontal transfer (Groisman and Ochman 1997, Ochman et al., 2000). *Salmonella* pathogenicity island-1 (SPI-1) is located at 63 centisomes on the *S. Typhimurium* chromosome, contains at least 35 genes and is approximately 40 kb in size (Collazo and Galan, 1997). The regulation of SPI-1 genes is complex and involves many factors (Eichelberg and Galan 1999; Altier, 2005). At the molecular level transcriptional activators, *hilA*, *hilC*, *hilD* and *invF* encoded on SPI-1 are essential for SPI-1 induction (Bajaj et al., 1995; Boddicker et al., 2003; Darwin and Miller, 1999; Lucas and Lee 2001; Schechter et al., 2003). In turn expression of these activators is regulated by a number of global regulatory proteins including the nucleoid-associated proteins, FIS (Wilson et al., 2001; Kelly et
Fig 1.1. Schematic model of Salmonella survival in macrophage and subsequent dissemination. Salmonella serotypes that cause systemic infection are engulfed by macrophages and reside in a Salmonella containing vacuole (SCV) where they have the ability to survive and replicate. Salmonella pathogenicity island-2 is essential for survival and proliferation in macrophages. Subsequent migration of infected phagocytes to other organs of the reticuloendothelial system allows dissemination of Salmonella in the host. Figure adapted from Kelly (2005).
al., 2004), IHF (Mangan et al., 2006), Hha (Fahlen et al., 2001) H-NS (Schechter et al., 2003).

Salmonella pathogenicity island-2 (SPI-2) is located at 30.7 centisomes on the S. Typhimurium chromosome is 40 kb in size and contains 40 genes (Shea et al., 1996). SPI-2 is divided into two genetic elements a 25-kb region and a 15-kb region. It is the 25 kb region that is required for virulence (Fig 1.2) (Hensel et al., 1999). The SSrA/B two-component signal transduction system encoded on SPI-2 by the ssrA and ssrB regulatory genes is required for the activation of the SPI-2 TTSS (Feng et al., 2004; Walthers et al., 2007). It has been reported that SPI-2 is expressed prior to penetrating the intestine (Brown et al., 2005). The OmpR/EnvZ two compoment signal transduction system activates expression of SsrA/B, OmpR binds directly upstream of the ssrA promoter stimulating transcription (Feng et al., 2003, 2004; Lee et al., 2000). The transcriptional regulator SlyA also affects ssrA/B gene expression (Linehan et al., 2005, Navarre et al., 2005).

1.2.2 Salmonella in macrophage

On exposure to certain stimuli macrophages manufacture O$_2^-$ (superoxide) by the reduction of oxygen at the expense of NADPH (Babior, 2000). Most of the O$_2^-$ reacts with itself to form H$_2$O$_2$ (Babior, 2000). These compounds are not however used by the phagocytes to kill microbes rather they function as starting materials for the production of microbial oxidants such as oxidized halogens and oxidizing radicals (Babior, 1984). O$_2^-$ and H$_2$O$_2$ can participate in the Fenton reaction catalyzed by Fe (II) or Cu (I) to produce the hydroxyl radical (Fenton, 1894) (Section 1.6.3).

Secreted effector proteins are purported to have a role in preventing trafficking of the macrophage NADPH oxidase to the SCV enabling it to avoid exposure to the damaging effects of the respiratory burst (Gallois et al., 2001).

1.3 Stationary Phase

At the most basic level, populations of bacteria tend to go through a four-phase growth cycle (Novick, 1955). Lag phase occurs as the population is adapting to a new environment and the rate of death equals the rate of growth. Log phase arises as the
population has adapted to its new environment and exponential growth occurs, reproduction rates are greater than death rates. Stationary phase takes place as the population responds to a limiting factor such as nutrients, and a state of equilibrium is reached with growth rates equaling the death rates. Finally little or no reproduction occurs in the death phase and the population declines (Novick, 1955).

Adaptation to stationary phase has many implications for the cell. In stationary phase cells the ATP/ADP ratio changes as energy generating processes become progressively ineffective due to lack of essential substrates (Matin, 1991). This can affect the activity of DNA gyrase and in turn affect the activity of genes subject to control via negative supercoiling (Drlica 1992). During stationary phase imposed by limited nutrients non-sporulating bacteria such as Salmonella and E. coli respond by inducing systems responsible for the scavenging of extracellular nutrients and for protecting the cell against various other stresses (Matin, 1991). The best characterised model for nutrient starvation is represented by glucose starvation and the key regulator in the response to this response is the sigma factor RpoS (σ^5, σ^38). In exponential cells the level of RpoS is maintained at a low rate through the action of the protease ClpXP in conjunction with the response regulator RssB (Zhou et al., 2001). It is not know what signal induces the cell to disrupt the proteolysis when starvation is sensed. The expression of genes responsible for the production of enzymes involved in energy production in the tricarboxylic acid (TCA) cycle such as malate dehydrogenase, isocitrate dehydrogenase and succinate dehydrogenase is decreased in response to glucose-limiting conditions (Nyström, 1994), however the abundance of transport proteins increases (Wick et al., 2001).

Morphological changes also occur during stationary phase. The Dps protein is regulated by IHF and RpoS, during stationary phase levels of the protein increase through stationary phase until it reaches approximately 180,000 molecules per cell (Ali Azam et al., 1999). In stationary phase cells Dps induces significant compaction of the chromosomal DNA (Frenkiel-Krispin et al., 2001). Dps in complex with plasmid DNA is proposed to form stacked alternative layers with the DNA sequestered in a crystal lattice (Frenkiel-Krispin et al., 2001; Wolf et al., 1999).
Fig 1.2 Genetic structure and organization of *Salmonella* pathogenicity island-2 (SPI-2). The 25-kb virulence region of SPI-2 includes genes encoding a type III secretion apparatus (*ssa*, blue boxes), regulatory proteins (*ssr*, green boxes), effector protein (*sse*, orange boxes) and chaperones (*ssc* purple boxes). Figure adapted from Carroll (2003).
1.4 Genetic regulation

There are various stages at which expression of a gene can be regulated, from DNA-to-RNA transcription through to the posttranslational modification of a protein. Thus affording the cell a step-wise ability to regulate genetic regulation as the occasion arises.

1.4.1 Regulation of transcription

Transcription consists of three stages: initiation, elongation and termination. The $\sigma$ factor of the RNA polymerase holoenzyme binds to the promoter region (often located at $-35$ to $-10$ with respect to the transcription start site, $+1$) of the gene being transcribed this is the closed complex (Record et al., 1996). The RNA polymerase opens up the DNA helix to form an open complex, exposing the bases to create a single-stranded template or transcription bubble (Record et al., 1996). The first two ribonucleotides enter the complex and base-pair to the template strand. RNA polymerase forms the phosphodiester bond between them with the release of diphosphate. The $\sigma$ factor is released from the holoenzyme and the core RNA polymerase proceeds down the DNA template, as the transcription bubble moves down the DNA molecule the newly synthesized RNA is disassociated from the DNA and the DNA helix is reformed behind the transcription bubble (Record et al., 1996). Specific sequences in the DNA signal the end of the gene. Transcription ceases and RNA polymerase and the completed mRNA chain are released from the DNA (Record et al., 1996).

1.4.1.1 Promoter regions and $\sigma$ factors

To date six $\sigma$ factors have been identified in Salmonella each has a different promoter recognition site. RpoD or $\sigma^{D70}$ is the housekeeping $\sigma$ factor and is responsible for the transcription of the majority of genes in Salmonella. RpoS ($\sigma^{S^{38}}$) is responsible for the transcription of stationary phase genes, the other $\sigma$ factors RpoN ($\sigma^{N^{54}}$), FliA ($\sigma^{28}$), RpoH ($\sigma^{H^{12}}$) and RpoE, ($\sigma^{E^{24}}$) contribute to the control of nitrogen-regulated genes, various flagella genes, heat-shock genes and envelope stress related genes respectively (Gruber and Gross, 2003). The consensus sequence for $\sigma^{70}$ regulated promoters is TATAAT at the $-10$ site and TTGACA at the $-35$ box. No clear distinct consensus sequence has been found for $\sigma^{5}$ dependent promoters however primer
extension analysis of a number of $\sigma^5$ genes suggest a proposed consensus in the -10 region of TGN0-2CYATAM with a lack of conservation observed in the -35 promoter element (Lacour et al., 2004).

1.4.1.2 Transcription factors

Transcription factors can be activators or repressors. Repressors such as the LeuO protein can bind to non-coding sequences on the DNA strand that are close to or overlapping the promoter region (Repoila and Gottesman 2001), preventing RNA polymerase from binding to the DNA and thus precluding expression of the gene.

Other mechanisms of repression include confining RNA polymerase within a looped structure preventing elongation of the mRNA transcript as occurs in the H-NS mediated repression of the rrrBl ribosomal gene promoter (Dame et al., 2000). H-NS is composed of two distinct functional domains separated by a flexible linker region. The N-terminal domain contains the oligomerization activity of the protein while the C-terminal domain is responsible of the DNA binding function of the protein (Dorman 1999, 2004, 2007). H-NS binds to DNA regions of intrinsic curvature such as occur in the vicinity of promoters (Jauregui et al., 2003). With regard to the repression of the rrrBl ribosomal gene promoter the binding of RNA polymerase to the promoter region loops the DNA around the polymerase bringing two regions of intrinsically curved DNA upstream and downstream of the promoter closer together (Dame et al., 2000). H-NS dimers binds to these curved regions and oligomerise along the DNA in effect “zippering” the area and trapping the RNA polymerase at the initiation stage of transcription repressing the promoter (Dame et al. 2000). The nucleoprotein complex that is formed by H-NS and the DNA can be disrupted by environmental signals such as elevated temperature or positively acting transcription factors.

Both mechanisms of regulation are proposed for the virF gene in Shigella flexneri and enteroinvasive E. coli. Two regions of the virF promoter region have H-NS binding sites. Binding of H-NS to these sites and protein-protein interaction leads to the formation of the repression loop in the DNA preventing transcription (Prosseda et al., 2004). The H-NS-mediated repression of the promoter occurs at temperatures below
32°C. At elevated temperatures melting of the bend, between the two H-NS binding sites, occurs and the H-NS nucleoprotein repression complex is disrupted (Prosseda et al., 2004).

Activators enhance the interaction between RNA polymerase and the promoter. This enhancement can be achieved by direct contact between the transcription factor and RNA polymerase or indirectly by altering the conformation of the DNA around the promoter region. Many promoters that are subjected to repression by H-NS are also controlled by sequence specific transcriptional activators. Such activators can antagonise the repressive activity of H-NS they act as anti-repressors (Beloin and Dorman 2003, Falconi et al., 2001). Fis binding sites are also present at the virF promoter. The Fis protein can bind to one of the H-NS sites, such binding by Fis can hinder H-NS binding, contributing to the disruption of the repression complex. In addition the binding of Fis and subsequent bending of the DNA alters the proximity of the two H-NS sites to each other hindering the H-NS-H-NS interaction which also destabilises the repression loop derepressing transcription (Falconi et al., 2001).

1.4.1.3 DNA Supercoiling
The transcriptional activity of many promoters is strongly dependent on the negative superhelical density of chromosomal DNA (Dorman 1995, Hatfield and Benham 2002, Travers and Muskhelishvili 2005). The DNA isolated from prokaryotes is usually negatively supercoiled (Drlica, 1992). DNA gyrase introduces negative supercoils into the DNA in an ATP-dependent manner (Drlica, 1992; Steck et al., 1993). DNA topoisomerase I relaxes negatively supercoiled DNA in an ATP-independent manner (Drlica, 1992; Steck et al., 1993). The promoters for the genes encoding these topoisomerasases are sensitive to the levels of supercoiling in the cell (Menzel and Gellert, 1983). The increase of negative DNA supercoiling by DNA gyrase is countered by activation of topA expression and increased levels of topoisomerase I relaxing negative supercoiled DNA (Menzel and Gellert, 1983). Conversely a decrease in negatively supercoiled DNA activates expression of the gyrA and gyrB promoters and increases DNA gyrase production restoring negative supercoils into the DNA (Menzel and Gellert, 1983).
The activity of DNA gyrase is linked to the physiological condition of the cell as it is controlled by the ATP/ADP ratio in the cell (Drlica 1992). As ATP levels fall and ADP levels rise, the negative supercoiling ability of gyrase is inhibited due to its dependence on ATP to drive the thermodynamically unfavourable process (Drlica 1992). Such reductions in ATP levels can occur for example, in the transition from exponential phase of growth to stationary phase, and in the conversion from aerobic to anaerobic growth (Cortassa and Aon 1993; Jensen et al., 1995; Hsieh et al., 1991; Dorman et al., 1988).

The nucleoid-associated protein Fis is reported to have a role in "fine-tuning" the homoeostatic control mechanism of DNA supercoiling (Schneider et al., 2000). It has recently been shown that Fis and supercoiling collaborate in modulating expression of virulence genes during intracellular growth of S. Typhimurium (O'Croinin et al., 2006). One of the master regulatory gene of the SPI-2 pathogenicity island (Fig 1.2) ssrA, was shown to be upregulated by DNA relaxation and the increase in expression required Fis (O'Croinin et al., 2006).

1.4.2 Posttranscriptional regulation

Various mechanisms of posttranscriptional regulation occur in bacteria. It has recently been shown in Salmonella that in addition to regulating fliC transcription fljA also regulates FliC translation. It is proposed that this posttranscriptional control is mediated through the interaction of FljA with the 5' untranslated region of fliC (Bonifield and Hughes 2003).

One particular area of posttranscriptional regulation that is subject to a lot of interest lately is the observed regulation of translation and message stability by small noncoding RNAs (sRNAs). sRNAs function as an RNA directly, not as part of a message (Gottesman 2004). The sRNA transcript is small usually 80-100 nucleotides in length and most are more stable than mRNAs (Masse et al., 2003). sRNAs have been shown to have key roles in bacterial response to stress and regulation of factors important for virulence (Fig 1.3), (Altuvia et al., 1997, 1998; Majdalani et al., 2002; Opdyke et al., 2004; Repoila et al., 2003; Sledjeski et al., 1996).
The regulation of genes by sRNAs can be positive or negative (Fig 1.3). A long 5' region of mRNA occurs upstream of the rpoS start codon. The 5' region of the rpoS transcript forms an inhibitory stem loop that occludes the Shine Delgarno sequence of ropS and thus prevents translation of the message (Brown and Elliot, 1997). In response to various environmental stress conditions, two sRNAs DsrA and RprA can activate rpoS translation by base pairing to the 5' region of rpoS and prevent the formation of the cis-inhibitory structure (Fig 1.4) (Majdalani et al., 1998, 2002). The dsrA promoter is most active at temperatures below 30°C (Repoila and Gottesman, 2001). Low temperature expression of DsrA leads to expression of RpoS during exponential growth at lower temperatures (Sledjeskii et al., 1996). The rprA promoter is regulated via the phosphorelay cascade RcsC/YojN/RcsB. This cascade is responsible, amongst other functions, for regulation of one of the many promoters of ftsZ (Carballes et al., 1999). Cell surface stress has been suggested as the environmental signal leading to the activation of the sensor kinase RcsB (Majdalani et al., 2002). In contrast the sRNA OxyS negatively regulates RpoS as well as a number of other targets including the fhlA gene. FhIA activates synthesis of the formate hydrogenlyase complex in the presence of formate. It is postulated that metal cofactors for this could lead to H$_2$O$_2$-induced damage (Altuvia et al., 1997). Oxidative stress leads to the activation of the OxyR transcriptional regulator resulting in the synthesis of OxyS. OxyS negatively regulates the synthesis of two transcriptional regulators RpoS and FhIA, and itself acts as an antimutator, contributing to the protection of cells from oxidative damage mediated by OxyR (Altuvia et al., 1997; Zhang et al., 1998).

A major class of bacterial non-coding RNAs act by pairing to target mRNAs (Gottesman 2005). Many of these sRNAs act together with the RNA chaperone Hfq. Hfq stabilizes the sRNAs and mediates their interaction with the target mRNA. Hfq can promote base-pairing by altering the RNA structures enabling contact between the two complementary RNAs (Stortz et al., 2004). This base pairing changes the secondary structure of the RNA molecule making it more susceptible to RNase cleavage (Geissmann and Touati, 2004). Because the sRNA is degraded along with its target mRNA the impact that it has on the target mRNA is coupled to its continued
induction (Masse et al., 2003). Once the stress signals are removed induction ceases and the cell can return to its pre-stress condition.

1.4.3 Post-translational regulation by proteolysis

One area of post-translational regulation is the controlled degradation both of misfolded proteins and rapid removal of regulatory proteins from the cytoplasm with consequential global effects (Gottesman, 1996, 2003; Jenal and Hengge-Aronis 2003).

RpoS is ordinarily associated with the stationary growth phase or with certain stress conditions. There is however a basal rate of RpoS synthesis even in cells not subjected to stress. However the level of RpoS in such cells remains low because of rapid degradation (Lange and Hengge-Aronis, 1994). The ClpXP protease is responsible for the proteolysis of $\sigma^5$ (Schweder et al., 1996). Degradation of $\sigma^5$ by ClpXP requires the response regulator RssB (Zhou et al., 2001). RssB delivers $\sigma^5$ to ClpXP where $\sigma^5$ is unfolded and completely degraded (Muffler et al., 1996). Similarly the Dps protein during exponential growth is highly unstable due to degradation by ClpXP protease, however in this case the degradation occurs independently of the RssB protein (Stephani et al., 2003).

1.5 The bacterial nucleoid

DNA is organized into a compact molecule by the introduction of negative supercoiling. Further packaging of the DNA is dependent on the type of cell. Bacteria and eukaryotes have developed different strategies to store their genetic information in a small volume while still being able to function. In eukaryotic cells the organization involves genomic DNA in the form of chromatin (repeating units of histones and DNA) being packaged in the nucleus (Kornberg 1974). In bacterial cells the genomic DNA is associated with particular DNA-binding structural proteins, known as nucleoid-associated proteins to form a highly organized compact structure called the bacterial nucleoid (Ali Azam et al., 1999; Dame 2005). The size and shape of the nucleoid is determined by a number of factors including macromolecular crowding, DNA supercoiling and the interaction of the DNA with the nucleoid associated proteins (Robinow C and Kellenberger, 1994).
Fig 1.3. Small RNA regulators in global regulation cascades. sRNAs can be produced in response to a stress. Depicted here are three such stresses and responses. (a) RyhB is produced in response to the lifting of repression by Fur protein due to iron limiting conditions. RyhB increases rapidly, it pairs with its target mRNA's and causes their degradation. By reducing the synthesis of these non-essential Fe-binding and storage proteins, limiting Fe is available for essential cellular processes. (b) Oxidative stress leads to the activation of the OxyR transcriptional regulator resulting in the synthesis of the sRNA OxyS. OxyS negatively regulates the synthesis of two transcriptional regulators RpoS and FhlA, contributing to the protection of cells from oxidative damage mediated by OxyR (Altuvia 1997, Zhang 1998). (c) The dsrA promoter is active at temperatures below 30°C (Repoila 2001). Low temperature expression of DsrA leads to expression of RpoS and consequent activation of RpoS-dependent genes during exponential growth at lower temperatures (Sledjeskii 1996). Figure adapted from Gottesman (2005).
Fig 1.4 Translational regulation of rpoS by the sRNA DsrA. The 5' region of the rpoS transcript forms an inhibitory stem loop that occludes the Shine Delgarno sequence of rpoS and thus prevents translation of the message. By base pairing to the 5' region of rpoS the sRNA DsrA prevents the formation of the cis-inhibitory structure. DsrA-mediated translation of RpoS requires the RNA-binding protein Hfq. Figure adapted from Szymanski et al 2002.
1.5.1 **Nucleoid-associated proteins**

At least 12 nucleoid-associated proteins have been identified in *Salmonella*. It was believed that four of these proteins, Fis (factor for inversion stimulation), H-NS (histone-like nucleoid structuring protein), HU (heat-unstable nucleoid protein) and IHF (integration host factor) were the major components of the nucleoid (Pettijohn *et al.*, 1996). However it has been shown that the protein composition of the nucleoid changes during growth phase. In exponential growth phase the most abundant of these proteins are Fis and HU, whilst in stationary growth phase the most abundant of these proteins are Dps and IHF. Levels of H-NS remain steady throughout the growth phases (Ali Azam *et al.*, 1999). It has been shown that IHF, HU, H-NS and Dps are found to be uniformly dispersed within the entire nucleoid, while Fis appears to be irregularly distributed within the nucleoid, being found upstream of highly expressed genes in rapidly growing cells such as rRNA and tRNA genes (Azam *et al.*, 2000).

1.5.2 **Fis**

Fis is an 11.2 kDa DNA-binding protein that was first identified as a stimulator of inversion of the Hin invertible DNA element, responsible for phase-variable expression of the H1 and H2 flagella antigens in *S. Typhimurium* (Johnson *et al.*, 1986). Fis can influence gene expression through its ability to modulate the degree of DNA supercoiling in the cell (Muskhelishvili and Travers, 2003), and has been described as a local topological homeostat (Rochman *et al.*, 2002). Fis binds to a highly degenerative consensus sequence, KNNYRNNWNNYRNNM (W = A/T, R=A/G, Y = C/T, K = G/T, M = A/C, N = any base) and localizes to 6-10 distinct loci within the nucleoid of rapidly growing cells (Azam *et al.*, 2000). Fis has been shown to have a global role in the transcriptional control of metabolism and type three secretion systems in *S. Typhimurium*. Among the genes regulated by Fis are those contributing to flagella biosynthesis and motility, genes involved in virulence located in the pathogenicity islands SPI-1, SPI-2 SPI-3 and SPI-5, and genes encoding components of various metabolic pathways (Kelly *et al.*, 2004).

1.5.3 **HU**

In *S. Typhimurium* HU is predominantly found in the form of a heterodimer consisting of two similar subunits HUα and HUβ, encoded by the *hupA* and *hupB*
genes respectively are unlinked on the chromosome and are subject to differential regulation (Kano et al., 1988, 1986). CRP positively regulates both genes, but Fis positively regulates the hupA gene while negatively regulating the hupB gene (Claret and Rouviere-Yanic, 1997). Accordingly, through the growth phases of the cell the form of HU present varies from homomeric HUα2 complexes during early exponential growth phase, to heteromeic HUαHUβ complexes from mid-to-late exponential growth phase to homomeric HUβ2, complexes in stationary growth phase (Claret and Rouviere-Yaniv, 1997). HU has an apparent disassociation constant (Kd) of 25 nM for non-curved DNA and a Kd of 51 nM for curved DNA (Talukder 1999). It binds DNA in a sequence independent manner and displays a high affinity for irregular DNA such as gapped kinked or nicked DNA (Talukder et al., 2000). HU can restrain negative supercoils, however overexpression of the HU protein does not induce increased compaction of the nucleoid (Rouviere-Yaniv et al., 1979; Skoko et al., 2004; van Noort et al., 2004).

1.5.4 IHF

IHF was first identified as a host factor for the integrative recombination of phage λ (Craig and Nash 1984). Similarly to HU, the IHF protein in S. Typhimurium is mainly found in the form of a heterodimer consisting of two similar subunits IHFα and IHFβ, encoded by the ihfA and ihfB genes respectively (Aviv et al., 1994). The ihfA and ihfB genes are not in an operon and are found at distinct locations on the chromosome and, like hupA and hupB, are also subject to differential regulation (Aviv et al., 1994). The level of IHF increases as cells undergo the transition from the growth phase to stationary phase and it has a Kd of 37 nM for a specific DNA sequence (Ali Azam et al., 1999, Azam and Ishihama 1999). The consensus sequence for IHF is 5'-WATCAANNNNTTR-3' (W = A/T, R = A/G and N = any base) (Craig and Nash, 1984). For transcription regulation, IHF appears to make a direct contact with the RNA polymerase as well as modulating DNA conformation (Azam et al., 2000). IHF has recently been shown to affect genes involved in motility and chemotaxis, virulence and genes involved in the transition to stationary phase in S. Typhimurium (Mangan et al., 2006).
1.5.5 H-NS

H-NS is a small (15.4 kDa) protein that was first identified as a heat-stable transcription factor (Jacquet et al., 1971). Because of its ability to condense DNA in vivo and in vitro in a manner reminiscent of the histones present in eukaryotes it was designated histone-like nucleoid structuring protein. H-NS is a highly pleiotropic regulator of gene expression and has been shown to regulate up to 10% of the genes in S. Typhimurium (Lucchini et al., 2006; Navarre et al., 2006). It binds preferentially to curved DNA, which is commonly found at promoters (Jauregui et al., 2003). H-NS’s role in regulation is chiefly as a repressor although there are rare instances where it functions as an activator of gene expression (Schroder and Wagner 2002). Mutations in hns are lethal in Salmonella unless accompanied by compensatory mutation in other regulatory loci such as rpoS or phoP (Navarre et al., 2006). It has recently being shown that H-NS preferentially binds to regions of DNA that have GC content that is lower than the rest of the S. Typhimurium genome (Lucchini et al., 2006; Navarre et al., 2006). Laterally acquired genes often have a GC/AT ratio that is different to the rest of the S. Typhimurium genome. This observation has lead to the hypothesis that one of the functions of H-NS is to selectively silence horizontally acquired genes, thus preventing inappropriate expression of these genes that may be detrimental to the cell (Lucchini et al., 2006; Navarre et al., 2006). This selective silencing is not confined to H-NS. The R27-like plasmid of S. flexneri, carries the sfh gene encoding an H-NS like protein Sfh (Beloin and Dorman, 2003). It has been reported that the presence of Sfh can aid the horizontal transfer of DNA (Doyle et al., 2007). Sfh binds to A-T rich areas on the plasmid, such binding of the Sfh protein is postulated to prevent the titration of H-NS away from the genome of the recipient towards the plasmid. The disturbance to the recipient is therefore minimal and the plasmid can be maintained (Doyle et al., 2007).

1.6 Dps

Dps is a small (19 kDa,) basic protein that was initially identified through the examination of three-day-old starved cultures of E. coli (Almiron et al., 1992). It was designated DNA-binding protein from starved cells or Dps. It is also present in Salmonella and is proposed to globally regulate gene expression in addition to its
protective role in the cell during particular stresses (Almiron et al., 1992; Halsey et al., 2004; McClelland et al., 2001).

1.6.1 The structure of Dps
Under conditions of either nutritional or oxidative stress *E. coli* produces high levels of Dps which binds DNA with no apparent sequence specificity (Almiron et al., 1992) and in stationary phase cells induces significant compaction of the chromosomal DNA (Frenkiel-Krispin et al., 2001). These properties make Dps a member of the class of bacterial nucleoid-associated proteins. Electron microscopy has shown that Dps in the absence of DNA forms a roughly spherical dodecamer ~ 90 Å in diameter and that Dps in complex with plasmid DNA forms hexagonally packed two-dimensional arrays (Frenkiel-Krispin et al., 2001) (Fig 1.4).

1.6.2 DNA binding by Dps
Dps binds DNA with low affinity (kd ~ 175 nM for both curved and non-curved DNA) and has no apparent sequence recognition specificity (Azam and Ishihama 1999). In *E. coli* the DNA binding properties are thought to be associated with the presence of a flexible N-terminal tail that contains three positively charges lysine residues and one arginine residue that can hook the negatively charged DNA backbone (Grant 1998). Bacteria lacking the specific properties of the *E coli* N-terminal tail do not display DNA binding capabilities. In *Agarobacterium tumefaciens*, the positive N terminus is present, however unlike the situation in *E. coli*, the N terminus is not disordered but rather is blocked onto the protein through several interactions including salt bridges (Ceci et al., 2003). In *Streptococcus mutans*, the Dpr (Dps-like peroxide resistance) protein does not interact with DNA; the long N-terminal tail does not contain positively charged residues (Yamamoto et al., 2002). HP-NAP (*H pylori* neutrophil-activating protein) from *Helicobacter pylori* and Dlp-1Dlp-2 from *Bacillus anthracis* lack the N-terminal extension and do not bind to DNA (Zanotti et al., 2002, Papinutto et al., 2002).

1.6.3 Ferritins and other iron storage proteins
Initially based on protein sequence alignments the Dps family of proteins was predicted to be similar to ferritins and bacterioferritins (Evans et al., 1995). Iron is
essential for most bacterial species as it functions as a co-factor in different proteins and is involved in various cellular functions (Andrews et al., 2003). Free iron in cells is extremely toxic since it catalyses the generation of reactive oxygen species such as the hydroxyl radical OH• during the Fenton reaction (Fenton, 1894).

\[
O_2 + H_2O_2 \xrightarrow{Fe(II) \text{ or } Cu(I)} \bullet OH + OH + O_2
\]

Iron is generally stored in ferritins that sequester the metal in a non-toxic yet bioavailable form. Iron is incorporated into the central cavity of ferritins by oxidation of Fe \(^{2+}\) followed by formation of a microcrystalline ferrihydrite-phosphate core. The conversion of Fe \(^{2+}\) to Fe \(^{3+}\) is catalyzed by a ferroxidase centre that is found in the subunits in ferritins and bacterioferritins (Andrews 1998) and between subunits in Dps proteins (Ren et al., 2003).

The ferritins are a widely distributed group of iron storage proteins. The structures of several ferritins have been solved by X-ray crystallography including the heme-binding bacterioferritin from \textit{E. coli} (Bfr) (Frolow et al., 1994). The Bfr monomer is a four-helix bundle, which oligomerizes such that 24 bacterioferritin monomers associate to form a large, roughly spherical, particle with octahedral symmetry (Frolow et al., 1994). The bacterioferritin particle measures \(~\)125 Å in diameter and has a \(~\)90 Å diameter hollow core that can store up to 2,000 iron atoms. Bacterioferritins have a ferroxidase activity, which is linked to the ability to efficiently incorporate ferrous iron into the mineralized core.

Like the bacterioferritin oligomer, the Dps dodecamer is a hollow ball with pores (Fig 1.5). However, the dodecamer displays tetrahedral symmetry with a central core measuring 45 Å (Grant et al., 1998) and the iron storage capability is not as considerable as bacterioferritin. Dps is capable of accommodating \(~\)500 Fe atoms (Ilari et al., 2000, 2002).

**1.6.4 Protection from oxidative stress by Dps**

When first described the protective action of Dps on DNA was attributed to physical association between the two macromolecules since the formation of the Dps-DNA
complex abolishes oxidative cleavage both *in vitro* and *in vivo* (Martinez *et al.*, 1997; Wolf *et al.*, 1999). More recently *E. coli* Dps was shown to possess iron and H$_2$O$_2$ detoxification capacity and this novel property was proposed to act together with physical association to DNA to achieve its protection against oxidative assault (Zhao *et al.*, 2002). It was proposed that iron incorporation was a multi-step process involving Fe$^{2+}$ binding, Fe$^{2+}$ oxidation, nucleation and growth of the mineral core as in classical ferritins. However in contrast to ferritins O$_2$ was not an efficient oxidant of Fe$^{2+}$, rather Fe$^{2+}$ oxidation was most efficiently accomplished by H$_2$O$_2$ (Zhao *et al.*, 2002).

1.6.5 *Regulation of dps gene expression*  
In cells exposed to oxidative stress during exponential growth *dps* transcription is induced by the redox-sensitive protein OxyR which activates the housekeeping RNA polymerase sigma factor RpoD or $\sigma^{70}$ (Altuvia *et al.*, 1994). The *dps* promoter is regulated by RpoS $\sigma^5$ (the stationary phase sigma factor) and IHF in stationary phase (Altuvia *et al.*, 1994). It is proposed that the *dps* promoter is recognized by both $\sigma^5$-holoenzyme and $\sigma^{70}$-holoenzyme, the promoter has a -10 sequence that conforms closely to the canonical -10 sequence for $\sigma^{70}$ (TATACT compared to TATAAT), however to date no corresponding -35 sequence has been found. It is further proposed that there is a need for IHF to enhance the binding of $\sigma^5$-holoenzyme and OxyR to enhance the binding of $\sigma^{70}$-holoenzyme explaining how the two holoenzymes can act at the same promoter (Altuvia *et al.*, 1994, Lomovskaya *et al.*, 1994).

1.6.6 *Regulation of Dps Protein*  
Dps protein levels are controlled by the ClpXP and ClpAP proteases. During exponential growth Dps is highly unstable due to degradation by ClpXP protease (Stephani *et al.*, *et al.*, 2003). This degradation occurs independently of the RssB protein, which acts as a recognition factor for proteolysis of $\sigma^5$ during growth (Muffler *et al.*, 1996; Schweder *et al*. 1996; Zhou *et al.*, 2001). ClpAP maintains ongoing Dps synthesis in long-term starved cells indirectly through an unknown mechanism (Stephani *et al.*, 2003).
Fig 1.5 Crystal structure of (A) Dps (B) Ferritin

1.7 Aim of this study

The description of Dps as a nucleoid-associated protein with an involvement in stationary phase adaptation, give rises to the possibility that along with other nucleoid-associated proteins it is a global regulator of gene expression. Consequently I set out to test the hypothesis that Dps is a global regulator of gene expression in *S. Typhimurium*, perhaps with a specific contribution to play in stationary phase adaptation.
Chapter 2

Materials and Methods
Chapter 2 Materials and Methods

2.1 Chemicals and reagents
The supplier of each chemical or reagent used in this study is given in parenthesis after the product. DNA restriction and modifying enzymes were obtained from Roche Molecular Biochemicals or New England Biolabs. GATC Biotech performed custom automated sequencing. Several molecular biology kits were used in this study. The basic principle of each kit is briefly described in the appropriate sections below.

2.1.1 Growth media, Antibiotics, X-Gal and IPTG
Materials for preparing growth media were obtained from Difco, Oxoid or Sigma. Suitable media were sterilized by autoclaving at 120 °C for 20 min prior to use or storage at room temperature. Additional solutions not suitable for autoclaving were sterilized by filtration through 0.2 μm Filtropur filters (Sarstedt). Unless indicated all quantities listed below are for the preparation of 1 litre of medium in AnalaR® ddH₂O. Media were supplemented with the appropriate antibiotics as required. Media agar was allowed to cool to 50 °C before the addition of the appropriate antibiotic.

LB broth and LB agar plates

LB (Luria Bertani) agar plates were used throughout this study for revival of bacterial strains from frozen stocks unless otherwise indicated. It was also used for general culturing of strains and selection of transformants and transductants. Bacterial strains were routinely grown in LB broth unless otherwise indicated.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB broth</td>
<td>10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl</td>
</tr>
<tr>
<td>LB agar</td>
<td>10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl, 15 g agar</td>
</tr>
</tbody>
</table>
Motility agar:

Characterization of motility was performed with swarm plates as outlined in Macnab (1986). The plates were centrally inoculated with equal numbers of bacteria and incubated at 37°C for 8 h. The motility of the strains was determined by measuring the radius of the swarming ring.

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

Green agar:

Green agar plates were used following P22-mediated transduction to obtain transduced isolates of S. Typhimurium that were free of phage. Pseudo-lysogens appear dark green due a lowered pH caused by bacterial lysis whereas phage-free colonies appear light green.

Green agar 8 g tryptone, 1 g yeast extract, 5 g NaCl, 15 g agar, 0.625 g Alizarin Yellow

After autoclaving and cooling to 50°C:

21 ml 40% glucose (w/v), 4 ml 2% Alineline Blue (w/v) are added.

R2A agar:

R2A agar is a rich medium with low nutrient concentrations and was used as a solid medium for reviving strains from frozen stocks prior to sampling on phenotypic microarrays. (Difco)

R2A agar 18.2g R2A agar (Difco)
MOPS minimal medium: 10 X

MOPS minimal medium is a near-neutral pH, culture medium for Enterobacteria. It is used as a chemically defined medium and can be supplemented with a specific carbon source. 10 X Mops minimal medium was prepared as previously described by Neidhardt et al (1974) and stored in aliquots of 50 ml at –20 °C until required.

MOPS minimal medium: 1 X

100.0 ml 10 X MOPS minimal medium
10.0 ml 132 mM K$_2$HPO$_4$
10.0 ml 40 % carbon source (w/v)
2.5 ml 200 mM L-histidine
877.5 ml AnalaR® ddH$_2$O

Solution adjusted to pH 7.2 and filter sterilized.

MOPS minimal medium agar

870 ml AnalaR® ddH$_2$O and 15 g agar (Oxoid) were mixed and autoclaved. The solution was cooled to 50 °C, and the following solutions, and antibiotics if required were added.

100.0 ml 10 X MOPS minimal medium
10.0 ml 132 mM K$_2$HPO$_4$
10.0 ml 40 % carbon source (w/v)
2.5 ml 200 mM L-histidine

Antibiotics X-Gal and IPTG

All stock antibiotic solutions were stored in aliquots at –20 °C and those prepared in water were sterilized by filtration through 0.2 μm Filtropur (Sarstedt.) Carbenicillin, kanamycin and spectinomycin were prepared as 50 mg /ml stock solutions in AnalaR® ddH$_2$O and used at a final concentration of 50 μg/ml.. Chloramphenicol
was prepared as a 25 mg/ml stock solution in 100% ethanol, and used at a final concentration of 25 μg/ml. Rifampicin was prepared fresh on day of use as a 250 mg/ml stock solution in 100% methanol and used at a final concentration of 250 μg/ml.

X-gal (5-bromo-4-chloro-3-indoly-β-D-galactoside), a chromogenic substrate for β-galactosidase was prepared as a 20 mg/ml stock solution in N, N-dimethyl formamide and stored in the dark at –20 °C. X-gal was used in agar plates at a final concentration of 20 μg/ml.

IPTG (Isopropyl-β-D-thiogalactopyranoside) a highly stable synthetic analog of lactose was used in conjunction with X-Gal to determine the lac phenotype in blue/white colony screening. IPTG was prepared as a 2 M stock in AnalaR® ddH₂O and used at a final concentration of 20 mM in agar plates.

2.2 Bacterial strains and culture conditions

All bacterial strains used in this study were derivatives of Salmonella enterica serovar Typhimurium SL1344 or Escherichia coli K-12 and are listed in Table 2.1. Bacterial strains were maintained as permanent stocks in 15% (v/v) glycerol in LB broth and stored at –70 °C.

Culture conditions

Bacterial strains were routinely grown at 37 °C in 250 ml flasks with shaking unless otherwise indicated. Overnight cultures were grown by inoculating single colonies into 5 ml of LB broth containing appropriate antibiotic in sterile test-tubes. Late exponential and stationary cultures were grown by spinning down equal numbers of bacteria from overnight cultures and resuspending the pellet in 1 ml of fresh LB. 250 μl of the resuspension was used to inoculate 25 ml of fresh LB. To maintain conditions as much as possible between experiments 25 ml cultures were incubated in a C76 Water Bath Shaker (New Brunswick Scientific) at 37 °C and 250 rpm.

2.3 Plasmids and oligonucleotides

Plasmids used in this study are listed in Table 2.2. Any necessary details of plasmid constructions will be described in the appropriate results section.
Table 2.1  Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Typhimurium</td>
<td>SL1344rpoS::kan</td>
<td>Keane 2002</td>
</tr>
<tr>
<td>CJD2090</td>
<td>SL1344, promoterless gfp+, Cm'</td>
<td>Hautefort, 2003</td>
</tr>
<tr>
<td>JH3008</td>
<td>SL1344dps::cat</td>
<td>This work</td>
</tr>
<tr>
<td>JTS004</td>
<td>JTS004 pJTS503</td>
<td>This work</td>
</tr>
<tr>
<td>JTS005</td>
<td>SL1344 pJTS503</td>
<td>This work</td>
</tr>
<tr>
<td>JTS006</td>
<td>SL1344 pJTS504</td>
<td>This work</td>
</tr>
<tr>
<td>JTS007</td>
<td>SL1344 pJTS505</td>
<td>This work</td>
</tr>
<tr>
<td>JTS008</td>
<td>SL1344 pJTS504</td>
<td>This work</td>
</tr>
<tr>
<td>JTS009</td>
<td>SL1344 pJTS505</td>
<td>This work</td>
</tr>
<tr>
<td>JTS010</td>
<td>SL1344 pJTS505</td>
<td>This work</td>
</tr>
<tr>
<td>JTS011</td>
<td>SL1344 pssrA-gfp</td>
<td>This work</td>
</tr>
<tr>
<td>JTS012</td>
<td>SL1344 Virulent wild type rpsL hisG</td>
<td>Hoiseth and Stocker, 1981</td>
</tr>
</tbody>
</table>

| SL1344dps  | SL1344dps::kan     | S. Lucchini |
| SL1344fis  | SL1344fis::cat     | Kelly 2004  |
| SL1344hns  | SL1344hns::kan     | J Hinton    |
| TH6232     | Δhin7517::FRT fljBA off FliC⁺ | K. T Hughes |
| TH6233     | Δhin7518::FRT fljBA on FliC  | K. T Hughes |

E coli

| BL21(DE3)p | B F dcm ompT hsdS(rB·mB) gal λ(DE3) [pLysS | Stratagene |
| LysS       | Cm' ]                                        |
| DH5α       | supE44ΔlacU169(φ80lacZΔM15)hsdR17 recA1 endA1 gyrA96 thi-relA1 | Promega |
| JTS001     | DH5α pJTS501                                  | This work |
| JTS002     | XL-1 blue pJTS501                             | This work |
| JTS003     | BL21(DE3)pLysS pJTS502                        | This work |
| MC4100     | F λ, araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC ptsF25 rbsR | Lab Stock |
XL-1 blue \( \text{recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac} \) Stragene

\[ [\text{F}^{\prime}\text{proAB lacI^q AZM15 TnJ0(Tc^{R}\text{})}] \]

\^Antibiotic resistance markers are abbreviated as follows: \( \text{Cm}^{R} \) resistance to chloramphenicol, \( \text{Tc}^{R} \), resistance to tetracycline
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics^a</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYC184</td>
<td>p15A replicon, Cm^R Tc^R</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pBluescript II KS</td>
<td>pMB1 replicon, Ap^R Sequencing plasmid</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pET19b</td>
<td>PSC101 replicon low copy number Spc^R</td>
<td>Lerner 1990</td>
</tr>
<tr>
<td>pJTS501</td>
<td><em>dps ORF downstream of the IPTG inducible promoter in PBluescript II KS</em></td>
<td>This work</td>
</tr>
<tr>
<td>pJTS502</td>
<td>PET19b expressing recombinant 6xHis-*dps</td>
<td>This work</td>
</tr>
<tr>
<td>pJTS503</td>
<td><em>dps ORF downstream of the arabinose inducible promoter in pBAD24</em></td>
<td>This work</td>
</tr>
<tr>
<td>pJTS504</td>
<td><em>dps promoter region upstream of promoterless gfp gene in pZep08</em></td>
<td>This work</td>
</tr>
<tr>
<td>pJTS505</td>
<td><em>dps ORF downstream of the IPTG inducible promoter in pCL1920</em></td>
<td>This work</td>
</tr>
<tr>
<td>pssrA-gfp</td>
<td>ssrA promoter region cloned upstream of promoterless gfp gene in pZep08</td>
<td>O’Cronin et al 2006</td>
</tr>
<tr>
<td>pZep08</td>
<td>gfp promoterless trap vector, Ap^R Cm^R Kn^R</td>
<td>Hautefort et al 2003</td>
</tr>
</tbody>
</table>

^aAntibiotic resistance markers are abbreviated as follows: Ap^R resistance to ampicillin, Cm^R resistance to chloramphenicol, Spc^R resistance to spectinamycin, Kn^R, resistance to kanamycin
Oligonucleotide sequences and nomenclature are listed in Table 2.3. Oligonucleotides were purchased from MWG-Biotech, Germany.

2.4 Creation of the \textit{dps} mutant JTS004

The allelic replacement technique based on the method of Datsenko and Wanner (2000) was used to construct the \textit{dps} mutant JTS004. Primer pair dwdpsfor and dwdpsrev (Table 2.3) were designed such that they had homology internally to the \textit{cat} gene of pACYC184, which confers resistance to chloramphenicol, with 60bp overhangs that had homology to the DNA directly upstream and downstream of the open reading frame of \textit{dps} (Section 2.14). pACYC184 was used as the template for the PCR. Following PCR, the resulting amplicon was gel purified and the linear DNA was transformed by electroporation into electrocompetent SL1344 harbouring the arabinose inducible, temperature sensitive pKOBEGA plasmid (Sections 2.5.2). Transformants displaying resistance to chloramphenicol were restreaked onto fresh chloramphenicol selective plates and incubated at 42°C to ensure transformants were free of plasmid. Finally P22 transduction was performed to transduce the mutation into a fresh SL1344 background.

2.5 Transformation of bacterial cells with plasmid DNA

Two methods were used to transform cells with plasmid DNA. Typically, recipient \textit{E. coli} cells were made competent by treatment with calcium chloride and transformed with a heat-shock triggering uptake of plasmid DNA. \textit{S. Typhimurium} cells typically were made electrocompetent and transformed by high-voltage electroshock treatment.

2.5.1 Preparation and transformation of calcium chloride-competent cells

An overnight culture of the strain to be made competent for transformation was used to inoculate 100 ml of LB broth and grown to an OD 600nm 0.3 – 0.5. The cells were incubated on ice for 30 min and then pelleted by centrifugation (Sorvall RC5C Plus) at 6,000 rpm for 10 min at 4°C. The bacterial pellet was resuspended in 50 ml of ice-cold 200 mM CaCl$_2$ and incubated on ice for 30 min. Cells were again harvested as above and resuspended in 10 ml ice-cold 200 mM CaCl$_2$ with 15% (v/v) glycerol. 24
Following another 30 min incubation on ice, cells were again collected and resuspended in 2 ml 200 mM CaCl₂ with 15% (v/v) glycerol. Cells were decanted into aliquots of 100 μl and used directly or stored at −70 °C.

DNA (0.1-1 μg) to be transformed in a volume not exceeding 10 μl, was added to the 100 μl aliquot of competent cells and left on ice for 30 min allowing the DNA to contact the surface of the bacterial cells. The tubes were then placed in a 42 °C water bath for 45 sec and returned to the ice for 2 min to allow cells time to adjust. This heat-shock treatment allows uptake of the plasmid DNA through the CaCl₂-induced competent bacterial membrane by an unknown mechanism (Mandel and Higa, 1970.) 1 ml pre-warmed LB broth was added to the culture which was then incubated at 37 °C for 1 h with shaking at 200 rpm to allow phenotypic expression of the plasmid borne antibiotic resistance marker. Tubes were spun down at 1.5 x g for 1 min. Most of the supernatant was discarded and the cell pellet resuspended in the remainder ~ 100 μl of the supernatant. This transformation mix was plated onto an appropriate selective agar plate. Cells to which no DNA had been added were treated in the same way and served to act as a negative control for contamination. Following overnight incubation at 37 °C, single colony transformants were purified on fresh selective agar plates.

2.5.2 Preparation and transformation of electro-competent cells

An overnight culture of the strain to be made competent for transformation was used to inoculate 100 ml of LB broth and grown to an OD 600nm 0.3 – 0.5. The cells were incubated on ice for 30 min. The cells were then pelleted by centrifugation (Sorvall RC5C Plus) at 6,000 rpm for 10 min at 4 °C. The bacterial pellet was resuspended in 100 ml of sterile ice-cold H₂O and incubated on ice for 30 min. Cells were again harvested as above and resuspended in 40 ml ice-cold 10% (v/v) glycerol and incubated on ice for 30 min. Following another centrifugation the cells were resuspended in 5 ml ice-cold 10% (v/v) glycerol and incubated on ice for 30 min, pelleted and finally resuspended in 2 ml of 10% (v/v) glycerol. Cells were decanted into aliquots of 100 μl and used directly or stored at −70 °C.

The DNA to be electroporated (50-200 ng in 8 μl sterile water) was added to 100 μl aliquot of electrocompetent cells and left on ice for 10 min. The mixture was then
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>catfor</td>
<td>AACTTTCCACCATAATGAAAT</td>
</tr>
<tr>
<td>catrev</td>
<td>AATTTCGGCATTCCATCCGC</td>
</tr>
<tr>
<td>dpscatfor</td>
<td>GGCCCCACTGGAACATGCG</td>
</tr>
<tr>
<td>dpscatrev</td>
<td>CGCCAGCGGCATCAGCACC</td>
</tr>
<tr>
<td>dpsfor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GAGCATATGAGTACCCGCTAAACTG</td>
</tr>
<tr>
<td>dpsrev&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CATGGATCCGATTTATTCGATGTG</td>
</tr>
<tr>
<td>dwdpsfor</td>
<td>TTAATTACCTGGGACACAAACATCAAGAGGATATGAG</td>
</tr>
<tr>
<td>dwdpsrev</td>
<td>TACCTTCCTGCAACTCGAAGTATTCAGGGTAGAGAGTAG</td>
</tr>
<tr>
<td>pBaddpsfor&lt;sup&gt;d&lt;/sup&gt;</td>
<td>GAGTCTAGAAGTACCCGCTAAACTG</td>
</tr>
<tr>
<td>pBaddpsrev&lt;sup&gt;e&lt;/sup&gt;</td>
<td>GAGAAAGCTTGATTTATTCGATGTG</td>
</tr>
<tr>
<td>pCLdpsfor&lt;sup&gt;f&lt;/sup&gt;</td>
<td>CATCTGCAGTACCGGGGCCG</td>
</tr>
<tr>
<td>pCLdpsrev&lt;sup&gt;g&lt;/sup&gt;</td>
<td>CGCGGATCCGATTTATTCGATGTG</td>
</tr>
<tr>
<td>pZepdpsfor&lt;sup&gt;h&lt;/sup&gt;</td>
<td>CGGGCGGCCGCCCTCAATGGGTCTACCTCG</td>
</tr>
<tr>
<td>pZepdpsrev&lt;sup&gt;i&lt;/sup&gt;</td>
<td>CGGTCTAGACGCCTCGGCCCATAGGTCC</td>
</tr>
<tr>
<td>RT-aceAfor</td>
<td>AGGCTATATCAAACAGCCTG</td>
</tr>
<tr>
<td>RT-aceArev</td>
<td>GCCAGGAAGTAATCCACATA</td>
</tr>
<tr>
<td>RT-aceBfor</td>
<td>TGTATCTGGATCAACACAGA</td>
</tr>
<tr>
<td>RT-aceBrev</td>
<td>CGAATCTGCCAATTCCTTC</td>
</tr>
<tr>
<td>RT-hnsfor</td>
<td>GAGCGAAGCABTTAAGTTTACG</td>
</tr>
<tr>
<td>RT-hnsrev</td>
<td>ATCAGGAATCTTTCACTGG</td>
</tr>
<tr>
<td>RT-livGfor</td>
<td>CAATGTATCGCTGGAATT</td>
</tr>
<tr>
<td>RT-livGrev</td>
<td>ACCAGCAGATTTCACATCAC</td>
</tr>
<tr>
<td>RT-livHfor</td>
<td>ATTATCGGCATGATCAACTT</td>
</tr>
<tr>
<td>RT-livHrev</td>
<td>GTAGGTTGGCAGAGATGG</td>
</tr>
<tr>
<td>RT-livJfor</td>
<td>TAAAAAGTTATGGCAGAGAGG</td>
</tr>
<tr>
<td>RT-livJrev</td>
<td>GACCGATAACATCCTGATG</td>
</tr>
<tr>
<td>RT-livMfor</td>
<td>ATGAAACCGATGCAATT</td>
</tr>
<tr>
<td>RT-livMrev</td>
<td>AGCTXTCATGCGTAGAGAG</td>
</tr>
<tr>
<td>RT-Mdhfor</td>
<td>CAGAICTCTCCCTGTAGC</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequence</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>RT-Mdhrev</td>
<td>CTGCACCAGGGTTTTTCAC</td>
</tr>
<tr>
<td>RT-mscLfor</td>
<td>GCATTCGTGTAAGATTTTGTC</td>
</tr>
<tr>
<td>RT-mscLrev</td>
<td>AAGCTTAATGGCAACAAAGA</td>
</tr>
<tr>
<td>RT-rpoSfor</td>
<td>GACTCAGCTTACCTTGTTG</td>
</tr>
<tr>
<td>RT-rpoSrev</td>
<td>AGTTGCTCTGCAATTTCCTTC</td>
</tr>
<tr>
<td>RT-STM1618for</td>
<td>GCTATTGAGCTTTTTGGCTA</td>
</tr>
<tr>
<td>RT-STM1618rev</td>
<td>CAAAAGCAGTCTCCATCCCTT</td>
</tr>
<tr>
<td>RT-SucCfor</td>
<td>CATGAATATCAGGCAAAAACA</td>
</tr>
<tr>
<td>RT-SucCrev</td>
<td>GTTTGATAGGTCACCAGACG</td>
</tr>
<tr>
<td>RT-SucDfor</td>
<td>GGGAAATAATGTCCGTTTTA</td>
</tr>
<tr>
<td>RT-SucDrev</td>
<td>GGGACGTAGATAACGGATG</td>
</tr>
<tr>
<td>SWfor</td>
<td>CGA TTT ATT GGT TCT TGA AA</td>
</tr>
<tr>
<td>SWrev</td>
<td>CCG ATA CAT CCA GTG TAG TA</td>
</tr>
</tbody>
</table>

*a* Restriction enzyme cleavage sites are underlined.

*b* Primer tailed with restriction site for *NdeI*

*c* Primer tailed with restriction site for *BamHI*

*d* Primer tailed with restriction site for *NotI*

*e* Primer tailed with restriction site for *XbaI*

*f* Primer tailed with restriction site for *HindIII*

*g* Primer tailed with restriction site for *PstI*
transferred to a pre-chilled electroporation cuvette (Cell projects, 2mm gap width). The cuvette was placed in the gene pulsar chamber (Bio-rad) and an electroshock delivered. Immediately 1 ml pre-warmed LB was added and the mixture transferred to a sterile eppendorf and incubated at 37°C for 1 h with shaking at 200 rpm allowing phenotypic expression of the plasmid borne antibiotic resistance marker. Tubes were spun down at 1.5 x g for 1 min Most of the supernatant was discarded and the cell pellet resuspended in the remainder ~ 100 μl of the supernatant. This transformation mix was plated onto an appropriate selective agar plate. Cells to which no DNA had been added were treated in the same way and served to act as a negative control for contamination. Following overnight incubation at 37°C, single colony transformants were purified on fresh selective agar plates.

2.6 Transduction with bacteriophage P22

During this study bacteriophage P22 was used to carry out generalised transduction from a donor to a recipient S. Typhimurium strain. P22 is a temperate dsDNA phage and specifically recognises and binds to the O-antigen on the outer membrane of S. Typhimurium. After binding, linear DNA is injected into the host. This DNA must be circularised by a homologous recombination event between the direct repeats at the two ends of the chromosome. The DNA is replicated in the host cell initially by θ-replication and then by rolling circle replication, which generates long concatamers of dsDNA P22 DNA. These concatamers are resolved by a phage-encoded endonuclease that cleaves the DNA at pac sites found 44 kb apart. This linear dsDNA is packaged into new phage particles that are released from the host once 50-100 particles have been produced.

Occasionally the phage will package a piece of host genome in to the phage head instead of phage DNA. These transducing phage on release from the host are capable of attaching to another cell and injecting the DNA into the recipient. The DNA is not capable of replicating and will be lost unless it can be integrated into the recipient genome by recombination.

In this study the P22 HT105/1 int-201 derivative was used. This derivative was used as it has a high transducing frequency due to its endonuclease having a lower
specificity for the *pac* sequence and thus a high proportion of the phage heads carry chromosomal DNA

### 2.6.1 Preparation of P22 phage lysate

The donor strain was grown overnight in 5 ml of LB broth, containing appropriate antibiotic, at 37°C with shaking. This overnight culture was used to inoculate 5 ml of fresh broth at 1:100 dilution. The fresh culture was incubated at 37°C with shaking for 90 min. 10 μl of P22 phage stock was added and the mixture incubated for a further 4 h when 500 μl of chloroform was added and the culture vortexed vigorously. Following the addition of chloroform the culture was incubated at 4°C for 1 h. Cellular debris was removed by centrifugation at 4,300 x g for 20 min. The supernatant was decanted to a clean tube and stored over chloroform at 4 °C until required.

### 2.6.2 P22 phage transduction

Generalised transduction was carried out as follows. The recipient strain was grown overnight in 5 ml of LB broth at 37°C with shaking. 100 μl of the prepared lysate was mixed with 100 μl of the recipient strain. The mixture was incubated at 30°C for 1 h without shaking. 800 μl of LB broth was added and the culture was then incubated at 37°C with shaking for 1 h. Transduced cells were selected for by plating on LB agar plates with the appropriate antibiotic incubated at 37°C overnight. True lysogens were distinguished from pseudo-lysogens by three repeated single colony purifications on Green agar plates with appropriate antibiotic. Pseudo-lysogens appear dark green due a lowered pH caused by bacterial lysis whereas phage free colonies appear light green.

### 2.7 Oxidative stress assay

Bacterial killing by H₂O₂ was measured using overnight cultures grown in LB at 37°C as described previously (Buchmeier *et al.*, 1997) Overnight cultures of SL1344 and JTS004 were standardised to 10⁷ cfu/ml and resuspended in 5 ml of 0.5 mM H₂O₂ in Phosphate Buffered Saline (PBS) or 5 ml PBS. The cultures were incubated at 37°C with shaking for 2 h. The number of viable cells was determined by serial dilution and plating on LB agar and survival percentages was calculated.
2.8 **Acid stress assay**

Bacterial survival at pH 2 was measured using overnight cultures grown in LB at 37°C as described previously (Finkel *et al.*, 2004). Overnight cultures of SL1344 and JTS004 were standardised to $10^7$ cfu/ml and resuspended in 5 ml of PBS pH 2. The cultures were incubated at 37°C with shaking for 3 h. The number of viable cells was periodically determined by serial dilution and plating on LB agar and survival percentages were calculated.

2.9 **Spectrophotometric assays**

2.9.1 **Monitoring bacterial growth**

The growth of bacterial cultures was monitored by measuring the optical density of the cultures at a wavelength of 600nm ($OD_{600}$). For routine measurement of $OD_{600}$, 0.05–1 ml of the culture was transferred to a disposable cuvette (Sarstedt) and brought to a final volume of 1 ml by the addition of LB broth. Change in $OD_{600}$ was measured in a Genysis 10uv spectrophotometer against 1 ml of LB as a blank.

2.9.2 **Determination of nucleic acid concentration**

The concentration of DNA and RNA was determined spectrophotometrically by measuring the absorbance at 260 nm. Samples were diluted 1:60 in Sigma ultra pure water and transferred to a quartz cuvette (Chandos). Following measurement the concentration of nucleic acid was determined according to the following formulae:

$$OD_{260\text{ nm}} \text{ value of } 1 \text{ corresponds to } 50 \mu g/ml \text{ of double stranded DNA}$$

$$40 \mu g/ml \text{ of single stranded DNA or RNA}$$

2.9.3 **Determination of protein concentration by the Bradford assay**

The concentration of protein was determined using a Bio-Rad Protein Assay that measures the differential colour change (shift in absorbance from 465 to 595 nm) of Coomassie Brilliant Blue G-250 when protein binding occurs and is based on the method of Bradford (1976). A standard curve was generated by measuring known concentrations of Bovine Serum Albumin (BSA) at 595 nm. The test sample was
serially diluted and also measured at 595 nm. Comparing the resulting measurements against the standard curve determined the protein concentration.

2.10 Assays of gfp reporter gene expression by flow cytometry
Throughout this study flow cytometric analysis was used to monitor gene expression using a green fluorescent protein (GFP)-based assay, in which the gfp gene is placed under the regulatory control of the promoter of a target gene. The activity of the target promoter is determined by assaying the level of fluorescence produced.

For measurement of GFP in Salmonella, samples were immediately fixed in 4% (w/v) formaldehyde (Sigma) freshly prepared in phosphate-buffered saline (PBS), and kept in the dark at 4°C until analysis (Hautefort et al., 2003). Fluorescence was measured in a Beckman Coulter Epics-XL flow cytometer Analysis was carried out using the Expo32 ADC program. Each assay was performed in duplicate and the mean values determined from at least three independent experiments.

2.11 Preparation of nucleic acids

2.11.1 Small-scale isolation of high purity plasmid DNA
The Qiaprep Spin Miniprep Kit (Qiagen) was used to extract plasmid DNA from 5 ml of culture according to the protocol provided. The Qiaprep miniprep procedure uses the modified alkaline lysis method of Birnboim and Doly (1979). Bacteria are lysed under alkaline conditions (NaOH/SDS) in the presence of RNaseA, and the lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step. The high salt concentration causes denatured proteins, chromosomal DNA, cellular debris and SDS to precipitate, while the smaller plasmid DNA renatures correctly and stays in solution. After lysate clearing by centrifugation, the supernatant is ready for purification on the Qiaprep silica membrane through washing and desalting. The plasmid DNA is eluted in 50 μl elution buffer (10 mM Tris-Cl, pH 8.5).

2.11.2 Large-scale isolation of high purity plasmid DNA
The Qiagen Plasmid Midi Kit was used to extract plasmid DNA from 100 ml of overnight bacterial cultures according to the protocol provided. Purification is based
on a modified alkaline lysis procedure similar to that described in Section 2.11.1, followed by binding of plasmid DNA to Qiagen Anion-Exchange resin under appropriate low-salt and pH conditions. A medium salt wash removes RNA proteins and low-molecular-weight impurities. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. The DNA precipitate is resuspended in 100 µl AnalalR® ddH₂O

2.11.3 Purification of chromosomal DNA
Genomic DNA for use in Southern blotting and as template in polymerase chain reaction (PCR) was isolated using the Bacterial Genomic DNA Purification Kit (Edge Biosystems). 5 ml of overnight culture was used to isolate chromosomal DNA according to the protocol provided. The sample was centrifuged to obtain a pellet and the supernatant was discarded. The pellet was resuspended in Spheroplast Buffer. The resuspension was incubated at 37 °C for 10 min when a mix of lysis buffers was added followed by a further incubation at 65 °C for 5 min. Advamax™ Beads and Extraction buffer were added to the mixture followed by a rapid vortex. The beads bind and clump cellular debris and denatured proteins, isoproponol was added to precipitate the DNA into the supernatant. The sample was then centrifuged to pellet this debris and the chromosomal DNA from the supernatant was washed in 70% ethanol to desalt the sample followed by drying in a Speedvac and final resuspension in 100 µl AnalalR® ddH₂O.

2.11.4 Isolation of RNA
Special precautions were used when working with RNA due to its chemical instability and the ubiquitous presence of RNases. Unlike DNases, RNases do not need metal ion co-factors and maintain activity even after prolonged boiling or autoclaving (Sambrook 1989). For this reason gloves were always worn, sterile disposable plastics were used and electrophesis tanks for RNA analysis were cleansed with 1% SDS, rinsed with H₂O then rinsed with absolute ethanol and finally soaked in 3% H₂O₂ for 10 min. Before use the tanks were rinsed with diethyl pyrocarbonate (DEPC)-treated water. All solutions used were prepared with DEPC-treated water (0.1% mixed overnight then autoclaved), which inactivates RNases by covalent modification.
RNA was isolated using the SV Total RNA Isolation System (Promega) according to the protocol provided. The procedure combines the disruptive and protective properties of guanidine thiocyanate (GTC) and β-mercaptoethanol to inactivate the ribonucleases present in cell extracts. SDS in association with GTC disrupts the nucleoprotein complexes allowing the RNA to be released into solution. Dilution of cell extracts in the presence of high concentrations of GTC causes selective precipitation of cellular proteins to occur, while the RNA remains in solution. After centrifugation, precipitated proteins and cellular debris are pelleted and the supernatant contains the RNA. The RNA is selectively precipitated with ethanol and binds to the silica surface of the spin columns. RNase free DNase I is applied directly to the silica membrane to digest contaminating genomic DNA. The RNA is washed and desalted (60 mM potassium acetate, 10 mM Tris-Hcl, pH 7.5, 60% ethanol). RNA was eluted in 100 μl RNase Free H2O (Sigma) and stored at −70 °C until required.

2.12 Manipulation of DNA in vitro

2.12.1 Restriction endonuclease cleavage of DNA
0.5-2.0 μg of Plasmid or purified PCR product was cleaved with 10 U of restriction enzyme in a 30 μl volume containing the reaction buffer supplied with the enzyme and bovine serum albumin (BSA) if appropriate. For double digests involving simultaneous cutting of DNA by two endonucleases a suitable buffer was chosen in which both enzymes had sufficient activity according to the manufacturer’s guidelines. If no single buffer was found to satisfy the buffer requirements of both enzymes then sequential digestion was carried out followed by DNA precipitation after the initial digest and then digestion with the second enzyme. Reaction temperatures were dependant on the enzyme used and reactions were incubated for 1-2 h.

2.12.2 Phosphatase treatment of restriction endonuclease-cleaved DNA
Phosphatase treatment of restriction endonuclease-cleaved DNA results in the removal of 5' phosphate groups from the DNA. Since phosphatase-treated fragments lack the 5' phosphoryl termini required by ligases, they cannot self-ligate (Sambrook 1989).
This property was used to decrease the vector background in cloning strategies. Alkaline phosphatase was used to dephosphorylate 5'-phosphoryl groups when a blunt-ended cloning was performed. Vector DNA was digested with restriction enzymes for 1-2 h, 1 U Alkaline Phosphatase, shrimp (Roche) and 1X supplied dephosphorylation buffer was added followed by incubation at 37°C for 1 h.

2.12.3 Purification of linear DNA
Linear DNA fragments were purified for cloning, or for the preparation of labelled probes using the HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Genomics). Linear fragments were purified directly or from agarose gels. If necessary DNA fragments were separated by electrophoresis through a 0.7% (w/v) TAE agarose gel containing 1 µg/ml ethidium bromide. The desired DNA fragment was visualised through a brief exposure to U.V. light and excised with a sterile scalpel. The gel slice was melted in a buffer that provided a suitable environment where DNA was selectively bound with high affinity to a glass fiber matrix, for PCR products no melting was required but the procedure was essentially the same. Following washing off contaminants the purified DNA fragments were eluted by addition of low salt elution buffer.

2.12.4 Ligation of DNA molecules
T4 DNA ligase catalyses the formation of phosphodiester bonds between neighbouring 3'hydroxyl- and 5'-phosphate ends in double-stranded DNA in the presence of ATP. Bacteriophage T4 DNA ligase (Roche) was used throughout this study to clone digested insert DNA into appropriately digested vectors according to the supplied protocol. Briefly a molar ratio of vector and fragment of 1:3 for sticky ends and 1:5 for blunt end ligation were mixed with 1X supplied buffer and 2 U T4 DNA ligase, in a reaction volume of 30 µl. For sticky ends the reaction mixture was incubated at 4°C overnight while for blunt ends the incubation was carried out at room temperature (~ 20°C). Typically 10 µl were transformed in the appropriate E. coli strain.
2.12.5 **Ethanol precipitation of DNA/RNA**

Two volumes of 100% ethanol and 0.1 volumes of 3.5 M sodium acetate (pH 5.2) were added to the nucleic acid to be precipitated. The contents were inverted to mix and incubated overnight at -70°C, before centrifugation at 16,100 x g for 1 h at 4°C. The pellet was washed in 500 µl of 70% ethanol, air-dried and resuspended in a suitable volume of AnalaR® ddH₂O for DNA and RNase free water (Sigma).

2.13 **Polymerase Chain Reaction (PCR)**

The polymerase chain reaction (PCR) was used for the amplification of DNA for preparation/confirmation of fragments during cloning strategies and for generating probes for Southern and Northern blots analyses. The PCR method is based on the procedure first outlined by Saiki *et al.* (1988) and involves double-stranded DNA being denatured to single stranded templates that can be acted on by a thermostable DNA polymerase primed from oligonucleotides each complementary to one end of the target sequence to amplify the DNA.

2.13.1 **Amplification of DNA**

Two different thermostable polymerases were used in this study. *Taq* DNA polymerase (New England Biolabs) is a DNA polymerase isolated from *Thermus aquaticus* YT1 that possesses a 5'→3' polymerase activity. It lacks however a proofreading 3'→5' exonuclease activity and was routinely used for PCR where simple detection of an amplification product or estimation of the product size was required. *Pfu* DNA polymerase (Promega) is a DNA polymerase isolated from *Pyrococcus furiosus* DSM3638. *Pfu* DNA polymerase catalyses the DNA-dependent polymerisation of nucleotides into duplex DNA in the 5'→3'direction in the presence of magnesium ions. The enzyme also exhibits 3'→5' exonuclease (proofreading) activity. Base misinsertions that may occur infrequently during polymerisation are rapidly excised by the proofreading activity of the polymerase. *Pfu* DNA polymerase was used for amplification of DNA fragments for cloning purposes.

PCR reactions were carried out by mixing 5 µl 10X *Pfu/Taq* buffer 0.2 mM of each dNTP (Roche), 100 pmol of each oligonucleotide primer, 1 U of *Pfu/Taq* polymerase, 10-100 ng template DNA and AnalaR® ddH₂O to a final volume of 50 µl in a 500 µl
thin-walled PCR tube. In addition 1-3 mM MgSO_4 was added to the *Pfu* based reactions only. Reactions were set-up on ice and on addition of the DNA polymerase immediately placed into the PTC-200 Peltier Thermal Cycler. Unless otherwise stated the reaction cycles were as follows:

94°C, 5 min (denaturation)  
oligo. annealing temperature^a^, 1 min  
72°C, 1-3 min^b^ (extension)  
94°C, 1 min (denaturation)  
steps 2-4 repeated, 30 cycles  
72°C, 10 min (final extension and renaturation)

^a^The annealing temperature was typically set 5°C below the theoretical melting temperature (Tm) of the oligonucleotides being used. The Tm was calculated using the formula Tm = 2 x (A+T) + 4 x (G + C), where A, T, G and C refer to the base composition of the oligonucleotide (Sambrook and Russell, 2001).  
^b^Extension time depended on the expected length of the PCR product (~ 30 sec – 2 min / kb)

2.13.2 Reverse Transcriptase-PCR (RT-PCR)  
Reverse Transcription Polymerase Chain Reaction (RT-PCR) was used to determine the relative level of abundance and stability of RNA transcripts of specific genes of interest. RT-PCR analyses were performed using the OneStep RT-PCR kit (Qiagen) according to the protocol provided. Reverse transcription and PCR are carried out sequentially in the same tube. All components required for both reactions are added during setup, and there is no need to add additional components once the reaction has been started. Reverse transcription is based on the ability of the enzyme, reverse transcriptase, to synthesis a DNA strand that is complementary to an RNA template. The newly synthesized complementary DNA (cDNA) acts as template for PCR as described in section 2.13.2.1.
2.13.2.1 cDNA synthesis and amplification

The OneStep RT-PCR kit contains an optimized combination of Omniscript Reverse Transcriptase, Sensicript Reverse Transcriptase and HotStarTaq DNA Polymerase. The reverse transcriptases provide highly efficient and specific reverse transcription and are designed for reverse transcription of RNA amounts greater than and less than 50 ng of RNA respectively. During reverse transcription, HotStarTaq DNA Polymerase is completely inactive and does not interfere with the reverse-transcription reaction. After the reverse transcription step reactions are heated to 95°C for 15 min to activate HotStarTaq DNA Polymerase and to simultaneously inactivate the reverse transcriptases. RT-PCR reactions were carried out by mixing 10 μl 5X OneStep RT-PCR buffer, 400 μM of each dNTP , 0.6 μM of each oligonucleotide primer, 2 μl of OneStep RT-PCR Enzyme Mix, 1.2 μg RNA template (Section 2.11.4) and RNase free H₂O (Sigma) to a final volume of 50 μl in a 500 μl thin-walled PCR tube. Reactions were set up on ice and following addition of template RNA were placed in the PTC-200 Peltier Thermal Cycler. Routinely the reaction cycles were as follows:

50°C, 30 min (reverse transcription)
95°C, 15 min (activation DNA polymerase, inactivation reverse transcriptases)
94°C, 30 sec (denaturation)
oligo. annealing temperature, 30 sec
72°C, 1-3 min (extension)
steps 3-5 repeated, 25-30 cycles
72°C, 10 min (final extension and renaturation)

The annealing temperature was typically set 5°C below the theoretical melting temperature (Tm) of the oligonucleotides being used. The Tm was calculated using the formula Tm = 2 x (A+T) + 4 x (G + C), where A, T, G and C refer to the base composition of the oligonucleotide (Sambrook and Russell, 2001).

Extension time depended on expected length of the PCR product (~ 1 min per 500 bp)

Range of cycles was performed for each transcript of interest to optimize RT-PCR reaction and avoid saturation.
RT-PCR products were gel electrophoresised through TAE 1% w/v agarose gels containing 1 μg/ml ethidium bromide and were visualised through a brief exposure to U.V. light.

2.14 RNA stability assay
The stability of mRNA was determined based on the ability of the antibiotic rifampicin to inhibit transcription. Cells were cultured to exponential phase (OD 600 nm = ~1.8) or late stationary phase (OD 600 nm = ~ 3.5) and treated with rifampicin (250 μg/ml) to inhibit transcription. Total RNA was then isolated (Section 2.11.4) in a time course experiment and used as the template for RT-PCR as described (Section 2.13.2.1)

2.15 Gel electrophoresis

2.15.1 Agarose gel electrophoresis
Electrophoresis through agarose gels was routinely used to separate nucleic acid molecules for size and concentration analysis and for purification of DNA fragments. The size and percentage of the gel depended on the number of samples and resolution necessary, mini gels 8.4 cm x 5.9 cm required 25 ml Tris-acetate (TAE) agarose, while midi gels 16.6 cm x 11.3 cm required 100 ml of TAE agarose. The gel solution was prepared from Agarose MP (multi purpose agarose, Roche) melted in 1 X TAE buffer (40 mM Tris, 1 mM EDTA, 0.114% (v/v) glacial acetic acid). Ethidium bromide was added before the gel was poured to give a final concentration in the gel of 1 μg/ml. Ethidium bromide intercalates both DNA and single-stranded RNA that has folded back onto itself providing local base pairing for the dye to intercalate, it fluoresces strongly in long wavelength UV light allowing the nucleic acid to be visualised. Samples were mixed with Blue/Orange 6X loading dye (Promega) and loaded against a known DNA size ladder, 1 kb or 100 bp (Promega) depending on the size of the fragment expected. Gels were typically run at 90 V and recorded by photography using a UVP camera coupled to a thermal imaging system.
2.15.2 SDS-PAGE

Proteins were separated by discontinuous polyacrylamide gel electrophoresis as described previously (Sambrook 1989). The discontinuous buffer system uses buffers of different pH and composition in the stacking and separating gels. Consequently protein migration through the large pores in the stacking gel (5% acrylamide) is fast and the proteins become concentrated into a narrow band. However, migration through the narrow pores of the separating gel (12% acrylamide) is according to size. Both gels were prepared with 0.1% SDS. Since most proteins bind SDS in a constant weight ratio, this leads to identical charge densities for denatured proteins, and allows proteins to migrate according to size, not charge.

Gel plates were cleaned before use and a 12% separating gel prepared by mixing 2 ml Protogel (National Diagnostics), 1.25 ml of 1.5-M Tris-HCl (pH 8.8), 50 µl 10% SDS, and 1.7 ml of AnalR® ddH₂O. Polymerization was catalyzed by the addition of 50 ml 10% (w/v) ammonium persulphate and 5 ml of TEMED. The solution was then poured between the plates until 1 cm below the tip of the comb and immediately overlaid with 200 µl isopropanol (excludes oxygen) and allowed to polymerize for 30 min. The 5% stacking gel was made by the mixing of 0.833 ml Protogel (National Diagnostics), 1.25 ml 0.5-M Tris-HCl (pH 6.8), 50 µl 10% SDS and 2.87 ml of AnalR® ddH₂O. The gel was electrophoresed in 1 x Tris-glycine running buffer (25-mM Tris-HCl, 250-mM glycine (pH 8.3), 0.1% (w/v) SDS). Prior to loading, protein samples (Section 2.17.1) were denatured at 95°C for 10 min and then centrifuged at 16,000 ×g for 30 sec. Electrophoresis was performed at 100 V for 60–90 min in the Mini-Protean system (Bio-Rad).

2.15.3 Staining of proteins

Gels were washed in AnalR® ddH₂O prior to overnight staining with Coomassie Brilliant Blue R-250 solution (0.25% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid). Gels were destained in Coomassie destain solution (45% (v/v) methanol, 10% (v/v) acetic acid). Following destaining protein bands appear blue against a clear background.
2.16 Northern blotting

The principle of northern blotting is that a transcript within immobilized RNA can be quantified after hybridization with a specific probe. This technique was used to investigate the relative level of abundance of RNA transcripts as a function of the presence or absence of Dps. The procedure involves resolution of an RNA sample by denaturing gel electrophoresis (Section 2.16.1) and then transfer of the resolved RNA to a nylon membrane (Section 2.16.2). The membrane is hybridized with a transcript-specific Digoxigenin (DIG)-labeled probe (Section 2.16.3) and then the RNA-DNA probe complex is detected by a chemiluminescent assay (Section 2.16.4).

2.16.1 Denaturing electrophoresis of RNA

RNA molecules can contain a high degree of secondary structure and are consequently electrophoresed under denaturing conditions. In this study, formaldehyde was used as the denaturant for agarose gel electrophoresis. Electrophoresis was done using 0.37 M formaldehyde agarose gel (1.2% (w/v) agarose) made with DEPC treated water. Samples of total RNA (5 μg), prepared as described in section 2.11.4 were precipitated as described in section 2.12.5 and the resulting pellet was resuspended in 3 volumes of denaturing solution (50% formamide, 1 x MOPS), followed by incubation at 65°C for 15 min, transfer to ice for 5 min, then mixing with 0.1 volumes of loading dye. Gels were run in 1X MOPS buffer containing 0.2 M formaldehyde at 100 V for 3–5 h.

2.16.2 Transfer of the resolved RNA to nylon membrane

After electrophoresis the resolved RNA was transferred to 0.45 μm Biodyne B nylon membrane (PALL) by overnight capillary transfer according to the method outlined in Sambrook (1989). RNA was immobilized on the membrane after transfer by UV-crosslinking.

2.16.3 DIG (Digoxigenin) probe synthesis

A DIG-labelled probe was synthesized by the polymerase chain reaction (Section 2.13.1) using the DIG DNA labeling mix (Roche Molecular Biochemicals). The mix contains 1mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP and 0.35 mM DIG-
dUTP. 5 μl of this mix was added to a 50 μl volume PCR reaction instead of standard dNTP mix. The synthesized PCR products incorporate the DIG-labeled nucleotides. The labelled probes were purified from an agarose gel slice as described in section 2.11.3 and eluted in 30 μl AnalAr® ddH2O. Typically 5-30 ng of probe was used per 1 ml hybridization solution. The probe was denatured by heating to 95°C for 10 min then incubated on ice prior to use.

2.16.4 Hybridisation and detection of DIG probe

Hybridization and detection of DIG labelled probes were carried out according to the protocol in the DIG Application Manual for Filter Hybridization (2003). Briefly the procedure involves overnight incubation of the probe with the nylon membrane at 50°C. Non-specific hybridized probe is removed by a series of stringency washes (decreasing salt concentration increasing temperature). Following 30 min incubation with blocking solution (DIG Wash and Block Buffer Set, Roche Molecular Biochemicals), the membrane is then incubated for 30 min with an Anti-DIG alkaline phosphotase-antibody conjugate (Anti-DIG-AP-conjugate Roche Molecular Biochemicals), which recognizes the immobilized DIG moiety that is incorporated into the probe. In the presence of a suitable chemiluminescent substrate (CDP-Star, Roche Molecular Biochemicals) the alkaline phosphatase-mediated enzymatic dephosphorylation reaction results in a luminescent signal that visualises hybridized molecules by autoradiography (Section 2.18).

2.17 Southern blotting

The principle of Southern blotting (Southern, 1975) involves separation of DNA by agarose gel electrophoresis, which is then denatured in situ and transferred to a nylon membrane (Section 2.16.2). Immobilised DNA is hybridised with a DIG labelled probe (Sections 2.16.3) that can be detected by a chemiluminescent immunoassay (Section 2.16.4). This sensitive technique was used to confirm the mutation of dps.

2.17.1 Electrophoresis, denaturation and transfer of DNA to nylon membrane

Total genomic DNA was isolated from bacterial strains as described in section 2.10.3 and ~3 μg of digested DNA was separated by electrophoresis (Section 2.15.1) through
a 1 % midi-sized agarose gel at 100 V for 6 h. The resolved DNA was then depurinated in situ (250-mM HCl, 10 min), denatured (1.5-M NaCl, 0.5-M NaOH, 45 min), and neutralised [1.5-M NaCl, 1.5-M Tris-HCl (pH 7.4), 45 min], prior to overnight capillary transfer to Biodyne B nylon membrane (PALL), according to the method outlined in Sambrook (1989). The in situ treatment of the DNA allows transfer to proceed more efficiently by cleaving larger fragments, and makes detection more sensitive by denaturing double-stranded DNA. DNA was immobilised on the membrane by UV-crosslinking.

2.18 Western immunoblot analysis

Western immunoblotting is a sensitive technique whereby proteins (antigens) are solubilised with SDS and 2-β-mercaptoethanol (Section 2.18.1) and separated by SDS-PAGE (Section 2.15.2), before being irreversibly transferred to a nitrocellulose membrane (Section 2.18.2). The membrane is incubated with an antigen-specific primary antibody, and the antigen–antibody complexes detected with a secondary antibody and revealed by a chemiluminescent assay (Section 2.18.3).

2.18.1 Preparation of total cellular protein extracts

Total protein extracts for SDS-PAGE and Western immunoblot analysis were prepared as described below. The OD_{600nm} of each culture was measured. A volume of cells, which corresponded to 2 ml of culture per 1 OD_{600nm} unit, was harvested. The pellets were then resuspended in 50 µl B-PER reagent (Pierce) supplemented with lysozyme (500 µg ml^{-1}) and DNase I (100 U ml^{-1}) and left at room temperature for 15 min. Protein concentration of the lysates was determined using the Bradford assay (Section 2.9.3). To samples 50 µl of 2 × SDS loading buffer (150-mM Tris-HCl (pH 6.8), 1.2% (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) β-mercaptoethanol) were then added and samples boiled at 95°C for 10 min. Typically 10 µl of this protein extract was used for immuno-detection in Western immunoblot analyses.

2.18.2 Transfer of proteins to nitrocellulose membrane

Following SDS-PAGE (Section 2.15.2), gels were electroblotted to 0.2-mm PROTAN nitrocellulose membrane (Schleicher and Schuell) using a Mini Trans-blot electrophoretic transfer cell (Bio-Rad) filled with transfer buffer (25-mM Tris, 192-
mM glycine, 20% (v/v) methanol) at 80 V for 2 h. Equal protein loading and consistent transfer to the nitrocellulose membrane were confirmed by staining the membrane with Ponceau S solution (2 g Ponceau S (Sigma), 1 ml glacial acetic acid) for 5 min followed by extensive washing with AnalaR® ddH$_2$O.

2.18.3 Detection of bound antigens

Nitrocellulose membranes were blocked overnight in blocking buffer [5% (w/v) nonfat dry milk, in phosphate-buffered saline (PBS)]. Anti-H-NS (1:1000, a generous gift from E. Bremer, University of Konstanz, Germany), anti-FliC and anti-FljB (1:1000, Becton Dickinson) anti-SsrB (Kelly 2004) or anti-RpoS (1:3000, a generous gift from R. Hengge, University of Berlin, Germany) antisera were diluted appropriately in blocking buffer and incubated with the membrane for 1 h at room temperature. The membrane was washed 3 x 10 min with PBS and then incubated in blocking buffer containing horseradish-peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signalling) for 1 h. The blot was washed as before, and in the presence of a suitable chemiluminescent substrate (Pierce SuperSignal), the HRP-mediated enzymatic reaction results in a luminescent signal that visualizes the antigen-antibody complex, which can by detected by autoradiography (Section 2.19). Typical exposures were from 30 sec to 15 min.

2.19 Autoradiography

In this study autoradiography was used to visualize and quantitate on X-ray film non-radioactive chemiluminescent emissions derived from alkaline phosphatase or horseradish peroxidase cleavage of chromogenic reagents (western, Southern, and northern blots). In each case Hyperfilm™ UV film (Amersham Biosciences) was used. When chemiluminescent photon emissions strike a silver halide crystal (X-Ray film is coated with silver halides suspended in gelatin), the crystal absorbs energy and releases an electron. This electron is attracted to a positively charged silver ion forming an atom of metallic silver. After an appropriate time the film was placed into a tray containing Kodak Readymatic™ Developer and replenisher for 3 min, a chemical solution that amplifies the signal by reducing exposed silver halide crystals to metallic silver. The film was washed briefly in water and then fixed in Kodak Readymatic™ fixer and replenisher for a further 3 min. The fixer serves to convert
any silver halide that was not reduced into soluble silver thiosulphate. Developed films were rinsed in a large volume of water and allowed to dry. For quantitative analysis of signal intensity several exposures of varying times were taken.

2.20 DNA microarray analysis

DNA microarray analysis was carried out to determine the *dps* regulon of *S.* Typhimurium during growth in LB broth and was performed as described previously (Clements *et al.*, 2002). Briefly total RNA was isolated and reverse transcribed to generate cDNA, this cDNA was labeled with Cy5 (red) dye and hybridised (together with Cy3 (green) labeled genomic DNA) to the array. Scanning of the slides was carried out the GenePix 4000A scanner. Fluorescent spot and local background intensities were quantified using BlueFuse software. Hybridisations of samples were carried out in triplicate and two independent experiments were carried out.

2.20.1 RNA isolation

Total RNA extracts were prepared by inoculating 250 ml flasks containing 25 ml volumes of L-broth with 1:100 volumes of overnight cultures of wild-type SL1344 and JTS004. Flasks were incubated at 37°C with shaking. At 4 h, 12 h and 22 h post subculture bacterial RNA was extracted. Volumes of culture corresponding to 4 OD\textsubscript{600} units for 4 h or 6 OD\textsubscript{600} units (i.e. 4ml or 6ml of OD\textsubscript{600} = 1) for 12 and 22 h were used. The appropriate volume of culture was transferred to a tube containing 0.2 volumes of phenol/ethanol mix (90 % (v/v) ethanol, 10% (v/v) phenol pH 4.3) and incubated on ice for at least 30 min. Incubation in phenol/ethanol helps stabilise RNA and prevent degradation (Tedin & Blasi). Post incubation on ice, cells were pelleted by centrifugation (Jouan MR23i) at 4,000 rpm for 10 min at 4 °C, supernatant was discarded and the pellets were resuspended in residual liquid present in the tubes and transferred to 1.5 ml Eppendorf tubes. The Eppendorf tubes were centrifuged at 16,000 xG and the supernatant was again discarded. Samples were frozen at −80 °C until required. RNA was isolated using the SV Total RNA Isolation System (Promega) (Section 2.11.4) and as described at http://www.ifr.ac.uk/safety/microarrays/protocols.html After elution the RNA was quantified as described in section 2.9.2, precipitated and resuspended at a concentration of 2 μg/μl in RNase free water (Sigma)
2.20.2 cDNA synthesis and Cy5-dye labeling

In a sterile microfuge tube 10 µg of RNA was incubated with 5 µg of random hexamer primers in a total volume of 12.4 µl using RNase free water (Sigma). To facilitate annealing of the primers to the RNA the mixture was heated to 70°C for 10 min and then cooled on ice 10 min, after which 12.6 µl of the following reaction mix was added: 3 µl 5 x buffer, 3 µl 0.1-M DTT, 0.6 µl dNTP mix, 2 µl dCTP-Cy5 (Amersham), and 4 µl SuperScript™ II (Invitrogen). Following overnight incubation in the dark at 37°C, 15 µl 0.1M NaOH was added and samples heated to 70°C for 15 min to hydrolyse the RNA. To this 15 µl 0.1 M HCl was then added to neutralize the samples and the resulting labelled cDNA purified using a Qiaquick PCR cleanup spin column (Qiagen) according to the guidelines provided and eluted in 2 x 50 µl RNase free water (Sigma).

2.20.3 Cy3-labelling of genomic DNA

Fluorescently labeled genomic DNA (0.4 µg) from SL1344 was used as a reference channel in each experiment. cDNA was labeled with cy5 while genomic DNA was labeled with cy3. 4 µg of EcoRI digested chromosomal DNA was added to a sterile microcentrifuge tube and the volume adjusted to 42 µl with RNase free water (Sigma). To this was added 40 µl of 2.5 X Random primer/reaction buffer from the Gibco Bioprime DNA labelling System. The mixture was boiled at 95 °C for 5 min then incubated on ice for a further 5 min. To the cooled mixture was added 10 µl 10 X dNTP mix (1.2 mM each dATP, dGTP, dTTP; 0.6 mM dCTP), 6 µl Cy3-dCTP and 1 µl Klenow. The mix was incubated at 37 °C in the dark overnight as for the RNA test samples. This volume of sample is enough for 10 microarrays. The labelled gDNA was purified using a Qiaquick PCR cleanup spin column (Qiagen) according to the guidelines provided, and eluted in 2 x 50 µl RNase free water (Sigma).

2.20.4 1,2-dichloroethane (DCE) blocking of microarray slides

DNA microarrays were printed on Corning CMT-GAPS-coated slides by a robotic DNA arrayer built in-house by Arthur Thompson, Sacha Lucchini and Bruce Pearson at the IFR. Each slide contains 2 X arrays. Each ‘Salsa’ Salmonella serovar microarray consists of 16 blocks of printed PCR products representing 5080
Salmonella genes including 4414 S. Typhimurium LT2a (and pSLT) genes, 155 S. Typhimurium DT104 specific genes, 229 S. Typhimurium SL1344 specific genes, 196 S. Enteritidis PT4 specific genes and 86 S. Gallinarum 287/91 specific genes http://www.ifr.ac.uk/Safety/Microarrays/default.html. Microarray slides were blocked as follows.

A diamond pencil was used to mark the edges of each array, on the reverse side, before the blocking procedure began. DNA was immobilised to the slides by crosslinking in a UV Strata linker using the autocrosslink setting. 1.5 g of succinic anhydride was added to 300 ml of anhydrous 1,2-dichloroethane, (DCE). As soon as the succinic anhydride had dissolved 3.75 ml of n-methylimidazol was added. Slides were incubated in this blocking solution with gentle agitation for 1 h at room temperature. After blocking the slides were washed with fresh DCE, 300 ml, for 3 min. The slides were quickly transferred to boiling water for 2 min to denature the DNA. Once denatured the slides were transferred to a slide chamber containing 300 ml 96% ethanol for 1 min. Finally the slides were transferred to a clean dry slide chamber lined with blotting paper and centrifuged at 1,200 rpm (Jouan MR23i) for 5 min to dry completely.

2.20.5 Hybridisation of DNA to slides
Each cDNA sample and the gDNA sample were dried down in a speed vac. A master mix of gDNA was set up (the dried gDNA sample was resuspended in 9.75 µl RNase free water (Sigma) X times the number of cDNA samples). 9.75 µl of the resuspended gDNA was used to resuspend each cDNA test sample. To this the following were added 1.125 µl E.coli tRNA, 2.25 µl 20 X SSC, 0.36 µl Hepes buffer, 0.338 µl 10% (w/v) SDS, 1.5 µl Denhardt’s solution. Samples were incubated at 100 °C for 2 min then cooled at room temperature for 10 min. The tubes were centrifuged at maximum speed for 5 min in a microcentrifuge and the supernatant was transferred to a clean tube. This centrifugation step was repeated. An array slide was placed in a hybridization chamber (Die-Tech) and 15 µl of the hybridization solution was carefully applied towards the edge of the array. The edge of a clean coverslip was placed on the edge of the array. A fine nosed forceps was positioned under the coverslip. Using the forceps the coverslip was slowly lowered down onto the
hybridization solution and the array taking care to exclude bubbles and ensuring even distribution of the solution across the array. 5 µl of 3 X SSC was applied on each corner of the slide to maintain the correct humidity inside the hybridization chamber. The hybridization chamber was sealed and placed in a water bath at 63°C overnight.

The following day the hybridization chambers were opened and the slides washed as follows. Arrays were washed twice in wash solution (2 X SSC, 0.1% SDS) 5 min at 63°C, with gentle agitation to remove coverslips. Followed by two further 5 min washes to remove SDS from the slides firstly in 1 X SSC at room temperature and finally by two 5 min washes in 0.2 X SSC also at room temperature. After the final wash the slides were dried by centrifugation 1,200 rpm (Jouan MR23i) for 5 min.

In this study two biological replicates and two technical replicates of RNA were used.

2.20.6 Scanning of slides and microarray data handling
After hybridization, microarray slides were scanned using a GenePix 4000A scanner (Axon Instruments.). Fluorescent spot intensities and local background data were quantified using the BlueFuse software package (BlueGnome, Oxford.). Data were passed through quality control procedures outlined in Eriksson et al (2003). Data that passed the quality controls were saved in .gpr file format, which were then converted to .txt text format. The .txt files were imported into Microsoft Excel and using a custom designed macro program (S. Lucchini), the cDNA data were normalized against the genomic DNA data. Data was then imported into the microarray analysis program GeneSpring 7.3 (Silicon Genetics). Only genes whose expression ratio showed at least a 1.5-fold difference [false discovery rate (FDR) ≤0.10] were considered as being statistically significant.

2.21 2-D PAGE

2.21.1 Protein sample preparation
Overnight bacterial cultures were diluted 1:100 into fresh broth and grown to mid exponential stage or late stationary stage. 100 ml of OD 600nm 1.0 units were harvested by centrifugation at 4,600 x g for 10 min at 4°C and the pellet was
resuspended in 10 ml of a low-salt wash buffer (3.0 mM KCl, 1.5 mM KH₂PO₄, 68 mM NaCl, 9.0 mM NaH₂PO₄) (www.expasy.org). The suspension was centrifuged again as before and washed a further three times with low-salt wash buffer. Following the final wash the bacterial pellet was resuspended in 200 μl of resuspension buffer (10 mM Tris-HCl (pH 8.0), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% (w/v) SDS and 1 tablet/10ml Complete Mini (protease inhibitor cocktail, Roche) and the bacterial samples were stored at -20°C in 40 μl aliquots. Each sample was thawed only once to minimise freeze-thaw damage. It should be noted that this method of sample preparation may result in an under-representation of membrane associated proteins.

2.2.1.2 Immobilized pH gradient (IPG) strip loading
To further solubilise proteins prior to first dimension separation the samples were incubated in a solution containing a high concentration of thiourea, urea, DTT and the detergent CHAPS. 40 μl of resuspension was thawed on ice 15 min and then mixed with 200 μl solubilisation buffer (7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 65 mM DTT, 0.0005% (w/v) bromophenol blue) and incubated at room temperature for 3 h. To ensure that proteins did not precipitate at their isoelectric point carrier ampholytes (pH 3-10) (BioRad) to a final concentration of 0.2% were added and the samples were again incubated at room temperature for 1 h. After removing insoluble proteins by centrifugation (10,000 x g 5 min) samples were loaded onto ProteoGel 4-7 IPG strips (Sigma-Aldrich), and passive rehydration was carried out overnight, in a sealed rehydration tray. Evaporation was minimised by adding 200 μl water to unused lanes in the tray and sealing the tray with parafilm. The rehydration was carried on as flat a surface as possible to ensure even distribution of the samples across the strips.

2.2.1.3 First dimension isoelectric focusing
Paper wicks were dipped in ddH₂O and placed onto the electrodes of a BioRad focusing tray. Rehydrated IPG strips were placed gel side down onto the paper wicks and 1 ml of mineral oil was added over each strip to prevent evaporation. Isoelectric focusing was performed at 150 V for 300 V hours and 6,000 V for 40,000 V hours, to separate the proteins according to their isoelectric point (pI).
2.21.4 Equilibration and second dimension SDS-PAGE

Before running the second dimension the IPG strips were incubated with gentle shaking for 10 min in 2.5 ml equilibration buffer (6 M Urea, 50 mM Tris-HCl pH 8.0, 30% (v/v) Glycerol, 2% (w/v) SDS, 0.0005% (w/v) bromophenol blue) and reducing agent DTT (25 mg/ml) followed by incubation for 10 min in an alkylating solution, 2.5 ml equilibration buffer and iodoacetamide (25 mg/ml). Each strip was then briefly dipped in 1x SDS-PAGE running buffer and then applied to the top a 12% (w/v) polyacrylamide resolving gel, overlaying the strip with 1% agarose overlay (1% (w/v) agarose 0.0005% (w/v) bromophenol blue). Electrophoresis was carried out at 200 V for 45 min to separate proteins based on their molecular mass. Resolved proteins were visualised using Coomassie Blue Stain before analysis was carried out.

2.21.5 Protein spot data handling

The raw 2-D electrophoresis gels were imaged using a BioRAD GS800 calibrated densitometer and further processed using the PDQuest™ software package. Spots were matched between the wild type and dps mutant gels.

2.21.6 Spot excision and identification

Protein spots showing different levels depending on Dps were excised from stained gels, and followed by in-gel digestion using the Trypsin Profile IGD Kit For In-Gel Digests (Sigma). Each excised gel piece was placed in an Eppendorf tube with 200 μl of destaining solution, to remove dye bound to the protein of interest, and incubated at 37°C for 30 minutes. The solution was discarded from the tube and replaced with fresh destaining solution. Following the second incubation in destaining solution the solution was again discarded and the gel was dried in a SpeedVac for 30 min. Trypsin to a final concentration of 8 ng/μl in 70 μl of Trypsin Reaction Buffer was added to the dried gel. Digestion was carried out at 37°C overnight. The solution containing the extracted tryptic peptides was transferred to fresh Eppendorfs. To increase the peptide yield 50 μl of Peptide Extraction Solution was added to the gel piece followed by a further incubation at 37°C for 30 min, the solution was removed and added to the initial tryptic peptides solution. The combined solution was dried in a SpeedVac until no liquid remained, and the resultant peptides were concentrated and desalted by
passing through a C18 ZipTip® (Millipore) that had been equilibrated with acetronitrile before MALDI-TOF analysis was carried out.

2.22 Tissue cell culture
Cell culture was performed to assess the effect that the absence of Dps has on invasion of epithelial cells and on survival in macrophage-like cells, and to assess the expression profile of dps in both epithelial and macrophage cell lines.

2.22.1 Epithelial cell invasion assays
Epithelial cells (CHO-K1 or CACO-II) were seeded 72 h prior to infection in 12-well plates at a density of $2 \times 10^5$ cells per well in culture medium [Dulbecco’s Modified Eagle’s Medium with F12 Nutrient mix supplemented with 10% (v/v) heat-inactivated foetal bovine serum]. The medium was removed from the monolayers. Bacteria from overnight L-broth cultures were harvested and resuspended in pre-warmed antibiotic-free culture medium and added to either CHO-K1 or CACO-II monolayers at a multiplicity of infection of 100:1. Plates were centrifuged at 600 xg for 5 min and then incubated at 37°C in 5% CO$_2$ for 1 h to allow invasion to occur. The culture medium was aspirated and cells were washed three times with PBS. The monolayers were incubated in fresh culture medium containing gentamicin (100 µg/ml) for 1 h to kill extracellular bacteria. The CHO-K1 monolayers were washed three times with PBS and then lysed with PBS containing 0.5 % (v/v) Triton X-100 (Sigma) to release the intracellular bacteria. An additional step was required to lyse the CACO-II monolayers. CACO-II monolayers were washed three times with PBS, incubated in trypsin-EDTA solution (Sigma) for 10 min at 37°C (to degrade protein matrix to produce a single-cell suspension) and then lysed with PBS containing 0.5 % (v/v) Triton X-100 (Sigma) to release the intracellular bacteria. Bacterial suspensions were serially diluted and spread onto L-agar plates or L-agar plates supplemented with chloramphenicol as appropriate. Plates were incubated for 24 h and then scored for colony forming units.
2.22.2 Macrophage survival assays
The experiment was carried out in the murine macrophage-like J774 cell line (American Type Culture Collection Manassas, VA, USA). Cells were seeded 72 h prior to infection in 12-well plates at a density of $2 \times 10^5$ cells per well in culture medium [Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum]. Bacteria from overnight L-broth cultures were harvested and resuspended in pre-warmed antibiotic-free culture medium and added to the monolayers at a multiplicity of infection of 100:1. Plates were centrifuged at 600 $\times$ g for 5 min and then incubated at 37°C in 5% CO$_2$ for 1 h to allow phagocytosis of the $Salmonella$ to occur. The culture medium was then aspirated. The monolayers were washed three times with PBS and incubated in fresh culture medium containing gentamicin (100 $\mu$g/ml) for 1 h to kill extracellular bacteria. The gentamicin-containing medium was aspirated and cells washed three times with PBS. The monolayers were then either lysed with PBS containing 0.5 % (v/v) Triton X-100 (Sigma) or incubated in fresh culture medium containing a minim concentration of gentamicin (20 $\mu$g/ml) and lysed 4, 6 or 8 h post-infection. After incubation, cells were washed three times with PBS and lysed with PBS containing 0.5 % (v/v) Triton X-100 (Sigma) to release the intracellular bacteria. Cell lysates were serially diluted and spread onto L-agar or L-agar plates supplemented with chloramphenicol as appropriate, incubated for 24 h and then scored for colony forming units.

2.22.3 Intracellular expression profile of $dps$
Cells were grown prior to infection in tissue-culture flasks in culture medium, until confluence was achieved. The medium was removed from the tissue culture flask, rinsed with DMEM that was also removed and replaced with 10ml Tripsin to dissolve the monolayer. The flask was incubated for 15 min at 37°C in 5% CO$_2$ and checked under the microscope to ensure monolayer had come away. The flask was gently agitated and the medium poured into a sterilin and centrifuged @ 1,200 rpm for 5 min. The cell pellet was resuspended in 5 ml of fresh medium and the number of cells present counted in a haemocytometer and further resuspended to give a concentration of $1 \times 10^6$ cells /ml. The assay was carried out in flow cytometer tubes using a concentration of $3 \times 10^5$ cells /tube, therefore 300 $\mu$l of resuspended cells was added to each tube.
Bacteria from overnight L-broth cultures containing the pZepdps plasmid were harvested and resuspended in pre-warmed antibiotic-free culture medium and added to the flow cytometer tubes at a multiplicity of 100:1, this represented time 0. Control tubes were also set up with no added bacteria. The tubes were incubated for 45 min at 37°C in 5% CO₂.

The tubes were removed from the incubator and washed with 3 ml PBS, the tubes were centrifuged @ 1,200 rpm for 5 min and the supernatant carefully decanted off in one movement. The pellet was resuspended in 500 μl Tissue Culture Medium supplemented with 100 μl/ml gentamycin by vortexing and the tubes incubated again until 2 h from time 0 was up. After the appropriate incubation time the cells were again washed in PBS and the cells fixed by resuspending the pellet in 300 μl PBS 2% (v/v) formaldehyde. The tubes containing fixed cells were incubated in the dark at 4°C prior to being read in the flow cytometer (Section 2.10). For incubation periods longer than 2 h, following washing in PBS the pellets were resupended in 500 μl Tissue Culture Medium supplemented with 20 μl/ml gentamycin by vortexing and the tubes incubated again until the appropriate incubation time was reached. Cells were fixed as for the 2 h period.

2.23 Phenotypic Array
To investigate a possible role for Dps in the regulation of metabolism, the ability of the dps mutant to oxidize defined carbon sources in the presence of a redox indicator – tetrazolium dye – was assessed by utilizing the Phenotype MicroArray™ system (Biolog).

2.23.1 Culture preparation
R2A agar plates were used to revive wild-type and dps mutant strains from frozen stocks to obtain single colonies. Single colonies of each strain were subsequently re-inoculated on fresh R2A agar plates 24 h before the commencement of the assay. The bacteria were removed from the plate with sterile swabs before being suspended in broth (Biolog). The OD₆₀₀nm of each suspension was measured and the strains were
standardized to each other by the further addition of broth followed by $\text{OD}_{600\text{nm}}$ measurement to ensure equal loading of the panels.

2.23.2 Phenotype microarray panel loading

The Phenotype MicroArray™ system (Biolog) panel consists of 96 wells containing defined carbon sources in the presence of the redox indicator – tetrazolium dye. 100 µl of suspension was inoculated into each of the 96 wells. The plates were incubated for 24 h at 37 °C before being read.

2.23.3 Phenotype microarray panel reading

The ability of each strain to oxidize a particular carbon source was indicated by a colour change in the redox indicator. If no oxidation occurs the dye remains colourless, a negative control or blank is present in well A1. If oxidation occurs a purple colour is produced. To quantify the relative change in colour of the dye observed, the plates were placed in a plate reader and the $\text{OD}_{540\text{nm}}$ was measured. Data were normalized by subtracting the measurement of the negative control from each measurement. Mean values were determined from three independent experiments.

2.24 Relative fitness assay

The ability of *Salmonella* to utilise specific sole carbon sources was further assessed by relative fitness assay in MOPS minimal medium supplemented with 0.2% (w/v) defined carbon source as indicated. The fitness of JTS004 relative to wild-type in the presence of an iron chelating agent was determined by competitive assay in LB containing 2 µm 2,2-dipyridyl. In all cases to insure that fitness was not affected by the presence of the chloramphenicol acetyltransferase (*cat*) gene, strain JH3008 (Hautefort *et al.*, 2003) was used as a control. Fitness can be defined as the average contribution of one genotype to the next or succeeding generations compared to that of other genotypes. The fitness of JTS004 relative to wild-type in the presence of defined carbon sources or in the presence of the iron chelating agent 2,2-dipyridyl was determined by simultaneously inoculating 25 ml fresh growth medium with $10^5$ cells of each competitor, which had been preconditioned in the growth medium for 24 h. The ratio of the two competing strains was determined at time zero and after 24 h growth by viable counts after plating serial dilutions of the inoculum in parallel on LB (to measure total c.f.u) and LB with cml (to determine mutant c.f.u). Relative fitness
$(W)$ was expressed as the ratio of the Malthusian parameters of the two strains being compared (Dahlberg and Chao, 2003) and calculated as follows:

\[
W_{ij} = \log_2 \left( \frac{N_j(1)}{N_j(0)} \right) \\
\log_2 \left( \frac{N_j(1)}{N_j(0)} \right)
\]

$N_j(0)$ and $N_j(1)$ = initial and final density of test strain, respectively

$N_j(0)$ and $N_j(1)$ = initial and final density of common competitor strain, respectively

Each assay was performed in triplicate and the mean values were determined from at least three independent experiments.
Chapter 3

Characterisation of a *dps* knockout mutant of *Salmonella enterica* serovar Typhimurium
3.1 Introduction

The *S. Typhimurium* *dps* gene has 85% homology with *dps* in *Escherichia coli* at the nucleotide level and its product has 95% homology in its amino acid sequence with *E. coli* Dps (McClelland *et al.*, 2001). The *E. coli* *dps* gene promoter is recognised by both the RNA polymerase vegetative holoenzyme Eσ\(^70\) and by the stationary phase holoenzyme Eσ\(^38\). The promoter has a sequence that conforms closely to the consensus -10 sequence for σ\(^70\) (TATACT compared to TATAAT), however to date no corresponding -35 sequence is evident (Altuvia *et al.*, 1994). Unusually both sigma factors recognize the same region in the *dps* promoter site (Lomovskaya *et al.*, 1994). Binding sites for the transcription factors IHF (concerned with the transcription of the *dps* gene during the stationary phase of growth) and OxyR (concerned with the control of transcription during oxidative stress) are located upstream of the promoter and it is thought that selection of the specific RNA polymerase holoenzyme relies on the interaction of the appropriate transcription factor with its binding site (Altuvia *et al.*, 1994; Lacour *et al.*, 2004).

Since its discovery it has been postulated that Dps has a regulatory role (Almiron *et al.*, 1992). During the initial investigation of the protein it was found that *E. coli* cells lacking Dps show dramatic changes in the pattern of proteins synthesised during starvation, however none of the proteins were identified during the study (Almiron *et al.*, 1992). Studies in *Salmonella enterica* serovar Typhimurium have shown that *dps* expression is induced following *Salmonella* ingestion by macrophages and that Dps promotes survival in murine macrophages and enhances *Salmonella* virulence in mice (Eriksson *et al.*, 2003; Halsey *et al.*, 2004).

To date the majority of studies into Dps have concentrated on the identification of Dps homologues throughout the bacterial world and confirmation that these homologues protect the cell from the effects of oxidative damage and other stresses (Ceci *et al.*, 2003; Chen *et al.*, 1995; Dundon *et al.*, 2002; Gupta *et al.*, 2002). Additional studies have concentrated on an analysis of the structure of Dps and in particular its similarity to ferritins (Grant *et al.*, 1998; Illari *et al.*, 2002; Reindel *et al.*, 2002; Yamamoto *et al.*, 2002). One further focus of study has been investigations in to the interaction
between Dps and DNA that results in the formation of tightly packed and highly ordered biocrystals (Minsky et al., 2002; Wolf et al., 1999).

The purpose of this study was to examine a \textit{dps} mutant in \textit{S} Typhimurium for phenotypic differences compared to the wild-type, to analyse \textit{dps} promoter activity and to investigate the role of Dps in the intracellular survival of SL1344.
3.2 Results

3.2.1 Examination of SL1344dps

To examine the effect of Dps on gene expression in \textit{S. enterica} it was necessary to mutate the \textit{dps} gene to prevent its expression. In the first instance I was kindly provided with a \textit{dps} mutant derivative of SL1344 by the BBSRC Institute of Food Research, Norwich, UK. This SL1344dps mutant had been constructed through an adaptation of the Datsenko and Wanner method whereby the open reading frame had been deleted and replaced with an antibiotic resistance cassette, (the \textit{aph} gene conferring resistance to kanamycin). The phenotypic effects of the \textit{dps} mutation were assessed. Published findings in \textit{E. coli} indicated a 20-fold decrease in viability of a \textit{dps} mutant when the bacteria were subjected to oxidative stress (Martinez and Kolter 1997). Overnight cultures of wild-type SL1344 and SL1344dps were resuspended in fresh broth and grown to stationary phase. The cultures were subjected to oxidative stress through the addition of 40 mM H$_2$O$_2$ or 50 mM H$_2$O$_2$ for 10 min (Section 2.7.1). The number of viable cells was determined by serial dilution and plating on LB-agar or LB-agar containing kanamycin as appropriate, and survival rates were calculated relative to the control culture with no H$_2$O$_2$ (Fig 3.1 A). Unexpectedly the \textit{dps} mutant displayed a higher survival rate than that of the wild-type at both concentrations of H$_2$O$_2$ tested. At 40 mM H$_2$O$_2$ the wild type survival rate was 12\% and the corresponding survival rate for the \textit{dps} mutant was 28\%. At 50 mM H$_2$O$_2$ the wild type survival rate was 5\% and the corresponding survival rate for the \textit{dps} mutant was increased from the 40 mM level at 28\% H$_2$O$_2$.

The IFR SL1344dps strain was further analysed by an acid stress test. Overnight cultures of wild-type SL1344 and SL1344dps were resuspended in fresh broth and grown to stationary phase. The cultures were subjected to acid stress through the resuspension of the bacteria in PBS at pH 2. The number of viable cells was determined by periodic serial dilution and plating on LB-agar or LB-agar containing kanamycin as appropriate, and survival rates were calculated. There was a decrease in survival rate in the \textit{dps} mutant of approximately two-fold compared to the wild-type \textit{Salmonella} over the time points tested (Fig 3.1 B). The results were again at variance with previously published work relating to \textit{E. coli}. Analysis of \textit{E. coli} 0157:H7
suggests a ten-fold decrease in survival for *dps* mutants under the same conditions (Choi *et al.*, 2000). It appeared therefore that SL1344*dps* was able to withstand conditions of oxidative or acid stress despite the presumed absence of Dps.

The IFR *dps* mutant was finally analysed through molecular methods. Total genomic DNA was isolated from strains SL1344 (wild-type) and SL1344*dps* (*dps*) (Section 2.11.3). The genomic DNA was digested with *BsrG*I and *Hinc*II, these enzymes were chosen as their recognition sites are external and flanking to the *dps* open reading frame and are not present in the *aph* gene. The digested total DNA was electrophoresed on agarose gels and probed for the *dps* gene using the primer pair *dps*for and *dps*rev (Table 2.3), these primers were used to amplify the open reading frame of *dps* (Section 2.13). Southern blot analysis (Section 2.17) revealed there was a target for hybridisation in the mutant (Fig 3.2). As the primers used to generate the mutant and those used to generate the probe are nested, elimination of the *dps* locus should deprive the probe of a hybridisation target. It appeared therefore that the IFR SL1344*dps* strain was not a *dps* knockout mutant, the mutant retained its protective role to some extent when subjected to oxidative and acid stress.

### 3.2.2 Construction of JTS004

The *dps* knockout mutant strain JTS004, was constructed by an allelic replacement technique based on the method of Datsenko and Wanner (2000) (Section 2.4). Primer pair *dw*dpsfor and *dw*dpsrev (Table 2.3) were designed such that they had homology internally to the *cat* gene of pACYC184, which codes for chloramphenicol-acetyltransferase, flanked by regions directly upstream and downstream of the open reading frame of *dps* (Fig 3.3 A,B). Following PCR, the resulting amplicon was gel purified and transformed by electroporation into SL1344 harbouring the pKOBEGA plasmid (Section 2.5.2). Homologous recombination resulted in an insertion event disrupting the *dps* gene (Fig 3.3 C). In this study the *dps::cat* allele was confirmed to be a mutant for *dps* by PCR, Southern blotting (Section 3.2.3), sequencing and by phenotypic testing (Section 3.2.5).

### 3.2.3 Genetic confirmation of the *dps* mutation

57
Fig 3.1. Investigation of SL1344dps from the IFR. (A) Histogram showing % survival of wild-type (pink) and SL1344dps (blue) following incubation in 40 mM or 50 mM H$_2$O$_2$ for 10 min. Results are averages of two independent experiments carried out in duplicate and error bars represent standard deviation. (B) Graph shows % survival of wild-type SL1344 ♦ and SL1344dps ■ following incubation in PBS pH 2 for indicated time. Results are averages of two independent experiments carried out in triplicate and error bars represent standard deviation.
Fig 3.3 Generation of the *dps* mutant JTS004. A) Genetic organization of the region of DNA around the *dps* gene. Positions of primer pairs and dwdpsfor and dwdpsrev (Table 2.3) are indicated. The blue arrows signify regions of homology flanking the gene. B) Genetic organization of the cloning vector and source of the chloramphenicol resistance cassette cat. Primer pair dwdpsfor and dwdpsrev (Table 2.3) were designed such that they had homology internally to the cat gene of pACYC184, which codes for chloramphenicol-acetyltransferase, flanked by regions directly upstream and downstream of the open reading frame of *dps* as highlighted in A. C) Following PCR amplification the linear product was gel purified and transformed by electroporation into Wild-type SL1344 harbouring the pKobega plasmid. Homologous recombination resulted in an insertion event disrupting the *dps* gene.
Initially PCR analysis of wild-type SL1344 and its otherwise isogenic \textit{dps} mutant JTS004 was carried out. Primer pair \textit{dpsfor} and \textit{dpsrev} (Table 2.3) were used to amplify the open reading frame of \textit{dps} (Section 2.13). An amplicon in the wild-type was found migrating at a size consistent with the published size of the \textit{dps} gene (515 bp) (McClelland \textit{et al.}, 2001); no product was recovered from the mutant strain (Fig 3.4 B).

Total genomic DNA was isolated from strains SL1344 (wild-type) and JTS004 (\textit{dps}). As described in the genetic analysis of the IFR \textit{dps} strain, the genomic DNA was digested with \textit{BsrGI} and \textit{HincII}, enzymes whose recognition sites flank the \textit{dps} open reading frame and are not present in the \textit{cat} gene. The digested total DNA was electrophoresed on agarose gels and probed for the \textit{dps} and the \textit{cat} gene through Southern blotting (Section 2.17). The \textit{dps} specific probe detected a signal in the wild-type consistent with the expected size of the digested fragment and a signal in the \textit{dps} mutant shifted to approximately 1.5 kb indicating that an insertion event had occurred (Fig 3.4 B). Analogous to the investigation of the IFR SL1344\textit{dps} the primer pair used to generate the probe \textit{dpsfor} and \textit{dpsrev} and the primer pair \textit{dwdpsfor} and \textit{dwdpsrev} used to generate the mutant are nested (Fig 3.4 A). The \textit{S. Typhimurium} element of the upstream primer \textit{dwdpsfor} stops at the start of the open reading frame whereas the \textit{dps} element of \textit{dpsfor} begins at the start codon of the gene with a comparable orientation of the reverse primers concerning the end and downstream region of the \textit{dps} gene. If a complete deletion of the ORF had occurred then no signal would be detected by the probe in the mutant strain. The \textit{cat} specific probe failed to detect a signal in the wild-type and detected a signal in the \textit{dps} mutant of equivalent size to that detected by the \textit{dps} probe indicating that the insertion event that occurred, resulted in the insertion of the chloramphenicol resistance gene into the \textit{dps} gene. The wild-type \textit{dps} signal migrated at just below 1 kb, while the signal in the \textit{dps} mutant migrated at approximately 1.5 kb. The \textit{cat} gene from pACYC184 is approximately 600 bp (Alton \textit{et al.}, 1979); as the shift observed between wild-type and mutant was approximately 600 bp it appeared that the chloramphenicol resistance cassette had become incorporated into the \textit{dps} ORF.
3.2.4 Effect of the \textit{dps} mutation on growth of \textit{S} Typhimurium in batch culture

The growth rates of wild-type strain, SL1344 and its \textit{dps} mutant derivative were compared in LB medium at 37°C, by measuring the optical density of the culture at 600 nm over a 12 h time course (Fig. 3.5 A). The generation time during the exponential phase of growth for the wild-type strain was 27 min and 26 min for the \textit{dps} mutant. Therefore there is no substantial growth defect associated with the \textit{dps::cat} knockout mutant when grown in rich medium. As Dps is a protein primarily associated with the stationary phase and starvation conditions, the growth rates of SL1344 and JTS004 were compared in MOPS minimal medium with glucose as the sole carbon source. The \textit{dps} mutant displayed a longer lag phase prior to exponential growth and ultimately did not reach the same OD\textsubscript{600nm} as the wild-type (Fig. 3.5 B). The generation time during the exponential phase of growth for the wild-type strain was 48 min but it was 76 min for the \textit{dps} mutant.

3.2.5 Phenotypic confirmation of the \textit{dps} mutation

A key characteristic of \textit{dps} mutants is their reduced ability to survive when oxidative stress is encountered, for this reason SL1344 and JTS004 were subjected to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) stress assays (Section 2.7.2). Stationary phase cultures were incubated in 5 ml PBS 0.5 mM H\textsubscript{2}O\textsubscript{2} or 5 ml PBS for 2 h without shaking and the survival rate was determined relative to the PBS control and compared. The results revealed a decrease in viability of the \textit{dps} mutant strain when compared to wild-type of approximately 14-fold, 2.25% survival of the \textit{dps} mutant compared to 33.75% for wild-type. These results were similar to previously published findings in \textit{S. Typhimurium} that showed a 15-fold decrease in viability of SL1344\textit{dps} when subjected to 2 h incubation in 1 mM H\textsubscript{2}O\textsubscript{2} (Halsey \textit{et al.}, 2004).

To further confirm that JTS004 was indeed a \textit{dps} knockout mutant an acid stress assay was carried out (Section 2.8). It has previously been shown that \textit{dps} mutants are more susceptible to acid stress than wild-type cells (Nair and Finkel, 2004). Stationary cultures of SL1344 and JTS004 were standardised to 10^7 cfu/ml and resuspended in 5 ml of PBS pH 2. The cultures were incubated at 37°C with shaking for 3 h. The number of viable cells was periodically determined by serial dilution and plating on
FIG 3.4. Molecular examination of SL1344 and JTS004. A) Genetic organization of the region of DNA around the $dps$ gene. Positions of primer pairs dpsfor and dpsrev and dwdpsfor and dwdpsrev (Table 2.3) are indicated. The red arrows and blue arrows signify regions of homology with the gene and regions of homology flanking the gene respectively. B) PCR analysis of wild-type SL1344 (lane 1) and its otherwise isogenic $dps$ mutant JTS004 (lane 2), using primer pair dpsfor and dpsrev to amplify the open reading frame of $dps$. No product was recovered from the mutant strain. C) Southern blot analysis of SL1344 and JTS004. Genomic DNA was harvested from SL1344 and JTS004, digested with $BsrG\text{i}$ and $Hinc\text{II}$ and probed with a dig-labelled $dps$ specific probe using the primer pair dpsfor and dpsrev. Arrows indicate position of migration of molecular size markers, white asterixes indicate position of migration of hybridised targets. The $dps$ specific probe detected a signal in the wild-type consistent with the expected size of the digested fragment and a signal in the $dps$ mutant shifted to approximately 1.5 kb indicating that an insertion event rather than a deletion event had occurred. MM = Molecular size marker.
A. 

![Diagram A](image1)

B. 

![Diagram B](image2)

C. 

![Diagram C](image3)
LB-agar or LB-agar containing chloramphenicol as appropriate. Survival rates were calculated relative to time point 0, the time at which the cultures were resuspended in the PBS pH 2 (Fig 3.6). The results showed an increase in susceptibility to acid stress in the \( \text{dps} \) mutant strain when compared to wild-type, a six-fold decrease in survival was observed at 45 min 26% of wild-type cells survived in comparison to 6% of \( \text{dps} \) mutant cells.

3.2.6 Complementation of \( \text{dps} \) in trans

To insure that the phenotypes observed in JTS004 were a consequence of the mutation in \( \text{dps} \), a \( \text{dps} \) complementation plasmid was constructed. The \( \text{dps} \) ORF was PCR amplified using the primer pair \( p\text{CLdpsfor} \) and \( p\text{CLdpsrev} \) (Table 2.3) and cloned into the low-copy plasmid vector \( p\text{CL1920} \) to create plasmid \( p\text{JTS505} \) (Table 2.2). The amplified \( \text{dps} \) ORF fragment was flanked with \( Psl \) and \( B\text{amHI} \) restriction sites as the 5' and 3' ends respectively. After digestion with \( Psl \) and \( B\text{amHI} \), the fragment was cloned into the \( Psl/B\text{amHI} \) digested \( p\text{CL1920} \) (Section 2.12.4). The order of these restriction sites in the plasmid \( p\text{CL1920} \) ensured the correct orientation of the \( \text{dps} \) ORF. Ligated plasmids were transformed into \( E. \text{coli} \) XL1-Blue cells and selected on LB agar containing spectinamycin (Section 2.5.1). Plasmid DNA was harvested and sequenced from the colonies recovered to confirm that the \( \text{dps} \) ORF was inserted correctly into \( p\text{CL1920} \). PCR analysis and restriction enzyme digests were also employed to confirm \( p\text{JTS505} \).

3.2.7 Phenotypic confirmation of the complementation of \( \text{dps} \) in trans

Plasmid \( p\text{JTS505} \) was transformed into JTS004 generating strain JTS009 (Table 2.1). To confirm the functionality of \( p\text{JTS505} \), the comparative sensitivity of the wild-type, \( \text{dps} \) mutant and complemented mutant to \( \text{H}_2\text{O}_2 \) was tested. Stationary phase cultures were incubated in 5 ml PBS 0.5 mM \( \text{H}_2\text{O}_2 \) for 2 h without shaking and the survival rate compared (Section 2.7.2). Partial alleviation of the \( \text{dps} \) mutation was observed indicating that \( p\text{JTS505} \) was functional (Fig 3.7). It could therefore be used to confirm that phenotypes shown by JTS004 were attributable to the mutation of \( \text{dps} \) if they were relieved by the presence of \( \text{dps} \) in trans.
3.2.8 Construction of a *dps* promoter fusion plasmid

Transcription of the *dps* gene is under the control of RpoS and IHF during stationary phase, and OxyR during oxidative stress (Altuvia *et al.*, 1994). An investigation was carried out to study the activity of the *dps* promoter in the wild-type and *dps* mutant backgrounds during growth in LB broth. Accordingly a *dps* transcriptional fusion was constructed. A 615bp region of DNA encompassing the promoter region of *dps* was amplified by PCR using the oligonucleotides pZepdpsfor and pZepdpsrev (Table 2.3), cloned into the *gfp* reporter plasmid pZep08, to generate plasmid pJTS504 (Table 2.2, Fig 3.8). The fragment and vector pZep08 were both digested with *XbaI* and *NotI* to allow directional ligation of the *dps* promoter upstream of the promoterless *gfp* gene in pZep08. Ligated plasmids were transformed into *E. coli* XL-1 Blue cells and selected on LB plates containing carbenicillin (Section 2.5.1). To confirm correct insertion of the *dps* promoter into the vector, plasmid DNA was isolated from transformants and sequenced.

3.2.9 *dps* promoter activity is not subject to autoregulation

Transcriptional activity of the *dps* promoter was examined throughout the growth cycle in LB by flow cytometric analysis using the pJTS504 reporter plasmid (Section 3.2.8). The newly constructed *dps* promoter fusion plasmid pJTS504 was transformed into wild-type SL1344 and its *dps* isogenic mutant, JTS004 to create strains JTS008 and JTS007 respectively (Table 2.1). Samples for flow cytometric analysis were harvested throughout the growth curve and assessed for fluorescence. The results of the transcriptional assays revealed that there is a pattern of *dps* expression similar to that seen for the Dps protein (Ali Azam *et al.*, 1999). *dps* expression is lowest at early exponential phase and increases steadily as the culture approaches late stationary phase (Fig 3.9). The relatively high levels of fluorescence seen at the earliest time points during the lag phase of growth may be because the samples were subcultured from stationary phase cultures. Dps is a stationary phase protein its expression is highest at late stationary phase and the fluorescence observed may be residual from the initial subculturing. It was observed that *dps* expression levels were neither elevated nor depressed in the *dps* mutant compared to the wild-type strain throughout the growth curve indicating that Dps does not autoregulate its own expression in the conditions tested.
Fig 3.6. **Phenotypic testing of JTS004.** Graph shows % survival of wild-type SL1344 ♦ and the *dps* mutant JTS004 ■ following incubation in PBS at pH 2 for the indicated times. Results are averages of two independent experiments carried out in triplicate and error bars represent the standard deviation.
Fig 3.7 Phenotypic confirmation of the complementation of $dps$ in trans. Histogram shows % survival for wild-type SL1344, its otherwise isogenic $dps$ mutant JTS004 and the complemented mutant JTS009 following incubation for 2 h in PBS, 0.5 mM $H_2O_2$. Results are averages of two independent experiments carried out in duplicate and error bars represent standard deviation.
Fig. 3.8. Cloning of the *dps* promoter region into plasmid pZep08. A) A 615 bp region of DNA incorporating the *dps* promoter region was amplified by PCR using primer pair pZepdpsfor and pZepdpsrev. B) This amplicon was digested with NotI and Xbal and ligated into plasmid pZep08 upstream of the promoterless *gfp* gene as indicated to generate plasmid pJTS504.
**Fig 3.9.** *dps* is not subject to autoregulation. Columns represent the activity of the *dps* promoter monitored using the *gfp* reporter gene in plasmid pJTS504 in wild-type SL1344 (green) and its otherwise isogenic *dps* mutant (orange). Curves represent the growth of wild-type SL1344 (triangles) and the *dps* mutant (squares). The experiment was carried out in triplicate on two independent occasions. Data is representative and the error bars represent the standard deviations of individual experiments.
3.2.10 Intracellular characterisation of Dps

3.2.10.1 Effect of Dps on invasion/uptake

*S. Typhimurium* is a facultative intracellular pathogen that survives and proliferates within both epithelial cells and macrophages. Genes located within SPI-1 encode the structural, effector, chaperone and regulatory proteins responsible for the assembly and function of a type III secretion system that are required for invasion of epithelial cells (Mills *et al.*, 1995; Wallis and Galyov 2000). Genes located within SPI-2 encode a different type III secretion system as well as regulatory, chaperone and effector proteins that are required for survival and replication in macrophages (Cirillo *et al.*, 1997; Hensel *et al.*, 1998; Hensel 2000; Ochman *et al.*, 1996). Genes found on the *S. Typhimurium* virulence plasmid are also required for intracellular pathogenicity and systemic infection (Guilig *et al.*, 1990; Guilig and Doyle 1993; Hensel *et al.*, 1995).

To determine if Dps had a role to play in the invasive ability of SL1344, epithelial cell invasion assays were performed. Cultured Chinese Hamster Ovary (CHO) –K1 epithelial cells were infected with early exponential or stationary phase LB cultures of wild-type SL1344 and JTS004 at a multiplicity of infection (M.O.I) of 100:1. So-called SPI-1 inducing growth conditions include low oxygen, high osmolarity, high pH and early exponential phase growth (Lee and Falkow 1990; Schiemann and Sharpe 1991). The comparison between the invasive ability of SL1344 and JTS004 was carried out using cultures grown to early exponential or stationary phase in LB broth 5 ml in a 10 ml test tube at pH 7. 3 h post infection epithelial cells were lysed, serially diluted and spread onto LB-agar or LB-agar plates supplemented with chloramphenicol as appropriate, incubated for 24 h and then scored for numbers of colony forming units. In contrast to the situation observed with other nucleoid associated proteins such as Fis (Kelly *et al.*, 2004), and IHF (Mangan *et al.*, 2006) no significant difference in invasion of CHO-K1 epithelial cells was seen using either early exponential or stationary phase cultures of SL1344 and JTS004 (Fig 3.10).

Macrophage uptake assays were performed to determine whether Dps could affect the uptake of SL1344 into macrophage. J774 murine macrophage-like cells were infected with early exponential or stationary phase LB cultures of wild-type SL1344 and
JTS004 (M.O.I 100:1). 3 h post infection epithelial cells were lysed, serially diluted and spread onto LB-agar or LB-agar plates supplemented with chloramphenicol as appropriate, incubated for 24 h and then scored for numbers of colony forming units. The wild-type strain's ability to be uptaken into macrophages was observed to be slightly superior compared to the \textit{dps} mutant when exponential cultures were used, $4.8 \times 10^5$ cfu/ml were recovered from the macrophages infected with wild-type compared to $4.5 \times 10^5$ cfu/ml from macrophages infected with the \textit{dps} mutant (Fig 3.11). In contrast, when stationary phase cultures were used a 2.5 fold decrease in uptake of JTS004 compared to wild-type was observed. $4.5 \times 10^5$ cfu/ml were recovered from the macrophages infected with wild-type compared to $1.8 \times 10^5$ cfu/ml from macrophages infected with the \textit{dps} mutant (Fig 3.11). Because macrophages unlike epithelial cells are phagocytic it could be argued that the decreased uptake of JTS004 by J774 macrophages was not important from a virulence point of view. Therefore a more relevant model would be a macrophage survival assay. Such an assay was undertaken.

\subsection*{3.2.10.2 Effect of Dps on \textit{Salmonella} survival in macrophage}

Equal concentrations of stationary phase SL1344 and JTS004 cells were used to infect J774 macrophage-like cells (M.O.I 100:1) (Section 2.22.2) in 12-well plates. 2 h post infection epithelial cells were lysed and the intracellular bacteria harvested. This procedure was repeated at 4, 6 and 8 h post-infection. Cell lysates were serially diluted and spread onto LB-agar or LB-agar plates supplemented with chloramphenicol as appropriate, incubated for 24 h and then scored for colony forming units. From the histogram (Fig 3.12) it can clearly be seen that Dps has a role to play in the survival of \textit{Salmonella} in macrophages. At 2 h post infection the mutant has a survival rate of 78\% of that of the wild-type this drops to 22\% at 4 h, increases slightly to 23 \% at 6 h and finally decreases again to 11\% at 8 h post infection. It is clear that the \textit{dps} mutant is severely compromised in its ability to survive in the macrophage particularly at 8 h post infection. The reduction in survival rates indicates that cells lacking Dps are less able to survive in macrophages than when Dps is present. These data are consistent with the study carried out by Halsey \textit{et al} who found there was an approximately 30\% decrease in the survival of \textit{dps} mutant \textit{Salmonella} in periodate-elicited murine peritoneal macrophages 3 h post infection.
Fig 3.10. The effect of the *dps* mutation on *S. Typhimurium* invasion of CHO-K1 epithelial cells. CHO-K1 cells were infected with wild-type SL1344 (green) and the *dps* mutant JTS004 (orange) for 1 h. Cells were then washed in PBS and incubated for 2 h with medium containing gentamycin (100 μg/ml) medium to kill the extracellular bacteria. The cells were then lysed with 0.5% Triton X 100 and the intracellular bacteria harvested and plated on LB agar plates to determine number of colony forming units per ml. The values represent the means and standard deviations of two independent experiments carried out in triplicate.
Fig 3.11. The effect of the $dps$ mutation on $S$ Typhimurium uptake by J774 macrophage-like cells. J774 cells were infected with wild-type SL1344 (green) and the $dps$ mutant JTS004 (orange) for 1 h. Cells were then washed in PBS and incubated for 2 h with medium containing gentamycin (100 μg/ml) medium to kill the extracellular bacteria. The cells were then lysed with 0.5% Triton X 100 and the intracellular bacteria harvested and plated on LB agar plates to determine number of colony forming units per ml. The values represent the means and standard deviations of two independent experiments carried out in triplicate.
Fig 3.12. The effect of the *dps* mutation on intracellular survival. J774 macrophage-like cells were infected with stationary phase LB cultures of SL1344 and JTS004 at a MOI of 100:1 for 1 h. Cells were then washed and incubated for 1 h with gentamycin-containing (100 μg/ml) medium to kill the extracellular bacteria. The cells were then washed and incubated in fresh medium containing a minimal concentration of gentamycin (20 μg/ml) for different time intervals (2, 4 and 6 h). Finally the cells were lysed with 0.5% Triton X-100 and plated on LB agar plates to determine numbers of colony forming units. The values represent the means and standard deviations of three independent experiments carried out in triplicate.
increasing slightly to approximately 33% at 6 h post infection and decreasing to approximately 32% at 12 h post infection (Halsey et al 2003).

3.2.10.3 Intracellular expression of *dps*

To confirm the finding that Dps is necessary for intracellular survival in the macrophage the intracellular levels of *dps* expression was measured using the Gfp reporter plasmid construct pJTS504. The relative level of *dps* expression was unchanged in wild-type SL1344 or *dps* mutant background during growth in rich medium (Fig 3.9), it was not however investigated if the lack of autoregulation would be observed in the rather different environments found in macrophages and epithelial cells. To limit the number of variables in the experiment the intracellular expression levels were measured in wild-type background only.

Macrophage-like cell line J774 and a Caco-II epithelial cell line were infected with the wild-type SL1344 strain harbouring the *dps* promoter fusion plasmid – JTS504 (Table 2.1) at a MOI of 100:1 in flow cytometer tubes (Section 2.22.3). Both cell lines were infected with equal concentrations of JTS007 and at an equal MOI. After the appropriate incubation time the cells were washed in PBS and fixed by resuspending the pellet in 300 µl PBS 2% (v/v) formaldehyde. The tubes containing fixed cells were incubated in the dark at 4°C prior to being read in the flow cytometer (Section 2.10). The level and profile of expression of *dps* was markedly different between the epithelial cell line Caco-II and the macrophage-like cell line J774 (Fig 3.13). The level of *dps* expression in Caco-II cells remained steady over the first six hours of the experiment and decreased at the 8-h time point, the expression levels were also relatively lower when compared to those in the macrophage. The arbitrary mean channel fluorescence observed, which gives an indication of expression levels were 10-fold higher in the macrophage through all time points. The level of *dps* expression in the macrophage increases steadily over the course of the experiment reaching a peak at the final time point. The expression profile was similar to that observed in batch culture (Fig 3.5 A).
3.2.10.4 Effect of Dps on SPI-2 regulators Fis, ssrA and ssrB

It has been demonstrated that the macrophage-induced SPI-2 virulence genes require the nucleoid-associated protein Fis for full activity (Kelly et al., 2004). To investigate if the requirement of Salmonella for Dps during intramacrophage survival was mediated through Fis, the levels of Fis protein were monitored in both wild-type and dps mutant background (Fig 3.14). Under standard aeration conditions Fis is maximally expressed very early on in the growth phase with undetectable levels at 3 h post subinoculation it was therefore necessary to use lag phase cultures to assess any effects (Osuna et al., 1995). The level of Fis protein detected did not change between wild-type and dps mutant after 30 min or 1 h of growth. This was not an ideal assay because Dps is a stationary phase protein; accordingly a different approach was required.

Previous studies have shown that the two-component signal transduction system encoded on SPI-2 by the genes ssrA and ssrB are responsible for the activation of the structural virulence genes of the pathogenicity island and are required for survival in the macrophage (Cirillo et al., 1998; Hensel 1998). The ssrA gene encodes an inner membrane histidine sensor kinase and SsrB is the response regulator (Ochman et al., 1996). Expression of SsrA/B in batch culture is usually most evident in the stationary phase of growth, when Dps would be most evident. To investigate if Dps was in some way modulating the ssrA and ssrB regulatory genes two separate approaches were used. Transcriptional analysis was undertaken to measure the expression of ssrA in wild-type and dps mutant backgrounds (fig 3.15 A). Western blot analysis was utilized to measure the levels of SsrB in wild-type and dps mutant backgrounds (Fig 3.15 B). Neither the expression of ssrA or SsrB was affected by the presence or absence of Dps. Taken together the data indicated that the requirement of the cell for Dps during macrophage survival is not mediated through an effect on SPI-2 genes.
FIG 3.13. Intracellular expression of dps. Time course analysis using the gfp reporter plasmid pJTS504 with gfp under the control of the dps promoter. A) dps expression in Caco-2 cells. B) dps expression in J774 macrophage-like cells. Data from representative experiments are shown. The values represent the means and standard deviations of triplicate samples.
Fig 3.14. The intracellular levels of Fis do not change in the \textit{dps} mutant. Total protein from \textit{S. Typhimurium} wild-type strain SL1344 and its \textit{dps} mutant derivative JTS004 were harvested following 30 min and 1 h of growth in LB broth at 37°C and analysed by Western immunoblot with a Fis-specific antibody.
Fig 3.15 Effect of the \textit{dps} mutation on SPI-2 regulators \textit{ssrA} & \textit{SsrB}. A) Time course analysis using the \textit{gfp} reporter plasmid \textit{pssrA-gfp} with \textit{gfp} expression under the control of the \textit{ssrA} promoter in wild-type SL1344 and the \textit{dps} mutant. Wild-type SL1344 transformed with just the pZep08 plasmid vector acted as a negative control. Data from representative experiments are shown. The values represent the means and standard deviations of triplicate samples. B) Total protein from \textit{S. Typhimurium} wild-type strain SL1344 and its \textit{dps} mutant derivative JTS004 were harvested following overnight incubation in LB broth at 37°C and analysed by Western immunoblot with an SsrB- specific antibody.
3.3 Discussion

Following phenotypic and molecular analysis of the *dps* mutant SL1344 kindly received from the Jay Hinton lab at the BBSRC Institute of Food Research Norwich it appeared that SL1344*dps* retained enough of its locus to enable Dps perform its protective role in *S. Typhimurium* (Fig 3.1 – 3.2). Accordingly it was necessary to generate a new SL1344 *dps* mutant, JTS004. Custom automated sequencing, PCR analysis and Southern blotting confirmed that the *dps* gene was disrupted by the chloramphenicol acetyltransferase gene. The method undertaken to generate the *dps* mutant by allelic replacement should result in a complete deletion of the open reading frame replacing it with the antibiotic resistance cassette (Datsenko and Wanner, 2000). Initial PCR analysis seemed to confirm that this was the case (Fig 3.4 Panel B). However, not unlike the findings when the IFR SL1344*dps* strain was molecularly tested, subsequent Southern blot analysis confirmed that there was sufficient *dps* locus remaining to enable hybridization to occur when a whole gene probe was used (Fig 3.4 Panel C). Because the primers used to generate the mutant and those to generate the probe are nested as depicted in figure 3.4 Panel A elimination of the *dps* locus would deprive the probe prepared by *dpsfors* and *dpsrev* of a hybridization target. It would seem therefore that an insertion event had occurred rather than a deletion of the ORF.

Phenotypic confirmation of the mutation was carried out by means of oxidative or acid stress tests. In contrast to the findings observed with the IFR SL1344*dps* strain and correlating with previous Dps function studies the *dps* mutant displayed a severe drop in survival when exposed to H$_2$O$_2$ in comparison to wild-type, in addition the mutant was also deficient in survival when exposed to acid stress (Nair and Finkel, 2004). Oxidative stress is characterised by the production of hydroxyl radicals that can cause strand breaks in DNA through the oxidation of sugar and base moieties in the backbone of the DNA. Such hydroxyl radicals can form in an iron catalysed reaction between H$_2$O$_2$ and O$_2$. The ability of Dps to sequester iron in a ferritin like manner is thought to promote protection of DNA from attack by oxidising radicals, studies have shown that the preincubation of *dps* mutant *Salmonella* with the iron chelator 2',2-dipyridyl rescues the *dps* mutant strain from killing by 2 mM H$_2$O$_2$ (Halsey *et al.*, 2003). In addition its ability to bind DNA into a microcrystalline
structure can physically prevent exposure of the DNA to attacking molecules (Frenkel-Krispin et al., 2004). Complementation of the mutation of dps was achieved by providing *dps in-trans* on the IPTG controlled low-copy number plasmid pJTS505. Partial alleviation of the mutation was observed when the cells were exposed to 2 mM H$_2$O$_2$ for 2 h (Fig 3.6).

Having confirmed a *dps* knockout mutation the growth of SL1344 and JTS004 in LB at 37°C was monitored (Fig 3.5 A). There was no growth defect associated with a mutation in *dps* when cultured in rich medium. Doubling times of 27 min and 26 min were observed, there was no significant difference in the length of the lag phase or in the terminal OD measured. As Dps is primarily known as a protein important in stationary phase and nutrient starved cells lack of a clear phenotype during growth when nutrient levels are high was not unexpected. Growth was therefore also examined in MOPS minimal media with glucose as the sole carbon source (Fig 3.5 B). Overnight cultures of *S. Typhimurium* grown in LB broth were washed in PBS to remove any remaining nutrients that may have been in the LB broth. The washed cultures were inoculated into glucose MOPS. In this medium there were significant differences observed between wild-type and *dps* mutant. During exponential growth the generation time of the *dps* mutant was over 1.5 times longer then that of the wild-type. The mutant also displayed a longer lag phase prior to exponential growth and failed to reach the wild-type levels of cell density. These data demonstrate the significance of Dps in nutrient limiting conditions.

Some of the factors controlling *dps* expression are known already, in stationary phase cells *dps* is expressed in a $\sigma^5$- and IHF-dependent manner. In contrast in exponentially growing cells, *dps* is induced by treatment with H$_2$O$_2$, controlled by $\sigma^{70}$ in an OxyR-dependent manner (Altuvia et al., 1994, Weber et al., 2005). Dps is present in exponential phase, approximately 6,000 molecules of the protein exist in the *E.coli* W3110 cell during log phase rising to the peak of about 180,000 molecules per cell at the late stationary phase (Ali Azam et al., 1999). An investigation into the regulation of *dps* promoter activity as a function of growth phase was carried out. Fluorescence assays were performed on SL1444 and JTS004 harbouring pJTS504 at intervals after subculture into fresh LB broth to measure *dps* promoter activity during
growth of the bacteria. The results of the assay confirm that the levels of \textit{dps} expression increase through the growth phase of the cell reaching a maximum level of expression during stationary phase (Fig 3.5). Other nucleoid-associated proteins such as H-NS, StpA, Fis and Lrp negatively regulate their own expression (Free \textit{et al.}, 1995; Sonden \textit{et al.}, 1996; Kelly 2005; McFarland 2007). However the results of the transcription assays indicated that Dps does not autoregulate its own expression in the conditions tested. The \textit{dps} expression levels observed followed the same profile throughout the growth curve regardless of the background.

Because \textit{S. Typhimurium} is a facultative intracellular pathogen that survives and proliferates within both epithelial cells and macrophages studies were undertaken into the effects that the mutation of \textit{dps} would have on intracellular phenotype of the bacteria. Initially the invasive ability of JTS004 was tested, no significant difference in invasion of CHO-K1 epithelial cells was seen using either early exponential or stationary phase cultures of SL1344 and JTS004 (Fig 3.7). It has been reported that Dps is required for virulence, using a C3H/HeN murine model it was found that a \textit{dps} mutant \textit{Salmonella} was attenuated for virulence (Halsey \textit{et al.}, 2004). It should however be noted that the mice were infected intraperitoneally and not by the oral route therefore the effect that \textit{dps} has on epithelial invasion was not investigated. Through examination of the livers and spleens of mice infected with the \textit{dps} mutant it was indicated that the attenuation of virulence observed was due to a defect in intramacrophage survival (Halsey \textit{et al.}, 2004). It would appear therefore that unlike other nucleoid associated proteins such as Fis, IHF, HU and H-NS, Dps's influence on virulence did not occur through affecting the invasive abilities of \textit{Salmonella} (Kelly 2004 \textit{et al.}; Lucas \textit{et al.}, 2000; Mangan \textit{et al.}, 2006).

The effect of Dps on the uptake of \textit{Salmonella} into professional phagocytic cells was investigated. J774 cells were infected at an equal multiplicity of infection with wild-type and \textit{dps} mutant cells. It was found that there was a 2.5-fold decrease in uptake of the \textit{dps} mutant compared to the wild-type when stationary phase \textit{Salmonella} were used for infection but no difference was observed when \textit{Salmonella} in the exponential phase were used (Fig 3.11). As Dps is a stationary phase protein, one would expect any pertinent effects attributable to the mutation of \textit{dps} to be particularly apparent in
stationary phase cultures. The lack of effect at exponential phase may be due to the scarcity of Dps in the wild-type cells at that particular growth phase.

Longer term experiments were performed to assess the survival capabilities of the *dps* mutant in the macrophage. It has already been shown that there is a decrease in uptake by macrophage of the *dps* mutant (Fig 3.11), so a reduction in survival is not unexpected. However if Dps was only implicated in uptake/invasion in macrophages, then the percentage survival rate though lower than the wild-type would remain at a constant level to the wild-type through the time-points. In particular there is a severe reduction in survival rate between 2 h post infection where a survival rate of over 75% compared to the wild-type is observed to 8 h post infection where the *dps* mutant displays a survival rate of 11% compared to the survival of wild-type in the macrophage like cell line (Fig 3.12). These data clearly show the importance of Dps during intracellular survival in the macrophage.

Transcriptional analysis was also carried out to monitor the expression of *dps* in the intracellular environment. Wild-type SL1344 harbouring the GFP reporter plasmid pJTS504 (incorporating the *gfp* gene under the control of the *dps* promoter) was used to infect both epithelial and macrophage-like cells. Epithelial cells and macrophages have different biological functions and accordingly have different environments. The level and profile of expression of *dps* was markedly different between the epithelial cell line Caco-II and the murine macrophage-like cell line J774 (fig 3.9). The level of *dps* expression in Caco-II cells remains steady over the first six hours of the experiment and decreases at the 8 h time point; the expression levels are also relatively low when compared to those in the macrophage. A recent study into the intracellular gene expression pattern displayed by *Shigella flexneri* in HeLa epithelial and U937 macrophage-like human cells found that there was an induction of the *sitABCD* genes, encoding a putative iron transport system indicating that iron availability is restricted inside the cytosol of the host cell (Lucchini *et al.*, 2005). *Halsey et al* have shown that limiting iron can negate the effects of a *dps* mutation to some extent (Halsey *et al.*, 2004). This may account for the relatively low levels of expression of dps in the epithelial cell. It is important to note however that *Salmonella* and *Shigella* inhabit very different environments intracellularly. *Shigella* reside in the
host cytosol while *Salmonella* reside in a membrane bound compartment known as the *Salmonella* Containing Vacuole it is not clear if iron is limiting in the SCV.

The data obtained from the transcriptional analysis of JTS004 indicated that the level of *dps* expression in the macrophage increased steadily over the course of the experiment reaching a peak at the final time point in a profile similar to that observed in batch culture. Macrophages express two major enzymes involved in free radical production, NADPH phagocyte oxidase and inducible nitric oxide synthase, which produce superoxide and NO respectively (Vazquez-Torres 2000). Macrophages require iron as an essential co-factor for the induction of the NADPH dependent oxidative burst and for the production of the reactive nitrogen intermediates (Vazquez-Torres et al., 2000). Eriksson *et al* undertook a study into the expression profile of intracellular *Salmonella* during this study it was found that the majority of genes in the oxidative stress regulon were not upregulated in J774 macrophage-like cells, however six genes including *soxS*, *ipbA*, *ycfR*, *trxC*, *ibpB* and *shp* were induced in intracellular *Salmonella* indicating that the bacteria is exposed to some degree of oxidative stress within the SCV (Eriksson *et al*., 2003). Previous studies have shown that the transcriptional regulator SlyA is required for resistance to oxidative stress and is expressed in the intracellular environment (Buchmeier *et al*., 1997). The expression of *dps* within the macrophage showed an increase in expression in the J774 cells of between almost two-fold and over four-fold depending on the length of time growing within J774 cells (Eriksson *et al*., 2003). The bacterial cells are coming under severe stress in macrophage, attacks by oxidative species as well as low pH this may account for the relatively high expression levels of *dps* observed in the macrophage, both in comparison to the levels observed in the epithelial cells and in batch culture, from the start of the experiment and through the time course.

An important factor in the survival of *Salmonella* in macrophages is the type three secretion system (TTSS) encoded by genes present on the SPI-2 pathogenicity island. The SPI-2 system translocates effector proteins across the vacuolar membrane into the host cell cytosol. Effector proteins such as SifA, SpiC and SspH-2 are injected in to the cytosol where they alter host cellular functions to maintain the integrity of the SCV membrane, inhibit fusion of the SCV with the lysosome amongst other roles.
(Beuzon et al., 2000, Uchiya et al., 1999). The two-component signal transduction system SsrA-SSrB activates expression of the TTSS encoded on SPI-2. Fis has been shown to collaborate with DNA supercoiling to modulate expression of virulence genes during intracellular growth of Salmonella, indeed it binds to the ssrA promoter (Kelly et al., 2004, O'Croinin et al., 2006). In the first instance an investigation to see if Dps would influence Fis protein levels was undertaken. There was no effect on Fis levels after 30 min or 1 h of growth, although Dps is present in exponential cells its level is minimal so the lack of effect was not unexpected (Fig 3.14). A further investigation to assess if Dps was in some way modulating the ssrA and ssrB regulatory genes was carried out. It was found that Dps had no influence on the transcription levels of ssrA or on the protein levels of SsrB indicating that the ability of Dps to promote intracellular survival in the macrophage was not mediated through SPI-2 genes (Fig 3.15).

The data presented in this study defined phenotypic differences between the dps mutant and wild-type. It was also confirmed that dps promoter activity like the Dps protein levels are maximal at the stationary phase and during intracellular existence in the macrophage. In addition SPI-2 genes were not shown to be influenced by Dps during the intracellular survival of SL1344.
Chapter 4

Microarray transcriptomic analysis of the Dps regulon of *Salmonella enterica* serovar Typhimurium
4.1 Introduction

In addition to their role involving the organisation of the bacterial nucleoid, bacterial nucleoid-associated proteins play a part in the regulation of gene expression. They also influence other systems within the cell such as DNA replication and recombination. Various studies on distinct nucleoid-associated proteins have defined regulons, groups of genes whose expression is influenced by a common factor, for the individual protein examined. To date the best characterised include the regulons controlled by H-NS, Fis and IHF. H-NS is a highly pleiotropic regulator of gene expression and has been shown to regulate up to 10% of the genes in *Salmonella* (Lucchini et al., 2006; Navarre et al., 2006). H-NS has recently been shown to selectively silence foreign DNA with low GC content in *Salmonella* (Navarre et al., 2006) it binds preferentially to curved DNA, such intrinsically curved areas of DNA are commonly found at promoters (Jauregui et al 2003). Fis has been shown to have a global role in the transcriptional control of metabolism and type three secretion systems in *S. Typhimurium*. Among the genes regulated by Fis are those contributing to flagella biosynthesis and motility, genes involved in virulence located in the pathogenicity islands SPI-1, SPI-2 SPI-3 and SPI-5, and genes encoding components of various metabolic pathways (Gutierrez-Rios et al., 2007; Kelly et al., 2004). IHF has recently been shown to be required for the normal expression of genes involved in the expression of the three type three secretion systems in *S. Typhimurium* and their effector proteins as well as genes involved in the transition from the active growth phase to the stationary phase (Magan et al., 2006). *hilA* is a gene found on SPI-1 and it encodes an OmpR/ToxR transcriptional regulator that is an activator of the type three secretion system contributing to the invasive ability of *S. Typhimurium* (Mills et al., 1995, Wallis and Galyov 2000). Three nucleoid-associated proteins, H-NS, Fis and HU affect *hilA* expression in *S. Typhimurium*. H-NS represses *hilA* under low-osmolarity conditions and together with HU and Fis appears to affect the derepression of *hilA* by HilD (Schechter et al., 2003; Navarre et al., 2006). These and other similar studies emphasise the importance of the nucleoid-associated proteins in the regulation of gene expression.
When Dps was first characterised in *E. coli* it was found that cells lacking Dps showed dramatic changes in the pattern of proteins synthesised during starvation, however none of the proteins was identified during the study (Almiron et al., 1992).

DNA microarrays consisting of PCR (polymerase chain reaction) products complementary to every gene in a specific genome, deposited in an ordered grid onto specially coated glass microscope slides (Lucchini et al. 2001). DNA microarrays are used for the assessment of transcription at the genomic level. Comparison of gene expression profiling under various conditions can reveal how the organism responds to specific conditions. Imaging and computational analysis are then used to assess the relative transcript levels of each gene under the specific condition being tested. Gene expression profiles between two genotypes can also be compared using DNA microarrays. The wild-type expression profile is compared to the expression profile of its otherwise isogenic mutant derivative to reveal how the particular mutation influences the global transcription profile. The DNA analysis employed in this study was performed in collaboration with Professor Jay Hinton at the BBSRC Institute of Food Research (IFR), Norwich, UK to elucidate the global gene response of *S. Typhimurium* to the mutation in *dps*.

It was hypothesized that Dps might have a pleiotropic regulatory role in global regulation of gene transcription in *S. Typhimurium*. To this end a DNA microarray analysis was carried out to elucidate the Dps regulon of *S. Typhimurium*. 


4.2 Results

4.2.1 DNA microarray analysis to determine the Dps regulon

A DNA microarray analysis was carried out to elucidate the \textit{dps} regulon of \textit{S. Typhimurium} during growth in LB broth. DNA probes used in this study consisted of cDNA (PCR products) printed on Corning CMT-GAPS-coated slides by a robotic DNA arrayer built in-house by Arthur Thompson, Sacha Lucchini and Bruce Pearson at the IFR http://www.ifr.ac.uk/Safety/Microarrays/default.html. Each array consists of 16 blocks of printed PCR products consisting of 5080 \textit{Salmonella} genes including 4414 \textit{S. Typhimurium} LT2a (and pSLT) genes, 155 \textit{S. Typhimurium} DT104 specific genes, 229 \textit{S. Typhimurium} SL1344 specific genes, 196 \textit{S. Enteritidis} PT4 specific genes and 86 \textit{S. Gallinarum} 287/91 specific genes http://www.ifr.ac.uk/Safety/Microarrays/default.html.

Overnight cultures of wild-type SL1344 and its otherwise isogenic \textit{dps} derivative JTS004 were inoculated into 25 ml LB medium in 250 ml flasks and grown with shaking at 37°C without antibiotic selection. Total RNA was extracted from cultures at 4 h (corresponding to late-exponential), 12 h (corresponding to stationary) and 22 h (corresponding to late stationary phases) post inoculation (Fig 3.4 A, Section 2.20.1). The RNA was fluorescently labelled with Cy5-dye during reverse transcription into cDNA (Section 2.20.2). Genomic DNA from wild-type SL1344 was used as a reference channel in each experiment and was labelled with Cy3-dye (Section 2.20.3). Each fluorescently-labelled cDNA sample and the gDNA reference sample was hybridised to the microarray slide (Section 2.20.5).

After hybridisation the microarray slides were scanned using a GenePix 4000A scanner (Axon Instruments). Fluorescent spot intensities and local background data were quantified using the BlueFuse software package (BlueGnome, Oxford.). Data were passed through the quality control procedures outlined in Eriksson \textit{et al} (2003). Data that passed the quality controls were saved in .gpr file format, which were then converted to .txt text format. The .txt files were imported into Microsoft Excel and using a custom designed macro program (S. Lucchini), the cDNA data were
normalized against the genomic DNA data. Data was then imported into the microarray analysis program GeneSpring 7.3 (Silicon Genetics). Only genes whose expression ratio showed at least a 1.5-fold difference [false discovery rate (FDR) \( \leq 0.10 \)] were considered as being statistically significant (Section 2.20.6).

4.2.2 The global transcription profile

As a member of the nucleoid-associated family of proteins, it was expected that the absence of Dps would result in extensive changes in the global transcriptional profile of S. Typhimurium. The microarray data indicate that \( \textit{dps} \) is not expressed in the mutant strain showing that the gene had been inactivated. When the gene expression profile of SL1344 was compared with that of JTS004 it was clear that the absence of Dps affected the transcription of a limited number of S. Typhimurium genes (Fig 4.1).

From the microarray analysis, at the 12-h time-point 48 of the 4747 Salmonella coding sequences passed the statistical filter, set with FDR \( \leq 0.10 \) and showed differential levels of expression (Table 4.1, Fig. 4.1 (B)). Of these 25 displayed higher levels of expression in the absence of Dps and 23 genes showed decreased levels of expression. 13 of these 48 genes coded for genes of unknown function.

At the 4-h time-point 29 genes are affected, 12 genes have lower levels of expression 17 genes show higher levels of expression (Table 4.2, Fig. 4.1 (A)). At the 22 h only eight genes display differential levels of expression one gene STM1809 a putative cytoplasmic protein had lower levels of expression while the remaining seven genes showed higher levels of expression (Table 4.3 Fig. 4.1 (C)).

An overview of the genes affected by Dps was obtained by defining functional categories of the statistically significant genes based on the Kyoto Encyclopedia of Genes and Genomics (KEGG – www.genome.ad.jp/kegg/kegg2.html). It was clear that the vast majority of functional categories were unaffected by the absence of \( \textit{dps} \) (Table 4.4).
Fig 4.1 Impact of *dps* on the transcriptome of SL1344. Statistically filtered microarray data (FD R ≤ 0.10%) from wild-type SL1344 vs. the *dps* mutant JTS004 are presented in graphs for 4 h (A) (Table 4.2), 12 h (B) (Table 4.1) and 22 h (C) (Table 4.3). Each gene is represented as a coloured line. Gene expression values were normalized to the wild-type and fold differences are indicated on the Y-axis. An increase from left to right indicates higher expression in the absence of Dps, while a decrease from left to right indicates a decrease in expression. D. Relative expression colour bar generated by GeneSpring – red indicates an increase in expression, yellow indicates no change and blue indicates a decrease in expression.
<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>wt/dps expression ratio</th>
<th>p-value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>lower expression in JTS004</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aroK</td>
<td>0.195 0.236</td>
<td></td>
<td>shikimate kinase I</td>
</tr>
<tr>
<td>dps</td>
<td>0.0585 0.00282</td>
<td></td>
<td>stress response DNA-binding protein; starvation induced resistance to H$_2$O$_2$</td>
</tr>
<tr>
<td>flgK</td>
<td>0.633 0.031</td>
<td></td>
<td>flagellar biosynthesis, hook-filament junction protein 1</td>
</tr>
<tr>
<td>fliH</td>
<td>0.27 0.534</td>
<td></td>
<td>flagellar biosynthesis; possible export of flagellar proteins</td>
</tr>
<tr>
<td>leuD</td>
<td>0.595 0.443</td>
<td></td>
<td>3-isopropylmalate isomerase (dehydratase), subunit with LeuC</td>
</tr>
<tr>
<td>lipA</td>
<td>0.563 0.402</td>
<td></td>
<td>lipoate synthase, an iron-sulfur enzyme</td>
</tr>
<tr>
<td>livM</td>
<td>0.128 0.172</td>
<td></td>
<td>ABC superfamily (membrane), branched-chain amino acid transporter, high-affinity</td>
</tr>
<tr>
<td>orfX</td>
<td>0.466 0.585</td>
<td></td>
<td>putative cytoplasmic protein</td>
</tr>
<tr>
<td>pduE</td>
<td>0.643 0.525</td>
<td></td>
<td>Propanediol utilization: dehydratase, small subunit</td>
</tr>
<tr>
<td>pduT</td>
<td>0.403 0.324</td>
<td></td>
<td>Propanediol utilization: polyhedral bodies</td>
</tr>
<tr>
<td>prgH</td>
<td>0.567 0.41</td>
<td></td>
<td>cell invasion protein</td>
</tr>
<tr>
<td>PSLT06I</td>
<td>0.635 0.882</td>
<td></td>
<td>putative inner membrane protein</td>
</tr>
<tr>
<td>Gene</td>
<td>Score</td>
<td>LogP</td>
<td>Function</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PSLT093</td>
<td>0.554</td>
<td>0.571</td>
<td>conjugative transfer</td>
</tr>
<tr>
<td><em>stfE</em></td>
<td>0.475</td>
<td>0.0623</td>
<td>putative minor fimbrial subunit</td>
</tr>
<tr>
<td>STM1747</td>
<td>0.467</td>
<td>0.311</td>
<td>putative inner membrane protein</td>
</tr>
<tr>
<td>STM1809</td>
<td>0.53</td>
<td>0.573</td>
<td>putative cytoplasmic protein</td>
</tr>
<tr>
<td>STM1870</td>
<td>0.615</td>
<td>0.535</td>
<td>Homology to <em>recE</em> (exoVIII) in <em>E. coli</em></td>
</tr>
<tr>
<td>STM2731</td>
<td>0.632</td>
<td>0.329</td>
<td>Fels-2 prophage: similar to protein in phage 186</td>
</tr>
<tr>
<td>STM3035</td>
<td>0.665</td>
<td>0.551</td>
<td>putative periplasmic protein</td>
</tr>
<tr>
<td>STM3598</td>
<td>0.32</td>
<td>0.245</td>
<td>putative L-asparaginase</td>
</tr>
<tr>
<td>STM4080</td>
<td>0.355</td>
<td>0.255</td>
<td>putative ribulose-5-phosphate 3-epimerase</td>
</tr>
<tr>
<td>STM4216</td>
<td>0.498</td>
<td>0.0865</td>
<td>putative inner membrane protein</td>
</tr>
<tr>
<td><em>yhhP</em></td>
<td>0.306</td>
<td>0.189</td>
<td>small ubiquitous protein required for normal growth</td>
</tr>
<tr>
<td><em>yhhV</em></td>
<td>0.598</td>
<td>0.404</td>
<td>putative cytoplasmic protein</td>
</tr>
</tbody>
</table>

**higher expression in JTS004**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Score</th>
<th>LogP</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>acrA</em></td>
<td>1.549</td>
<td>0.751</td>
<td>acridine efflux pump</td>
</tr>
<tr>
<td><em>leuS</em></td>
<td>1.617</td>
<td>0.431</td>
<td>leucine tRNA synthetase</td>
</tr>
<tr>
<td><em>malQ</em></td>
<td>1.739</td>
<td>0.442</td>
<td>4-alpha-glucanotransferase (amylomaltase)</td>
</tr>
<tr>
<td><em>phpC</em></td>
<td>1.827</td>
<td>0.223</td>
<td>transglycosylase of penicillin-binding protein 1c</td>
</tr>
<tr>
<td>Gene</td>
<td>Fold Change</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>proP</td>
<td>2.418</td>
<td>0.409</td>
<td></td>
</tr>
<tr>
<td>proW</td>
<td>1.594</td>
<td>0.423</td>
<td></td>
</tr>
<tr>
<td>purE</td>
<td>1.58</td>
<td>0.601</td>
<td></td>
</tr>
<tr>
<td>rplA</td>
<td>1.716</td>
<td>0.265</td>
<td></td>
</tr>
<tr>
<td>rpoS</td>
<td>1.639</td>
<td>0.378</td>
<td></td>
</tr>
<tr>
<td>rtcA</td>
<td>7.056</td>
<td>0.534</td>
<td></td>
</tr>
<tr>
<td>STM0306</td>
<td>1.677</td>
<td>0.111</td>
<td></td>
</tr>
<tr>
<td>STM0344</td>
<td>2.019</td>
<td>0.238</td>
<td></td>
</tr>
<tr>
<td>STM0362</td>
<td>2.42</td>
<td>0.417</td>
<td></td>
</tr>
<tr>
<td>STM0557</td>
<td>9.132</td>
<td>0.266</td>
<td></td>
</tr>
<tr>
<td>STM0972</td>
<td>2.837</td>
<td>0.246</td>
<td></td>
</tr>
<tr>
<td>STM1300</td>
<td>1.786</td>
<td>0.226</td>
<td></td>
</tr>
<tr>
<td>STM2494</td>
<td>2.847</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>STM2789</td>
<td>3.033</td>
<td>0.426</td>
<td></td>
</tr>
<tr>
<td>STM4156</td>
<td>3.376</td>
<td>0.333</td>
<td></td>
</tr>
<tr>
<td>thrS</td>
<td>1.862</td>
<td>0.438</td>
<td></td>
</tr>
<tr>
<td>yffF</td>
<td>2.538</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>ygaE</td>
<td>1.511</td>
<td>0.522</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>yigW</td>
<td>1.936</td>
<td>0.327</td>
<td>putative hydrolase of PHP superfamily</td>
</tr>
<tr>
<td>yifC</td>
<td>1.869</td>
<td>0.127</td>
<td>putative glutathionylspermidine synthase</td>
</tr>
<tr>
<td>yqhE</td>
<td>2.079</td>
<td>0.444</td>
<td>2,5-diketo-D-gluconate reductase A</td>
</tr>
</tbody>
</table>
Table 4.2 4-h Gene Expression Difference 1.5-fold FDR 0.10

<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>wt/dps expression ratio</th>
<th>p-value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>lower expression in JTS004</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cheM</td>
<td>0.634</td>
<td>0.155</td>
<td>methyl accepting chemotaxis protein II, aspartate sensor-receptor</td>
</tr>
<tr>
<td>cheR</td>
<td>0.566</td>
<td>0.115</td>
<td>glutamate methyltransferase, response regulator for chemotaxis</td>
</tr>
<tr>
<td>dps</td>
<td>0.011</td>
<td>2.46e-20</td>
<td>stress response DNA-binding protein; starvation induced resistance to H$_2$O$_2$</td>
</tr>
<tr>
<td>flgH</td>
<td>0.59</td>
<td>0.132</td>
<td>flagellar biosynthesis, basal-body outer-membrane L (lipopolysaccharide layer) ring protein</td>
</tr>
<tr>
<td>fliH</td>
<td>0.603</td>
<td>0.135</td>
<td>flagellar biosynthesis; possible export of flagellar proteins</td>
</tr>
<tr>
<td>phpC</td>
<td>0.34</td>
<td>0.0751</td>
<td>transglycosylase of penicillin-binding protein 1c</td>
</tr>
<tr>
<td>PSLT093</td>
<td>0.653</td>
<td>0.623</td>
<td>conjunctive transfer</td>
</tr>
<tr>
<td>rplA</td>
<td>0.569</td>
<td>0.131</td>
<td>50S ribosomal subunit protein L1, regulates synthesis of L1 and L11</td>
</tr>
<tr>
<td>rplD</td>
<td>0.575</td>
<td>0.118</td>
<td>50S ribosomal subunit protein L4, regulates expression of S10 operon</td>
</tr>
<tr>
<td>spr</td>
<td>0.613</td>
<td>0.18</td>
<td>putative lipoprotein, suppresses thermosensitivity of prc mutants at low osmolality</td>
</tr>
<tr>
<td>STM0917</td>
<td>0.386</td>
<td>0.101</td>
<td>Fels-1 prophage; putative minor tail protein</td>
</tr>
<tr>
<td>STM1300</td>
<td>0.584</td>
<td>0.123</td>
<td>putative periplasmic protein</td>
</tr>
<tr>
<td>Gene</td>
<td>Fold Change</td>
<td>P Value</td>
<td>Function</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>-------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>yhhV</td>
<td>0.179</td>
<td>0.0388</td>
<td>putative cytoplasmic protein</td>
</tr>
<tr>
<td>ggt</td>
<td>1.501</td>
<td>0.181</td>
<td>gamma-glutamyltranspeptidase</td>
</tr>
<tr>
<td>hutH</td>
<td>2.289</td>
<td>0.000245</td>
<td>histidine ammonia lyase</td>
</tr>
<tr>
<td>kdtA</td>
<td>1.596</td>
<td>0.261</td>
<td>3-deoxy-D-manno-octulosonic-acid transferase (KDO transferase)</td>
</tr>
<tr>
<td>livM</td>
<td>2.111</td>
<td>0.000678</td>
<td>ABC superfamily (membrane), branched-chain amino acid transporter, high-affinity</td>
</tr>
<tr>
<td>pduT</td>
<td>1.536</td>
<td>0.181</td>
<td>Propanediol utilization: polyhedral bodies</td>
</tr>
<tr>
<td>proY</td>
<td>2.043</td>
<td>0.0583</td>
<td>putative ABC family, proline transporter</td>
</tr>
<tr>
<td>PSLT061</td>
<td>1.529</td>
<td>0.0211</td>
<td>putative inner membrane protein</td>
</tr>
<tr>
<td>STM0362</td>
<td>1.527</td>
<td>0.0449</td>
<td>putative cytoplasmic protein</td>
</tr>
<tr>
<td>STM0918</td>
<td>1.919</td>
<td>0.213</td>
<td>Fels-1 prophage; putative minor tail protein</td>
</tr>
<tr>
<td>STM2636</td>
<td>1.555</td>
<td>0.0168</td>
<td>Gifsy-1 prophage: similar to integrase in phage</td>
</tr>
<tr>
<td>STM2789</td>
<td>3.291</td>
<td>0.00039</td>
<td>putative cytoplasmic protein</td>
</tr>
<tr>
<td>STM2911</td>
<td>1.615</td>
<td>0.0133</td>
<td>putative permease</td>
</tr>
<tr>
<td>STM3595</td>
<td>2.526</td>
<td>0.263</td>
<td>putative phosphatase</td>
</tr>
<tr>
<td>STM4014</td>
<td>2.006</td>
<td>0.102</td>
<td>putative periplasmic protein</td>
</tr>
<tr>
<td>STM4156</td>
<td>1.578</td>
<td>0.0373</td>
<td>putative cytoplasmic protein</td>
</tr>
<tr>
<td>wcaK</td>
<td>1.562</td>
<td>0.0138</td>
<td>putative galactokinase in colanic acid gene cluster</td>
</tr>
<tr>
<td>ygaE</td>
<td>1.694</td>
<td>0.00631</td>
<td>putative transcriptional repressor (GntR family)</td>
</tr>
<tr>
<td>Systematic expression Name</td>
<td>wt/dps ratio</td>
<td>p-value</td>
<td>Function</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------</td>
<td>---------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>lower expression in JTS004</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>dps</em></td>
<td>0.0267</td>
<td>3.38e-9</td>
<td>stress response DNA-binding protein; starvation induced resistance to H$_2$O$_2$</td>
</tr>
<tr>
<td>STM1809</td>
<td>0.562</td>
<td>0.441</td>
<td>putative cytoplasmic protein</td>
</tr>
<tr>
<td><strong>higher expression in JTS004</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>leuD</em></td>
<td>2.282</td>
<td>0.0246</td>
<td>3-isopropylmalate isomerase (dehydratase), subunit with LeuC</td>
</tr>
<tr>
<td><em>lipA</em></td>
<td>1.88</td>
<td>0.211</td>
<td>lipoate synthase, an iron-sulfur enzyme</td>
</tr>
<tr>
<td><em>livM</em></td>
<td>2.251</td>
<td>0.0902</td>
<td>ABC superfamily (membrane), branched-chain amino acid transporter, high-affinity</td>
</tr>
<tr>
<td><em>pduT</em></td>
<td>2.509</td>
<td>0.333</td>
<td>Propanediol utilization: polyhedral bodies</td>
</tr>
<tr>
<td>STM0362</td>
<td>1.668</td>
<td>0.18</td>
<td>putative cytoplasmic protein</td>
</tr>
<tr>
<td>STM4041</td>
<td>1.696</td>
<td>0.183</td>
<td>putative inner membrane protein</td>
</tr>
</tbody>
</table>
4.2.3 Amino acid transport and metabolism

The results revealed that the functional category displaying the greatest number of Dps-regulated genes were genes involved in amino acid transport and metabolism. Four of the eight genes affected at the 22 h time point, 13 of the 48 genes with differential levels of expression at the 12 h time-point and six of the 29 gene affected at the 4 h time-point were involved in amino-acid transfer and metabolism. (Table 4.4).

4.2.3.1 Branched-chain amino acid transport

One gene in particular appears in all three lists. *livM* has a higher level of expression in the absence of *dps* at the 4 and 22 h time-points while it has a lower level of expression at the 12 h time point (Table 4.4).

Branched-chain amino acids are transported into *Escherichia coli* by a low-affinity system LIV-II and by two high affinity systems (Rahmanian et al., 1973). LivFGHMJ (LIV-I) and LivFGHMK (LS) are the two ATP-dependent high-affinity branched-chain amino acid transport systems and are members of the ATP Binding Cassette (ABC) Superfamily of transporters (Adams et al., 1990). They have shared membrane and ATP-binding components but have distinct periplasmic binding proteins, and differ in their binding specificity. LS is specific for the transport of leucine while LIV-I is a transporter for leucine, isoleucine and valine (Adams et al., 1990). LivJ and LivK are the two periplasmic amino acid-binding proteins that confer the specificity of the complexes, LivH and LivM are the membrane components and LivG and LivF are the ATP-binding components of the ABC transport complexes (Fig 4.2, Adams et al., 1990). LivJ binds L-leucine, L-isoleucine and L-valine with approximately equal affinity, whereas LivK binds D- and L-Leucine but neither isoleucine nor valine (Haney et al., 1992).

To determine if Dps is a regulator of *liv* genes RT-PCR analyses was performed on all six *liv* genes involved in the high affinity transport of branched chain amino acids (Fig 4.3 B). Total RNA was extracted from 12 h cultures of wild-type SL1344, the *dps* mutant JTS004 and the complemented mutant JTS009 and used as the template for RT-PCR. The gene STM1618 did not respond significantly to the presence or
absence of \textit{dps} in the DNA microarray experiments and was used as a negative control in all RT-PCR analysis.

The RT-PCR analysis revealed that, with the exception of \textit{livH}, the \textit{liv} genes were repressed in the \textit{dps} mutant and this repression was partially relieved by providing \textit{dps in trans} (Fig 4.3. B). The levels of \textit{livJ} transcript were barely detectable in any of the \textit{dps} samples tested.

To further test the observation that Dps may regulate branched-chain amino acid transport, direct competition assays were carried out (Section 2.24). Fitness can be defined as the average contribution of one genotype to the next or succeeding generations compared to that of other genotypes (Lenski, 1991). The fitness of JTS004 relative to wild-type in the presence of branched-chain amino acids was determined by simultaneously inoculating 25 ml fresh growth medium with $10^5$ cells of each competitor, which had been preconditioned in the growth medium for 24 h. The growth medium was MOPS minimal medium supplemented with 0.2\% (w/v) glucose together with L-leucine, L-isoleucine or L-valine (final concentration 0.5 mM) as indicated. The ratio of the two competing strains was determined at time zero and after 24 h growth by viable counts after plating serial dilutions of the inoculum in parallel on LB (to measure total c.f.u) and LB with cml. (to determine mutant c.f.u). The negative control in this case was represented by MOPS minimal medium, supplemented with 0.2\% (w/v) glucose with no additional amino acids added (Table 4.5).

It is clear from the results that the transport of branched-chain amino acids is influenced by Dps. In all cases the \textit{dps} mutant showed a lack of fitness compared to the wild-type SL1344 when branched-chain amino acids were supplied.

These data show for the first time that Dps has an influence on the metabolism of \textit{S. Typhimurium SL1344}. 

78
Table 4.4. Genes involved in amino-acid transport and metabolism affected by the *dps* mutation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>JTS004/SL1344 expression ratio&lt;sup&gt;6&lt;/sup&gt;</th>
<th>p-values as appropriate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 h</td>
<td>12 h</td>
</tr>
<tr>
<td><em>aroK</em></td>
<td>shikimate kinase I</td>
<td>ND</td>
<td>0.195</td>
</tr>
<tr>
<td><em>ggt</em></td>
<td>gamma-glutamyltranspeptidase</td>
<td>1.501</td>
<td>ND</td>
</tr>
<tr>
<td><em>hutH</em></td>
<td>histidine ammonia lyase</td>
<td>2.289</td>
<td>ND</td>
</tr>
<tr>
<td><em>leuD</em></td>
<td>3-isopropylmalate isomerase (dehydratase), subunit with LeuC</td>
<td>ND</td>
<td>0.595</td>
</tr>
<tr>
<td><em>leuS</em></td>
<td>leucine tRNA synthetase</td>
<td>ND</td>
<td>1.617</td>
</tr>
<tr>
<td><em>livM</em></td>
<td>branched-chain amino acid transporter</td>
<td>2.111</td>
<td>0.128</td>
</tr>
<tr>
<td><em>phpC</em></td>
<td>transglycosylase of penicillin-binding protein 1c</td>
<td>0.34</td>
<td>1.827</td>
</tr>
<tr>
<td><em>pduE</em></td>
<td>Propanediol utilization: dehydratase, small subunit</td>
<td>ND</td>
<td>0.643</td>
</tr>
<tr>
<td><em>pduT</em></td>
<td>Propanediol utilization: polyhedral bodies</td>
<td>1.536</td>
<td>0.403</td>
</tr>
<tr>
<td><em>proP</em></td>
<td>MFS family, low-affinity proline transporter</td>
<td>ND</td>
<td>2.418</td>
</tr>
<tr>
<td><em>proW</em></td>
<td>glycine/betaine/proline transport protein</td>
<td>ND</td>
<td>1.594</td>
</tr>
<tr>
<td><em>proY</em></td>
<td>putative APC family, proline transporter</td>
<td>2.043</td>
<td>ND</td>
</tr>
<tr>
<td><em>rtcA</em></td>
<td>RNA 3'-terminal phosphate cyclase (with b3419)</td>
<td>ND</td>
<td>7.056</td>
</tr>
<tr>
<td><em>thrS</em></td>
<td>threonine tRNA synthetase</td>
<td>ND</td>
<td>1.862</td>
</tr>
<tr>
<td><em>yhhV</em></td>
<td>putative cytoplasmic protein</td>
<td>ND</td>
<td>0.598</td>
</tr>
<tr>
<td>yifC</td>
<td>putative</td>
<td>glutathionylspermidine synthase</td>
<td>ND</td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
<td>--------------------------------</td>
<td>----</td>
</tr>
</tbody>
</table>

*ND = No statistically significant data

Blue gene expression ratios represent genes with lower expression in JTS004

Red gene expression ratios represent genes with higher expression in JTS004
LivJ and LivK are the periplasmic amino acid-binding proteins that confer the specificity of the complexes, LivH and LivM are the membrane components and LivG and LivF are the ATP-binding components of the ABC transport complexes. Yhhk is a putative acetyltransferase. Figure adapted from Adams 1990.
Fig 4.3. Dps affects LS and LIVII. RT-PCR analysis of liv gene transcripts in wild-type SL1344 (lane 1), dps mutant JTS004 (lane 2) and complemented mutant JTS009 (lane 3). Total RNA was extracted from 12 h cultures and used as template for RT-PCR. To control for loading RT-PCR analysis of STM1618 was performed as its expression does not alter significantly in the presence and absence of dps.
Table 4.5. Fitness measurements

<table>
<thead>
<tr>
<th>Suplemented amino acid</th>
<th>Relative mean fitness$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.034 +/- .05</td>
</tr>
<tr>
<td>L-leucine</td>
<td>0.89 +/- .02</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>0.76 +/- .02</td>
</tr>
<tr>
<td>L-valine</td>
<td>0.73 +/- .04</td>
</tr>
</tbody>
</table>

$^a$Fitness relative to the wild-type SL1344 (1)
4.2.4 Motility gene expression

The synthesis and function of the Salmonella flagella system requires the ordered expression of more than 50 genes. At the 4 h time point four genes involved in the motility of Salmonella were affected two of these genes were also affected at the 12 h time point while no genes involved in motility were found to be affected at the 22 h time point (Table 4.6 and Fig 4.3).

4.2.5 Regulator gene expression

Interestingly the level of expression of rpoS the stationary phase sigma factor was upregulated in the dps mutant at the 12-h time point by more than 1.5-fold (Table 4.1). It should be noted that although the level of expression of rpoS was also upregulated in the 4-h and 22-h time points the fold-difference was less than 1.5 in both cases. A 1.295-fold increase was shown at 4 h while the increase in expression at 22 h was 1.48-fold. The observation that rpoS expression was increased in the absence of dps led to the hypothesis that a degree of reciprocal regulation may be occurring between RpoS and Dps and this will be covered in chapter 5. dps is positively regulated by RpoS during stationary phase (Altuvia 1994).

4.2.6 Iron-regulated genes

No iron-regulated genes appeared to be significantly affected by the absence of dps. fur, the transcriptional repressor of iron-regulated genes, was 1.28-fold downregulated at the 12-h time point and virtually unchanged at the other time points (1.161-fold upregulated and 1.068-fold downregulated at 4 h and 22 h respectively). Neither of the two genes coding for the iron storage proteins ftmA and bfr coding for a ferritin FtnA and a bacterioferritin Bfr respectively were significantly affected at any of the time points. Although ftNB encoding a ferritin-like protein FtnB is just under the 1.5 threshold, being upregulated by 1.478-fold at the 12 h time point. lipA coding for an iron-sulphur enzyme involved in the biosynthesis of biotin and lipoic acid (Marquet et al. 2001) was upregulated by 1.88-fold at the 22 h time point but was unaffected at either 4 h or 12 h time points.
4.2.7 Virulence gene expression

In contrast to a number of recent studies (Mangan et al., 2006, Kelly et al., 2004) very few genes involved in virulence were affected by the absence of \textit{dps} (Table 4.7). Two genes on the \textit{Salmonella} plasmid, pSLT, had differential levels of expression. pSLT093 was downregulated by more than 1.5-fold at both 4 and 12 h time points, while pSLT061 was upregulated at the 4 h time point and downregulated at the 12 h time point. \textit{prgH}, coding for a cell invasion protein, and \textit{orfX} both associated with SPI-5 were both downregulated by more than 1.5-fold at the 12 h time point but were unaffected at either the 4-h or 22-h time points.

4.2.8 Stress response gene expression

The microarray data showed that few classical stress response genes were affected by the \textit{dps} mutant at the time points chosen for analysis. This result was surprising as Dps has been previously shown to protect the cell from a number of stresses including oxidative and thermal stress, acid and alkali shock and gamma and ultraviolet radiation (Martinez et al., 1997; Nair and Finkel 2004; Section 3.2.5).

These data suggest that Dps may not be functioning as a classic transcriptional regulator, at least under the conditions tested, leading to a new hypothesis that Dps may exert a global influence on gene expression post-transcriptionally.
Table 4.6 Motility genes affected by the *dps* mutation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>JTS004/SL1344 expression ratio$^a$</th>
<th>p-values as appropriate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 h</td>
<td>12 h</td>
</tr>
<tr>
<td><em>cheM</em></td>
<td>methyl-accepting chemotaxis protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.634</td>
<td>ND</td>
</tr>
<tr>
<td><em>cheR</em></td>
<td>response regulator for chemotaxis</td>
<td>0.566</td>
<td>ND</td>
</tr>
<tr>
<td><em>flgH</em></td>
<td>Flagellar biosynthesis, basal-body outer-membrane L ring protein</td>
<td>0.59</td>
<td>ND</td>
</tr>
<tr>
<td><em>flgK</em></td>
<td>flagellar hook-associated protein 1</td>
<td>0.685</td>
<td>0.633</td>
</tr>
<tr>
<td><em>fliH</em></td>
<td>flagellar assembly protein</td>
<td>0.603</td>
<td>0.27</td>
</tr>
</tbody>
</table>

$^a$ND = No statistically significant data

Blue gene expression ratios represent genes with lower expression in JTS004
Fig 4.4 The flagellar regulatory network. There are over 50 genes involved in flagellar biosynthesis. These genes are divided into at least 17 operons, which are subdivided into three temporally regulated transcriptional classes, early middle and late with respective promoters classified as class 1, 2 and 3. Flagellar biosynthesis is initiated by activation of the single class 1 promoter that transcribes the flhDC operon. FlhD and FlhC are the master regulators of the flagellar region, and their expression is required for activation of the class 2 promoters, which transcribe the middle genes. Middle gene operons encode proteins required for the structure and assembly of the hook basal body. Middle genes also encode the sigma factor FliA ($\sigma^{28}$) and the anti-sigma factor FlgM. FlgM binds to $\sigma^{28}$ and prevents transcription of the class 3 $\sigma^{28}$-dependent promoters until completion of the flagellum basal body and hook. On completion of the flagellum basal body and hook FlgM is excreted out of the cell allowing $\sigma^{28}$-dependent transcription of the class 3 promoters. The late genes encode proteins required for maturation of the flagellum and chemosensory system including the flagellin subunits FliC and FliB. (figure adapted from Chilcott and Hughes (2000))
Table 4.7 Virulence genes affected by the \textit{dps} mutation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>JTS004/SL1344 expression ratio\textsuperscript{a}</th>
<th>p-values as appropriate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSLT061</td>
<td>putative inner membrane protein</td>
<td>1.529 0.635</td>
<td>0.0211 0.882</td>
</tr>
<tr>
<td>PSLT093</td>
<td>conjugative transfer</td>
<td>0.635 0.554</td>
<td>0.623 0.571</td>
</tr>
<tr>
<td>\textit{prgH}</td>
<td>cell invasion protein</td>
<td>ND 0.567</td>
<td>n/a 0.411</td>
</tr>
<tr>
<td>\textit{orfX}</td>
<td>putative cytoplasmic protein</td>
<td>ND 0.466</td>
<td>n/a 0.585</td>
</tr>
</tbody>
</table>

\textsuperscript{a}ND = No statistically significant data

Blue gene expression ratios represent genes with lower expression in JTS004

Red gene expression ratios represent genes with higher expression in JTS004
4.3 Discussion

The description of Dps as a nucleoid associated protein with levels of expression rising from about 6,000 molecules per cell in exponential phase to a peak of about 180,000 molecules per cell at late stationary phase (Ali Azam et al., 1999), lead to the hypothesis that Dps would have a pleiotropic regulatory role in global regulation of gene transcription in S. Typhimurium. To investigate this hypothesis the effect of mutating the \textit{dps} gene on the SL1344 transcriptome was investigated by DNA microarray analysis.

Unexpectedly, mutating the \textit{dps} gene had very little influence on the SL1344 transcriptome. There did not seem to be a significant dissimilarity in the transcriptome of the \textit{dps} mutant compared with the wild-type at any of the time points tested.

The results revealed that the greatest number of Dps-regulated genes with known function were genes involved in amino acid transport and metabolism. One system in particular involved in the transport of branched-chain amino acid into the cell displayed a response to the absence of Dps. Although only \textit{livM} (one of the membrane components of the system) passed the statistical filter, all of the genes involved in the system were assessed for their response to the mutation of \textit{dps}. RT-PCR analysis revealed that all of the genes of the two ATP-dependent high-affinity branched-chain amino acid transport systems, LIV-I and LS, involved in the transport of L-leucine, L-valine, and L-isoleucine were affected by the mutation in \textit{dps} (Fig 4.2. B). With the exception of \textit{livH} all of the \textit{liv} genes were expressed at lower levels in the \textit{dps} mutant. It has previously been shown that all of the membrane component genes as well as at least one of the binding protein genes are essential for active transport of leucine (Adams et al., 1990). LivJ and LivK are the two periplasmic amino acid-binding proteins that confer the specificity of the complexes. The addition of excess L-leucine, L-valine, or L-isoleucine into the growth medium rendered the \textit{dps} mutant less fit than the wild-type when they were directly competed. It is proposed that the inability to efficiently transport these branched-chain amino acids into the cell necessitated the expenditure of energy to synthesise these amino-acids \textit{de}-
Such expenditure was unnecessary in the wild-type resulting in a fitness advantage. These data show for the first time that Dps has an influence on the metabolism of SL1344.

The lack of effect on stress response gene expression and iron-regulated genes in particular was surprising. Dps is capable of accommodating ~500 Fe atoms per dodecamer (Ilari et al., 2002), therefore it would not be unexpected to observe an increase in the expression levels of bfr and ftnA to compensate for the loss of an additional iron storage protein, however such an increase did not occur. It is also possible that the ability of Dps to protect the cell from various stresses is at a physical level rather than at the level of transcription regulation.

Collectively the findings of this study indicate that Dps does not greatly affect transcription at the genomic level at least under the conditions tested. The Dps-affected genes have not been examined for direct regulation by the Dps protein. It is possible that the effects seen in the transcriptomic data are due to an indirect influence. To date no further studies have been published defining the regulatory role of Dps. This leads to the question how is Dps exerting an impact on the profile of proteins synthesised during starvation as observed by Almiron et al., (1992)? It is possible that Dps may exert a global influence post-transcriptionally.
Chapter 5

2-D PAGE proteomic analysis of the Dps regulon of *Salmonella enterica* serovar Typhimurium
5.1 Introduction

There are various stages at which expression of a gene can be regulated, from DNA-to-RNA transcription through to the posttranslational modification of a protein. Nucleoid associated proteins such as Fis and H-NS can directly regulate transcription of genes through binding to the promoter regions of these target genes for example, ssrA in the case of Fis and the virF promoter of Shigella flexneri in the case of H-NS (Kelly et al., 2004, Prosseda et al., 2004).

This direct regulation is not confined to nucleoid associated proteins. In Salmonella the FljA transcriptional inhibitor regulates the flagellar gene fliC. The fljA gene forms part of an operon with fljB. In one orientation the fljBA promoter directs transcription of the fljBA operon and FljB flagellin is produced. The fljA gene is cotranscribed with the fljB gene and represses transcription of the fliC gene, thus ensuring that only one flagellin subunit type is produced at a time. It has recently been shown that in addition to regulating fliC transcription fljA also regulates FliC translation. It is proposed that this posttranscriptional control is mediated through the interaction of FljA with the 5' untranslated region of fliC (Bonifield et al., 2003). Thus fljA can be considered as both a transcriptional and posttranscriptional regulator of fliC.

Other posttranscriptional mechanisms of regulation include influences on mRNA stability, for example H-NS binds to rpoS mRNA and enhances its cleavage while the small RNA DsrA stabilises the rpoS mRNA (Brescia et al., 2003). Translation can also be influenced. Again using RpoS as an example, it has been shown that the RNA chaperone Hfq is essential for rpoS translation (Muffler et al., 1996). Hfq modulates rpoS expression by altering the binding of small RNAs such as DsrA, OxyS and RprA (Sledjeski et al., 2001, Zhang et al., 2002; Masse et al., 2003). HU and the small RNA, RprA both stimulate translation of rpoS (Balandina et al., 2001, Majdalani et al., 2002) while another small RNA OxyS represses the translation of the rpoS mRNA (Zhang et al., 1998).

RpoS is also regulated posttranslationally. In exponentially growing cells the RssB response regulator directly targets RpoS for degradation by the ClpXP protease (Zhou
et al., 2001). RssB delivers RpoS to ClpXP where RpoS is unfolded and completely degraded (Muffler et al., 1996).

Based on the microarray analysis (Chapter 4), Dps did not seem to be a global transcriptional regulator, at least under the conditions tested. It was postulated that the regulatory role observed for Dps could involve a posttranscriptional mechanism. To investigate if this was the case, two-dimensional polyacrylamide gel electrophoresis analysis was undertaken.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is a form of gel electrophoresis in which proteins are separated according to their charge (pI) by isoelectric focusing (IEF) in the first dimension and according to their mass by SDS-PAGE in the second dimension (O'Farrell 1975). In common with cDNA microarray technology it facilitates a comparison to be made directly between wild-type and mutant strains. The wild-type expression profile is compared to the expression profile of its otherwise isogenic mutant derivative to reveal how the particular mutation influences the global protein profile. cDNA microarray studies enable a comparison of cDNA to be made between samples. They can consequently, give an insight into the transcriptional regulation of particular genes and can contribute to the identity of members of specific regulons.

The changes that occur in transcript levels do not always correlate with changes in either protein amount or activity within cells. The expression pattern of sfh mRNA is reciprocal to the expression pattern of the Sfh protein in S. flexneri BS184 (Deighan et al., 2003). The sfh mRNA is abundant in early exponential phase whereas the Sfh protein levels are relatively low (Deighan et al., 2003). Conversely protein levels are relatively high at early stationary phase while the levels of mRNA are relatively low (Deighan et al., 2003). The levels of RpoS protein are low in the exponential stage of growth, yet relatively high levels of rpoS mRNA are present (Hengge-Aronis, 2002). There is a basal level of dps expression in log-phase cells; however, the Dps protein is degraded rapidly during exponential growth through the action of the ClpXP protease (Jeong et al., 2006,). Such posttranscriptional events are not highlighted in cDNA microarray analysis. 2-D PAGE has been used to study protein expression in bacteria,
yeast, drosophila and humans (Ames and Nikaido 1976, Ishiguro et al., 1976, Lambertsson et al., 1975, Ucer et al., 1975). 2-D PAGE with subsequent MALDI-TOF identification of particular proteins has contributed to the detection of novel colonization and virulence factors in S. Typhimurium including STM3117 (Shi 2006).

The protein analysis employed in this study was performed in collaboration with Dr Steven Smith of the Department of Clinical Microbiology, Trinity College, St. James Hospital, Dublin to elucidate the global protein response of S. Typhimurium to the dps mutation.
5.2 Results

5.2.1 2D-PAGE proteome analysis to determine the Dps regulon

Surprisingly, DNA microarray analysis seemed to indicate that Dps exerts only a minor influence on the SL1344 transcriptome. Therefore it was postulated that the role of Dps in genetic regulation in *S. Typhimurium* might be at the post-transcriptional level. To this end a study of the Dps proteome was undertaken. Based on the results of the microarray analysis two time points were chosen for investigation i.e. 4-h and 12-h. These time-points represent the exponential stage of growth and stationary phase respectively.

Overnight cultures of SL1344 and JTS004 were diluted 1:100 into fresh LB and grown for 4 h or 12 h at 37°C with shaking. Equal concentrations of the strains were harvested and washed three times in decreasing volumes of low-salt buffer before being resuspended in 200 µl of resuspension buffer and divided into 40 µl aliquots for storage at −20°C (Section 2.21.1). It should be noted that this sample preparation method may result in an under-representation of membrane-associated proteins. 5 µl of the pre-frozen sample from each strain was boiled for 10 min and mixed with 5 µl of Laemmli buffer before electrophoresis through a 12% (w/v) polyacrylamide gel was carried out to confirm integrity of the samples and to assess loading volumes (Section 2.15.2).

Proteins were separated in the first dimension according to their pi by isoelectric focusing and in the second dimension according to their mass by SDS PAGE (Section 2.21). The raw 2-D electrophoresis gels were imaged using a BioRAD GS800 calibrated densitometer and further processed using the PDQuest™ software package. Spots were matched between the wild type and Dps mutant gels. Protein spots showing different levels depending on Dps were excised from stained gels, followed by in-gel digestion using the Trypsin Profile IGD Kit For In-Gel Digests (Sigma) (Section 2.21.5 & 6). Gwen Manning, at the TCD Proteomic Core Institute, carried out MALDI-TOF.
5.2.2 The protein expression profiles of SL1344 and JTS004 in stationary phase

14 proteins were highlighted, in a total of three pairs of gels analysed, that had different intensities between wild-type and \textit{dps} mutant. Five proteins that showed a change in intensity of at least 4-fold between wild-type SL1344 and its otherwise isogenic \textit{dps} mutant JTS004 were identified (Fig. 5.1). Protein (a) was identified in the wild-type gel as malate dehydrogenase. As this protein was only observed in the wild-type gel with the corresponding area of the mutant gel failed to produce an identifiable protein sample. Protein (b) was identified in both gels as succinyl-CoA synthetase beta chain, while protein (e) was identified also in both gels as succinyl-CoA synthetase alpha chain. Protein (c) was identified in the wild-type gel as glycerol kinase, but the corresponding spot from the mutant gel failed to be identified. Protein (d) was determined to be the galactose-binding component of an ABC transport system responsible for the uptake of galactose. Once again only the spot in the wild-type was successfully identified. These proteins showed higher levels in the wild-type strain than in the \textit{dps} mutant suggesting that the expression of these proteins is probably upregulated by Dps.

Glycerol kinase (protein c) is an enzyme involved in the uptake and metabolism of glycerol. The enzyme catalyzes the MgATP-dependent phosphorylation of glycerol to yield sn-glycerol 3-phosphate (Lin, 1976). Both malate dehydrogenase (protein a) and succinyl-CoA synthetase (proteins b and e) are enzymes involved in the tricarboxylic-acid (TCA) cycle responsible for the conversion of malate to oxaloacetate and of succinyl-CoA to succinate respectively (Fig 5.2).

These data confirm the findings from the transcriptional microarray studies that Dps has a role to play in carbon utilization and energy production in SL1344.

5.2.3 Impact of the \textit{dps} mutation on carbon catabolism in \textit{S} Typhimurium

To further examine the effect of the \textit{dps} mutation on metabolism, the ability of JTS004 to oxidize defined carbon sources in the presence of a redox indicator was assessed using the Phenotype MicroArray™ system from Biolog (Section 2.23). These are 96-well microtiter plates containing different carbohydrates lyophilized on
the bottom of each well in the presence of the redox indicator – tetrazolium dye (Table 5.1). The ability of each strain to catabolise a particular carbon source was indicated by a colour change in the redox indicator. If no oxidation occurs the dye remains colourless, a negative control or blank is present in well A1. If oxidation occurs a purple colour is produced. To quantify the relative change in colour of the dye observed, the plates were placed in a plate reader and the OD490nm was measured. Data were normalized by subtracting the measurement of the negative control from each measurement. Mean values were determined from three independent experiments (Table 5.2). The results revealed that JTS004 is less able to catabolise the carboxylic acid, mono-methyl-succinate, the 8-carbon carbohydrate N-acetyl-D-glucosamine, the monosaccharides D-galactose and α-D-glucose, and the disaccharide D-lactose (Table 5.3). JTS004 showed better ability to oxidize two of the amino acids, glycyll-L-glutamic acid and glycyll-L-aspartic acid and two of the carboxylic acids L-malic acid and mucic acid as well as the surfactant Tween 20 (Table 5.3). There appeared to be no significant difference between the ability of JTS004 to oxidize glycerol.

*Salmonella* lacking any of the enzymes of the citric acid cycle are unable to use acetate as a sole carbon and energy source (Cronan and LaPorte 1996). To investigate if the mutation in *dps* would have an effect on carbon utilization, wild-type SL1344 and *dps* mutant JTS004, were grown in MOPS minimal medium with sodium acetate (0.2% w/v) as the sole carbon source. As can be seen from Fig 5.3, both wild-type and *dps* mutant are able to grow in MOPS minimal medium with acetate as the sole carbon source. These data indicate that the levels of TCA enzymes produced in the mutant are sufficient to allow JTS004 to use acetate as the sole carbon source. However, it was felt that this method of testing the relative ability to utilise defined carbon sources was quite crude. For this reason pairwise growth competition was chosen to further investigate the initial findings of the 2D-PAGE analysis.

The ability of *Salmonella* to utilise specific sole carbon sources was further assessed by relative fitness assay in MOPS minimal medium supplemented with 0.2% (w/v) defined carbon source as indicated (Section 2.24). To ensure that fitness was not affected by the presence of the chloramphenicol acetyltransferase (*cat*) gene, strain JH3008 (Hautefort 2003) was used as a control in all cases (Table 2.1). The fitness of
Fig 5.1. The protein expression profile of SL1344 and JTS004 in stationary phase. 2-D gel electrophoresis of stationary phase wild-type SL1344 (A) and its $dps$ derivative JTS004 (B). Cells were grown in LB broth without antibiotic to stationary phase. Isoelectric focusing was performed with IPG strips ranging from pH 4 to pH 7 and second dimensional separation was accomplished on 12% polyacrylamide gels. Blue boxes indicate protein spots that are changed in wild-type and the $dps$ mutant and whose identity was successfully confirmed through MALDI-TOF analysis.
Fig. 5.2. Outline diagram of the Tri-carboxylic acid (TCA) cycle. Genes whose expression was influenced by the mutation in \( \text{dps} \) are coloured blue.
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Negative control</td>
<td>L-arabino</td>
<td>N-acetyl-D-glucosamine</td>
<td>D-saccharic acid</td>
<td>succinic acid</td>
<td>D-galactose</td>
<td>L-aspartic acid</td>
<td>L-proline</td>
<td>D-alanine</td>
<td>D-trehalose</td>
<td>D-mannose</td>
<td>dulcitol</td>
</tr>
<tr>
<td>B</td>
<td>D-serine</td>
<td>D-sorbitol</td>
<td>glycerol</td>
<td>L-fucose</td>
<td>D-glucuronic acid</td>
<td>D-glucosamine</td>
<td>D.L.-α-D-glucuronic acid</td>
<td>D.L.-xylitol</td>
<td>L-lactic acid</td>
<td>formic acid</td>
<td>D-mannitol</td>
<td>L-glutamic acid</td>
</tr>
<tr>
<td>C</td>
<td>D-glucose-6-phosphate</td>
<td>D-galacturonic acid-γ-lactone</td>
<td>D.D.-malic acid</td>
<td>D-ribose</td>
<td>tween 20</td>
<td>L-rhamnose</td>
<td>D-fructose</td>
<td>acetic acid</td>
<td>α-D-glucose</td>
<td>maltose</td>
<td>D-melibiose</td>
<td>thymidine</td>
</tr>
<tr>
<td>D</td>
<td>D-asparagine</td>
<td>D-aspartic acid</td>
<td>D-glucosaminic acid</td>
<td>α-keto-glutaric acid</td>
<td>α-keto-butyric acid</td>
<td>α-methyl-D-galactoside</td>
<td>α-D-lactose</td>
<td>lactulose</td>
<td>sucrose</td>
<td>uridine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>L-glutamine</td>
<td>M-tartric acid</td>
<td>D-glucose-1-phosphate</td>
<td>D-fructose-6-phosphate</td>
<td>tween 80</td>
<td>α-hydroxy-glutaric acid</td>
<td>α-hydroxy-butyric acid</td>
<td>β-methyl-D-galactoside</td>
<td>adonitol</td>
<td>maltotriose</td>
<td>2-deoxyadenosine</td>
<td>adenosine</td>
</tr>
<tr>
<td>F</td>
<td>Glycyl-L-aspartic acid</td>
<td>citric acid</td>
<td>M-inositol</td>
<td>D-threonine</td>
<td>fumaric acid</td>
<td>bromo-succinic acid</td>
<td>propionic acid</td>
<td>mucic acid</td>
<td>glycolic acid</td>
<td>glyoxylic acid</td>
<td>D-cellobiose</td>
<td>inosine</td>
</tr>
<tr>
<td>G</td>
<td>Glycyl-L-glutamic acid</td>
<td>tricarballylic acid</td>
<td>L-serine</td>
<td>L-threonine</td>
<td>L-alanine</td>
<td>L-alanyl-glycine</td>
<td>acetoacetic acid</td>
<td>N-acetyl-β-D-mannosamine</td>
<td>mono methyl succinate</td>
<td>methyl pyruvate</td>
<td>D-malic acid</td>
<td>L-malic acid</td>
</tr>
<tr>
<td>H</td>
<td>Glycyl-L-proline</td>
<td>p-hydroxyphenylacetate</td>
<td>m-hydroxyphenylacetate</td>
<td>tyramine</td>
<td>D-psicose</td>
<td>L-lyxose</td>
<td>glucuronamide</td>
<td>pyruvic acid</td>
<td>L-galacturonic acid</td>
<td>D-galacturonic acid</td>
<td>phenylethylamine</td>
<td>2-aminoethanol</td>
</tr>
</tbody>
</table>
Table 5.2 OD$_{540nm}$ values* for the carbon sources outlined in table 5.1

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.000</td>
<td>1.028</td>
<td><em>0.160</em></td>
<td>1.791</td>
<td>3.042</td>
<td><em>0.453</em></td>
<td>1.444</td>
<td>1.312</td>
<td>1.564</td>
<td>1.017</td>
<td>1.041</td>
<td>ND</td>
</tr>
<tr>
<td>B</td>
<td>1.147</td>
<td>1.183</td>
<td>0.903</td>
<td>0.975</td>
<td>1.144</td>
<td>1.211</td>
<td>1.368</td>
<td>0.563</td>
<td>0.966</td>
<td>2.165</td>
<td>0.789</td>
<td>0.822</td>
</tr>
<tr>
<td>C</td>
<td>1.274</td>
<td>0.955</td>
<td>1.103</td>
<td>1.219</td>
<td><em>3.091</em></td>
<td>0.646</td>
<td>0.600</td>
<td>1.567</td>
<td><em>0.268</em></td>
<td>0.999</td>
<td>1.622</td>
<td>0.882</td>
</tr>
<tr>
<td>D</td>
<td>1.084</td>
<td>0.733</td>
<td>0.653</td>
<td>1.085</td>
<td>0.850</td>
<td>0.629</td>
<td>1.044</td>
<td>1.005</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.877</td>
</tr>
<tr>
<td>E</td>
<td>1.156</td>
<td>ND</td>
<td>1.029</td>
<td>0.973</td>
<td>0.921</td>
<td>0.724</td>
<td>0.870</td>
<td>1.320</td>
<td>ND</td>
<td>1.146</td>
<td>0.920</td>
<td>1.033</td>
</tr>
<tr>
<td>F</td>
<td><strong>2.405</strong></td>
<td>0.950</td>
<td>ND</td>
<td>1.084</td>
<td>1.506</td>
<td>1.896</td>
<td>0.928</td>
<td><strong>3.071</strong></td>
<td>ND</td>
<td>ND</td>
<td>0.689</td>
<td>0.961</td>
</tr>
<tr>
<td>G</td>
<td><strong>2.937</strong></td>
<td>1.000</td>
<td>0.953</td>
<td>0.873</td>
<td>2.115</td>
<td>1.209</td>
<td>0.787</td>
<td>1.251</td>
<td><strong>0.457</strong></td>
<td>1.151</td>
<td>ND</td>
<td><strong>3.803</strong></td>
</tr>
<tr>
<td>H</td>
<td>1.329</td>
<td>1.979</td>
<td>1.893</td>
<td>1.000</td>
<td>1.049</td>
<td>ND</td>
<td>8.526</td>
<td>0.730</td>
<td>ND</td>
<td>1.853</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Values represent the average JTS004/SL1344 OD$_{540nm}$, value of three independent experiments. Well A1 represents the negative control. ND – No oxidation occurred in the well. Red values represent carbon sources whose OD$_{540nm}$ in the *dps* mutant was less than 2 fold that of the wild-type. Blue values represent carbon sources whose OD$_{540nm}$ in the *dps* mutant was more than 2-fold that of the wild-type.
Table 5.3 Carbon sources whose catabolism was affected\(^a\) in JTS004

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Grid reference</th>
<th>Fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>More oxidation in JTS004</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycyl-L-aspartic acid</td>
<td>F1</td>
<td>2.4</td>
</tr>
<tr>
<td>Glycyl-L-glutamic acid</td>
<td>G1</td>
<td>2.9</td>
</tr>
<tr>
<td>Tween 20</td>
<td>C5</td>
<td>3.1</td>
</tr>
<tr>
<td>Mucic acid</td>
<td>F8</td>
<td>3.1</td>
</tr>
<tr>
<td>L-malic acid</td>
<td>G12</td>
<td>3.8</td>
</tr>
<tr>
<td><strong>Less oxidation in JTS004</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-acetyl-D-glucosamine</td>
<td>A3</td>
<td>6.3</td>
</tr>
<tr>
<td>D-galactose</td>
<td>A6</td>
<td>2.21</td>
</tr>
<tr>
<td>α-D-glucose</td>
<td>C9</td>
<td>3.7</td>
</tr>
<tr>
<td>Mono methyl succinate</td>
<td>G9</td>
<td>2.2</td>
</tr>
</tbody>
</table>

\(^a\)Cut of point was a 2-fold difference between OD\(_{500nm}\) of SL1344 and JTS004
Fig 5.3. **JTS004 has no growth defect associated when growing on acetate as the sole carbon source.** *S. typhimurium* SL1344 and its otherwise isogenic *dps* mutant JTS004 were grown overnight in 5 ml LB broth. Following overnight incubation the cultures were washed in PBS, suspended in MOPS minimal medium and subcultured into 25 ml volumes of fresh MOPS minimal medium supplemented with sodium acetate in 250 ml non-baffled conical flasks and grown at 37°C with shaking. Growth of the two strains was monitored by OD$_{420}$ nm readings at various time points post inoculation. The log of the OD$_{420}$ nm values were plotted against time. The above experiment was carried out in triplicate on two occasions and representative data are shown. Error bars represent the standard deviation of a single experiment.
JTS004 relative to wild-type in the presence of defined carbon sources was determined by inoculating 25 ml fresh growth medium with $10^6$ cells of each competitor. The bacteria had been preconditioned in the growth medium for 24 h prior to subinoculation. The ratio of the two competing strains was determined at time zero and after 24 h growth by viable counts after plating serial dilutions of the inoculum in parallel on LB (to measure total c.f.u) and LB with cml. (to determine mutant c.f.u) (Fig 5.4).

In contrast to previous work in this study, where there was a growth defect associated with JTS004 when glucose was the sole carbon source (Section 3.2.4) there was no apparent decrease in the fitness of JTS004 relative to wild-type. Both the $dps$ mutant and the "neutral" chloramphenicol-resistant strain had fitness values close to 1 (0.987 and 0.992 respectively) (Fig 5.4). It should be noted that the cultures used to perform the time course analysis had been seeded from washed overnight cultures that had been inoculated into LB broth. The cultures used for all of the fitness work relating to defined carbon sources had been grown directly from glycerol stocks into MOPS minimal medium so as to ensure no possible contamination from the carbon sources contained in LB broth.

When the sodium salts of succinate or acetate were used as the sole carbon source, there was a marked decrease in fitness of the $dps$ mutant relative to the wild-type SL1344. The decrease in fitness was over 20% in the case of acetate and was just under 25% in the case of succinate (Fig 5.4). The neutral chloramphenicol-resistant strain had fitness values close to 1 (1.04 and 1.08 respectively) indicating that the fitness deficit was directly or indirectly as a result of the mutation in $dps$ not the presence of the chloramphenicol resistance cassette.

In *E. coli* the full TCA cycle is seen only during aerobic growth on acetate or fatty acids, growth during these conditions requires induction and function of the glyoxylate shunt to replenish the dicarboxylic acid intermediates consumed in amino acid biosynthesis (Cronan and LaPorte 1996). During aerobic growth on glucose a branched biosynthetic form of the TCA cycle is used. The full TCA cycle is not required under these conditions because the bulk of energy is derived from glycolysis.
(Amarasingham and Davis, 1965). It was postulated that the $dps$ mutant would be more likely to participate in the glyoxylate shunt, which converts isocitrate to malate bypassing a number of steps in the TCA cycle including the conversion of succinyl-CoA to succinate by succinyl-CoA synthetase (Fig 5.2).

Accordingly the fitness of JTS004 was assessed when sodium glyoxylate was the sole carbon source. JTS004 proved to have an increase in fitness of over 25% compared to the wild-type using glyoxylate while there was no significant difference in the fitness of JH3008 when compared to wild-type SL1344 (Fig 5.4).

To see if this observed phenotype could be explained by transcriptional control of selected genes, $sueC$ $mdh$ $aceA$ and $aceB$ (Fig 5.2), of the TCA and glyoxylate bypass RT-PCR analysis was carried out (Section 2.13.2). RNA was extracted from overnight cultures and used as the template for the RT-PCR (Section 2.1.4). The results indicated that there was a decrease in expression of all four genes in the $dps$ mutant but only in the stationary phase, at exponential growth phase no difference in expression level was observed for any of the genes (Fig 5.5). As a control for loading the expression levels of STM1618 was also analysed by RT-PCR and expression levels were not found to change between wild-type and $dps$ mutant at either growth phase.

5.2.4 Effect of the mutation of $dps$ on growth in low iron concentration

Iron is a cofactor of the TCA cycle enzymes aconitase, (citrate to isocitrate) and succinate dehydrogenase (succinate to fumarate), and fumarase (fumarate to malate) (Fig 5.2). As I had shown that JTS004 was less fit than the wild-type when succinate or acetate was the only available carbon source, and since Dps is able to bind iron, I wanted to see what effect limiting the concentration of iron in the medium would have. Initially a comparison was made using bacteria grown in LB (Section 2.23).

When $dps$ mutant cells were transferred to iron-limited conditions in rich medium, fitness was not affected relative to wild-type cells (Fig 5.6). This was interesting as each Dps dodecamer has the ability to sequester $\sim$500 Fe $2^+$ atoms (Ilari et al., 2002), and thus its absence could have an effect on the amount of iron available to the cell to
Fig 5.4. The effect of the *dps* mutation on the fitness of *S. Typhimurium* growing in defined carbon sources. The ability of *Salmonella* to utilise specific sole carbon sources was assessed by relative fitness assay in MOPS minimal medium supplemented with 0.2% (w/v) defined carbon source as indicated. The fitness of JTS004 or JH3008 relative to wild-type in the presence of defined carbon sources was determined by simultaneously inoculating 25 ml fresh growth medium with $10^5$ cells of each competitor, which had been preconditioned in the growth medium for 24 h. The ratio of the two competing strains was determined at time zero and after 24 h growth by viable counts after plating serial dilutions of the inoculum in parallel on LB (to measure total c.f.u) and LB with cml (to determine mutant c.f.u). Experiments were carried out in triplicate the error bars indicate standard deviations based on three independent experiments.
Fig 5.5. The effect of the $dps$ mutation on expression of selected genes of the TCA cycle and glyoxylate bypass. Lanes 1, 3, 5 and 7 wild-type SL1344. Lanes 2, 4, 6 and 8 $dps$ mutant JTS004. Total RNA was extracted from 4 h cultures, exponential phase and 12 h cultures, stationary phase and used as template for RT-PCR. The experiment was repeated on three occasions and typical data are shown.
Fig 5.6. Effect of the $dps$ mutation on the fitness of S. Typhimurium growing in the presence of the iron chelator 2,2-dipyridyl. The fitness of JTS004 relative to wild-type in the presence of an iron chelating agent was determined by competitive assay in LB containing 2 μm 2,2-dipyridyl (DP). Cells grown to stationary growth phase in LB were transferred to LB containing 2 μm DP (orange bars) or no iron-chelator (green bars) in equal concentrations. The ratio of the two competing strains was determined at time zero and after 24 h growth by viable counts after plating serial dilutions of the inoculum in parallel on LB (to measure total c.f.u) and LB with cml (to determine mutant c.f.u). Experiments were carried out in duplicate and error bars represent standard deviations based on three separate experiments.
participate in metabolism. However it was not possible to assess the initial concentration of iron present in the medium and it may well have been in excess to such an extent that there was still sufficient iron present even on addition of 2 μm 2,2-dipyridyl that would make the absence or presence of Dps irrelevant.

To assess if the effect of iron-limited conditions was more apparent when iron was chelated in a growth medium with defined initial iron concentration (0.01 mM), the relative fitness assay was attempted in MOPS minimal medium with acetate or succinate as the sole carbon source. However the dps mutant would not grow when acetate or succinate was the sole carbon source in the presence of the iron-chelator, 2,2-dipyridyl. When cultures were inoculated directly from glycerol stocks, growth occurred in wild-type SL1344 and the chloramphenicol resistant strain JH3008 within 48 h, but no growth was observed for JTS004 even after 144 h (data not shown). As a control cultures were inoculated into medium that did not contain the iron-chelator and growth was observed for all three strains within 12 h.

It is possible that the dual stresses of iron-limitation and a more oxidised carbon source was more than the dps mutant could cope with. I had shown that there was a neutral effect when glucose was the sole carbon source. Therefore to assess the effect of iron-limited conditions the growth of the wild-type and the dps mutant was monitored when stationary phase cells were transferred from MOPS minimal medium to MOPS minimal medium with or without 2 mM 2,2-dipyridyl (Fig 5.7). The wild-type and dps mutant inoculated into MOPS minimal medium with no chelator displayed no significant differences in their growth profile. In contrast to the situation observed when washed cultures from LB broth were used as the starting inocula, the lag phase for both strains of S. Typhimurium appeared to be somewhat extended. The exponential phase of growth did not commence until 12 h post inoculation, whereas exponential growth was observed for the washed cultures 3-4 h post inoculation (Fig 3.4.B).

When the dps mutant grown in MOPS minimal medium with 2 mM 2,2-dipyridyl was subinoculated into fresh medium, no growth occurred. The wild-type strain SL1344 grew under this regime. The lag phase observed for these conditions was the longest,
with exponential growth commencing 24 h post inoculation. In particular growth was severely attenuated in comparison to the wild-type and \textit{dps} mutant inoculated into MOPS minimal medium with no chelator. Exponential growth phase commenced in the cultures inoculated into MOPS minimal medium containing chelator between 12 and 24 h post inoculation. However the \textit{dps} mutant appeared to enter stationary phase at a much lower OD$_{600}$ and at an earlier time than the wild-type. The terminal OD reached by the \textit{dps} mutant inoculated from MOPS minimal medium with no chelator into fresh MOPS minimal medium containing chelator most resembled that of the wild-type coming from medium containing chelator into fresh medium also containing chelator (Fig 5.7).

5.2.5 The protein expression profile of SL1344 and JTS004 in exponential phase

The relative abundances of nine proteins were found to change in protein intensity of at least 6-fold between wild-type SL1344 and its otherwise isogenic \textit{dps} mutant JTS004 (Section 2.20.5). These proteins were excised and subjected to MALDI-TOF. Four were successfully identified (Fig. 5.8). Proteins (a) and (b) were expressed at higher levels in the \textit{dps} mutant, while proteins (c) and (d) were expressed at lower levels in the \textit{dps} mutant. Protein (a) was identified as the cell division protein FtsZ. Protein (b) was identified as the SodB superoxide dismutase. Proteins (c) was identified as FlgM, the anti-sigma factor responsible for the repression of the alternative sigma factor controlling expression of the late genes of the flagellar regulatory network. Protein (d) was identified as the nucleoid-associated protein H-NS (Fig 5.8).

5.2.6 The \textit{dps} mutation has no significant effect of on morphology of \textit{S. Typhimurium}

FtsZ, identified as being up-regulated in the \textit{dps} mutant is a structural homolog of tubulin (Lowe and Amos, 1998) and is an essential cell division protein. Assembly of FtsZ into a ring structure the Z ring (Bi and Lutkenhaus, 1991) at the future site of cell division is the first event in cell division. Overproduction of FtsZ results in the formation of filamentous bacteria. To investigate if this phenotype would be observed in the \textit{dps} mutant wild-type SL134 and JTS004 were grown overnight at 37°C with
Fig 5.7 Effect of the dps mutation on growth in low iron concentration. Cells grown to stationary growth phase in MOPS minimal medium or MOPS minimal medium containing 2 μM 2,2-dipyridyl (DP) were transferred (0 h) to MOPS minimal medium containing 2 μM or no DP. The optical density at 600nm was monitored. ♦, dps mutant cells (0 DP), ■ dps mutant cells (2 μM DP), ▲ wild-type cells (0 DP), × wild-type cells (2 μM DP), * wild-type cells from MOPS minimal medium containing 2 μM DP (2 μM DP). Experiments were carried out in triplicate the error bars indicate standard deviations based on two independent experiments.
Fig 5.8. The protein expression profiles of SL1344 and JTS004 in exponential phase. 2-D gel electrophoresis of stationary phase wild-type SL1344 (A) and its $dps$ derivative JTS004 (B). Cells were grown in LB broth without antibiotic to exponential phase. Isoelectric focusing was performed with IPG strips ranging from pH 4 to pH 7 and second dimensional separation was accomplished on 12% polyacrylamide gels. Coloured boxes indicate protein spots that are changed in wild-type and the $dps$ mutant and whose identity was successfully confirmed through MALDI-TOF analysis. Red boxes are protein spots that were upregulated in the $dps$ mutant. Blue boxes indicate protein spots that were downregulated in the $dps$ mutant.
shaking prior to Gram staining and examination by microscopy. The results revealed that the cells of JTS004 did not appear to be significantly more filamentous than the wild-type (data not shown).

5.2.7 The *dps* mutation has lower levels of H-NS than wild type SL1344

To confirm the finding that the H-NS protein had a reduced level of expression in the *dps* mutant JTS004, western blot analysis was undertaken (Section 2.17). Total protein was isolated from wild-type SL1344, *dps* mutant JTS004 and complemented mutant JTS009 following 4 h growth in LB broth at 37°C. Equal protein concentrations were loaded on an SDS-PAGE gel and analysed by western blot using anti-H-NS antiserum (Fig 5.9). Results revealed that the level of H-NS protein was strongly reduced in the *dps* mutant and this reduction was partially relieved by providing *dps in-trans*.

5.2.8 The *dps* mutant does not display reduced motility

H-NS has been shown to be a positive regulator of genes involved in the biogenesis of flagella and *hns* mutants are non-motile due to the absence of flagella (Hinton *et al.*, 1992). FlgM is a negative regulator of genes involved in the flagella biogenesis. It has been reported that the flagellar number produced in an *flgM* mutant is twice to three times as many as wild-type (Kutsukake *et al.*, 1994). Because both FlgM and H-NS have an influence on motility, analysis of the flagella system in *S. Typhimurium* was undertaken. In the first instance tests were performed on semi-solid swarming agar plates (Section 2.1.1). Equal numbers of bacteria from wild-type SL1344, and *dps* mutant JTS004 were used to inoculate the centres of semi-solid agar plates and incubated at 37°C for 8 h. Following incubation the diameter of the swarm ring was measured and compared (Fig 5.10). As a control for the experiment *S. Typhimurium fis* and *hns* mutants were also tested for their motility phenotypes. In agreement with previous studies the *hns* mutant was non-motile (data not shown) while the *fis* mutant displayed decreased motility (Hinton *et al.*, 1992, Kelly *et al.*, 2004). However, there was no significant difference in the diameter of the swarm rings produced by SL1344 or JTS004 indicating that motility was not affected by the *dps* mutation.
5.2.9  The effect of the \textit{dps} mutation on flagellin subunits

The production of the two distinct flagellin subunits, FliC and FljB, was assessed by western immunoblot (Section 2.17). Total protein was harvested from overnight bacterial cultures of SL1344 and JTS004 grown in LB broth at 37°C. Protein was also harvested from \textit{S} Typhimurium strains locked on and locked off for FljB and used as the positive and negative controls for FljB expression respectively. The results demonstrated a clear phenotype the \textit{dps} mutant did not produce the FljB flagellin subunit and produced increased levels of FliC when compared to the wild-type SL1344 (Fig 5.11).

RT-PCR analysis was carried out to see if this observed phenotype could be explained by transcriptional control of the \textit{fljB} and \textit{fliC} genes (Section 2.12.2). RNA was extracted from overnight cultures and used as the template for the RT-PCR (Section 2.1.4). There was decreased expression of \textit{fljB} in the \textit{dps} mutant and increased expression of \textit{fliC} compared to the wild-type (Fig 5.11). It was postulated that there must be a level of posttranscriptional regulation occurring, because if transcriptional regulation alone were responsible for the lack of detectible FljB in JTS004 then presumably the \textit{fljB} transcript would also be undetectable.

\textit{S}. Typhimurium undergoes phase variation to express alternatively two different flagellin subunit proteins, FljB or FliC. The flagella phase variation involves the inversion of approximately 16 kb of DNA containing the promoter of the \textit{fljBA} operon and the \textit{hin} gene encoding a recombinase (Zieg 1982). Fis facilitates Hin-mediated DNA exchange to generate a site-specific inversion. It was found that SL1344 could produce flagellar protein FljB or FliC alternatively but JTS004 could produce only FliC (Fig 5.11). A switch assay was undertaken to investigate if Hin-mediated DNA inversion was not possible in the \textit{dps} mutant strain (Section 2.24).

The assay performed takes advantage of the variable position of a recognition site for a restriction enzyme. When a 1.6 kb region of DNA encompassing the promoter region of the \textit{fljBA} operon is amplified by PCR and subsequently digested with the restriction enzyme \textit{SacI}, a distinct pattern of digestion fragments is observed, depending on the orientation of the promoter (Fig 5.12).
Fig 5.9. The intracellular level of H-NS is reduced in JTS004. The cellular content of H-NS was detected in wild-type SL1344 (Lane 1), $dps$ mutant JTS004 (Lane 2) and complemented mutant (Lane 3) by western blot as per section 2.17. Equal amounts of protein as normalized by the $\text{OD}_{600\text{nm}}$ value were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Levels of H-NS were detected using a polyclonal anti-body against H-NS.
Fig 5.10. Effect of the *dps* mutation on *S Typhimurium* motility. Motility of wild-type *dps* mutant and *fis* mutant strains was assayed on a semi-solid agar plates. Equal numbers of bacteria were inoculated into the centres of semi-solid swarming agar plate and incubated for 8 h at 37°C. The rate of spreading of rings of bacteria (swarming) on the plates was a measure of chemotaxis and motility.
Fig 5.11. Effect of the *dps* mutation on the intracellular levels of flagella proteins and on flagella transcription. (A) The cellular content of FliC or FljB was detected in wild-type SL1344 (Lane 1), *dps* mutant JTS004 (Lane 2) complemented mutant (Lane 3) positive control for FljB (Lane 3) and negative control for FljB (Lane 5) by western blot as per section 2.17. Equal amounts of protein as normalized by the OD$_{600nm}$ value were run on a SDS-PAGE and blotted onto a nitrocellulose membrane. Levels of FliC or FljB were detected using a polyclonal anti-body against FliC or FljB. (B) RT-PCR analysis of *fljBA* operon and *fliC* gene. Lanes 1, 3 and 5 wild-type SL1344. Lanes 2, 4 and 6 *dps* mutant JTS004.
Fig 5.12. Effect of the dps mutation on the hin switch of S. Typhimurium.

A. Diagram of the fljBA operon with the invertible DNA segment, enclosed by brackets containing the hin gene and promoter (P→). FljB+ the fljAB promoter is orientated towards the operon: FljB flagellin is produced along with FljA a repressor of FliC. FliC+ the fljBA promoter is orientated away from the operon: neither FljA nor FljB are produced allowing the production of the FliC flagellin. The orientation of the invertible segment was determined by PCR with primers flanking the region (SWfor and SWrev, Table 2.3) followed by digestion of the amplicon with SacI. The predicted sizes of the digestion fragments generated for the FljB+ and FliC+ positions are indicated below each orientation (figure adapted from Ikeda 2001). B. PCR amplification of the invertible hin region digested with SacI. MR. Molecular size marker, Lane 1 SL1344, Lane 2 JTS004.
The results of this assay were counterintuitive. A mixed population was observed in the wild-type with both orientations of the invertible element being apparent. As both FliC and FljB were detected in the wild-type by western blot this was not unexpected. The \textit{dps} mutant however seemed to have only one population and this had the invertible element fixed in the orientation to produce \textit{fljBA} (Fig 5.12).

The \textit{dps} mutation was transduced into naïve SL1344 (Section 2.5). Five of these transductants were tested by western blot. Three were unable to produce detectable levels of FljB, while two transductants were able to produced FljB (data not shown). It is not known why this occurred.

5.2.10 The \textit{dps} mutation has lower levels of RpoS than wild-type SL1344

It was shown that the \textit{dps} mutation has an effect on the transcription of \textit{rpoS}, the level of expression observed was upregulated at the 12-h time point by 1.639-fold (Section 4.2.5). As \textit{dps} is positively regulated by RpoS during stationary phase (Altuvia 1994) it was postulated that a degree of reciprocal regulation may be occurring between RpoS and Dps. To assess if the intracellular level of the RpoS protein would be similarly affected western immunoblot analysis was undertaken (Section 2.17). Total protein was isolated from wild-type SL1344, \textit{dps} mutant JTS004 and complemented mutant JTS009 following 12 h growth in LB broth at 37°C. Equal protein concentrations were loaded on an SDS-PAGE gel and analysed by western blot using anti-RpoS antiserum (Fig 5.13). Results revealed that the level of RpoS protein was strongly reduced in the \textit{dps} mutant and this reduction was relieved by providing \textit{dps} \textit{in-trans}. These data were in direct contrast to the data from the microarray analysis. To confirm the findings of the microarray analysis RT-PCR analysis was carried out. RNA was extracted from overnight cultures and used as the template for the RT-PCR (Section 2.11.4). There was increased expression of \textit{rpoS} in the \textit{dps} mutant compared to the wild-type (Fig 5.14). It is clear therefore that the mutation in \textit{dps} has an effect both transcriptionally and posttranscriptionally on RpoS.

The findings of this study confirm for the first time that Dps has an influence on the protein profile in \textit{S. Typhimurium} as well as in \textit{E. coli} (Almiron 1992). These data
also for the first time suggest that Dps may affect gene expression in the exponential phase of growth even though Dps concentration is low in exponential phase.
Fig 5.13. The intracellular level of RpoS is reduced in JTS004. The cellular content of RpoS was detected in wild-type SL1344 (Lane 1), $dps$ mutant JTS004 (Lane 2) and complemented mutant (Lane 3) by western blot as per section 2.17. Equal amounts of protein as normalized by the OD$_{600nm}$ value were run on a SDS-PAGE and blotted onto a nitrocellulose membrane. Levels of RpoS were detected using a polyclonal anti-body against RpoS. Values below the image are densitometric analysis of the data and are expressed relative to the wild-type level of RpoS, the value of which was taken as 1.
SL1344   JTS004

*rpoS*

STM1618

Fig 5.14. The levels of *rpoS* transcript are increased in the *dps* mutant. RT-PCR analysis of *rpoS* mRNA in the stationary phase of growth. Wild-type SL1344, and *dps* mutant JTS004 were grown in LB broth at 37°C for 12 h. Total RNA samples were extracted and mRNA analysed by RT-PCR analysis with *rpoS*-specific primers. The gene STM1618 did not respond significantly to the presence or absence of *dps* in the DNA microarray experiments and was used as a loading control. The experiment was repeated on three occasions and typical data are shown.
5.3 Discussion

As it was clear from the microarray study that Dps had a minimal effect on the transcriptional profile of SL1344, an investigation into a possible posttranscriptional effect was undertaken.

In the first instance 2-D PAGE analysis was employed to identify possible direct or indirect targets for Dps regulation. Because each of the proteins found to be downregulated in the stationary 2-D PAGE analysis was involved in metabolism further investigations into the effect the \textit{dps} mutation on carbon metabolism were undertaken (Fig 5.1).

The capacity of wild-type strain SL1344 and \textit{dps} mutant JTS004 to metabolise 95 different carbon sources was tested using PM1 phenotype microarrays from Biolog (Table 5.1). It was interesting to note that the metabolism of both D-galactose and \(\alpha\)-D-glucose was down in the \textit{dps} mutant (Table 5.3). One of the proteins shown to be downregulated in the \textit{dps} mutant by 2-D PAGE was the galactose-binding component of an ABC transport system responsible for the uptake of galactose, The largest fold-difference (6.26-fold) between SL1344 and JTS004 was observed in the well containing N-acetyl-D-glucosamine. N-acetyl-D-glucosamine is an 8-carbon monosaccharide component of peptidoglycan. With regard to the carbon sources whose oxidation was found to be increased in the \textit{dps} mutant perhaps the most significant is L-malic acid or malate an intermediate in the full TCA cycle and the product of the glyoxylate shunt (Fig 5.2).

The tricarboxylic acid (TCA) cycle has two essential roles to play in metabolism. The cycle has a role to play in provision of energy for the cell. TCA cycle intermediates are required in the biosynthesis of several amino acids and heme (Cronan and LaPorte 1996).

In \textit{E. coli} the full TCA cycle is seen only during aerobic growth on acetate (a 2-carbon sugar) or fatty acids (Cronan and Laporte 1996). Growth during these conditions requires induction and function of the glyoxylate shunt, as it bypasses the two CO_2-
evolving steps of the TCA cycle (LaPorte et al., 1984). Strains lacking this pathway do not grow on acetate as a sole carbon source as the acetate carbon entering the TCA cycle would be lost as CO₂ and thus there would be no means to replenish the dicarboxylic acids consumed in amino acid biosynthesis (Fig 5.2). When acetate is the sole carbon source a junction or branch point is created at which isocitrate dehydrogenase and isocitrate lyase compete for their common substrate isocitrate. In the presence of acetate, isocitrate dehydrogenase is inactivated by phorsporylation (Stueland et al., 1988). The reactivation of isocitrate dehydrogenase to its non-phosphorylated form can occur on the addition of a preferred carbon source such as glucose or pyruvate rendering the glyoxylate bypass unnecessary (Cronan and LaPorte 1996).

The regulation of the TCA cycle and its glyoxylate bypass is complex and it is not possible to say if the influence that Dps has on individual components is direct or indirect. The fact that the dps mutant is less fit than wild-type when grown with acetate, could suggest that it would be less likely to operate the glyoxylate shunt (Fig 5.4). The results of the RT-PCR from stationary phase RNA indicating that the expression of the aceA and aceB genes encoding isocitrate lyase (converts isocitrate to glyoxylate and succinate) and malate synthase (converts glyoxylate to malate) respectively are reduced in the dps mutant supports this view (Fig 5.5).

Conversely, the dps mutant was more fit than wild-type when grown with glyoxylate (an intermediate of the glyoxylate bypass) as the sole carbon source (Fig 5.4). If only the expression of aceA and its product isocitrate lyase were reduced in the dps mutant than the supply of exogenous glyoxylate could confer a fitness advantage to the dps mutant. In order for the glyoxylate to be processed it requires conversion to malate through the action of the enzyme malate synthase, encoded by the aceB gene. Expression of aceB was also observed to be reduced in the dps mutant at stationary phase.

It is not inconceivable that the fitness deficit observed in the dps mutant during growth with acetate stems from an effect on isocitrate lyase or perhaps on isocitrate dehydrogenase. In the event of an effect on isocitrate dehydrogenase this could be
direct or indirect or indeed could be a direct or indirect effect on isocitrate dehydrogenase kinase (the enzyme responsible for the phosphorylation of isocitrate dehydrogenase). In any event the increased fitness observed when glyoxylate was the sole carbon source could indicate that malate synthase may be negatively regulated by Dps either directly or indirectly.

The fitness deficit observed in JTS004 when succinate is the sole carbon source cannot be explained by the observation that the enzyme succinyl-CoA synthetase may be down regulated in the $dps$ mutant (Fig 5.1). Succinyl-CoA synthetase is responsible for conversion of succinyl-CoA to succinate with the generation of ATP (Cronan and Laporte 1996). Succinate dehydrogenase is responsible for the conversion of succinate to fumarate but had not been highlighted as having been affected by the mutation in $dps$.

The ability of JTS004 to utilize malate as a sole carbon source was not tested, however the oxidation of malate was found to be increased in the $dps$ mutant through the phenotype microarray analysis (Table 5.3), which would seem to contradict the results of the 2-D PAGE. In the stationary 2-D PAGE analysis the protein spot attributed to this enzyme was not detected in the $dps$ mutant. However the ability of JTS004 to grow with acetate as sole carbon and energy source (Fig 5.3) implies that this enzyme is present in the $dps$ mutant albeit at levels insufficient to be identified using 2D-PAGE analysis (Cronan & LaPorte 1996).

Iron is a cofactor of some of the TCA cycle enzymes including succinate dehydrogenase, which is responsible for the conversion of succinate to fumarate (Fig 5.2). As I had shown that JTS004 was less fit than wild-type when succinate was the only available carbon source it was postulated that the mutation in $dps$ was manifesting itself through a reduction in the iron concentration in the cell and thus was affecting enzymes whose cofactor was iron. By also limiting the concentration of iron in the external medium I postulated that this additional stress would have an "additive" effect and the $dps$ mutant would be more severely compromised and therefore have an increased fitness deficit when succinate was the sole carbon source.
The \textit{dps} mutant would not grow when acetate or succinate were the sole carbon source in the presence of the iron-chelator, 2,2-dipyridyl (DP). Cultures were inoculated directly from glycerol stocks, growth occurred in wild-type SL1344 and the chloramphenicol neutral strain JH3008 within 48 h, but no growth was observed for JTS004 even after 144 h. As a control, cultures were inoculated into medium that did not contain the iron-chelator and growth was observed for all three strains within 12 h. It is probable that there was indeed an additive effect that was potentially lethal to the \textit{dps} mutant. Unfortunately the effect of the \textit{dps} mutation on \textit{S. Typhimurium} in these conditions could not be quantified.

A modification of the premise was tested using glucose as the sole carbon source in the presence of 2 \textmu{}m 2,2-dipyridyl. The time course experiment revealed that the wild-type and \textit{dps} mutant inoculated into MOPS minimal medium with no chelator displayed no significant differences in their growth profiles (Fig 5.7). The same starting cultures were used to inoculate MOPS minimal medium with 2 mM DP. The \textit{dps} mutant appeared to enter stationary phase at a much lower OD\textsubscript{600nm} and earlier than the wild-type in these growth conditions. It was not possible to grow the \textit{dps} mutant from MOPS minimal medium with 2 mM DP in fresh medium. It was however possible to reinoculate wild-type SL1344 from MOPS minimal medium with 2 mM DP into fresh medium. Growth was severely attenuated in comparison with the wild-type and \textit{dps} mutant inoculated into MOPS minimal medium with no chelator. The terminal OD\textsubscript{600nm} reached by the \textit{dps} mutant inoculated from MOPS minimal medium with no chelator into fresh MOPS minimal medium with 2 mM DP most resembled that of the wild-type coming from medium containing chelator into fresh medium also containing chelator.

It is possible that the wild-type SL1344 coming from the iron-limited culture medium is behaving in a manner similar to the \textit{dps} mutant. There would be limited iron in the medium available for transport into the cell and the internal stocks of iron could be diminishing with no way to replenish these stocks. The cell could be limiting its growth as a way to preserve its stocks of iron for essential pathways. Although Dps is not a major iron-storage protein, unlike for example the ferritins, the dodecamer has the ability to sequester approximately 500 atoms of iron (Andrews, 2003).
Dps the internal stocks of iron could be reduced and a similar limitation of growth and division could be occurring. Pairwise fitness assays using glucose as the sole carbon source indicated that there was no fitness deficit associated with the *dps* mutation compared to the wild-type. It is therefore clear that the addition of the iron chelator to the culture medium has a dramatic effect on the metabolism of JTS004.

Thus far the examination of the mutation of *dps* had focused on stationary phase cultures. The Dps protein is present during the exponential phase of growth but is observed to be degraded rapidly during exponential growth through the action of the ClpXP protease (Stpehani *et al.*, 2003). Microarray analysis revealed that the mutation in *dps* influenced the transcription of 29 genes at the 4-h time point. To investigate if the mutation of *dps* would have an impact on the proteome of *S. Typhimurium* during log-phase, 2-D PAGE analysis was performed using exponential cultures.

One of the proteins observed to be upregulated in the *dps* mutant was FtsZ (Fig 5.8). This prokaryotic tubulin homologue (Lowe and Amos, 1998) assembles into the Z ring at the future site of cell division (Bi and Lutkenhaus, 1991). Overproduction of FtsZ results in the formation of filamentous bacteria (Bi and Lutkenhaus, 1991). Microscopy results revealed, however, that the cells of JTS004 did not appear to be significantly more filamentous than the wild-type. It is possible that the increase in FtsZ observed in the *dps* mutant was insufficient to produce the filamentous phenotype. Another alternative is that the overproduction of FtsZ was compensated for by the alteration in production of another unknown factor. In *E. coli* the *min* system encodes three proteins: MinC, MinD and MinE. Studies have shown that MinC can interact with FtsZ and prevent polymerization thus inhibiting formation of the Z ring (Hu *et al.*, 1999). It is feasible that in JTS004 upregulation of FtsZ is countered by similar upregulation of MinC thereby leading to no change in morphology of the cell.

H-NS and FlgM, two of the proteins observed to be down regulated in the *dps* mutant JTS004 have effects on the motility of *S. Typhimurium*. The reduction in H-NS was confirmed by western immunoblot analysis (Fig 5.9); the effect on motility of the *dps*
mutation was tested. H-NS has been shown to be a positive regulator of genes involved in the biogenesis of flagella and hns mutants are non-motile due to the absence of flagella (Hinton et al., 1992).

It was found that the dps mutant was motile perhaps indicating that the reduction in H-NS was being compensated by the reduction in the anti sigma-28 protein, FlgM (Fig 5.10). The reduction in FlgM may be relieving the repression of σ^{28} allowing σ^{28}-dependent transcription of the class 3 promoters of the flagellar regulatory network (Fig 4.4).

*S. Typhimurium* is motile and has a peritrichous arrangement of approximately 6 to 10 flagella on its cell surface; there are over 50 genes involved in flagellar biosynthesis and function (Chilcott et al., 2000). These genes are divided into at least 17 operons, which are subdivided into three temporally-regulated transcriptional classes, early middle and late with respective promoters classified as class 1, 2 and 3 (Fig 4.4). Flagellar biosynthesis is initiated by activation of the single class 1 promoter that transcribes the fhlDC operon. FlhD and FlhC are the master regulators of the flagellar region. Their expression is required for activation of the class 2 promoters, which transcribe the middle genes. Middle gene operons encode proteins required for the structure and assembly of the hook basal body. Middle genes also encode the sigma factor FliA (σ^{28}) and its cognate anti-sigma factor FlgM.

FlgM binds to σ^{28} and prevents transcription of the class 3 σ^{28}-dependent promoters until completion of the flagellum basal body and hook. On completion of the flagellum basal body and hook FlgM is excreted from the cell allowing σ^{28}-dependent transcription of the class 3 promoters (Bonifield et al., 2003). FlgM is a negative regulator of genes involved in the flagella biogenesis. It has been reported that the flagellar number produced in a flgM mutant is twice to three times as many as wild-type (Stephani et al., 1994). The molecular mechanism by which H-NS influences flagellar biogenesis has not yet been identified. H-NS does not interact with the promoter region of the master regulators fhlDC (Kelly 2004 thesis). No direct interaction between HN-S and FlgM has been reported. Motility in pathogenic bacteria is considered to be a virulence factor necessary for colonization of a host.
organism or target organ (Schmitt et al., 2001). Kutsukake and Iino suggested that the over production of flagellar structures can cause growth retardation in batch culture, in addition the production of flagella and the resulting motility represent a drain on the cell's resources (Kutsukake and Iino, 1994; Chilcott and Hughes 2000). It is therefore necessary to balance the cost of producing flagella with the benefits afforded with their presence. It is possible therefore that Dps either directly or indirectly is influencing the production of either H-NS or FlgM in response to perturbations in the levels of FlgM or HN-S respectively.

H-NS has a number of roles to play in global regulation (Dorman 2004, 2007). Recent reports suggest that an additional role for H-NS is the ability to selectively silence portions of the genome (Lucchine et al., 2006; Navarre et al., 2006). H-NS like proteins such as Sfh found on the R27-like plasmid of S. Flexneri are postulated to minimize the impact of the arrival of the plasmid into a new host, thereby aiding horizontal transfer of genetic information (Doyle et al., 2007). It is intriguing, therefore that a reduction in the level of H-NS does not have a more significant effect on the phenotype of the \textit{dps} mutant. It is possible that decreased levels of H-NS antagonists also, are compensating for the reduction in H-NS observed in the \textit{dps} mutant. The lack of effect on the expression of Fis (Chapter 3) would suggest that Fis is not a candidate for such compensation in JTS004 unlike the situation observed in the regulation of \textit{virF} observed in \textit{S. flexneri} (Falconi et al., 2001).

Because the production of FliC is regulated both transcriptionally and posttranscriptionally an investigation was carried out to examine if the mutation in \textit{dps} would have any effect on flagellar regulation. The late genes encode proteins required for maturation of the flagellum and chemosensory system including the flagellin subunits FliC and FljB. These two antigenically distinct flagellin proteins are expressed alternatively through flagellar phase variation (Bonifield and Hughes 2003, McNab et al., 1996). The promoter for the \textit{fljBA} operon is located within an invertible DNA segment (Fig 5.11, McNab, 1996). In one orientation the promoter is situated directly upstream of the \textit{fljBA} operon and transcription of both \textit{fljB} and \textit{fljA} can occur leading to FljB production (Bonifield and Hughes 2003, McNab et al., 1996). The gene for the other flagellin subunit \textit{fliC} is located elsewhere on the chromosome, FljA
is a transcriptional regulator of the \textit{fliC} gene. Through the action of the Hin recombinase in conjunction with other protein such as HU and Fis site-specific recombination occurs between the two \textit{hix} sites flanking the promoter of the \textit{fljBA} operon (Bonifield and Hughes 2003, McNab \textit{et al.}, 1996). The orientation of the \textit{fljBA} promoter region changes and neither \textit{fljA} nor \textit{fljB} are transcribed and the promoter for \textit{fliC} is derepressed allowing production of the FliC flagellin subunit (Bonifield and Hughes 2003, McNab \textit{et al.}, 1996). This flagellar phase variation has been shown also to be mediated by a posttranscriptional control mechanism involving FljA (Bonifield and Hughes 2003). Transcription of \textit{fliC} the gene encoding phase 1 flagellin is positively regulated by $\sigma^{28}$.

The production of the antigenically distinct flagellin subunits was investigated. JTS004 could only produce the FljB flagellin no production of FliC was detected by western immunoblot (Fig 5.11). Further analysis through RT-PCR and DNA switch assays seemed to confirm that there was an effect on the flagellar system caused directly or indirectly by \textit{dps}. There was a decrease in expression of \textit{fljB} in the \textit{dps} mutant and an increase in expression of \textit{fliC} compared to the wild-type (Fig 5.11). It was postulated that the effect must be posttranscriptional as there was a detectible transcript of \textit{fljB} albeit at a reduced level. The DNA switch assay showed that both orientations of the promoter were present in the wild-type population. Western immunoblotting had previously shown that both FliC and FljB were being produced in the wild-type SL1344. The \textit{dps} mutant however seemed to have only one population with the promoter fixed in the orientation to produce \textit{fljBA}. This finding seemed to confirm that the influence that the mutation in \textit{dps} had on the flagellar system was posttranscriptional and may indeed be posttranslational.

The suggestion that Dps is a posttranscriptional regulator of gene expression was further investigated through analysis of both \textit{rpoS} transcript and RpoS protein levels. The \textit{rpoS} transcript was observed to be upregulated in the \textit{dps} mutant through DNA microarray analysis especially at the 12-h time point perhaps indicating that Dps negatively regulates RpoS (Table 4.1). RpoS is a positive regulator of Dps so it was postulated that mutual regulation between the two proteins could be occurring.
Western immunoblot analysis revealed that the intracellular level of RpoS was strongly reduced in the \textit{dps} mutant and this reduction was relieved by providing \textit{dps in-trans}, indicating that the observed phenotype was as a result of the mutation in \textit{dps} either directly or indirectly. An attempt was made to increase the production of RpoS indirectly through overproduction of Dps under the control of an arabinose inducible promoter. However, it was not possible to induce the production of the RpoS protein by this method during exponential phase. During a recent investigation into the reciprocal expression profile for Fis and RpoS during growth in the absence of aeration, the artificial overexpression of RpoS directly under the control of an arabinose inducible promoter was attempted (O'Croinin and Dorman, 2007). The transcript of \textit{rpoS} was observed to be increased under the control of the arabinose inducible promoter however no increase in protein was observed (O'Croinin and Dorman, 2007). Consistent with the findings of this study, the overexpressed sigma factor was only detected at late stages of growth in aerated cultures, conditions when the protein is naturally abundant. The authors postulate that the posttranscriptional mechanisms responsible for regulation of RpoS protein expression were vigorous enough to withstand the attempt to overwhelm them by upregulation of the \textit{rpoS} gene (O'Croinin and Dorman 2007). The level of production of RpoS in stationary phase could not be attributable to Dps as no discernible difference between uninduced and induced samples could be observed.

To confirm the results of the microarray analysis regarding the increase in \textit{rpoS} transcript especially in light of the decreased levels of RpoS protein, RT-PCR analysis was carried out. The level of expression of the \textit{rpoS} transcript was indeed increased in the \textit{dps} mutant (Fig 5.14). This raises a number of questions as to how Dps is regulating RpoS.

The regulation of RpoS is complex and occurs at a number of levels. Reduced growth rate increases the rate of \textit{rpoS} transcription 5- to 10-fold (Lange and Hengge-Aronis, 1994). The secondary structure of the \textit{rpoS} mRNA is thought to have an influence on the translation of the message (Takayanagi et al., 1994). Several small regulatory RNAs have an influence on the translation of \textit{rpoS} mRNA. OxyS represses translation while DsrA and RprA promote \textit{rpoS} translation (Zhang et al., 1998, Lease
Nucleoid associated proteins such as H-NS and HU have an antagonistic role in the regulation of \( rpoS \) translation. H-NS downregulates \( rpoS \) translation while HU promotes translation (Brescia \textit{et al.}, 2004, Balandina \textit{et al.}, 2001). During exponential growth the energy-dependent ClpXP protease degrades RpoS rapidly (Schweder \textit{et al.}, 1996). This proteolysis relies on the targeting of the RpoS protein by RssB a specific recognition factor (Becker \textit{et al.}, 1999).

The effect of Dps on both the \( rpoS \) transcript and on the intracellular levels of RpoS protein may be direct or indirect. Dps could be destabilizing the \( rpoS \) transcript directly or through an influence on any of the known regulators above, and indeed appears to regulate the intracellular level of H-NS. Dps could be affecting the levels of RssB thus decreasing the proteolysis of RpoS. Clearly the mutation in \( dps \) has an effect both transcriptionally and posttranscriptionally on RpoS. Further definition of the role of Dps in regulating both \( \sigma^{38} \) and H-NS will be covered in Chapter 6.

Collectively these data indicate that Dps has an effect on the central metabolism of S. Typhimurium in particular the tricarboxylic acid cycle and its glyoxylate bypass. In addition these data suggest that Dps affects gene expression in the exponential phase of growth even though Dps concentration is low in exponential phase. Finally the data indicate that the postulated regulatory role Dps has in S. Typhimurium may be at the posttranscriptional level rather than at the transcriptional level as is observed for many nucleoid-associate proteins such as Fis and IHF etc. This particular aspect of proposed regulation will be addressed in Chapter 6.
Chapter 6

The effect of Dps on global regulators RpoS and H-NS in S. Typhimurium
6.1 Introduction

The reciprocal relationship between the levels of \( rpoS \) transcript and RpoS protein observed in the \textit{dps} mutant (Sections 4.1.5 and 5.1.10), raised the question as to how Dps was regulating RpoS. It is possible that Dps is having an indirect influence on the levels of both \( rpoS \) and RpoS by influencing an sRNA in \textit{S. Typhimurium}.

sRNAs have been shown to have key roles in bacterial response to stress and regulation of factors important for virulence (Fig 1.3), (Altuvia \textit{et al.}, 1997, Altuvia \textit{et al.}, 1998, Majdalani \textit{et al.}, 2002, Opdyke \textit{et al.}, 2004, Repoila \textit{et al.}, 2003, Sledjeski \textit{et al.}, 1996).

\( rpoS \) is known to be regulated by at least two of these sRNAs, DsrA and RprA (Section 1.4.2).

Another sRNA, RyhB is involved in the regulation of genes contributing to iron metabolism. Iron levels need to be carefully regulated because abundant iron can cause damage, but it is also an essential nutrient: iron is present in the active site of iron-using enzymes which are involved in metabolic pathways such as the TCA cycle, respiration, DNA synthesis and synthesis of metabolites (Andrews 2003). Free iron in cells is extremely toxic since it catalyses the generation of reactive oxygen species such as the hydroxyl radical \( \text{OH}^- \) during the Fenton reaction when oxygen and hydrogen peroxide is present (Fenton, 1894; Imlay, 2003). When iron is plentiful, the ferric-uptake regulator protein (Fur) repressor binds \( \text{Fe}^{3+} \) and is active. The main physiological function of Fur is to repress the iron acquisition genes during periods of iron abundance (Andrews 2003). During iron limiting periods Fur is no longer expressed and a large number of genes in the Fur regulon are induced promoting the uptake of iron from the extracellular environment (Hantke 1981, 2001). In addition to its negative regulation of iron-uptake genes Fur positively regulates a number of genes. The positively regulated genes encode nonessential Fe-S proteins and ferritins, the cell stops making these proteins when iron is limiting (Hantke 2001). The mechanism of positive regulation by Fur has recently been identified; Fur represses the sRNA RhyB (Fig. 1.3) (Masse \textit{et al.}, 2002).
RyhB is produced in response to the lifting of repression by Fur protein due to iron limiting conditions. RyhB levels increase rapidly, it pairs with its target mRNAs’ and causes their degradation.

The Fe-S proteins, involved in the TCA cycle, aconitase, fumarase and succinate dehydrogenase are observed to be positively regulated by such a mechanism (Masse 2002). Data presented in Chapter 5 suggests that Dps is implicated in the modulation of these enzymes. The iron storage proteins bacterioferritin and ferritin are also regulated by RhyB (Masse et al.,2002). By reducing the synthesis of these non-essential Fe-binding and storage proteins, Fe is available for essential cellular processes (Andrews 2003).

The aim of this study was to further define the role of Dps in regulating σ^{38} and H-NS.
6.2 Results

6.2.1 The *dps* mutant JTS004 has higher levels of the major transcript of *rpoS*.

At least three promoters controlling *rpoS* transcription have been identified. The major promoter is located in the coding region of the *nlpD* gene upstream of *rpoS*, whereas the second and third closely spaced promoters are upstream of the *nlpD* gene, resulting in a polycistronic transcript of *nlpD* and *rpoS* (Lange and Hengge-Aronis 1994, Takayanagi et al., 1994). To assess the activity of these promoters northern blot analysis was carried out (Section 2.17). Total RNA was isolated from overnight cultures of wild-type SL1344 and the *dps* mutant JTS004, and electrophoresed through a denaturing 0.37 M formaldehyde agarose gel (1.2% (w/v) agarose). Following transfer the membrane was probed with a Dig-labelled probe specific to the *rpoS* gene amplified using the primer pair RT-rpoSf and RT-rpoSr (Table 2.3). Three different bands were identified in both samples with lengths of approximately 2.2 kb, 1.6 kb and 1 kb (Fig 6.1). The 1.6 kb band provided the most intense signal, which is consistent with the size of the transcript produced by the major *rpoS* promoter contained within the *nlpD* gene (Lange 1994). The intensity of this signal was also increased in the *dps* mutant compared to the wild-type confirming the results from the RT-PCR analysis (Chapter 5). The 2.2 kb signal is possibly the polycistronic message including the *nlpD* gene resulting from the activation of the weaker *nlpD* promoter (Lange 1994). It is not clear what the 1 kb signal represents; it may be a degraded message.

6.2.2 The effect of the *dps* mutation on the stability of *rpoS* mRNA is dependent on growth phase

Having confirmed that the expression of the *rpoS* transcript was observed to be higher in the *dps* mutant (Fig 5.15, Fig 6.1), while RpoS protein levels were observed to be lower in the *dps* mutant than wild-type (Fig 5.14) an investigation was undertaken into the stability of the *rpoS* mRNA in the *dps* mutant. It was postulated that the reduction in protein levels shown in the *dps* mutant could be accounted for by a decrease in the stability of the transcript.
The stability of the *rpoS* message was monitored in both the exponential and stationary phase of growth in JTS004, SL1344 and JTS009 (Section 2.15). Overnight cultures of wild-type SL1344, the *dps* mutant JTS004 and the complemented mutant JTS009 were subcultured into 25 ml LB medium in 250 ml flasks and grown with shaking at 37°C without antibiotic selection. At 4 h or 12 h subculture the cultures were treated with rifampicin, an antibiotic that blocks transcription. Total RNA was then extracted in a time course experiment and the stability of the *rpoS* message was monitored by RT-PCR (Fig 6.2 & 3).

The results were unexpected. The mRNA appears to be more stable in the *dps* mutant JTS004 in exponential phase (Fig 6.2). Conversely the mRNA appears to be more stable in the stationary phase wild-type SL1344 (Fig 6.3). It should be noted however that the impact of the mutation of *dps* on the intracellular levels of RpoS in exponential phase could not be quantified (Chapter 5).

**6.2.3 The mutation in *dps* has no effect on the level of expression of *hns*.**

Dps had an influence on the intracellular level of H-NS in *S. Typhimurium*, possibly upregulating the protein. I wanted to see if this regulation was at the transcriptional level. Accordingly RT-PCR analysis was undertaken (Section 2.14.2). Total RNA was extracted from wild-type SL1344 and *dps* mutant JTS004 and used as the template for RT-PCR (Fig 6.4. A). The results indicated that there was no significant change in expression in *hns* in the presence or absence of *dps*.

To further confirm this finding northern blot analysis was carried out (Section 2.17). Total RNA was isolated from 4 h of wild-type SL1344 the *dps* mutant JTS004 and the complemented mutant JTS009, and electrophoresed through a denaturing 0.37 M formaldehyde agarose gel (1.2% (w/v) agarose). Following transfer the membrane was probed with a Dig-labelled probe specific to the *hns* gene amplified using the primer pair RT-hnsf and RT-hnssr (Table 2.3). Only one band was identified in the three samples with a length of approximately 600 bp consistent with the *hns* transcript length. The intensity of the signal did not vary between the three strains of *S.*
Fig 6.1 Expression of rpoS from the major rpoS promoter is increased in dps mutant JTS004. Northern blot analysis of the rpoS message from wild-type strain SL1344 (Lane 1) and the dps mutant JTS004 (lane 2) grown in LB broth for 12 h. Total RNA was extracted and probes with an rpoS-specific probe. The positions of the molecular size markers (Lane MR) are indicated.
Fig 6.2. Stability of *rpoS* mRNA is maintained in the *dps* mutant at exponential phase. RT-PCR analysis of *rpoS* mRNA in the exponential phase of growth. Wild-type SL1344, *dps* mutant JTS004 and complemented mutant JTS009 were grown in LB broth at 37°C for 4 h and transcription subsequently inhibited by the addition of rifampicin (250 μg/ml) to the cultures. Total RNA samples were extracted at the indicated time (min) before (0), or after (5,10) rifampicin treatment. mRNA stability was then analysed by RT-PCR analysis with *rpoS*-specific primers. The above experiment was carried out twice and representative gels are shown.
Stability of \( rpoS \) mRNA is not maintained in the \( dps \) mutant at stationary phase. RT-PCR analysis of \( rpoS \) mRNA in the stationary phase of growth. Wild-type SL1344, \( dps \) mutant JTS004 and complemented mutant JTS009 were grown in LB broth at 37°C for 12 h and transcription subsequently inhibited by the addition of rifampicin (250 \( \mu g/ml \)) to the cultures. Total RNA samples were extracted at the indicated time (min) before (0), or after (5,10) rifampicin treatment. mRNA stability was then analysed by RT-PCR analysis with \( rpoS \)-specific primers. The above experiment was carried out twice and representative gels are shown.
Fig 6.4. The mutation in \textit{dps} has no effect on the transcription of \textit{hns}. (A) RT-PCR analysis of \textit{hns} mRNA in the exponential phase of growth. Wild-type SL1344 (Lane a), and \textit{dps} mutant JTS004 (Lane b) were grown in LB broth at 37°C for 4 h. Total RNA samples were extracted and mRNA analysed by RT-PCR analysis with \textit{hns}-specific primers. (B) Northern blot analysis of the \textit{hns} content from wild-type strain SL1344 (Lane a), the \textit{dps} mutant JTS004 (Lane b) and complemented mutant JTS009 (Lane c) grown in LB broth for 4 h. Total RNA was extracted and probes with an \textit{hns}-specific probe. The above experiment was carried out on three occasions and representative gels are shown. Values below the image are densitometric analysis of the data and are expressed relative to the wild-type level of \textit{hns} (A) and H-NS (B), the value of which was taken as 1.
Typhimurium tested indicating that the transcription of *hns* is not affected by the mutation in *dps* (Fig 6.4 B).

### 6.2.4 The mutation in *dps* has no effect on the stability of *hns* mRNA.

Having confirmed that the effect of the mutation in *dps* on the intracellular levels of H-NS was not at the transcriptional level, an investigation was undertaken into the stability of the *hns* mRNA in the *dps* mutant (Section 2.15). It was proposed that the reduction in protein levels shown in the *dps* mutant could be accounted for by a decrease in the stability of the transcript.

The stability of the *hns* message was monitored in both the exponential and stationary phase of growth in JTS004, SL1344 and JTS009. Overnight cultures of wild-type SL1344, the *dps* mutant JTS004 and the complemented mutant JTS009 were subcultured into 25 ml LB medium in 250 ml flasks and grown with shaking at 37°C without antibiotic selection. At 4 h or 12 h subculture the cultures were treated with rifampicin. Total RNA was then extracted in a time course experiment and the stability of the *hns* message was monitored by RT-PCR (Figs 6.5 & 6.6).

Intriguingly there was no significant influence on the stability of the mRNA at either growth phase perhaps indicating that the regulation observed in the *dps* mutant is at the posttranslational level.

The results of this study reveal that the role of Dps in regulating both $\sigma^{38}$ and H-NS is complex and warrants further investigation.
6.2.5 Discussion

The nature of RT-PCR analysis means that the probes used to amplify the message dictate the size of the amplicon; therefore polycistronic messages are not indicated through this technique. There are two species of rpoS of clearly different lengths. Monocistronic rpoS mRNA originates from the major rpoS promoter found within the nlpD gene. Polycistronic nlpD-rpoS mRNA originates from two closely spaced promoters upstream of nlpD (Lange and Hengge-Aronis, 1994, Takayanagi et al., 1994). To assess the activity of these promoters northern blot analysis was undertaken. The results indicated that the strongest signal for both the wild-type SL1344 and the dps mutant JTS004 occurred in the band at 1.6 kb consistent with a message being produced from the major rpoS promoter contained within the nlpD gene (Fig 6.1) (Lange and Hengge-Aronis, 1994). The intensity of this signal was also increased in the dps mutant compared to the wild-type confirming the results from the RT-PCR analysis (Chapter 5). Interestingly the larger approximately 2.2 kb signal was stronger in the wild-type than the dps mutant.

The observation that the rpoS mRNA transcript was more stable in the wild-type than the dps mutant in stationary phase seems to confirm the hypothesis that Dps could be either directly or indirectly stabilising the message ensuring translation and increased levels of protein. The temperature 37°C, at which the experiments were conducted, may preclude the sRNA DsrA from being influenced by Dps as low temperatures, below 30°C, stimulate DsrA. However, DsrA can play a minor role for rpoS translation in cells grown at 37°C (Repoila et al., 2001). Cell surface stress has been suggested as the environmental signal leading to the activation of the sensor kinase RcsB, stimulating promoters under the control of RcsB (Maldalani et al., 2002). It is not clear if cell surface stress is a factor in the dps mutant. The rprA promoter is regulated via the phosphorelay cascade RcsC/YojN/RcsB. Both DsrA and RprA pair with the rpoS mRNA 5' region preventing the formation of the inhibitory stem loop that occludes ribosomal binding, thus promoting translation (Majdalani et al., 1998, 2002). The third sRNA known to regulate rpoS, OxyS represses rpoS in response to oxidative stress. For OxyS to be responsible for the difference in stabilization observed in the dps mutant, Dps would be required to downregulate the sRNA. Such
Fig 6.5. Stability of hns mRNA is not affected by the mutation in dps at exponential phase. RT-PCR analysis of hns mRNA in the exponential phase of growth. Wild-type SL1344, dps mutant JTS004 and complemented mutant JTS009 were grown in LB broth at 37°C for 4 h and transcription subsequently inhibited by the addition of rifampicin (250 µg/ml) to the cultures. Total RNA samples were extracted at the indicated time (min) before (0), or after (5,10) rifampicin treatment. mRNA stability was then analysed by RT-PCR analysis with hns-specific primers. The above experiment was carried out twice and representative gels are shown.
Fig 6.6. Stability of *hns* mRNA is not affected by the mutation in *dps* at stationary phase. RT-PCR analysis of *hns* mRNA in the stationary phase of growth. Wild-type SL1344, *dps* mutant JTS004 and complemented mutant JTS009 were grown in LB broth at 37°C for 12 h and transcription subsequently inhibited by the addition of rifampicin (250 μg/ml) to the cultures. Total RNA samples were extracted at the indicated time (min) before (0), or after (5, 10) rifampicin treatment. mRNA stability was then analysed by RT-PCR analysis with *hns*-specific primers. The above experiment was carried out twice and representative gels are shown.
a scenario is unlikely; OxyR, which also regulates Dps in response to oxidative stress, regulates OxyS. Levels of RpoS decrease during oxidative stress perhaps due to the σ^70-dependence of oxidative stress response genes.

It is possible that none of these sRNAs are responsible for the increased stabilisation of the rpoS mRNA observed in the dps mutant. Dps could be acting directly with the rpoS message or indeed could be influencing the production of a novel sRNA that either stimulates rpoS translation in which case Dps would also upregulate this sRNA. In the event of Dps interacting with a repressor of rpoS translation, Dps would be downregulating this sRNA. It is not necessary for Dps to be influencing an sRNA Dps could be regulating another protein that has an influence on rpoS translation.

The observation that the RpoS mRNA transcript was more stable in JTS004 than SL1344 in exponential phase is perplexing. It appears that Dps could be destabilizing the rpoS mRNA. The impact of the mutation of dps on the intracellular levels of RpoS in exponential phase could not be quantified (Chapter 5), it is to be assumed however, that ClpXP and RssB would still be able to degrade RpoS in the exponential phase. The microarray results (Chapter 4) suggest that there is a modest increase in rpoS transcription at the 4-h time-point in the absence of Dps. A recent study has shown that overexpression of rpoS mRNA from a pBAD promoter in exponential phase does not result in an increase in RpoS protein (O’Croinin and Dorman 2007). RT-PCR shows that there appears to be no significant difference between the levels of rpoS transcript in the wild-type or the dps mutant at the point of addition of rifampicin. It is possible that Dps could be interacting with a different partner at exponential phase than that proposed for stationary phase, explaining the bimodal regulation observed.

To investigate the role of Dps in regulating H-NS intracellular levels in S. Typhimurium northern blot and RT-PCR analysis was carried out. The results seemed to indicate that the mechanism of regulation is occurring at the posttranscriptional level. The levels of expression of the hns transcript were unchanged regardless of the background.
When the stability of the *hns* transcript was monitored no difference in the levels of stability was observed between the three strains tested. It may be possible that extension of the time course analysis to beyond 10 min would result in more significant effects. The reduction in H-NS protein levels observed (Sections 5.1.6 and 5.1.7) in the *dps* mutant could suggest that the translation process is the target for Dps influence. Dps could also affect protein stability, although there is no evidence that H-NS is proteolytically cleaved.

These data confirm that Dps has an influence on two of the global regulators of *S. Typhimurium*, RpoS and H-NS – although the mechanism(s) remain undetermined.
Chapter 7

General Discussion
The main objective of this work was to define a role for Dps in genetic regulation in *Salmonella enterica* serovar Typhimurium. Following the creation of the *dps* mutant with consequent testing and confirmation, a microarray analysis of the effect of the *dps* mutation on the transcription of *S. Typhimurium* was carried out. The transcriptional analysis carried out by comparing the gene expression profile of the wild-type *S. Typhimurium* with its otherwise isogenic *dps* mutant yielded an intriguing result. Despite being designated a nucleoid-associated protein (Almiron *et al*., 1992; Azam *et al*., 2000, 2001) and given its close association with DNA and presence in such large numbers particularly in stationary cells, very few genes in the *S. Typhimurium* genome responded to its absence. It is possible that the absence of Dps was being compensated by other factors as yet unknown under the conditions tested.

Since it's discovery in 1992, it has been postulated that Dps is a regulator of gene expression in *E. coli* it was found that cells lacking Dps showed changes in the pattern of proteins synthesised during starvation, however none of the proteins was identified during the study (Almiron *et al*., 1992). To date few studies have been published regarding the role of Dps in genetic regulation. It has however, recently been shown in *E. coli* that Dps interacts with DnaA protein to impede initiation during oxidative stress conditions, thus hindering DNA replication (Chodavarapu *et al* 2008).

Of the Dps-regulated genes with known function the greatest number were those genes involved in amino acid transport and metabolism. One system in particular involved in the transport of branched-chain amino acid into the cell displayed a response to the absence of Dps. RT-PCR analysis revealed that all of the genes of the two ATP-dependent high-affinity branched-chain amino acid transport systems, LIV-I and LS, involved in the transport of L-leucine, L-valine, and L-isoleucine were affected by the mutation in *dps*.

It has previously been shown that all of the membrane component genes as well as at least one of the binding protein genes are essential for active transport of leucine (Adams 1990). LivJ and LivK are the two-periplasmic amino acid-binding proteins that confer the specificity of the complexes. The addition of excess L-leucine, L-
valine, or L-isoleucine into the growth medium rendered the $dps$ mutant less fit than the wild-type when they were directly competed. It is proposed that the inability to efficiently transport these branched-chain amino acids into the cell necessitated the expenditure of energy to synthesise these amino-acids de-novo. Such expenditure was unnecessary in the wild-type resulting in a fitness advantage. These data showed for the first time that Dps had an influence on the metabolism of SL1344.

2-D PAGE analysis of stationary S. Typhimurium revealed that two enzymes of the tricarboxylic acid (TCA) cycle were downregulated in the $dps$ mutant, malate dehydrogenase and succinylCo-A synthetase. The TCA cycle is an important source of energy and intermediates that are required in the biosynthesis of several amino acids and heme (Cronan and LaPorte, 1996). The impact of Dps on central metabolism specifically carbon utilization was investigated through RT-PCR, phenotypic microarrays and direct fitness assays. The $dps$ mutant proved to be able to grow when acetate was the sole carbon source, indicating that the deficiency in the enzymes was not sufficient to cause the $dps$ mutant to be nonviable in these conditions. However, a competitive disadvantage was displayed by the $dps$ mutant when it was competed directly with wild-type, when acetate or succinate was the sole carbon source. These data suggest that Dps could be influencing transport of $C_4$ dicarboxylates into the cell or could be increasing the expression of central metabolic enzymes to enhance the ability of S. Typhimurium to utilize available substrates more efficiently.

The regulation of the TCA cycle and its glyoxylate bypass is complex and it is not possible to say if the influence that Dps has on individual components is direct or indirect.

Iron is a cofactor of some of the TCA cycle enzymes. It was postulated that the mutation in $dps$ was manifesting itself through a reduction in the iron concentration in the cell and thus was affecting enzymes whose cofactor was iron. By also limiting the concentration of iron in the external medium I postulated that this additional stress would have an "additive" effect and the $dps$ mutant would be more severely compromised and therefore have an increased fitness deficit when the enzyme
responsible for conversion of a particular carbon source required iron as a cofactor. The enzyme responsible for converting succinate to fumarate, succinate dehydrogenase requires iron as a co-factor (Cecchini et al. 2002). The inability of the *dps* mutant to grow in iron-limited conditions when acetate or succinate was the sole carbon source could support this view.

When *dps* mutant cells were transferred to iron-limited conditions in minimal medium, with glucose as the sole carbon source an interesting phenotype was observed.

The terminal OD$_{600nm}$ reached by the *dps* mutant inoculated from MOPS minimal medium with no chelator into fresh MOPS minimal medium with 2 mM 2,2-dipyridyl most resembled that of the wild-type coming from medium containing chelator into fresh medium also containing chelator. It is possible that the wild-type SL1344 coming from the iron-limited culture medium is behaving in a manner similar to the *dps* mutant. There would be limited iron in the medium available for transport into the cell and the internal stocks of iron could be diminishing with no way to replenish these stocks. The cell could be limiting its growth as a way to preserve its stocks of iron for essential pathways. Without Dps the internal stocks of iron could be reduced and a similar limitation of growth and division could be occurring. Pairwise fitness assays using glucose as the sole carbon source indicated that there was no competitive disadvantage associated with the *dps* mutation compared to the wild-type. It is therefore clear that the addition of the iron chelator to the culture medium has a dramatic effect on the metabolism of JTS004.

These data confirmed that Dps has an influence on the metabolism of SL1344, however it is not possible to say whether this influence is direct or indirect.

Expression of the SPI-2 type three secretion system is controlled by a regulatory hierarchy that includes EnvZ and OmpR, SsrA-SsrB, SlyA and the nucleoid-associated proteins Fis, H-NS and YdgT (Lee et al., 2000; Garmedia et al., 2003; Linehan et al., 2005; Kelly et al., 2004; Lucchini et al., 2006; Navarre et al., 2006; Walthers et al., 2007). Western blot analysis and GFP analysis were performed to
determine the activity of the 2 regulatory genes encoded on SPI-2 responsible for the full activation of the SPI-2 virulence genes. This study confirmed that Dps is not mediating survival in the macrophage through activation of the SPI-2 virulence genes as no effect was seen on the expression of ssrA transcript or SsrB protein.

The lack of an effect on stress response gene expression and on iron-regulated genes in particular was surprising. Although Dps is not strictly an iron-storage protein it is capable of accommodating ~500 Fe atoms per dodecamer (Ilari et al., 2002). It would not be unexpected, therefore, to observe an increase in the expression levels of bfr and ftnA to compensate for the loss of Dps. However such an increase did not occur. It is also possible that the ability of Dps to protect the cell from various stresses is at a physical level rather than at the level of transcription regulation. The physical sequestering of DNA by Dps in the DPS-DNA co-crystal lattice could support this view (Freikiel-Krispin et al., 2001; Wolf et al., 1999).

It was clear from the microarray study that Dps had little effect on the transcriptional profile of SL1344; an investigation into the possibility of posttranscriptional effect was undertaken. In the first instance 2-D PAGE analysis was employed to identify possible targets for Dps regulation either directly or indirectly.

It is also possible that the impact of the dps mutation was being compensated by another factor, in the conditions tested. therefore the effect of Dps on two global regulators, H-NS and RpoS was tested both at the transcriptional level and at the posttranscriptional level.

The intracellular level of H-NS was tested at exponential phase and was found to be downregulated in the dps mutant. H-NS is a highly pleiotropic regulator of gene expression and has been shown to regulate up to 10% of the genes in S. Typhimurium (Lucchini et al., 2006; Navarre et al., 2006). It binds preferentially to curved DNA, which is commonly found at promoters (Dorman 2004; Dorman 2007). H-NS's role in regulation is chiefly as a repressor although there are rare instances where it functions as an activator of gene expression (Schroder and Wagner 2002). H-NS has also been shown to bind to the small RNA, DsrA and to rpoS mRNA influencing their
stability (Brescia 2004). Recent reports suggest that an additional role for H-NS is the ability to selectively silence portions of the genome (Lucchini et al., 2006; Navarre et al., 2006, 2007). H-NS like proteins such as Sfh found on the R27-like plasmid of S. flexneri are postulated to minimize the impact of the arrival of the plasmid into a new host, thereby aiding horizontal transfer of genetic information (Doyle et al., 2007).

To investigate the role of Dps in regulating H-NS intracellular levels in S. Typhimurium northern blot and RT-PCR analysis was carried out. The results seemed to indicate that the mechanism of regulation is occurring at the posttranscriptional level, or perhaps at the posttranslational level. Dps could possibly be influencing increased proteolysis of the H-NS protein.

Given the multitude of H-NS targets in S. Typhimurium it is interesting that a reduction in the level of H-NS does not have a more significant effect on the phenotype of the \textit{dps} mutant. It is possible that there is also a reduction in the levels of anti-H-NS antagonists compensating for the reduction in H-NS.

Western immunoblot analysis revealed that the intracellular level of RpoS was strongly reduced in the \textit{dps} mutant and this reduction was relieved by providing \textit{dps} \textit{in-trans}, indicating that the observed phenotype was as a result of the mutation in \textit{dps} either directly or indirectly. An attempt was made to increase the production of RpoS indirectly through overproduction of Dps under the control of an arabinose inducible promoter. However, it was not possible to induce the production of the RpoS protein by this method during exponential phase. During a recent investigation into the reciprocal expression profile for Fis and RpoS during growth in the absence of aeration, the artificial overexpression of RpoS directly under the control of an arabinose inducible promoter was attempted (O'Croinin and Dorman 2007). It was shown that it was possible to increase transcription of \textit{rpoS}; however no subsequent increase of RpoS was observed in exponential phase. Consistent with the findings of this study, the overexpressed sigma factor was only detected at late stages of growth in aerated cultures, conditions when the protein is naturally abundant. The authors postulate that the posttranscriptional mechanisms responsible for regulation of RpoS...
protein expression were vigorous enough to withstand the attempt to overwhelm them by upregulation of the rpoS gene (O'Croinin and Dorman 2007).

The effect of Dps on both the rpoS transcript and on the intracellular levels of RpoS protein may be direct or indirect. The observation that the rpoS mRNA transcript was more stable in the wild-type than the dps mutant in stationary phase seems to confirm the hypothesis that Dps could be either directly or indirectly stabilising the message thus ensuring translation and increased levels of RpoS protein.

Regulation of factors important for virulence and responses to stresses has been shown to be influenced by small noncoding RNAs (sRNAs) (Altuvia et al., 1997, 1998; Majdalani et al., 2002, Opdyke et al., 2004, Repoila et al., 2003, Sledjeski et al., 1996). It is apparent that this area of posttranscriptional regulation is becoming more important.

It is possible that Dps could be acting directly with the rpoS message or indeed could be influencing the production of a novel sRNA that either stimulates rpoS translation in which case Dps would also upregulate this sRNA. In the event of Dps interacting with a repressor of rpoS translation, Dps would be downregulating this sRNA. It is not necessary for Dps to be influencing an sRNA; Dps could be regulating another protein that has an influence on rpoS translation.

The data presented in this study raise a number of questions that have yet to be answered regarding the role of Dps in genetic regulation in S. Typhimurium. Additional experiments could be carried out to further define this role. To determine if Dps is acting directly or indirectly to influence transcription electrophoretic molecular shift assays (EMSA) could be performed using probes for the genes identified in this study. Additionally it would be possible using competitive EMSA to assess if the addition of a potential competitor for binding eg H-NS would have an influence on the binding to the DNA target of Dps. Furthermore EMSA using RNA as the target rather than DNA could identify if the posttranscriptional effect of Dps on the production of the rpoS message is direct or indirect. Similarly to determine if Dps is acting in concert with another protein yeast two-hybrid assays could be performed.
using H-NS and RpoS. With regard to the influence of Dps on the carbon metabolism of the cell, enzymatic assays could determine if the effect of Dps on the individual TCA pathway components is manifesting itself as an increase or decrease of the particular enzyme.

The data presented in this study show that Dps has a modest influence on the transcriptome of S. Typhimurium. They also show that Dps has an influence either directly or indirectly at the posttranscriptional level particularly in the case of carbon metabolism and in the regulation of two global regulators in S. Typhimurium. With regard to the observed regulation of RpoS and H-NS it is clear that the lack of Dps must be compensated by another factor, perhaps an sRNA, given that the reduction in the intracellular levels of both these proteins does not result in more severe perturbations to the cell.

Nucleoid-associated proteins have roles to play in global regulation of transcription. Dps has been classed as a nucleoid-associated protein. This work shows that Dps affects global gene expression but not at the level of transcription setting it apart from the other nucleoid-associated proteins. Acting predominantly at the posttranscriptional level Dps is thus defining a new class of nucleoid-associated protein.
Bibliography


