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Characterisation of the Redox Protein Thioredoxin from the Gastric Pathogen *Helicobacter pylori.*

A Thesis Submitted for the Degree of
Doctor of Philosophy (Ph.D.)

by

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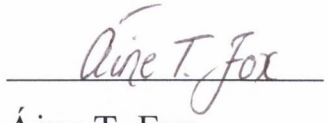
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Áine T. Fox

SUMMARY

Gastric colonisation by the Gram-negative bacterium *Helicobacter pylori* is the primary cause of peptic ulceration, type B chronic gastritis, gastric adenocarcinoma, and is linked to the progression of gastric MALT lymphoma. *H. pylori* infection elicits a host immune response and this results in an oxidative burst from macrophages and polymorphonuclear leukocytes recruited to the site of infection. As a microaerophile, *H. pylori* is particularly susceptible to the effects of reactive oxygen metabolites. Many studies have highlighted the vital role played by sulphydryl groups (-SH) in response to oxidative stress and the thioredoxin (Trx) and glutaredoxin (Grx) systems play a major role in protecting cells from oxidative damage. *H. pylori* is significantly different from other prokaryotes in terms of deficiency in its complement of thiol-dependent redox active proteins. The bacterium is equipped with only one of the two major, almost ubiquitous, intracellular disulphide reducing systems, the Trx system and lacks the Grx system. Thioredoxins (Trxs) are small redox proteins present in almost all sequenced prokaryotic and eukaryotic cells which share a similar three-dimensional structure and a possess a highly conserved -CGPC- catalytic motif. *H. pylori* possess two distinct thioredoxin proteins, one of which, Trx2 has a unique redox active site, -CPDC-. The results of this study provide further insight into the role of the redox protein Trx2 in the gastric pathogen, *H. pylori*.

Initially, the *trx2* gene from *H. pylori* was cloned and overexpressed in an *Escherichia coli* expression host strain. The recombinant protein was purified to homogeneity and determined to possess thiol-oxidoreductase activity by an insulin reduction assay. Polyclonal antibodies were raised against the recombinant protein and Western blot analysis showed that the protein is cytoplasmically expressed in two *H. pylori* reference strains. Crystals of recombinant *H. pylori* Trx2 were subjected to X-ray crystallography anaysis. This study presents preliminary structural data that has been obtained for this purified protein for the first time. These data are being utilised to construct a high resolution three-dimensional structural model of this unusual redox enzyme which will facilitate elucidation of the *in vivo* functions of this redox enzyme. Secondly, mutants of the *H. pylori trx2* gene were constructed by insertional inactivation in reference *H. pylori* strains to test whether Trx2 contributes to oxidative stress management in the *H. pylori*. Phenotypic analysis of the mutant strains revealed that *H. pylori* Trx2 has a role in oxidative stress resistance in the bacterium in response stress generated by organic

hydroperoxides. Trx2 is also capable of providing reducing equivalents to two cellular *H. pylori* enzymes involved in the detoxification of reactive oxygen species, thiol peroxidase and bacterioferritin comigratory protein. This study revealed that Trx2 has a role in peroxide stress resistance in the pathogen and supports the postulate that *H. pylori* Trxs have different functional roles within the bacterium.

Thirdly, this study sought to investigate the paradigm that secreted factors from *H. pylori* may influence the gastric inflammatory response. Trx2 has been previously identified as one of thirteen proteins selectively released by the bacterium. The significance of this finding is intriguing in terms of host-pathogen interaction and persistence of infection. Recombinant *H. pylori* Trx2 was shown to be capable of modulating the activity of certain gastric epithelial cell proteins involved in mediating the host inflammatory response, IκB, NF-κB and AP-1, in the gastric epithelial cell line, AGS. Finally, *H. pylori* Trxs interact with a wide spectrum of proteins within the cell. As the Trx system constitutes the only thiol-dependent reduction-oxidation system present in *H. pylori*, interacting proteins of components of this system are of interest. An approach which exploits a thiol-specific fluorescent tag combined with proteomics to flag proteins reduced by *H. pylori* Trx2 *in vitro* was used to isolate potential interacting proteins of *H. pylori* Trx2 in the bacterium and in gastric epithelial cells. In addition, three mutant forms of *H. pylori* Trx2 were generated in which two active site cysteines were mutated via site-directed mutagenesis to serine residues individually (C28S, C31S), and in combination (C28S/C31S). These were used in an affinity/proteomics approach to stabilise Trx-substrate complexes from *H. pylori* lysates and gastric epithelial cell lysates in order to isolate Trx interacting proteins. The approach utilised in this study could permit construction of a metabolic framework to understand pathways and processes which may be importance in virulence and long-term persistence for the pathogen.

In conclusion, Trx2 structure/functional relationships and the role of Trx2 in oxidative stress management and immunomodulation in *H. pylori* are explored in this study. Recent investigations in various cell types have amplified the role of Trx beyond that of a simple high capacity hydrogen donor, the significance of this paradigm for the gastric pathogen, *H. pylori*, is considered.

DEDICATION

To my parents, David and Teresa.

“All great truths begin as blasphemies” – George Bernard Shaw (1856-1950)

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TABLE OF CONTENTS

Table of Contents	**Page**
PREFACE	
Title	I
Declaration	II
Summary	III
Acknowledgements	VII
Table of Contents	IX
Abbreviations	XVII

CHAPTER I: GENERAL INTRODUCTION

1.1 *HELICOBACTER PYLORI*	**1**
1.1.1 *Helicobacter pylori*; The Pathogen	1
1.1.2 Historical Perspective	2
1.1.3 Taxonomy	3
1.1.4 *H. pylori* and Host Environmental Ecology	4
1.1.5 Epidemiology of *H. pylori* Infection	4
1.2 *HELICOBACTER PYLORI* CELLULAR PROCESSES	**6**
1.2.1 *H. pylori* Physiology and Metabolism	6
1.2.2 *H. pylori* Macromolecule Biosynthesis	6
1.2.3 Cell Division and Morphology	7
1.2.4 Regulation of Gene Expression	8
1.2.5 Environmental Sensing and Chemotaxis	8
1.2.6 Functional Characterisation of the *H. pylori* Genome	9
1.3 *HELICOBACTER PYLORI*; MOLECULAR ASPECTS OF INFECTION	**10**
1.3.1 Motility	10
1.3.2 *H. pylori* Lipopolysaccharide	11
1.3.3 Pathogenicity Determinants: Cytotoxin Associated Antigen	

and Vacculolating Cytotoxin 12
1.3.4 Urease 14
1.3.5 Adhesins 15

1.4 *HELICOBACTER PYLORI* AND GASTROINTESTINAL DISEASE 16
1.4.1 *H. pylori* Disease Associations and Clinical Outcomes 16
1.4.2 Infection Eradication; Clinical Indications 17
1.4.3 *H. pylori* Antimicrobial Therapy 17
1.4.4 Control of *H. pylori*: Vaccination 18

1.5 *HELICOBACTER PYLORI* AND HOST INFLAMMATORY RESPONSE 19
1.5.1 *H. pylori*; Activation of the Inflammatory Cascade 19
1.5.2 The Th1/Th2 Response and *H. pylori* Infection 19

1.6 OXIDATIVE STRESS AND THE GASTRIC NICHE 20
1.6.1 Reactive Oxygen Species 20
1.6.2 Reactive Oxygen Species and the Inflammatory Response 21
1.6.3 Reactive Oxygen Species-Induced Membrane and Protein Damage 22
1.6.4 Cellular Antioxidant Enzyme Systems 23
1.6.5 Superoxide dismutase and Catalase 24
1.6.6 Peroxidases 25
1.6.7 Regulation of Cellular Responses to Oxidative Stress 26
1.6.8 Sensing Stress by Disulphide Bond Formation 27

1.7 THE THIOREDOXIN SYSTEM 28
1.7.1 Disulphide Bond Formation 28
1.7.1.1 Chemistry of Disulphide Bond Formation 28
1.7.1.2 Disulphide Bond Reducing Pathways in the Cytoplasm 29

1.7.2 Thioredoxin; A Thiol-Oxidoreductase 30
1.7.2.1 Thioredoxin 30
1.7.2.2 Catalytic Mechanism 31
1.7.2.3 Thioredoxin Structure and Structure/Function Relationships 32

1.7.2.4 Non-Classical Thioredoxins 34
1.7.2.5 Thioredoxin Reductase 34
1.7.3 The Thioredoxin Superfamily 36
1.7.3.1 The Thioredoxin Fold; A Common Bond 36
1.7.3.2 Glutathione and Glutaredoxins 37
1.7.3.3 Protein Disulphide Isomerase 38
1.7.3.4 Peroxiredoxins 39
1.7.3.5 The Thioredoxin Superfamily: Specificity Versus Redundancy 40

1.7.4 Redox Regulation of Transcription Factors by Thioredoxin 41
1.7.4.1 Eukaryotic Cells: Oxidative Stress and Thiol Redox State 41
1.7.4.2 Oxidative Stress and Gene Expression 42
1.7.4.3 Redox Regulation of NF-κB 44
1.7.4.4 Additional Redox Regulated Transcription Factors 46

1.8 SUMMARY 47

CHAPTER II: MATERIALS AND METHODS

2.1 Chemicals 49
2.2 Antibodies and Oligonucleotides 49
2.3 *Helicobacter pylori* Culture 50
2.3.1 *H. pylori* Strains and Growth Conditions 50
2.3.2 *H. pylori* Total Lysate Preparation 50
2.4 Cell Culture 50
2.4.1 Cell Line Source and Culture Reagents 50
2.4.2 Cell Culture Maintenance 51
2.4.3 Cell Freezing and Revival 51
2.4.4 Cell Enumeration and Viability 51
2.4.5 Co-culture of Gastric Epithelial Cells with Recombinant Thioredoxin 52
2.4.6 MTT Assay 52
2.5 Cell Protein Preparation 53
2.5.1 Cell Fraction and Preparation of Cytosolic Fractions 53

2.5.2 Determination of Protein Concentration 53
2.6 Polyacrylamide Gel Electrophoresis 54
2.6.1 Sample and Molecular Weight Standard Preparation 54
2.6.2 SDS-PAGE and Gradient SDS-PAGE 54
2.6.3 Two-dimensional Gel Electrophoresis 55
2.6.4 Western Immunoblotting 56
2.6.5 Immunoblot Detection and Development 57
2.7 Electrophoretic Mobility Shift Assay 57
2.7.1 Preparation of Nuclear Fractions 57
2.7.2 Consensus Oligonucleotide Labelling 58
2.7.3 EMSA DNA Protein Binding Reaction 59
2.7.4 Gel/Buffer Preparation and DNA-Protein Complex Electrophoresis 59
2.7.5 Positive Controls for NF-κB and AP-1 Activation 60
2.8 DNA Manipulation 61
2.8.1 Preparation of *H. pylori* Genomic DNA 61
2.8.2 *E. coli* DNA Manipulation 61
2.8.3 Preparation of Competent *E. coli* Cells for Transformation 61
2.8.4 DNA Amplification by PCR 62
2.9 Insertional Mutagenesis 62
2.9.1 Construction of *H. pylori* Thioredoxin 2 Isogenic Mutants by Insertional Mutagenesis 62
2.9.2 Natural Transformation of *H. pylori* 62
2.10 Site-directed Mutagenesis 63
2.10.1 Construction of Mutated Redox-active Site *H. pylori* Thioredoxins 63
2.11 Recombinant Protein Purification 65
2.11.1 Expression and Purification of recombinant His-tagged *H. pylori* Thioredoxin 2. 65
2.11.2 Factor Xa Digestion and His Tag Removal 65
2.11.3 Ion Exchange Chromatography 66
2.11.4 Thioredoxin Activity Assay 66
2.11.5 Spin Chromatography 67
2.11.6 Immuno-affinity Purification of Anti-Thioredoxin 2 Antibodies 67
2.12 Identification of Thioredoxin 2 Targets; Affinity Selection and Thiol -Specific Labelling 68

2.12.1 Preparation of Sepharose 4B for Ligand Coupling 68
2.12.2 Preparation of Thioredoxin-Immobilised Resin 68
2.12.3 Collection of Target Proteins by Immobilised Thioredoxin 2 Resin 68
2.12.4 Coomassie Blue G-250 Staining (for Mass Spectrometry) 68
2.12.5 Fluorescent Labelling of Proteins and Identification of Labelled Proteins 69
2.12.6 Reconstitution of Recombinant Proteins with the Thioredoxin system Using a Fluorescent Labelling System 69

CHAPTER III: MOLECULAR CLONING, OVEREXPRESSION, PURIFICATION AND CHARACTERISATION OF AN UNUSUAL *H. PYLORI* THIOREDOXIN

3.1 INTRODUCTION 71
3.1.1 Thiol-disulfide Oxidoreductases 71
3.1.2 *H. pylori* Thiol-Disulphide Redox Chemistry 72

3.2 OBJECTIVES 73

3.3 RESULTS 73
3.3.1 Initial Cloning of *H. pylori trx2* Gene from *H. pylori* 73
3.3.2 Sequence Comparison of *H. pylori* Thioredoxin 2 with Other Thioredoxins 73
3.3.3 Overexpression of the *H. pylori trx2* Gene and Purification by Immobilised Metal Ion Affinity Chromatography 75
3.3.4 Functional Characterisation of *H. pylori* Thioredoxin 2. 75
3.3.5 Enzymatic Activity of Recombinant *H. pylori* Thioredoxin 2 Protein 76
3.3.6 Removal of Recombinant Thioredoxin 2 Histidine-Tag via Factor Xa Digestion 76
3.3.7 Overexpression and Purification of Recombinant *H. pylori* Thioredoxin 2 in Alternate Expression Host Strains 77
3.3.8 Overexpression of an Alternate *H. pylori* Thioredoxin 2 Cloning Construct 77

3.3.9 Purification of Recombinant *H. pylori* Thioredoxin 2 using Ion Exchange Chromatography 77
3.3.10 Disulphide Oxidoreductase Activity of Non-tagged Recombinant *H. pylori* Thioredoxin 2 78
3.3.11 Determination of *H. pylori* Thioredoxin 2 Three-Dimensional Structure 78

3.4 DISCUSSION **79**

CHAPTER IV: ROLE OF THE THIOREDOXIN SYSTEM IN MEDIATING RESISTANCE TO OXIDATIVE STRESS IN *H. PYLORI*

4.1 INTRODUCTION **85**
4.1.1 Oxidative Stress in the Gastric Niche 85
4.1.2 Redox Homeostasis 85

4.2 OBJECTIVES **86**

4.3 RESULTS **86**
4.3.1 Disruption of the *trx2* Gene in *H. pylori* by Insertional Mutagenesis 86
4.3.2 Growth Characteristics of *H. pylori* Thioredoxin 2 Mutant Strains 87
4.3.3 Sensitivity of *H. pylori* Strains to Oxidative Stress 88
4.3.4 Disruption of *H. pylori trx2* and Resistance to Hydrogen Peroxide in a Selected *H. pylori* Strain 90
4.3.5 Identification of Thioredoxin 2 Interacting Proteins in *H. pylori* 90
4.3.6 Thioredoxin-Dependent Reduction of *H. pylori* Thiol Peroxidase 91
4.3.6 Thioredoxin-Dependent Reduction of *H. pylori* Bacterioferritin Comigratory Protein 91

4.4 DISCUSSION **92**

CHAPTER V: *H. PYLORI* THIOREDOXIN AND HOST INFLAMMATION; REDOX-ASSOCIATED IMMUNOMODULATION AND THIOREDOXIN 2.

5.1 INTRODUCTION **99**

5.1.1 The *H. pylori* Secretome 99

5.1.2 Thioredoxin and Transcription Factors 99

5.2 OBJECTIVES **100**

5.3 RESULTS

5.3.1 Effect of *H. pylori* Thioredoxin 2 on IκB Expression in Gastric Epithelial Cells 101

5.3.2 Effect of *H. pylori* Thioredoxin on Constitutive NF-κB in Gastric Epithelial Cells 101

5.3.3 Effect of Redox-Inactive *H. pylori* Thioredoxin 2 on IκB Expression in Gastric Epithelial Cells 102

5.3.4 Effect of *H. pylori* Thioredoxin 2 on AP-1 Expression in Gastric Epithelial Cells 103

5.3.5 Effect of *H. pylori* Thioredoxin 2 on AP-1 DNA-Binding Activity in Gastric Epithelial Cells 103

5.3.6 *H. pylori* Thioredoxin 2 and MAP Kinase Signalling in Gastric Epithelial Cells 104

5.3.7 Effect of *H. pylori* Thioredoxin 2 on Gastric Epithelial Cell Viability 104

5.4 DISCUSSION **104**

CHAPTER VI: IDENTIFICATION OF INTERACTING PROTEINS OF *H. PYLORI* THIOREDOXIN 2

6.1 INTRODUCTION **112**

6.1.1 Thioredoxin; A Pleiotropic Molecule 112

6.2 OBJECTIVES **113**

6.3 RESULTS **114**

6.3.1 Identification of *H. pylori* Thioredoxin Interacting proteins via a Proteomic Approach Using a Fluorescent Thiol-Specific Probe 114
6.3.2 Identification of Thioredoxin 2-Gastric Epithelial Cell (AGS) Interacting Proteins via a Proteomic Approach Using a Fluorescent Thiol-Specific Probe 115
6.3.3 Construction of Redox-Active Site Mutants of *H. pylori* Recombinant Thioredoxin 2. 116
6.3.4 Overexpression and Purification of Redox Active Site Mutants of *H. pylori* Recombinant Thioredoxin 2 116
6.3.5 Non-Reducing SDS-PAGE Analysis of *H. pylori* Thioredoxin 2 Redox Active Site Mutants 117
6.3.6 Affinity Trapping of *H. pylori* Thioredoxin 2 Interacting Proteins 117
6.3.7 Gradient SDS-PAGE Analysis and Identification of *H. pylori* Thioredoxin 2 Interacting Proteins 118
6.3.8 2-D PAGE Analysis of *H. pylori* Thioredoxin 2 Interacting Proteins 119
6.3.9 Affinity Trapping of Thioredoxin 2 Interacting Proteins in Gastric Epithelial Cells 119
6.3.10 Isolation of Gastric Epithelial Cell Thioredoxin 2 Interacting Proteins 120

6.4 DISCUSSION 120

CHAPTER VII: GENERAL DISCUSSION 127

REFERENCES 138

APPENDICES 175
Appendix A: Reagents and Buffers
Appendix B: Publications and Presentations

ABBREVIATIONS

2-D PAGE	2-dimensional polyacrylamide gel electrophoresis
Amp	ampicillin
AO	acridine orange
AP-1	activator protein-1
APS	ammonium persulphate
Asp	aspartic acid
Bcp	bacterioferritin co-migratory protein
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CagA	cytotoxin-associated gene A
cag PAI	*cag* pathogenecity island
Cm	chloramphenicol
CM	carboxymethylcellulose
Cys	cysteine
DEAE	diethylaminoethyl
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E_0'	standard redox potential
ECACC	European Collection of Animal Cell Cultures
EB	ethidium bromide
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunoassay
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK1/2	extracellular signal related kinase ½
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
Gly	glycine
GSH	glutathione
HBSS	Hank's Balanced Salt Solution
HCL	hydrochloric acid
His	histidine
HIV	Human Immunodeficiency Virus
H_2O_2	hydrogen peroxide
HRP	horse radish peroxidase
Hsp	heat shock protein
Ig	immunoglobulin
IκB	inhibitor of κB
IκB-α	Ikappa B-alpha
IKK	Ikappa B kinase complex
IPTG	isopropyl-β-D-thiogalactopyranoside
JNK	c-Jun NH_2-terminal kinase
IL-1β	interleukin-1 beta
LB	Luria-Bertani medium
Kan	kanamycin

kb	kilobase pairs
kDa	kilodaltons
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
mBrB	monobromobimane
$MgCl_2$	magnesium chloride
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-Tetrazolium, inner salt; MTS)
NaCl	sodium chloride
NaOH	sodium hydroxide
NCTC	National Collection of Type Cultures
NCTCC	National Cell and Tissue Culture Collection
NF-κB	nuclear factor kappa B
NP-40	nonidet P-40
mV	millivolts
O^{-}_2	superoxide anion
OD	optical density
OH^{-}	hydroxyl radical
OMP	outer membrane protein
ORF	open reading frame
PBMCs	peripherial blood mononuclear cells
PBS	phosphate buffered saline
pI	isoelectric point
PKC	protein kinase C
PMA	phorbol-12-myristate-13 acetate
PMSF	phenyl methyl sulphonyl fluoride
Pro	proline
PVDF	polyvinylidene difluoride
PAGE	polyacrylamide gel electrophoresis
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RNA	ribonucleic acid
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
Ser	serine
SOD	superoxide dismutase
TAE	Tris-acetate EDTA buffer
TBE	Tris-acetate EDTA buffer
TBS	Tris buffered saline
TE	Tris-EDTA buffer
TEMED	N,N,N', N'-tetramethyl ethylene diamine
TIGR	The Institute of Genomic Research
Tpx	thiol peroxidase
TR	thioredoxin reductase
Tris	Tris (hydroxymethyl) aminomethane
Trx	thioredoxin
UV	ultra-violet

WHO	World Health Organisation
VacA	vacuolating cytotoxin A

CHAPTER I
GENERAL INTRODUCTION

CHAPTER I

General Introduction

1.1 *HELICOBACTER PYLORI*

Since the establishment of modern medicine, the search for the cause of peptic ulcer disease has engaged generations of physicians and pathologists. In 1983, Warren and Marshall (Warren and Marshall, 1983) described a spiral-shaped, Gram-negative, microaerophilic bacterium which they named *Campylobacter pyloridis* (Marshall *et al.*, 1984). The isolation and culture of this bacterium launched a medical revolution the field of gastroenterology and has changed the medical and scientific perception of the gastric environment and gastric disease since that time.

1.1.1 *Helicobacter pylori*; The Pathogen

Helicobacter pylori is a Gram-negative, microaerophilic, spiral bacillus, 3-5 μm long and about 0.5 μm in diameter **[Figure 1]**, which was originally assigned taxonomically to the genus *Campylobacter*. However, as subsequent 16S rRNA gene sequencing showed, this bacterium appeared not to be a true member of the *Campylobacter* genus (Romaniuk *et al.*, 1987), and with this observation leading to the establishment of a new genus, *Helicobacter* (Goodwin *et al.*, 1989). In excess of 20 species of the genus *Helicobacter* have been described thus far and these are not exclusively colonists of humans but colonise a diverse range of host species in the vertebrate kingdom (Fox and Lee, 1997). The relationship between *H. pylori* and host is very complex. Nowhere is the relationship between eukaryotic and prokaryotic cells as large, intimate and diverse and clinically relevant as in the human gastrointestinal tract. A breakdown in this relationship can result in the manifestation of disease, and *H. pylori* represents an ideal example of this paradigm.

The investigation of the microbiological, biochemical and genetic properties of *H. pylori* has been considerably boosted by the determination of the complete genome sequence (Tomb *et al.*, 1997) and the release of a second genome sequence (Alm *et al.*, 1999) has facilitated examination of the strain-strain variation in *H. pylori* for the first time. Thus the scientific investigation of the pathogen is proceeding at a unique time with a unique advantage. Genomic analysis has provided an excellent framework for further exploration and characterisation *H. pylori* and its role in human pathogenesis.



Fig. 1. Electron micrograph of *H. pylori.* A single *H. pylori* bacterium from a cultured human isolate. 400 X magnification. (Reproduced from the Canadian *H. pylori* Website)

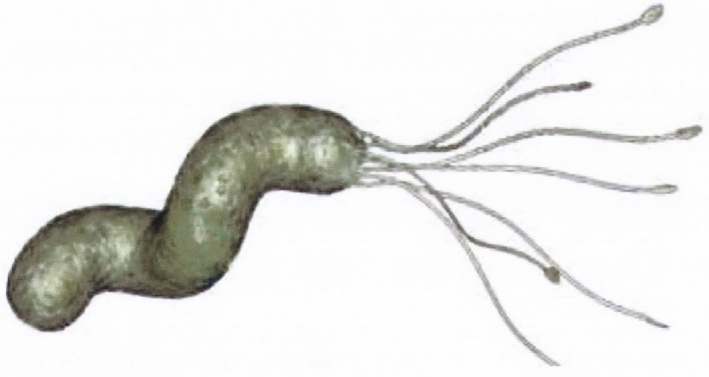


Fig. 1 (a). *H. pylori* high resolution schematic. (Reproduced from Marshall, L. 1997)

1.1.2 Historical Perspective

The first recorded case of gastric ulcer was reported over 400 years ago (Donati, 1586), and for much of the past the origins of peptic ulcer disease remained a mystery and highly speculative. However, a paradigm emerged, which until relatively recently in the history of peptic ulcer disease, gained widespread recognition and acceptance in the field of gastroenterology. That a phenomenon of gastric secretion, gastric acid overproduction, was responsible for varying gastric pathologies seemed logical when formal studies of the regulation of gastric secretion originated. The emergence of the microbiological era, during which the relationship between bacterium and human disease was established, saw the development of an alternate theory for the cause of peptic ulcer disease, one which pointed towards a bacterial theory of peptic ulcer. Spiral microorganisms have been described by histologists in animal stomachs in the latter part of the 19th century and the discovery of these morphologically distinct microorganisms soon followed in humans (Jaworski, 1889). The development of electron microscopy facilitated the full characterisation of active superficial gastritis which showed *H. pylori* attached to gastric epithelial cells (Steer, 1975) and resulted again in a pathological role for bacteria in gastric ulceration being postulated. However, the old acid-ulcer paradigm still prevailed and medical and scientific communities remained blinded by the established dogma that the normal stomach is a sterile environment, except for transiently present flora of oral origin until the early 1980's.

A revolution in perception of gastric disease occurred when a letter by Warren and Marshall described a close correlation between *H. pylori* and gastritis (Warren and Marshall, 1983). *H. pylori* was isolated and cultured for the first time in 1983, although at that time there was residual reluctance to accept the bacterium as the cause of peptic ulcer disease. Two documented independent ingestion studies undertaken with *H. pylori* occurred around this time, the first, an unusual attempt to fulfil Koch's postulates for the newly identified bacterium, which resulted in chronic colonisation of the human volunteer (Marshall *et al.*, 1985). The other study reported elimination of acute infection following ingestion of *H. pylori* by a human volunteer (Morris and Nicholson, 1987). Less extreme epidemiological studies (Hirschl *et al.*, 1986; Johnston *et al*, 1986) appeared to strengthen the correlation between duodenal ulceration and infection and widespread acceptance of the link between *H. pylori* and ulceration occurred in the scientific community around this time.

In addition to gastritis and ulceration, gastric malignancy has been widely described in historical medical literature and the evocation of chronic inflammation as a cause of gastric cancer had become widespread in the 19th century (Nepveu, 1821). The sequence of changes which give rise to metaplastic gastric epithelial cells is recognised as beginning with chronic gastritis, progressing to mucosal atrophy, finally culminating in intestinal metaplasia (Correa *et al.*, 1975). With *H. pylori* accepted as a causative agent of chronic gastritis, infection with this bacterium was well placed to become recognised as a significant initiating event in the metaplastic chain. This causative link was first reported in the scientific literature in 1991 (Forman *et al.*, 1991; Parsonnet *et al.*, 1991). In addition, at this time epidemiological investigations into the development of malignant gastric lymphoma of the MALT-type linked *H. pylori* with a risk of development of this pathology (Wotherspoon *et al.*, 1991). The prevalence of suggestions of a possible etiopathological role of *H. pylori* in gastric malignancy and low-grade MALT lymphoma lead in 1994 to the bacterium being designated a class I carcinogen by a Working Group of the International Agency for Research on Cancer (IARC, 1994) after assessment of available scientific evidence. Throughout modern medical history *H. pylori* has represented a paradigm of the mutability of medico-scientific opinion and continues to be so today as the role of *H. pylori* gastric malignancy is still a subject of some considerable dispute.

1.1.3 Taxonomy

Members of the genus *Helicobacter* are Gram-negative, non-spore forming bacilli and are generally capable of motility by virtue of single or multiple bipolar sheathed flagella. The genus was proposed when the traditional practise of grouping spiral Gram-negative bacteria isolated from the mammalian gastrointestinal tract as *Campylobacter* proved inaccurate based on extensive analysis of 16S rRNA sequences, analysis of fatty acid profiles and enzymatic activities, DNA hybridisation and growth characteristics (Goodwin *et al.*, 1989; Paster *et al.*, 1991). This robust genus currently comprises twenty formally named species and features of the genus include the possession of unusual morphological features and occupation of characteristic ecological niches in the gastrointestinal tract of mammals as diverse as humans, ferrets, birds and cheetahs. *Helicobacter* species have been isolated from both the gastric and enteric (intestinal) tract and as such display a niche preference. *H. pylori* is a gastric *Helicobacter* which colonises the corpus and antrum of the human stomach, residing in the mucus layer adjacent to the epithelium. It has been shown recently that tissue tropism exhibited by *H. pylori* may be directed by its interaction

with human single domain trefoil protein TFF1 which seems to be a receptor for *H. pylori* as it co-localizes with MUC5AC in gastric epithelia with which the bacterium also associates (Clyne *et al.*, 2003). The authors hypothesise that TFF1 is a receptor for *H. pylori* on the gastric mucosa possibly explaining why *H. pylori* only colonises the stomach. Not exclusively a human colonist, *H. pylori* is capable of infecting other mammals, among them, cats (Fox *et al.*, 1995) and non-human primates (Handt *et al.*, 1995). The isolation and characterisation of *Helicobacter* species from other mammals has contributed to a recognition of the importance of these organisms as members of the gastrointestinal flora.

1.1.4 *H. pylori* and Host Environmental Ecology

The human gastric tract represents a unique ecological niche for microflora and the stomachs bacterial residents have become the subject of much interest since the assignation of a microbial etiology to peptic ulcer disease. An understanding of the natural ecology of the stomach is essential to the understanding of bacterial adaptation to the host environment and also to the consequences of its presence. The gastric environment is a most demanding one, with high acidity, peristalsis, gastric emptying and mucus and cell shedding and turnover. *H. pylori* is found in the acid-secreting portion of the stomach, the fundus, although its natural niche appears to be the mucus lined surface of the non-acid secreting area close to the pyloric sphincter, the antrum. In this area, the pH is less acidic than in the central part of the stomach, the corpus where acid secreting cells are abundant. *H. pylori* resides primarily in the gastric mucus layer of the gastric epithelial cells (Hessey *et al.*, 1990). The mucus layer is rich in urea and bicarbonate which can efficiently buffer the acid pH from the lumen, and provides *H. pylori* with a protected niche conducive to long term colonisation of the gastric mucosa (Blaser, 1993). Microbial colonisation of a host locale affects the surrounding tissue in several ways.

Most prominently through the occupation of niches, utilisation of resources and excretion, all of which can be considered as signals to the host. This signalling is not however a one-way process as the host can also be considered to signal the microbe. This signalling can occur in the form of pressure, chemical milieu and temperature, all of which can be detected by a microbe in a host environment. Host defence molecules may also be considered as part of this signalling process. It is unlikely that the signals from both the host and the microbe are completely uncoordinated in the case of *H. pylori* and humans considering their long co-evolution. The persistence of *H. pylori* in the host could be

thought of as having reached equilibrium, a state that can only be successfully established in the presence of negative feedback. This signalling model may help to explain the extraordinary persistence of *H. pylori* infection in particular, and microbial persistence in general. Understanding the relationship between *H. pylori* and host may also aid in the understanding of diverse disease processes such as chronic inflammation, oncogenesis and hormonal disregulation which may be relevant to modern epidemic problems such as diabetes and obesity (Atherton and Blaser, 2004).

1.1.5 Epidemiology of *H. pylori* Infection

The epidemiology of *H. pylori* infection is a matter of intensive debate and three of the most important epidemiological questions concerning mode of spread, acquisition of infection and risk factors have not been completely elucidated. New epidemiological data requires continuous reassessment and reinterpretation of the existing information concerning prevalence rates and disease associations. Humans appear to be the primary natural reservoir of *H. pylori* infection, however, other sources have been suggested. The route of transmission of infection is postulated to be either oral-oral, faecal-oral (Lee *et al.*, 1991) or gastro-oral (Goodman and Correa, 1995) and transmission by children within families common. These features resemble observations made for other species of enteric pathogen. Vehicular transmission by food or water may also contribute to infection transmission (Ozturk *et al.*, 1996), however, these vehicles are variable over different geographical locations and therefore the impact of this type of transmission is difficult to assess. The majority of infections with *H. pylori* occur in children (Cave *et al.*, 1996) however, a paucity of information regarding initial acquisition of infection in this population means that the epidemiology of *H. pylori* in children in not completely understood. Certain socio-economic risk factors correlate with prevalence of *H. pylori* infection, including low socio-economic status and low education levels (Malaty *et al.*, 1996). High rates of seropositivity in children have been reported in developing countries and the incidence of infection is estimated to be between 3% and 10% in paediatric populations (Torres *et al.*, 2000). Overall, *H. pylori* represents one of the most common infections of human beings with approximately 50% of the global population estimated to be infected (Brown *et al.*, 2000).

H. pylori infection is characterised by geographical subdivisions, an observation which is also true for several other human pathogens such as *Haemophilus influenzae* (Musser *et*

al., 1990) and *Mycobacterium tuberculosis* (Kremer *et al.*, 1999). However, the spread of this bacterium in human populations is not epidemic in nature and due to its long persistence within the host can be seen as a valuable epidemiological marker capable of yielding information about its hosts migration patterns over long time periods. Recent investigations show that *H. pylori* may be divided into seven major populations and subpopulations with distinct geographical distributions, populations which derive their gene pools from ancestral populations which arose in Central Asia, Africa and East Asia (Falush *et al.*, 2003). The worldwide distribution of infection may be attributed to several known human migratory fluxes which occurred in the past. A fortuitous feature of the genetic makeup of *H. pylori*, sequence diversity, allows the recognition of distinct populations after centuries of coexistence in diverse geographical locations. The high sequence diversity seen in this pathogen compared with other bacterial pathogens (Achtman *et al.*, 1999), and which is approximately 50-fold greater than that seen in humans (Li *et al.*, 1991), facilitates the investigation of human migrations using techniques such as residual short linkage disequilibrium. From a medical perspective, the elucidation of population pattern subdivision is relevant as infection prevention through vaccination approaches must take into account global strain diversity. Recent data suggests that *H. pylori* prevalence is decreasing globally (Parsonnet, 1995). A consequence of this may be a decrease in the rate of peptic ulcer disease, however, as this infection continues to disappear it may alter the pattern of gastric disease globally.

1.2 *HELICOBACTER PYLORI*; CELLULAR PROCESSES

1.2.1 *H. pylori* Physiology and Metabolism

Several features of the metabolic pathway of *H. pylori* have been elucidated from examination of the genome sequence of the pathogen (Tomb *et al.*, 1997). The sequencing of a second strain of the bacterium facilitated a detailed examination of the physiology of *H. pylori* as a result of the genomic comparison of the two sequenced strains, 26695 and J99 (Doig *et al.*, 1999). *H. pylori* appears to have a limited capacity to acquire sugars from its environment and appears to use glucose as the only source of carbohydrate (Mendz and Hazell, 1993). There also seems to be a deficit in sugar uptake systems suggesting a limited ability for sugar catabolism, and also that the bacterium does not use sugars as a major source of energy. The glycolytic/gluconeogenic metabolic axis appears not to be utilised for catabolic energy production but rather for anabolic biosynthesis (Hoffman *et*

al., 1996). Peptidoglycan, aromatic amino acid, phospholipid, cofactor and fatty acid biosynthesis derive from acetyl-CoA of intermediates in the glycolytic pathway. The pyruvate metabolism of *H. pylori* is characteristic of that of a microaerophilic organism, where pyruvate is dissimilated to produce acetate, formate, succinate and lactate (Mendz *et al.*, 1994). The *H. pylori* tricarboxylic acid pathway is similar to that used by *E. coli*. Urea, ammonia, alanine, serine and glutamate are all used by *H. pylori* as nitrogen sources. Ammonia assimilation, necessary due to the action of the urease enzyme, is carried out by the glutamine synthase enzyme. Finally, four-respiratory electron generating enzymes have been identified from genomic analysis, NADH-ubiquinone oxidoreductase complex, glycerol-3-phosphate dehydrogenase, D-lactate dehydrogenase and a hydrogenase complex, all participating in the pathogens respiratory process (Tomb *et al.*, 1997). *H. pylori* is the first bacterial species to have two completed genome sequences available for comparison and both sequenced strains appear to have almost identical metabolic potential.

1.2.2 *H. pylori* Macromolecule Biosynthesis

The enzymes responsible for DNA replication have been identified in *H. pylori* as DnaE, DnaN, DnaX, DnaQ and HolB, all functional subunits of the DNA polymerase III holoenzyme. Although this holoenzyme is smaller than that of *E. coli*, it is similar to the holoenzymes of other bacteria (Cole *et al.*, 1998; Deckert *et al.*, 1998). Homologues of the majority of genes involved in initiation and DNA chain elongation have been identified in *H. pylori*. In addition, both sequenced genomes possess numerous genes with similarity to DNA restriction and modification enzymes, a proportion of which are strain specific (Akopyants *et al.*, 1998; Alm *et al.*, 1999). Many of the genes required for the initiation and elongation of fatty acid biosynthesis and phospholipid synthesis were represented by genomic homologues. Also, genes involved in peptidoglycan synthesis and by *H. pylori* are represented by a full complement of the necessary genes, starting with the synthesis of UDP-*N*-acetylmuramic acid and finishing with undecaprenol lipid carrier linked UDP-disaccharide pentapeptide. Some of the genes involved in the recycling of this macromolecule are present although from examination of the genome it is clear that others previously identified as required for this process are not, so it remains unclear whether recycling is carried out by the bacterium (Tomb *et al.*, 1997).

1.2.3 Cell Division, Morphology and Protein Secretion

The basic mechanisms of replication and cell division and secretion of *H. pylori* are similar to those of *H. influenzae* and *E. coli*, however, there are important differences (Tomb *et al.*, 1997). Genome analysis of *H. pylori* strains has identified several genes involved in bacterial cell division, including *ftsZ* and *ftsA*. The FtsW protein, which functions in cell division is represented by two proteins with significant sequence homology, RodA (Ikeda *et al.*, 1990), and may be a functional homologue of FtsW in *H. pylori*. In *E. coli*, two proteins implicated in the determination of cell morphology, MreB and RodA, involved in the elongation process, have representative homologues in *H. pylori* (Ishino *et al.*, 1986; Doig *et al.*, 1999). The *H. pylori* genome is missing an orthologue of the secretory chaperonin, SecB, however the SecA-dependent secretory pathway is present. The set of bacterial chaperones in *H. pylori* (DnaK, DnaJ, CbpA, GrpE, GroEL, GroES and HtpG) are regulated differently than in *E. coli* due to the fact that *H. pylori* does not possess the sigma factors that up-regulate chaperone synthesis in *E. coli*, heat shock σ^{32} and stationary phase σ^{S}. *H. pylori* has two specialised protein export systems, one is flagella associated, and is composed of the Fli and Flh orthologues. The other system is involved with the *cag* pathogenicity island. *H. pylori* also does not appear to possess a type IV signal peptidase and orthologues of the dsbABC system which function in the surface structure assembly, pilin maturation and DNA transformation (Tomb *et al.*, 1997).

1.2.4 Regulation of Gene Expression

Bacteria can regulate the transcription of their genes in a variety of different methods in response to a wide range of environmental stimuli including cell density, pH, nutrient availability and target tissue contact. The global regulatory proteins found in other bacteria such as *E. coli* are found to be less abundant in *H. pylori* (Tomb *et al.*, 1997), which suggests that the regulation of gene expression may be less complex than in some other bacterial pathogens. Both sequenced strains of *H. pylori* possess only three identifiable sigma factors, which provide promoter recognition specificity to the DNA polymerase III holoenzyme. There is σ^{70} (*rpoD* gene), the general housekeeping sigma factor, σ^{28} (*fliA* gene), the flagellar specific sigma factor and the σ^{54} factor (*rpoN* gene) which controls the expression of several different gene classes and is susceptible to environmental regulation (Merrick, 1993). In addition to sigma factor-mediated gene regulation, *H. pylori* can modulate gene expression by the mechanism of slipped-strand repair as it possess several

genes which contain dinucleotide repeats and homopolymeric tracts (Alm *et al.*, 1999; Saunders *et al.*, 1998). Furthermore, antigenic frameshifts or pseudogenes are present in both sequenced genomes as well as in-frame termination codons which could be exploited by the bacterium as a further mechanism of gene expression regulation (Doig *et al.*, 1999)

1.2.5 Environmental Sensing and Chemotaxis

To colonise and persist in its particular niche a bacterium must be capable of sensing and responding to deleterious environmental signals. Two-component systems are composed of a membrane-associated sensor kinase and a partner response protein. In this system, the sensor kinase uses an amino terminal 'input domain' to sense the local environment. This is followed by autophosphorylation of a conserved histidine residue in its 'transmitter domain'. The phosphate is then transferred to an amino terminal aspartic acid residue in the 'receiver domain' of the response regulator. A carboxy terminal DNA-binding 'activator domain' of the response regulator is then activated by a conformational change induced by these upstream events. In this way environmental signals are translated into changes in gene expression to counter environmental insults. Analysis of the *H. pylori* genome sequence has resulted in the identification of four ORFs encoding sensor histidine kinases and six ORFs encoding response regulators (Beier and Frank, 2000). In other enteric bacterial species, two-component systems are well defined and several of the identified proteins in *H. pylori* are homologous to the model CheA sensor kinase and the CheY response regulators (Eisenbach, 1996). These two-component systems are responsible for the control of chemotaxis generally and this is also observed in *H. pylori*. Mutagenesis of the response regulators and senor kinases of *H. pylori* have shown that the two-component system elements are essential for chemotaxis and colonisation (Foynes *et al.*, 2000). The genome sequence of *H. pylori* has revealed the presence of a novel set of chemotaxis genes including three cheV paralogues. A recent study, in which each of the three cheV paralogues were insertionally inactivated in *H. pylori* strain 26695 to determine their importance in the chemotactic signal-transduction pathway of the bacterium has also shown that only CheV1 was critical for chemotaxis, indicating a specific role distinct from the other paralogues in the signal-transduction pathway (Pitman *et al.*, 2001). These workers also showed that none of the CheV proteins could substitute for the loss of CheW, as an *H. pylori* cheW null mutant was non-chemotactic.

In addition to the use of two-component systems for environmental sensing, bacteria have developed quorum sensing systems which facilitates sending of environmental factors such as cell density and plays an important role in the formation and maintenance of bacterial biofilms and intra/interspecies communication. Quorum sensing involves the production of autoinducers or homoserine lactones. Homoserine lactone binding to cognate sensor proteins in response to environmental signals initiates signalling cascades which terminate in gene expression changes (Bassler, 1999). Evidence has emerged of such a system in *H. pylori*, as the genome has been shown to encode a *luxS* homologue (Joyce *et al.*, 2000), however, deletion of the autoinducer homologue had no notable effect and the role that the protein might have in environmental sensing/communication remains undefined.

1.2.6 Functional Characterisation of the *H. pylori* Genome

The genome of *H. pylori* strain 26695 consists of a circular chromosome of 1,667,867 base pairs with an average G+C content of 39% (Tomb *et al.*, 1997). *H. pylori* strain 26695 was originally isolated from a patient in the U.K. with gastritis and this strain was shown to colonise piglets and elicit an immune and inflammatory response (Akopyants *et al.*, 1995). In addition it is a toxigenic and transformable strain. Thirty-six tRNA species were identified following genomic analysis, and arranged into seven gene clusters plus twelve single genes. Two separate sets of 23S-5S and 16S rRNA genes were identified. Intergenic regions of the chromosome were commonly found to have repeat families with an identity in excess of 97% and a variance in length from 0.4 to 3.8 kb. The presence of two insertion sequences (IS605 and IS606) was revealed and were found to code for two divergently transcribed transposases, TnpA and TnpB, respectively. Evaluation of ORFs identified 1590 predicted coding sequences of which 1091 were accredited with biological roles after searching against a non-redundant protein database. The predicted genes had an average size, comparable to other prokaryotes, of 954 base pairs. Of the proteins predicted, over 70% have a calculated isoelectric point (pI) of greater than 7.0, compared with approximately 40% in *E. coli* and *Haemophilus influenzae*. The basic amino acids, arginine and lysine, occur twice as frequently as in *E. coli*, possibly reflecting the adaptation of *H. pylori* to the hostile acid environment of the gastric niche. Genomic analysis also suggested that systems for homologous recombination, post-replication mismatch, excision and repair are present in *H. pylori* (Tomb *et al.*, 1997).

Although, the two sequenced strains of *H. pylori* appear to have almost identical metabolic potential, isolates of *H. pylori* harvested from different individuals appear to be highly polymorphic and strain variation has also been observed within a single human host. A recent study has revealed that within the ecological niche of a single host, remarkable genetic differences exist among single colony isolates of *H. pylori* (Israel *et al.*, 2001). Following nucleotide sequence analysis of such isolates it appears that the pathogen has the capacity to integrate exogenous DNA. The authors postulate that this ability is consistent with a model of continuous microevolution within its cognate host.

1.3 *HELICOBACTER PYLORI*; MOLECULAR ASPECTS OF INFECTION

1.3.1 Motility

The ability of *H. pylori* to move is central to its success as a colonist and pathogen of the human gastric tract. Previously classified as a species of the *Campylobacter* genus, the flagella is one of the components of *H. pylori* which distinguishes it from its former category. Flagellar motility is an essential requirement of the ability of *H. pylori* to generate and facilitate movement and to colonise the viscous layer covering the gastric mucosa. The powerful flagella give *H. pylori* significantly greater motility than, for example, *E. coli* (Morgan *et al.*, 1996). The structural features of *H. pylori* flagella have been extensively studied as motility is considered an important virulence determinant, more correctly a colonisation factor. Evidence from several lines of inquiry show that less motile strains are less able to colonise or survive in the host than more motile strains of the pathogen. It has been shown that the degree of motility of *H. pylori* strains correlates with the degree of infectivity in gnotobiotic piglets (Eaton *et al.*, 1989). The general organisation of the physical structure of the *H. pylori* flagellum conforms with those of the intensively studied model enteric bacteria, *Salmonella typhimurium* and *E. coli* (Macnab, 1996). *H. pylori* possess multiple (2-6 per cell) sheathed flagella, invariably polar, each flagellum consisting of a basal body hook and flagellar filament (Geis *et al.*, 1989). The flagellar filament, a copolymer of two flagellin subunit species, flaA and flaB, is linked to the flagellar basal body by a hook encoded by the *flaE* gene (Schmitz *et al.*, 1997). The membrane sheath enveloping each filament, which is a bilayered membrane containing proteins, lipids and lipopolysaccharides, protects the filaments from the gastric acid environment of the gastric lumen (Suerbaum *et al.*, 1993). The *flaA* and *flaB* genes are unlinked on the chromosome and preceded by different promoters (Schmitz *et al.*, 1997).

H. pylori flagellar gene regulation represents a departure from the models of transcriptional regulation of motility genes in the most intensively studied organisms such as *S. typhimurium*. Transcriptional control of two of the *H. pylori* flagellar genes (*flaB* and *flgE*) has been shown to be exerted by the alternative sigma factor σ^{54}, whereas *flaA* was found to be transcribed under the control of a promoter displaying a σ^{28} consensus sequence (Suerbaum *et al.*, 1993). A current model postulates that *H. pylori* may vary the ratio of its flagellar proteins, FlaA and FlaB in response to changes in environmental conditions (Suerbaum, 1995). *H. pylori* can therefore be considered representative of a bacterial group where transcription of motility genes is controlled by more than one alternative sigma factor. The annotated sequence of the *H. pylori* genome (Tomb *et al.*, 1997) includes at least 40 proteins which may contribute to motility in this pathogen, however, the basic flagellar structure is common to other bacteria with few variations which may reflect niche adaptation and the idiosyncrasies of a organism with a small genome. Flagellar proteins of various enteric bacteria have been shown to elicit a host inflammatory response as they constitute major surface exposed antigens. A recent study in which the authors hypothesised that *H. pylori* may escape detection by the pattern recognition receptor Toll-like receptor 5, has shown that that *H. pylori* FlaA is not proinflammatory and also not secreted, which allows it to evade TLR-5 mediated immunity. In contrast, FliC from *Salmonella typhimurium* (similar to FlaA) results in potent induction of TLR5-mediated IL-8 production and rapid clearance of *S. typhi* from the host (Gerwirtz *et al.*, 2003).

1.3.2 *H. pylori* Lipopolysaccharide

The surface-exposed lipopolysaccharide (LPS) plays an important role in *H. pylori* pathogenesis (Moran, 1996). In other enteric bacterial pathogens, LPS represents a highly potent immunogenic molecule which can be responsible for severe and acute inflammatory responses, however, the LPS of *H. pylori* is significantly less immunogenic than that of other bacteria (Baker *et al.*, 1994). Recently, *H. pylori* LPS has been demonstrated to less biologically active when compared with *E. coli* LPS in its ability to induce the expression of cyclooxygenase 2 and synthesis of prostaglandin E2 (Smith *et al.*, 2003). The authors suggest that this may provide one mechanism by which *H. pylori* minimizes the inflammatory response in the gastric mucosa, facilitating chronic infection. When the genome of *H. pylori* was examined, genes for synthesis of the lipid A molecule, the core region and the O antigen were found (Tomb *et al.*, 1997). Comprehensive, detailed structural analysis of *H. pylori* LPS has revealed some unique features of the molecule

which may account for certain aspects of *H. pylori*-induced pathogenesis (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1996; Monteiro *et al.*, 1998). The O-chain polysaccharide structure of *H. pylori* strain NCTC11637 is composed of a type-2 N-acetyl-lactosamine chain of various lengths which may be terminated at the non-reducing end by Lewis blood group epitopes. The O antigen of many *H. pylori* isolates is known to mimic the human Lewis^x (Le(x)) and Lewis^y (Le(y)) blood group antigens which mimic human cell surface glycoconjugates and glycolipids (Moran, 1996), which may account for reduced immunogenicity of this particular LPS molecule. Although, it remains to be formally established if the O-chain of *H. pylori* LPS contributes to protective immunity generation. The roles of molecular mimicry and phase variation of *H. pylori* Lewis antigen expression in both persistent infection and pathogenesis of this important human gastric pathogen have been widely examined (Appelmelk *et al.*, 2000; Wang *et al.*, 2000). Although putative molecular mechanisms affecting Le(x) and Le(y) expression have been investigated previously, a recent report has identified an environmental trigger, pH, which appears to induce phase variation of Le(x) and Le(y) in *H. pylori* (Moran *et al.*, 2002). The authors postulate that acid-induced phase variation can aid adaptation of the bacterium to its ecological niche. *In vitro* and *in vivo* studies from various groups have suggested that *H. pylori* LPS Le(x) antigens mediate bacterial adhesion, however, a recent study has suggested that *H. pylori* LPS plays a distinct but minor role in adhesion (Mahdavi *et al.*, 2003).

1.3.3 Pathogenicity Determinants: Cytotoxin Associated Antigen and Vaculolating Cytotoxin

H. pylori virulence determinants include moieties such as urease and flagella, however, they are not involved in the direct causation of gastroduodenal disease. Toxin elaboration is a common bacterial virulence determinant responsible for overt pathology in the host. *H. pylori* by virtue of its niche adjacent to the gastric epithelium has the potential to cause damage to the gastric mucosa, and this capability of inflicting cell damage was first described by Leunk (Leunk *et al.*, 1986). This study noted the ability of *H. pylori* to induce vacuolisation of epithelial cell membranes. Further investigation resulted in the cloning, purification and characterisation of VacA, a vacuolating cytotoxin (Cover *et al.*, 1992; Telford *et al.*, 1994). *H. pylori* infection of cultured epithelial cells can result in the induction of gastric cell lesions and the cytotoxin VacA is reported to be responsible for this effect (Ghiara *et al.*, 1995). Different alleles of the *vacA* gene have been reported

(Atherton *et al.*, 1995) and epidemiological studies with humans have correlated disease outcome with the level of expression of the *vacA* gene (Stephens *et al.*, 1998).

In addition to VacA, the cytotoxin associated gene, *cagA*, was identified. The type IV secretion system is used by the bacterium to deliver CagA into the mammalian cell cytosol. CagA sequence analysis shows that CagA contains both tyrosine phosphorylation motifs and cyclic adenosine 3′, 5′-monophosphate (cAMP)-dependent phosphorylation motifs. CagA becomes tyrosine phosphorylated at different sites (Backert *et al.*, 2001; Stein *et al.*, 2002) and induces changes in the tyrosine phosphorylation sites of eukaryotic proteins (Puls *et al.*, 2002). *In vitro* investigations suggest that the CagA protein becomes phosphorylated by members of the Src-kinase family (Stein *et al.*, 2002; Selbach *et al.*, 2002) and downregulates the phosphatase SHP-2 (Higashi *et al.*, 2002). Downregulation of SHP-2 is believed to induce abnormal cell growth and promote gastric cancer (Ferber, 2001). Molecular genetic analyses of *H. pylori* has revealed that approximately 50% to 60% of strains have a 40-kb DNA segment called the *cag* pathogenicity island (PAI), which encodes a multi-component type IV secretion system (Akopypants *et al.*, 1998). The cagA gene is locate at the one end of the PAI which is replete with genes that elicit the production of interleukin (IL)-8 by gastric epithelial cells (Censini *et al.*, 1996).

H. pylori may be subdivided into two types. Type I strains, containing the *cag* PAI, exhibit increased virulence, since they are predominantly associated with severe gastric disease, while type II strains, lacking the *cag* PAI, are frequently isolated from asymptomatic carriers. Parsonnet *et al.* (1997) showed that infection with CagA-positive *H. pylori* was associated with a 5.8-fold increased risk for gastric cancer, and that *H. pylori* infected individuals without antibodies to CagA showed a markedly decreased (2.2-fold) risk for gastric cancer. Also, of clinical significance is that most strains from individuals with overt disease produce both virulence determinants, VacA and CagA, in contrast to strains isolated from asymptomatic carriers (Cover *et al.*, 1996; Covacci *et al.*, 1997).

1.3.4 Urease

Urease production is a defining characteristic of the gastric *Helicobacters* and robust urease activity is a characteristic feature of *H. pylori*, indicating the importance of urease for gastric colonisation. The bacterium produces large quantities of urease, approximately 5% to 6% of total cell protein (Mobley, 1995). *H. pylori* urease is a multimeric, 550 kDa

cytosolic metalloenzyme that requires two nickel ions in each of the six active sites for catalytic activity (Fulkerson *et al.*, 1998; Mobley, 1995). The enzyme consists of six copies of two structural subunits, UreA and UreB. Urease catalyses the hydrolysis of urea to ammonia and carbamate, the latter decomposing to yield an additional ammonia molecule and carbonic acid in the gastric mucosa. *H. pylori* is not an acidophile and does not survive at low pH. The presence of ammonia in the gastric mucosal environment facilitates survival of the bacterium through the neutralisation of gastric acid and increasing the pH in the immediate microenvironment. The development of achlorhydria and neutral pH allow the bacteria to swim through the mucus layer to the gastric epithelium where the environment is less harsh. Therefore, urease appears to be essential for the initial gastric colonisation, but not necessary for continued colonisation (Mobley, 1995; Mobley, 1996). On the *H. pylori* genome the genes encoding urease are located as a single 6.13 kb gene cluster on the chromosome (Clayton *et al.*, 1990; Labigne *et al.*, 1991). Seven contiguous genes have been designated *ureABIEFGH* are required for active enzyme synthesis (Cussac *et al.*, 1992; Hu *et al.*, 1992). Urease mutants have been constructed by allelic exchange mutagenesis but pathogenesis studies with these mutants have shown that they are avirulent in animal models of *H. pylori* infection (Ferrero *et al.*, 1992). As urease is absolutely necessary for colonisation of the gastric mucosa, it has been recognised as a critical virulence determinant.

H. pylori urease is a nickel-containing enzyme. It has been shown that *H. pylori* regulates the expression and activity of its urease enzyme as a function of the availability of the co-factor nickel (van Vliet *et al.*, 2001). The authors also reveal that the *H. pylori* ferric uptake regulator, Fur (HP1027; TIGR annotation), is involved in the modulation of urease expression in response to nickel and that regulation of *H. pylori* urease is nickel-responsive. Furthermore, the same workers demonstrated that the HP1338 protein, an ortholog of the *E. coli* nickel regulatory protein NikR, mediates nickel-responsive transcriptional induction of urease expression in *H. pylori* (van Vliet *et al.*, 2002). Transcriptional and mutational analysis of the *H. pylori* urease promoter has recently been reported. The study demonstrates the modular structure of the *H. pylori* urease promoter (Davies *et al.*, 2002). Finally, this enzyme has provided a means for testing for the presence of the bacterium in individuals, forming the basis for various *H. pylori* testing procedures which are in widespread use today.

1.3.5 Adhesins

The establishment *H. pylori* infection in the hostile gastric environment is dependent on motility, however, survival and long-term colonisation require that the pathogen balances its motility with attachment to host cells by adherence to facilitate nutrient acquisition. Bacteria unable to adhere to the epithelium tend to be removed by shedding of the surface cells and mucus layer (Borén *et al.*, 1993), therefore *H. pylori* has evolved a very specific association with the gastric epithelium and adhesion is considered a virulence factor for colonisation of the human stomach. Bacterial adherence to any epithelial surface occurs through a network of surface molecules which are responsible for bacterial tissue tropism (Falk *et al.*, 1994). Adhesins recognise cellular receptors such as cell surface proteins, glycolipids and glycoproteins. *H. pylori* exhibits a distinct tissue tropism and two subsets of interaction processes occur which depend on whether the bacterium is present in the mucus layer or actually adhered to the gastric epithelial cell surface. In the mucus layer the bacterium interacts with the highly glycosylated high molecular weight mucin molecules, where sialyated-, fucosylated- and sulphated-carbohydrate epitopes are available as attachment sites (Wadström *et al.*, 1996). If the bacterium penetrates the mucus layer, attachment to epithelial lining occurs through specific binding sites. Type I *H. pylori* strains which contain the *cag* PAI stimulate bacterial transmembrane signalling and cell activity. Upon activation of the type IV secretion apparatus encoded by *cag* PAI, CagA is secreted into cells and the result is pedestal formation beneath the bacterium which proceeds through actin polymerisation over a local area (Segal *et al.*, 1999). Genes within the *H. pylori cag* PAI also promote attachment and effacment which is a strategy used by other enteric pathogens in colonisation process (Guruge *et al.*, 1998).

Genome sequence analyses of *H. pylori* strains has resulted in the identification of approximately 30 genes encoding putative outer membrane proteins, a proportion of which share conserved sequence motifs in the N- and C-terminal regions. These ORFs code for a variety of adhesins, porins and transport proteins (Tomb *et al.*, 1997; Alm *et al.*, 1999). Included among these are the adherence associated proteins, AlpA and AlpB (Odenbreit *et al.*, 1999), the blood group antigen binding adhesion, BabA (Ilver *et al.*, 1998), the HopZ adhesin protein (Peck *et al.*, 1999) and the family of HOP porins (Exner *et al.*, 1995; Hancock *et al.*, 1998). A recent study has shown in real time using a relevant animal model (rhesus macaque model) that *H. pylori* regulates OMP expression *in vivo* using both antigenic variation and phase variation (Solnick *et al.*, 2003). *H. pylori* recovered from

challenged monkeys had deleted babA and in some cases the babA gene was replaced by babB, an uncharacterised OMP, related to babA. Antigenic variation and phase variation therefore appear to be emerging as 'real' mechanisms of immune evasion. The further study and validation of *H. pylori* adhesions and host receptors should contribute to the understanding of the molecular cross-talk between pathogen and host thus revealing the relationships of these molecules with overt disease causation.

1.4 *HELICOBACTER PYLORI* AND GASTROINTESTINAL DISEASE

1.4.1 *H. pylori* Disease Associations and Clinical Outcomes

Growing scientific evidence supports the association of *H. pylori* infection with peptic ulcer, chronic active, chronic persistent, and atrophic gastritis in adults and children, antral adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. However, other gastrointestinal diseases such as non-ulcer dyspepsia and gastroeosophageal reflux disease (GORD), have not been shown to be conclusively linked to the to the presence of the bacterium in the gastrointestinal tract (Graham and Yamaoka, 1998). Most people with infection have an asymptomatic chronic gastritis without ulcers, cancer or lymphoma. The distribution is a pan gastritis in contrast to duodenal ulcer disease associated antral-predominant gastritis. *H. pylori* is a major etiologic factor leading to peptic ulcer disease, and about 6% to 20% of persons infected with *H. pylori* are found to have the disease. Approximately 90% to 95% of duodenal ulcers and 70% to 75% of gastric ulcers are attributable to infection with *H. pylori* (Mitchell and Hazell, 1996). Recurrence of ulcer after successful eradication of *H. pylori* is approximately 2% to 4% and this rate rises to about 80% if the infection persists (Schutze *et al.*, 1993). The link between lymphoma and *H. pylori* is well recognised with the bacterium present in 72% to 98% of low-grade MALT lymphomas. *H. pylori* was designated a class I carcinogen by the World Health Organisation in 1994 and two distinct cancers are associated with infection, gastric lymphoma and adenocarcinoma (IARC, 1994). Infection is thought to create an environment that facilitates a chain of neoplastic changes in the gastric mucosa culminating in adenocarcinoma. Infected persons have a 2.7- to 12-fold increased risk of developing lymphoma and gastric cancer compared with uninfected individuals (Kuipers *et al.*, 1995).

1.4.2 Infection Eradication; Clinical Indications

If an ulcer is related to infection with *H. pylori*, ulcer healing in conjunction with eradication of the bacteria represents a curative approach which is unlikely to be complicated by recurrence of the ulcer (Van der Hulst *et al.*, 1997). If an individual has either active duodenal or gastric ulcers or a documented history of peptic ulcer disease, then they represent candidates for *H. pylori* testing. The association between duodenal ulcer disease and *H. pylori* is well documented and *H. pylori* eradication virtually cures duodenal ulcer disease, with an approximately 5% relapse rate at one year. Infection with *H. pylori* has been shown to increase the relative risk of non-cardia gastric cancer (Forman, 1998) and this has important implications from a public health perspective especially in developing countries where there are high infection acquisition rates in early childhood. In addition to peptic ulcer disease, testing for *H. pylori* infection is indicated for gastric MALT lymphoma and following resection of gastric cancer (Uemura *et al.*, 1997). Rarely, re-infection can occur when an individual is treated for *H. pylori* infection, therefore, retesting may be advisable after completion of a treatment regimen (Malfertheiner *et al.*, 2002) and retesting is advisable in cases of more complicated peptic ulcer disease.

1.4.3 *H. pylori* Antimicrobial Therapy

Over the last decade, important advances have permitted the ready and accurate diagnosis of *H. pylori* infection. Similarly, antimicrobial therapy has improved to the stage that safe and well tolerated short term regimens are greater than 90% effective. Combination antibiotic therapy is the usual course of treatment for infection however the efficacy of these treatments is being eroded by the rise in frequency of antibiotic resistant isolates of *H. pylori* (Osato *et al.*, 2001; Jenks and Edwards, 2002). Currently, peptic ulcer disease is the main indication for treatment for *H. pylori* infection, and *H. pylori* therapy appears to be the preferred course in the treatment of first presentation of a peptic ulcer with infection. However, the observed decline in the prevalence of *H. pylori* infection in developed countries and the resultant decline in *H. pylori*-associated diseases may be the result of steadily improving public health measures preventing infection in childhood. The advent of antibiotic regimens appears to have played only a quantitatively minor role. Indeed, the use of indiscriminate *H. pylori* antimicrobial therapy with a view to secondary prevention of gastric cancer must be weighed against the current trend in *H. pylori* antibiotic resistance presently emerging (Ling *et al.*, 1996).

1.4.4 Control of *H. pylori*: Vaccination

H. pylori colonises the gastric mucosa of approximately half the worlds adult population with rates rising up to 90% in developing countries, therefore this infection represents an attractive candidate from therapeutic intervention by vaccination. Current eradication regimes based on combination antibiotic therapy, although very successful, by virtue of global socio-economic patterns do not represent a suitable worldwide infection eradication approach. Therefore, vaccination remains an attractive cost effective global solution to curbing *H. pylori* infection. Several *H. pylori* antigens have been shown to confer protection against *H. pylori* infection in animal models, including, urease B subunit, HspA/HspB, VacA, CagA and catalase (Corthesy-Theulaz *et al.*, 1995; Ferrero *et al.*, 1995; Michetti *et al.*, 1994; Radcliff *et al.*, 1997; Dubois *et al.*, 1998). However, successful animal model vaccines have only limited relevance in the development of a human vaccine. The ideal antigen candidates or more likely subunit vaccines should be immunogenic, conserved across strains and non-toxic to the host (Kimmel *et al.*, 2000; Del Giudice *et al.* 2001) and the availability of genome sequence data for two *H. pylori* strains should enhance the search for suitable antigen candidates (Lee, 1998).

1.5 *HELICOBACTER PYLORI* AND HOST INFLAMMATORY RESPONSE

1.5.1 *H. pylori*; Activation of the Inflammatory Cascade

One of the tenets of pathogenicity is the ability of a pathogen to circumvent the hosts defence systems (Falk, 1994). Many *H. pylori* patients are unable to eradicate the bacterium suggesting that *H. pylori* may have evolved a means to influence the host immune response. Failure to clear infection leads to the development of chronic gastritis, the platform for a range of gastric pathologies including gastric cancer. *H. pylori* infection is characterised by a local inflammatory infiltrate composed of macrophages and neutrophils. The secretion of IL-8 in response to the presence of the pathogen by the gastric epithelium (Crabtree *et al.*, 1993) is crucial for the host response to infection as this cytokine serves as a chemotactic factor for neutrophils, expanding the inflammatory cascade **[Figure 2]**.

1.5.2 The Th1/Th2 Response and *H. pylori* Infection

The cytokine response of the host remains mixed until stimulated by polarising signals from the microenvironment. This results in the differentiation of the response to either the

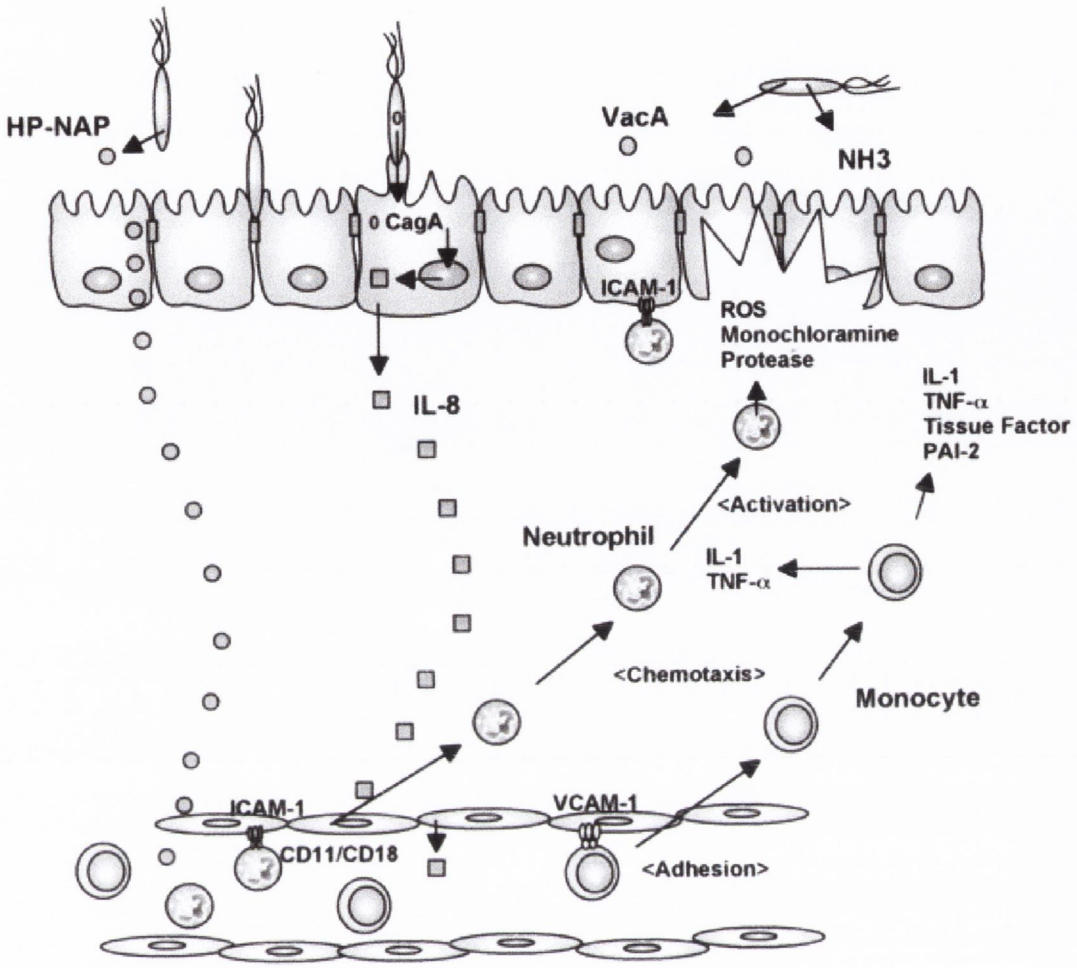


Fig. 2. Adhesion molecules, cytokine, and chemical mediators in leukocyte-endothelial cell interactions, and inflammation-associated virulence factors for gastric mucosal cells. CagA: cytotoxin association gene A protein; HP-NAP: *Helicobacter pylori*-neutrophil activating protein; ICAM-1: intracellular adhesion molecule 1; IL-1: interleukin 1; IL-8: interleukin 8; PAI-2: plasminogen activator-2; ROS: reactive oxygen species; TNF-α: tumor necrosis factor-α; VacA: vacuolating cytotoxin gene A protein; VCAM-1: vascular cell adhesion molecule 1. (Reproduced from Naito and Yoshikawa, 2002)

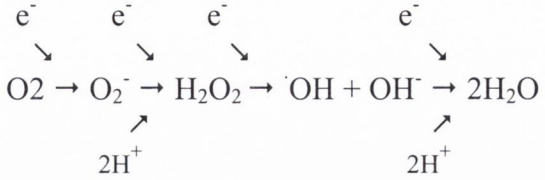
Th1 or Th2 pathway. Recently, it has become clear that the intestinal mucosa has a unique subset of CD4+ T cells that secrete TGF-beta (Th3 cells) that provide help for IgA (Weiner, 2001). These cells have downregulatory properties for Th1 cells and other immune cells and therefore play an important role in the active suppression of oral tolerance and IgE response. Mucosal and systemic T-helper cell responses to *H. pylori* are predominantly of the Th1 type, characterised by a predominance of interferon (IFN)-γ producing $CD4^+$ T-cells and resultant low IgG1 and high IgG2a levels (Fan *et al.*, 1994; Mohammadi *et al.*, 1996; D'Elios *et al.*, 1997). It has been shown that gastric T-cells produce IFN-γ, IL-2 and low levels of Th2 cytokines, IL-4 and IL-5 (Bamford *et al.*, 1998) and this observation suggests that a deficiency of Th2 response by the host results in an inability to initiate bacterial clearance. Although *H. pylori* can induce a specific cellular and humoral immune response, it is capable of suppressing the immune response and this suppression of the host immune mechanism is postulated to contribute to disease pathogenesis (Knipp *et al.*, 1993). Due to its location in the gastric tissue *H. pylori* is exposed to an environment rich in inflammatory infiltrates and reactive oxygen species (ROS) as a consequence of the host immune response to infection. *H. pylori* induces monocytes and polymorphs to release reactive oxygen species *in vitro*, and in the gastric and duodenal mucosa of infected patients (Drake *et al.*, 1998; Nielsen and Anderson, 1992) Host responses and pathogen induced alterations to the gastric environment combined with deficiencies in host antioxidants such as glutathione (GSH) and antioxidant proteins (*e.g.* gastric peroxidase) encourages the persistence of damaging oxidative and carcinogenic agents. In addition, *H. pylori* secretes O_2 thus providing a direct role for the bacterium in modulating the gastric oxidative environment.

1.6 OXIDATIVE STRESS AND THE GASTRIC NICHE

1.6.1 Reactive Oxygen Species

Molecular oxygen (O_2) has an even number of electrons, but of these, two unpaired electrons exist in its molecular orbitals. This molecule is generally unreactive with other compounds except radicals, therefore oxidation of molecular oxygen is limited to the acceptance of electrons and when this occurs the reactivity of the molecule increases. With the gain of one, two or three electrons, formation of the superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) respectively, can result. If conditions are very acidic the hydroperoxyl radical ($HOO^{\cdot}$) can form. The stepwise reduction of

molecular oxygen via 1-electron transfers, producing and connecting the aforementioned oxygen species can be summarised in the following reaction:



O_2^- is capable of oxidising thiols, ascorbate, tocopherol and catecholamines (Hill, 1978; Machino and Fridovich, 1987) and can attack proteins containing $(Fe\text{-}S)_4$ clusters (Gardner and Fridovich, 1991). One of the most relevant reactions of O_2^- is its spontaneous dismutation to H_2O_2 and O_2, and in a neutral aqueous environment it spontaneously dismutates to form H_2O_2 (Fridovich, 1989). The ability to reduce transition metals and transition metal complexes is also a feature of H_2O_2. *In vivo*, these transition metals are generally Fe^{3+} and Cu^{2+} although other transition metals may also be reduced (Brunori and Rotilio, 1984). H_2O_2 also reacts with organic molecules however, the exact nature of these reactions remain unresolved as hydrogen peroxide rapidly reacts with contaminating metals to form more reactive species and these by-products obscure the role of H_2O_2 in oxidation reactions. Most significantly, the reaction of H_2O_2 with reduced iron or copper ions results in the formation of hydroxyl radicals ($OH^{\cdot}$) in the Fenton reaction (Cadenas, 1989). $OH^{\cdot}$ is highly reactive and will react with almost all biomolecules at diffusion-limited rates (Singh and Singh, 1982).

Reactive oxygen species are generated from a variety of source including various compounds and enzymes. The four-electron reduction of molecular oxygen to water is a sequential process enzymatically catalysed by the membrane-associated enzymes of the respiratory chain. In *E. coli* it has been demonstrated that the major sources of O_2^- include the autoxidation of NADH dehydrogenase, succinate dehydrogenase, and D-lactate dehydrogenase (Imlay and Fridovich, 1991). An additional enzyme proposed to produce significant amounts of O_2^- is glutathione reductase, which uses NADH as an electron source (Imlay and Fridovich, 1991). The generation of O_2^- may also occur non-enzymatically by the autoxidation of several cellular components, among them, catechols, thiols, ubiquinols and flavins. Sources of H_2O_2 include both spontaneous and superoxide

dismutase (SOD)-catalysed dismutation of O_2^-, and also several oxidases such as D-amino acid oxidase. Sources of OH^- are numerous including tryptophan residue photo-absorbtion. It is apparent that an abundance of reactive oxygen species occur naturally in cells, both intracellularly and extracellularly, and as these species are highly reactive, cells must maintain a strong defence against damage arising from these sources if they are to survive.

1.6.2 Reactive Oxygen Species and the Inflammatory Response

Reactive oxygen species (ROS) are formed and degraded by all aerobic organisms as an adaptation to the evolution of molecular oxygen. In excessive quantities, ROS lead to a state of oxidative stress which is deleterious to normal growth and survival. ROS readily react with a vast array of biomolecules and this leads to a chain reaction event, the formation of free radicals. Intracellular free radicals are low molecular weight molecules with an unpaired electron, and the terms ROS and free radical can be used interchangeably. The termination of a ROS-initiated chain reaction within a cell is dependent on the elimination of unpaired electrons, and for this event to occur, a free radical must interact with an additional free radical or with a free radical scavenger. Free radical scavengers are primary antioxidants which can act as a final electron acceptor or electron sink. Despite adverse affects on biomolecules which will be reviewed subsequently, ROS serve as an important facet of cellular defence against infection and therefore ROS may be seen to have a physiological role, defence of the host against invading bacterial pathogens. When phagocytes are activated during the inflammatory response, these cells can produce ROS in sufficient amounts to kill intruding microorganisms (Thomas *et al.*, 1988). *H. pylori* upon colonisation of the gastric environment secretes immunogenic products which recruit macrophages and polymorphonuclear leukocytes to the site of infection (Rieder *et al.*, 1997) .

ROS, due to their extremely high reactivity have the capacity to induce damage to biomolecules, which may influence protein function. In addition, ROI are potentially toxic have been shown to be mutagenic (Marnett, 2000; Mates *et al.*, 1999), an effect that is primarily orchestrated through chemical modification of DNA. DNA damage can occur through base modification, for example, purine oxidation, DNA cleavage or DNA-protein cross-linking. The effects of oxidative stress on membranes and proteins are numerous and varied.

1.6.3 Reactive Oxygen Species-Induced Membrane and Protein Damage

Oxidative damage to membranes can occur through either lipid or membrane protein modification, and lipid peroxidation constitutes one of the most intensively investigated areas of ROS induced damage (Ylä-Herttuala, 1999; Steinberg, 1997). Peroxidation of lipids by oxidative stress has been demonstrated *in vivo* and *in vitro*. In both systems, lipid peroxidation can be initiated by singlet oxygen, the hydroxyl radical and the hydroperoxyl radical, all of which are formed during periods of oxidative stress. The membrane constituent, polyunsaturated fatty acids, are the most susceptible targets of free radical attacks, due to their multiple double bonds. The process of lipid peroxidation occurs in three stages, initiation, propagation and termination, and this chain reaction probably begins with the abstraction of a hydrogen from a polyunsaturated fatty acid to form a lipid radical ($L^{\cdot}$). This moiety reacts with molecular oxygen to form a lipid peroxyl radical ($ROO^{\cdot}$), which proceeds to attack additional polyunsaturated fatty acids present in the lipid membrane to form a fatty acid hydroperoxide (ROOH). The fatty acid hydroperoxides eventually break down into lipid peroxyl radicals which can initiate new rounds of peroxidation causing further damage to the membrane structural integrity (Kappus, 1985). The end products of lipid peroxidation include ketones, alkanes, aldehydes and epoxides (Benedetti *et al.*, 1984; Kappus, 1985) and these products and their intermediates have been shown to be mutagenic. End products such as epoxides and aldehydes are capable of reacting directly with DNA causing intra- and interstrand crosslinks (Summerfield and Tappel, 1983) and base alkylation (Segerback, 1983). As a result of the extremely damaging consequences of ROS exposure during an inflammatory response, bacteria have evolved mechanisms to defend against this threat, including the alteration of the lipid contents of their cell membranes. As the rate of lipid peroxidation is directly proportional to the number of unsaturated carbon double bonds, bacteria generally have saturated or monounsaturated fatty acids present in their membranes (Kappus, 1985). However, despite these physiological adaptations to defend against lipid peroxidation and subsequent membrane damage, bacteria are still vulnerable to oxidative lipid damage. Oxidative stress by virtue of protein damage can result in the generation of modified and partially inactivated cellular enzymes and also denatured, non-functional proteins if damage is irreversible. ROS have been shown to interact with several amino acid residues *in vitro* (Butterfield *et al.*, 1998). Interactions between proteins and oxygen radicals can lead to the conversion of arginine and proline residues into carbonyl derivatives. Oxidation of methionine and cysteine residues forms methionine sulfoxide derivatives and disulphide

derivatives, respectively (Amici *et al.*, 1989). These modifications can result in the formation of inactive enzymes or indeed to the targeted degradation of such enzymes. Many bacterial enzymes are sensitive to oxidative damage and for metalloenzymes, the $(Fe\text{-}S)_4$ centres appear to be the target of superoxide radical attack (Gardner and Fridovich, 1991). This metalloenzyme family represents a group of enzymes that seem to be particularly susceptible to reactive oxygen species.

1.6.4 Cellular Antioxidant Enzyme Systems

Defences against the damaging effects of reactive oxygen species can be divided into two broad classes, preventative and reparative, and the major cellular antioxidant systems can be divided into two major groups, enzymatic and non-enzymatic. The enzymatic components can directly scavenge ROS or alternately may produce non-enzymatic antioxidants. In certain circumstances, the basal scavenging capacity of the cell is overwhelmed by the concentration of ROS, which gives rise to an oxidative stress condition. To survive this outcome, prokaryotic and eukaryotic cells are equipped with a variety of strong defences against ROS threats, including superoxide dismutases (SODs) which dismutate O_2^- to H_2O_2, catalases which transform H_2O_2 into H_2O and O_2, and alkylhydroperoxidases, which are capable of reducing certain toxic organic hydroperoxides such as *tert*-butyl hydroperoxide, cumene hydroperoxide and lipid hydroperoxides. Under experimental conditions, the condition of oxidative stress can be artificially induced by the addition of hydrogen peroxide or superoxide radical generators to a growing culture. In addition to chemical induction of oxidative stress, mutagenesis of genes involved in the oxidative stress response can also occur under these conditions. In *E. coli*, SOD mutants have been constructed in which the endogenous level of O_2^- is elevated 2.5×10^3-fold over the endogenous level of O_2^- in wild-type cells (Carlioz and Touati, 1986). The above mentioned enzymes are ubiquitous in bacteria and provide the prokaryotic cells with the enzymatic machinery vital for detoxification of ROS.

1.6.5 Superoxide dimutase and Catalase

Superoxide dismutases (SOD) were the first ROS-metabolising enzymes described (McCord and Fridovich, 1969) and there are three types of SOD based on the metal ligands bound, copper, iron or manganese. These transition metals facilitate electron transfer by the enzyme and the enzyme itself is encoded by the *sodA* and *sodB* genes. Iron binding SOD is the most common SOD found in bacterial species with the other isoenzymes either

rarely or never represented in prokaryotes. The presence of SOD in the cell facilitates the reduction of steady-state concentration of oxygen by approximately three orders of magnitude (Steinman, 1982). A novel type of superoxide scavenging enzyme has recently been described which can catalyse the direct reduction of superoxide but thus far has only been identified in a limited number of bacterial species (Lombard *et al.*, 2000; Jenney *et al.*, 1999; Lombard *et al.*, 2000). Although SODs are responsible for the dismutation of the toxic superoxide radical, the production of hydrogen peroxide as a by-product of this reaction does not reduce the potential of oxidative damage within the cell to proteins or membranes as H_2O_2 is also toxic by virtue of its radical forming role via the transition metal-catalysed Fenton reaction (Fridovich, 1999). The heme-containing enzyme catalase (Aebi, 1974) lowers the risk of this secondary damage by H_2O_2 dismutation, one of its primary antioxidant roles within the cell in addition to detoxification of substrates such as phenols and alcohols through the coupled reduction of H_2O_2 (Halliwell, 1999). The catalytic activity of catalase is extremely efficient and these enzymes can eliminate H_2O_2 from the cell with extraordinary rapidity, 6 million molecules of H_2O_2 converted to water and O2/min/molecule catalase, and the enzyme does not have a requirement for ATP. Catalase binds NADPH which provides protection from inactivation and increases catalytic efficiency (Kirkman and Gaetani, 1984; Kirkman *et al.*, 1999).

1.6.6 Peroxidases

Peroxidases constitute another enzymatic member of the oxidative stress defence repertoire. The cellular function of this class of enzymes is the destruction of H_2O_2. However, peroxidases are dependent on the supply of reducing power from either NADH or NADPH. Glutathione (GSH) is an abundant intracellular antioxidant, present in millimolar concentrations in all living aerobic cells and functions in the maintenance of a strong reducing environment within the cell. In addition to acting as a sulphydryl buffer, glutathione can facilitate detoxification of ROS through one of two mechanisms, via conjugation reactions catalysed by glutathione S-transferases (Armstrong, 1997) or directly through glutathione peroxidase (GPx)-catalysed reactions with H_2O_2. GSH will react with H_2O_2, O_2^- or $HOO^{\cdot}$ to form a stable glutathione radical ($GS^{\cdot}$) which dimerises with another $GS^{\cdot}$ to form oxidised glutathione (GSSG). Finally, glutathione reductase (GR) will transfer an electron from NADPH to GSSG to re-form reduced GSH (Meister and Anderson, 1983). Glutathione peroxidases can catalyse the reduction of H_2O_2 using GSH as a substrate, thus constituting an additional important defence against the deleterious effects

of H_2O_2 on membranes, proteins and nucleic acid. Glutathione peroxidases can also reduce additional peroxides to alcohols. Another enzyme that has an important protective function is alkylhydroperoxide reductase (Ahp) which is capable of reducing organic hyroperoxides within the cell (Jacobson *et al.*, 1989). Peroxiredoxins, enzymes capable of directly reducing various alkylhydroperoxides and other peroxides, such as H_2O_2, have been described in the literature (Chae *et al.*, 1999(a); Chae *et al.*, 1999(b); Kim *et al.*, 1988). The non-enzymatic components of cellular antioxidant systems constitute a wide array of low molecular weight compounds which are of importance in ROS defence. These compounds include selenium compounds, ascorbic acid, α-tocopherol, R-α-lipoic acid, ubiquinones and menaquinones and function through interactions with other cellular reducing systems. Thus, members of the cellular antioxidant system each play a crucial role as contributors to a cells total protection against oxidative damage.

1.6.7 Regulation of Cellular Responses to Oxidative Stress

Catalase, superoxide dismutase and glutathione represent antioxidant defences that are present in all aerobic organisms. These enzymatic and non-enzymatic systems function in the prevention of oxidative damage to cellular membranes, proteins and genetic material. Key to the success of cellular antioxidant defence is the ability of enzymatic systems to adjust and respond to the continuous flow of environmental cues that organisms receive. The coordinate expression of multiple defence functions can be described as an oxidative stress response, and the best characterised oxidative stress response is from *E. coli*. In this organism, the stress response is controlled by the OxyR and SoxR transcriptional activators (Hidalgo and Demple, 1996). The OxyR system is activated by the presence of H_2O_2, and in response to an elevated flux of H_2O_2, the OxyR protein activates transcription of the OxyR regulon genes. The SoxR system is activated in the presence of superoxide generating compounds, and the gene products of the *SoxR* gene and *SoxS* gene are essential for the inducibility of the SoxR regulon genes (Wu and Weiss, 1991). When these systems are activated by the appropriate cellular conditions, cellular resistance to oxidative stress is greatly increased (Hidalgo and Demple, 1996). The transcriptional activators OxyR and SoxR are present in a latent form in cells which are not experiencing oxidative stress, where they await activation if ROS exceed critical levels. When activated, OxyR induces in excess of ten genes in response to H_2O_2 (Storz and Imlay, 1999; Christman *et al.*, 1985), including *oxyS* (a small, non-translated regulatory RNA), *katG* (hydroperoxidase I), *aphC* (alkylhydroperoxide reductase), *gorA* (glutathione reductase) and *grxA* (glutaredoxin).

Activated SoxR induces soxS expression, generating additional SoxS protein which activates various target genes such as *sodA* (Mn-SOD), *fpr* (ferredoxin:NADPH oxidoreductase) and *fumC* (fumerase) (Storz and Imlay, 1999; Koh *et al.*, 1996). In certain microorganisms, OxyR homologues appear to be absent, and proteins such as PerR, a homologue of the ferric uptake regulator, Fur, of enteric bacteria. These homologues can act as peroxide sensors probably mediated via metal catalysed oxidation of the protein or a change in the oxidation state of a bound metal ion (Bsat *et al.*, 1998). The biochemical signalling events which lead to the activation of these proteins have been a focus of intense investigation for some time. The mode of activation of SoxR has been identified and the protein appears to undergo reversible one-electron oxidation of its iron-sulphur centres (Hidalgo *et al.*, 1997; Gaudu *et al.*, 1997). More recently, the biochemical events leading to activation of OxyR were described. OxyR appears to be reversibly activated by the formation of an intramolecular disulphide and this activation is balanced by reduction through the thiol donors, glutaredoxin and GSH (Zheng *et al.*, 2000).

1.6.8 Sensing Stress by Disulphide Bond Formation

A major factor in the response to a changing external environment is the ability to regulate protein activity. The investigation of protein regulation has lead to the characterisation of several mechanisms which function in this area including phosphorylation, ligand-binding leading to allosteric changes and protein-protein interactions. However, another mechanism, disulphide bond formation, has been widely postulated as a covalent modification that results in the regulation of protein activity. Not new, the concept of regulation of enzyme activity by thiol-disulphide exchange was originally proposed over 30 years ago and the importance of this mechanism is only beginning to be understood.

When bacteria encounter environments with high levels of hydrogen peroxide and other reactive oxygen species, they encounter the deleterious effects of oxidative stress. A key event that results in the exposure of a bacterium to this type of stress is during the formation of oxidative species by host immune cells during an inflammatory response. As discussed previously, ROS can induce membrane, protein and DNA damage. Microorganisms can respond to the stress that unwanted disulfide bond formation in cytosolic molecules causes in two ways, by enzymatic elimination of the oxidant and by the restoration of redox homeostasis. Recently, the importance of disulphide bond formation was underscored by observations that the activation of the important prokaryotic

oxidative stress responsive transcriptional activator, OxyR, was dependent on this regulatory event (Zheng *et al.*, 1999), thus providing the first well characterised example of oxidative stress response activation by disulphide bond formation. Activation of OxyR induces peroxidase expression and oxidant clearance from the cytosol. Moreover, the expression of cellular disulphide reductants is induced thus contributing to the restoration of the normal reducing conditions which exist within the 'unstressed' cell. The induced reductants contribute also to the deactivation of OxyR by virtue of disulphide bond reduction within the protein.

Thus, the mechanism of disulphide bond formation is a key signal in the response of an organism to oxidative stress. Cellular disulphide reducing systems are vital in the maintenance of redox homeostasis within the cell which facilitates the regulation of protein activity through disulphide bond formation. In addition to the glutaredoxin-glutathione pathway, one of the most important systems involved in maintaining the reducing thiol-disulphide status of the cytosol is the thioredoxin system (Prinz *et al.*, 1997).

1.7 THE THIOREDOXIN SYSTEM

1.7.1 Disulphide Bond Formation

1.7.1.1 Chemistry of Disulphide Bond Formation

The formation of disulphide bonds between correct pairs of cysteine residues is vital for the folding and stability of many proteins. Disulphide bond formation can occur in one of two ways, either by enzymatic catalysis (Givol *et al.*, 1965; Katzen and Tietze, 1966; Freedman and Hawkins, 1977) or alternately by uncatalysed thiol-disulphide exchange (Wetlaufer *et al.*, 1977; Saxena and Wetlaufer, 1970). The formation of disulphide bonds between cysteine residues is a posttranslational modification that is highly dependent on a proteins' conformation. For a disulphide bond to be formed between two cysteine residues, the cysteine residues must have their α carbon atoms at a distance of 4 to 9 Å of each other and the adjoining peptide backbones must be in the correct orientation with respect to the side-chain atoms (Richardson, 1981; Thornton, 1981). The *in vivo* mechanism of protein disulphide bond formation is less well understood and the majority of information regarding this chemical process has arisen from *in vitro* studies of bond reduction and re-formation in intact proteins. Investigations have revealed that which Cys residues are linked by disulphide bonds depends on both protein conformation and the redox potential

of the environment (Creighton, 1978). Once disulphides are formed, the physical conformation that facilitated the bonding reaction is stabilised, thus the stability of disulphide bonds and protein conformation are closely linked and interdependent. Spontaneous formation of disulphide bonds cannot occur in the absence of an appropriate electron acceptor (A) as in the following reaction, where 2-SH represents 2 free thiol groups:

$$2\ \text{-SH} + A \leftrightarrows \text{-S-S-} + AH_2$$

The range of acceptors which can be utilised is wide including the classical oxidant, O2, but the *in vivo* donor and acceptor is the most relevant to study disulphide bond formation. Glutathione fulfils this role as it interacts chemically with all thiols and disulphides and is present in millimolar concentrations in most cells. The rate of the disulphide reaction is dependent on a number of factors including ionic strength, temperature, the nature of the groups attached to the sulphur atoms and pH (Shaked *et al.*, 1980), in addition to the electrostatic environment. When a disulphide reagent is added to a reduced protein, two sequential thiol-disulphide exchange reactions are required to form one protein disulphide, the first of which is a simple chemical reaction between one Cys thiol group and the disulphide reagent, to generate the mixed disulphide. The second step involves the reaction of the second Cys thiol group with the mixed disulphide to form the protein disulphide. The rate of formation of such a protein disulphide is determined by the reaction rate of the first step. Trapping of species of the multi-step process of disulphide bond formation is possible by a number of mechanisms including, protonation of thiol groups by acidification (acid-trapping), reaction of thiols with iodoacetic acid or iodoacetamide or by the use of other thiol blocking agents such as N-ethylmaleimide. These approaches allow the many intermediates of the pathway to be examined as distinct chemical moieties facilitating elucidation of the process and kinetics of disulphide bond formation. Understanding this pathway is key to understanding the thiol-oxidoreductase enzymes that catalyse the process of bond formation.

1.7.1.2 Disulphide Bond Reducing Pathways in the Cytoplasm

Disulphide bonds play a number of roles in protein structure and activity. The role of disulphide bonds in protein function may be categorised into four distinct classes. Firstly, disulphide bonds can act as enhancers of protein stability (e.g. β-lactamase), and indeed their formation may be intrinsically linked to the folding pathway for a particular protein

(e.g. bovine pancreatic trypsin inhibitor) (Creighton, 1997; Darby *et al.*, 1995; Schultz *et al.*, 1987). Secondly, those proteins whose enzymatic activity requires the formation of a covalent bond between two cysteines, i.e. reductases, and this bond comprises a necessary feature of their catalytic cycle (e.g. ribonucleotide reductase) (Laurent *et al.*, 1964). Thirdly, proteins which have a regulatory role and can be reversibly activated by the formation of a disulphide bond in response to environmental conditions (e.g. OxyR) (Zheng *et al.*, 1998). Fourthly, a recently described group of proteins which although disulphide bonds do not form part of the final protein, these moieties are necessary for the promotion of the correct folding pathway of such proteins (Robinson and King, 1997). The formation of stable disulphide bonds occurs within a cellular compartment such as the cell envelope in Gram negative prokaryotes (Bardwell, 1994), and in eukaryotes where cells are highly compartmentalised, this activity occurs within the endoplasmic reticulum (Hwang *et al.*, 1992). As the cytoplasmic compartment of both prokaryotes and eukaryotes is a reducing environment, cytosolic proteins have evolved to maintain their cysteines reduced in the native form (Derman and Beckwith, 1991). In contrast cellular proteins destined for export have evolved to be more stable when appropriate cysteines are joined in disulphide bonds. Although formation of disulphide bonds in a protein can occur spontaneously, the rate of disulphide bond formation in this way is several fold slower than the rate of formation *in vivo* (Saxena and Wetlaufer, 1970). Hence, the rate of formation *in vivo* must be subject to enzymatic catalysis. Investigations have revealed that disulphide bond reduction, formation and isomerisation *in vivo* are indeed catalysed processes (Anfinsen, 1973).

The enzymatic catalysts involved belong almost exclusively to the thioredoxin superfamily, and all retain features of the superfamily prototype, thioredoxin, in particular the active site motif, Cys-X_1-X_2-Cys (where X denotes any amino acid), and a thioredoxin-like fold (Martin *et al.*, 1993). The most comprehensive investigations into disulphide bond reducing pathways in prokaryotes have been undertaken in *E. coli*, where the thiol reducing activity of the cytoplasm is maintained by the concerted actions of two thiol reducing systems, the glutaredoxin system and the thioredoxin system.

1.7.2 Thioredoxin; A Thiol-Oxidoreductase

1.7.2.1 Thioredoxin

Thioredoxin is a small (M_r 12 000), highly thermostable, ubiquitous protein which is characterised by the presence of a redox active disulfide/dithiol within a highly conserved active site sequence, -Cys-Gly-Pro-Cys- (Holmgren, 1985; Holmgren, 1989; Gleason and Holmgren, 1988; Eklund *et al.*, 1991). Thioredoxin exists in either of two states, reduced (Trx-$(SH)_2$), where thioredoxin has a dithiol moiety or oxidised (Trx-S_2), with a disulphide moiety. The oxidised form of the protein comprises of half-cysteine residues joined by an intramolecular disulphide bridge, and therefore has the ability to participate in various redox reactions through the reversible oxidation of the active centre dithiol. Thioredoxin and the associated flavoenzyme thioredoxin reductase constitute a powerful thiol-dependent reduction-oxidation system that can catalyse the reduction of certain disulphides by NADPH. Thioredoxin was first described in the scientific literature nearly forty years ago when it was originally purified from *E. coli* as a hydrogen donor cofactor in the biosynthesis of deoxyribonucleotides by the essential enzyme ribonucleotide reductase (Laurent *et al.*, 1964). The name thioredoxin was assigned to the small protein as its function was dependent on the cyclic reduction-oxidation of a single -S-S- group (Laurent *et al.*, 1964).

This enzyme is a true example of a multifunctional protein, which has a role in many vital biological phenomena, which span fields of interest as diverse as virology and photosynthesis. Accumulating evidence from multiple cell types implicates a central role for thioredoxin in cell division, radical scavenging, transcription, DNA replication, detoxification, embryogenesis, protein folding, intracellular signalling, chemotaxis, gene transcription, regulation of enzyme activity, regulation of photosynthetic activity (Calvin cycle) and maintenance of the intracellular redox balance (Arner and Holmgren, 2000). The secreted form of mammalian thioredoxin 1 has recently been classified as a redox proto-oncogene due to its association with some solid human tumors where it displays mitogenic activity (Pekkari *et al.*, 2003). The importance of this enzyme is also underscored by its ubiquitous nature, being present in almost all prokaryotes and eukaryotes (Holmgren, 1985; Holmgren, 1989; Gleason and Holmgren, 1988; Eklund *et al.*, 1991). However, the most thoroughly investigated and characterised thioredoxin comes from the organism in which it was first discovered, *E. coli*. Since then, thioredoxins from many prokaryotes have been isolated and both functionally and structurally characterized.

In *E. coli*, thioredoxin is necessary for the assembly of filamentous bacteriophages and the replication of T7 bacteriophage but is not essential for DNA synthesis and growth (Collet and Bardwell, 2002; Debarbieux and Beckwith, 1999; Aslund and Beckwith, 1999). Of note, *E. coli* thioredoxin can also act as an oxidant *in vivo*, promoting the formation of disulfide bonds in the cytoplasm, suggesting that thioredoxins may possess dual redox activities (Stewart *et al.*, 1998). Most recently, it has been shown that *E. coli* thioredoxin (and thioredoxin reductase) can function as a molecular chaperone in addition to its thiol-disulfide reductase activity (Kern *et al.*, 2003).

1.7.2.2 Catalytic Mechanism

The oxidized form of thioredoxin contains a disulfide bridge (-S-S-) that is reduced to two sulphydryls (-SH) by NADPH via the FAD containing thioredoxin reductase. This is known as the thioredoxin system and it is composed of three components, thioredoxin, NADPH and thioredoxin redustase. The reduced form of thioredoxin is a very efficient catalyst for the reduction of disulfide bonds in target proteins (i). Reducing equivalents are transferred to a disulfide target ($X\text{-}S_2$). Oxidized thioredoxin is then regenerated to the reduced form in the presence of thioredoxin reductase (ii):

$$\text{thioredoxin-}(SH)_2 + \text{protein-}S_2 \xrightarrow{\text{spontaneous}} \text{thioredoxin-}S_2 + \text{protein-}(SH)_2 \quad \textbf{(i)}$$

$$\text{thioredoxin-}S_2 + NADPH + H^+ \xleftrightarrow{\text{thioredoxin reductase}} \text{thioredoxin-}(SH)_2 + NADP^+ \quad \textbf{(ii)}$$

The first reaction is spontaneous and therefore thioredoxin itself can be regarded as an enzyme (Holmgren, 1979). The redox potential (E'_0 value) of H^+ + NADPH/$NADP_+$ at pH 7.0 and 25°C is –325 mV, with the redox potential of *E. coli* thioredoxin ($Trx\text{-}(SH)_2$/$Trx\text{-}S_2$) determined as –270 mV (Holmgren, 1985; Gleason and Holmgren, 1988) which makes *E. coli* thioredoxin one of the most reducing proteins known. Reaction (ii) is fully reversible and may be used to determine the E'_0 value of a particular thioredoxin.

1.7.2.3 Thioredoxin Structure and Structure/Function Relationships

In its capacity as a thiol-oxidoreductase, thioredoxin can interact with a wide range of proteins in order to facilitate electron transport in substrate reduction or regulation of activity by virtue of thiol-redox control (Holmgren, 1985). These operations rely on the

seemingly simple mechanism of reversible oxidation of half cysteine residues to a disulphide with the concomitant transfer of two protons and two electrons. The final result of this chemical sequence is the conversion of a disulphide to a dithiol. The correlation of this catalytic process to the structure of the thioredoxin molecule has been a focus of investigation since the elucidation of the amino acid sequence of *E. coli* thioredoxin 35 years ago (Holmgren, 1986). The *E. coli* enzyme was shown to be a polypeptide of 108 amino acids with the active site sequence, -Trp-Cys-Gly-Pro-Gly-, which is now the consensus active site sequence for thioredoxin. The objective of observing the three-dimensional structures of both reduced and oxidised thioredoxin has been realised, with the high resolution structures of *E. coli* and human thioredoxins being reported in the literature (Qin *et al.*, 1994; Jeng and Dyson, 1994). The first three-dimensional thioredoxin structure was obtained for oxidised *E. coli* thioredoxin in 1975 (Holmgren *et al.*, 1975). X-ray crystallography data revealed that the protein was a compact globular protein, possessing a high degree of secondary structure with five β-strands surrounded by four α-helices. The active site disulphide was shown to be located on a unique protrusion at the C-terminal end of a β-strand (β_2) followed by a long α-helix. The current structure of *E. coli* thioredoxin has been refined to a level of 1.7 Å resolution (Katti *et al.*, 1990) **[Figure 3]**. Examination of the structure of thioredoxin has lead to a proposed mechanism of enzyme/substrate interaction (Kallis and Holmgren, 1980), which encompasses several concepts. The surface of reduced thioredoxin has a hydrophobic surface area where binding to the substrate protein occurs to form a enzyme/substrate complex, and within this complex a hydrophobic environment is established. The thiolate of Cys32, which is acting as a nucleophile, attacks the target protein to form a covalently-linked mixed disulphide transition state. The Cys32 residue becomes deprotonated, and this chemical moiety then undergoes nucleophilic attack by Cys35 resulting in the formation of a disulphide in thioredoxin and the generation of a dithiol in the target protein **[Figure 4]**. All of the electron-transfer steps and target protein binding are facilitated by conformational changes in thioredoxin itself and the target protein.

It appears that for *E. coli* thioredoxin the structures of the oxidised and reduced form of the protein are very similar, with identical backbones except in the vicinity of the redox active site. High resolution NMR studies revealed that the side chain of the Cys32 residue is tilted towards the solution in the reduced form. This is postulated to facilitate the increase in sulphur-sulphur distance upon the formation of the two sulphydryl groups (Dyson *et al.*,

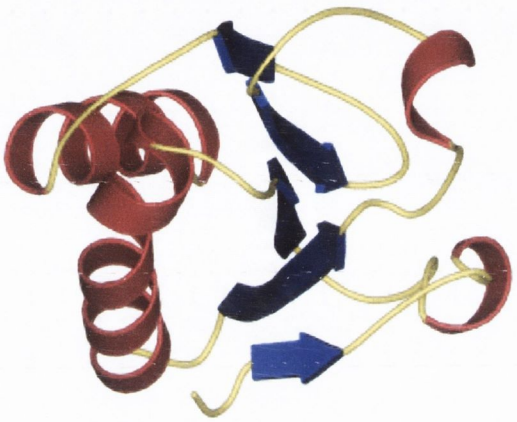


Fig. 3. Cartoon drawing of *E. coli* thioredoxin (PDB code: 2TRX). Schematic shows the conserved β-strands (blue) surrounded by α-helices (red).

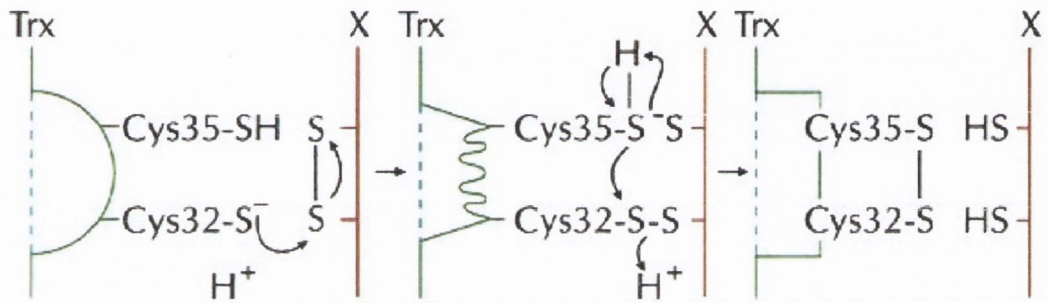


Fig. 4. Proposed mechanism of thioredoxin-catalysed protein disulfide reduction. Reduced Trx (Trx-SH_2) binds to a target protein via its hydrophobic surface area. Nucleophilic attack by the thiolate of oxidised cysteine results in the formation of a transient mixed disulfide, which is followed by the nucleophilic attack of the deprotonated thiol generating Trx-S2 and the reduced protein. This reaction cycle results in conformational changes in both Trx and the target protein. (Reproduced from Martin, 1995)

1989). Other changes can be observed in the hydrogen bonding patterns of the two forms of the enzyme. The hydrophobic surface of thioredoxin is also postulated to play an important role in determining target specificity (Huber *et al.*, 1986). The aim of further investigations into structure/function relationships for thioredoxin appears to centre on whether the differences in function of the two oxidation states, which despite highly similar active site surfaces, are induced by local conformational flexibility around the disulphide loop (Stone *et al.*, 1993). Indeed, a higher rate of mobility has been observed for the reduced form of thioredoxin. Despite several differences, the active site surfaces of *E. coli* and eukaryotic thioredoxins are highly conserved, this being demonstrated by the very similar kinetics of redox activity observed for both thioredoxins. The investigation of thioredoxin/target protein interactions constitutes the main focus of study for this protein at present, and it is becoming apparent that thioredoxin can exploit a chaperone-like mechanism of conformational changes to interact with and bind a very diverse range of proteins to promote disulphide reduction.

1.7.2.4 Non-Classical Thioredoxins

As a rule the active site sequence -Trp-Cys-Gly-Pro-Cys- is highly conserved in thioredoxins from both prokaryotes and eukaryotes. However, this consensus active site sequence can be modified in certain thioredoxins. Thioredoxins from anaerobic, amino acid utilising bacteria have been shown to possess a modified consensus sequence at the active site (Harms *et al.*, 1998). The thioredoxins of such bacteria did not react with thioredoxin reductase of *E. coli* and lead the authors to suggest that these thioredoxins might form an evolutionary separate group. Other investigations from a structural standpoint have revealed further examples of deviation from the consensus active site sequence. A study by Ren *et al.* (1998) looked at the crystal structure of an archael disulphide oxidoreductase with an unusual active site motif. Interestingly, all members of this putative group of archaebacterial thioredoxins have unique -C-X-X-C- motifs. It seems that this evolutionary ancient group appear to have relaxed their requirement for an absolutely conserved sequence -Trp-Cys-Gly-Pro-Cys- active site for redox function. Unusual active site thioredoxin variants are also found in eubacteria. *H. pylori* possess two thioredoxins, one of which harbours an unusual redox active site sequence (Tomb *et al.*, 1997). The relevance of such sequence deviations in terms of structure/function relationships for this enzyme constitutes a key focus of current investigations.

1.7.2.5 Thioredoxin Reductase

Thioredoxin reductase is a flavoprotein which catalyses the reduction of thioredoxin by NADPH (Moore *et al.*, 1964; Williams, 1995), and is a member of a family of dimeric enzymes that catalyse the transfer of electrons between pyridine nucleotides and disulphide/dithiol compounds. Members of this family also promote catalysis via FAD (flavin adenine dinucleotide) and a redox active disulfide (Dai *et al.*, 1996; Williams, 1992; Brown *et al.*, 1996; Ben-Menachem *et al.*, 1997; Bult *et al.*, 1996), and includes proteins such as glutathione reductase and lipoamide dehydrogenase. However, thioredoxin reductase is unusual from an evolutionary standpoint, in that the structure and mechanism of the enzyme changes depending on the source of the enzyme. Thioredoxin reductase represents an enzyme where the same reaction is catalysed by more than one structure and mechanism (Arscott *et al.*, 1997; Luthman and Holmgren, 1982), and therefore has been categorised into two classes since this feature was first observed (Holmgren, 1989). *E. coli* thioredoxin reductase typifies members of the first class, the low molecular weight type and the second class is designated the high molecular weight type and this type is predominantly eukaryotic in origin, found in mammals, *Drosophila melanogaster* and *Plasmodium falciparum*. Members of the low molecular weight type are also found in plants and fungi. The low molecular weight type thioredoxin reductase has a subunit size of 35 000 kDa, while the high molecular weight type has a subunit size of 55 000 to 60 000 kDa. Thioredoxin reductases contain a redox active disulphide and a FAD in each subunit (Luthman and Holmgren, 1982). High molecular weight thioredoxins contain a C-terminal peripheral redox centre, a third redox active group in each subunit, that communicates with the C-terminal redox active catalytic site (Holmgren, 1989). This additional redox centre is located on a flexible C-terminal extension, which explains another characteristic of large thioredoxin reductases, their unusual broad substrate specificity (Gromer *et al.*, 1998). In humans, this third group is a selenylsulphide, which means that human thioredoxin reductase is classed as a selenoenzyme.

Thioredoxin reductase has been isolated from many sources as the thioredoxin/thioredoxin reductase system is found ubiquitously in both prokaryotes and eukaryotes, however, many studies of thioredoxin reductase have been carried out with low molecular weight thioredoxin reductases of bacterial and yeast origin. The *E. coli* thioredoxin reductase has an Mr of 70 000 and the crystal structure of this enzyme has been refined to a 2.0 Å resolution (Kuriyan *et al.*, 1991; Waksman *et al.*, 1994). *E. coli* thioredoxin reductase is

specific for its homologous thioredoxin, but shows a degree of reactivity with other prokaryotic thioredoxin molecules, such as *Anabaena* thioredoxin (Holmgren, 1989; Gleason and Holmgren, 1988; Eklund *et al.*, 1991; Nikkola *et al.*, 1993). In mammalian cells, selenium containing thioredoxin reductase has been linked to critical components of cell metabolism such as ribonucleotide reductase (Thelander and Reichard, 1979), transcription factor activity (Spyrou *et al.*, 1995; Handel *et al.*, 1995; Kim and Stadtman, 1997), and thiol peroxidase (Chae *et al.*, 1994) among others. Mammalian thioredoxin reductase will also reduce thioredoxins from other species in addition to low molecular weight compounds such as selenite (Bjornstedt *et al.*, 1995), vitamin K (Holmgren, 1985) and DTNB (Holmgren, 1977).

1.7.3 The Thioredoxin Superfamily

1.7.3.1 The Thioredoxin Fold; A Common Bond

Members of the thioredoxin superfamily are generally responsible for introduction and isomerisation of disulphide bonds that are present in secreted proteins. The reducing environment of the cytosol can have profound effects on protein folding and activity. Secreted proteins have evolved to possess increased stability when the cysteine residues within the protein are joined in disulphide bonds, whereas, cytosolic proteins have evolved to maintain cysteine residues reduced in the native form. Thioredoxin is a member of the thioredoxin superfamily, and exerts it action by a disulphide exchange reaction utilising a Cys-X_1-X_2-Cys active site. Other members of the thioredoxin superfamily are responsible for the introduction and isomerisation of disulphide bonds that are often present in secreted proteins. Additionally, thioredoxin superfamily members share another common feature in addition to the redox active sequence motif. The overall structure in which this short sequence motif is found corresponds to what is called a thioredoxin-like fold (Martin, 1995). This feature was named after the protein in which it was first observed and is a distinct structural motif consisting of a four stranded β-sheet and three flanking α-helices **[Figure 5]**. It has been identified in the three-dimensional structures of proteins from the five thioredoxin superfamily classes, thioredoxin (Holmgren *et al.* 1975; Katti *et al.*, 1990), glutaredoxin (Xia *et al.*, 1992; Bushweller *et al.*, 1994), glutathione S-tranferase (Reinemer *et al.*, 1991), DsbA and glutathione peroxidase (Martin *et al.*, 1993) **[Figure 6]**. However, the thioredoxin fold originally described upon the elucidation of the structure of thioredoxin itself had an additional β-strand and α-helix, therefore, the fold that is common to the five protein families represents a smaller version that that originally seen in *E. coli*

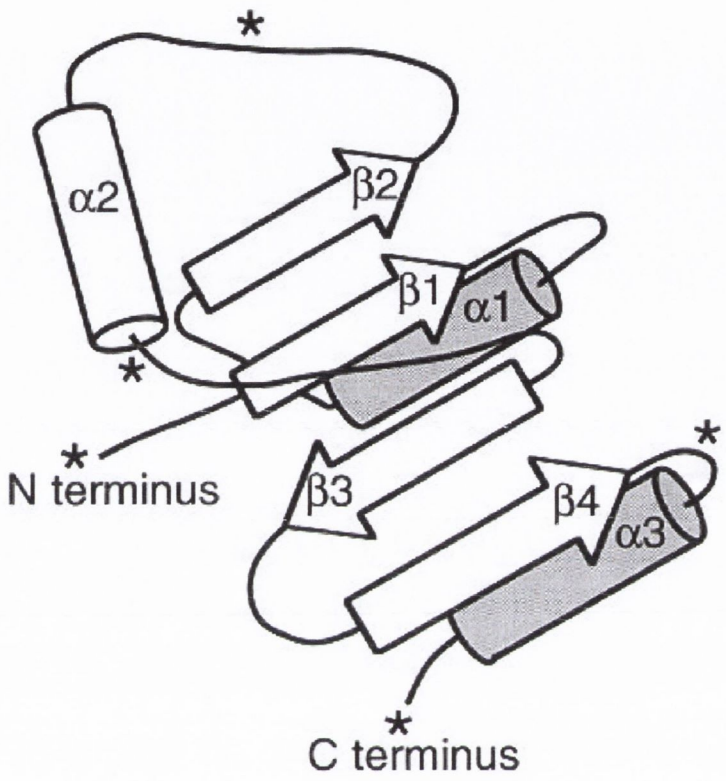


Fig. 5. The thioredoxin fold. The placement of the helices with respect to the central β-sheet is shown. The shaded helices, α1 and α3, in this orientation are depicted behind the sheet. Points in the fold at which insertions of residues are found are indicated by asterisks. (Reproduced from Martin, 1995)

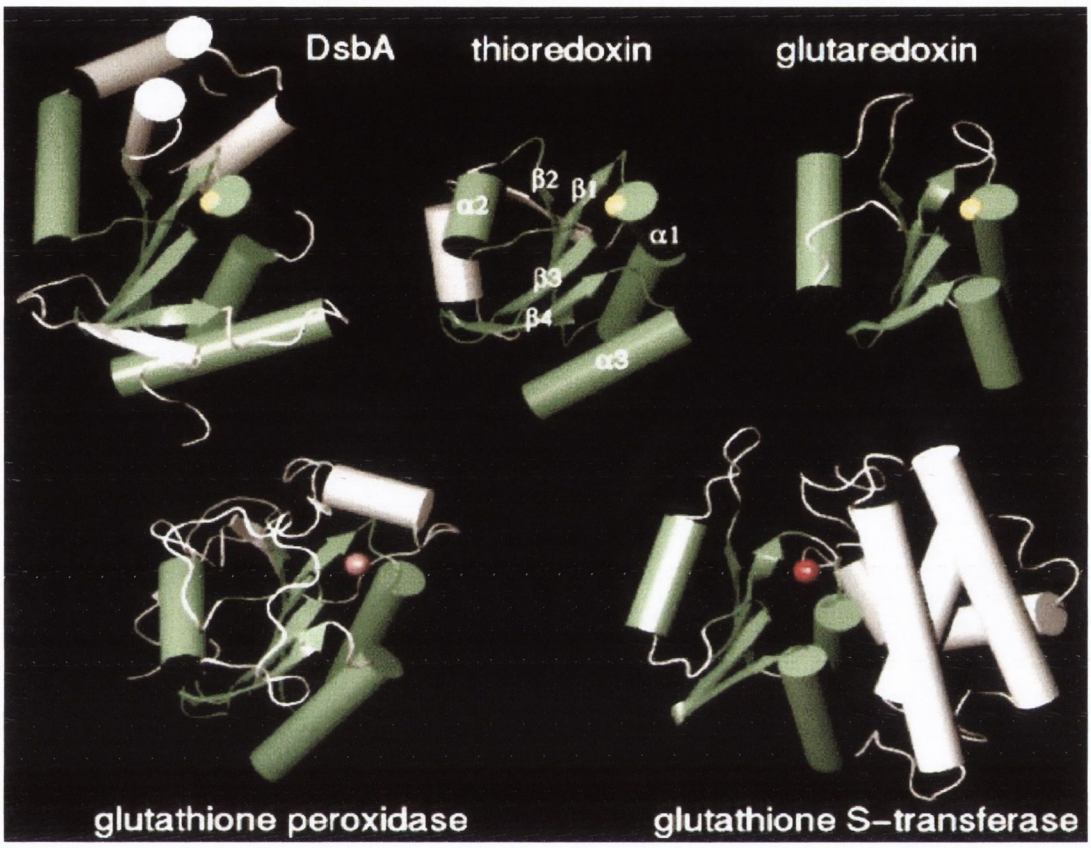


Fig. 6. Structural comparison of the five thioredoxin fold proteins. Structures of DsbA, thioredoxin, glutathione peroxidase and glutathione S-transferase are shown, with those parts of the structure that form part of the thioredoxin fold motif depicted in green. (Reproduced from Martin, 1995)

thioredoxin (Holmgren *et al.* 1975; Katti *et al.*, 1990). Proteins from the five classes of the thioredoxin superfamily, although they do not have close overall structural similarities as observed from sequence identity comparisons, nor indeed, common biological or catalytic function, all are redox proteins. Three members, thioredoxin, glutaredoxin and DsbA all possess the C-X-X-C motif, whereas, glutathione S-transferase and glutathione peroxidase do not, although both proteins do interact with the cysteine containing substrate, glutathione. So, in effect, the basic common bond of members of the superfamily is cysteine chemistry, however, all may also be defined as members of a family by virtue of a structural relationship represented by the thioredoxin fold.

Thioredoxin fold architecture has been described previously (Eklund *et al.*, 1984). When the structures of thioredoxin fold containing proteins are aligned, it appears that the cysteine substrate interacting atom of such proteins is usually positioned in the same relative location in space. That the substrate interacting atom of each of the five classes corresponds in location so much is notable, as the active site residues differ across the protein classes and arise from different regions of the thioredoxin fold (Eklund *et al.*, 1984). The average thioredoxin fold generally comprises approximately 80 residues, but this number may vary according to extra residues which are present in the folds of some family members. The overall similarities of the thioredoxin folds of the five protein classes can be compared through constrained structural fitting procedures. After such analysis it appears that the protein classes can be split into two branches, one in which thioredoxin, glutaredoxin and DsbA share similar fold residues and the other in which glutathione S-transferase and glutathione peroxidase are more similar and have shorter secondary-structure elements (Martin, 1995). In all five proteins no significant areas of amino acid sequence are conserved, but local regions of similarity may be highlighted through sequence alignment. All members of the thioredoxin superfamily represent variations on a central theme. The existence of a common ancestor could be anticipated at least for thioredoxin, glutaredoxin and DsbA and this ancestor may have been protein which had a low level of specificity for substrate proteins and could have interacted with a wide variety of differing substrates, perhaps a thioredoxin-like protein, from which glutathione S-transferase and glutathione peroxidase may have gone on to evolve separately. Indeed, a 'glutathione-binding protein' ancestor has been proposed (Sinning *et al.*, 1993). It is clear however, that the thioredoxin superfamily represents a unique group of protein classes

based on reactive cysteines all with a similar structural motif, the thioredoxin-like fold, and play an important role in cellular thiol-redox chemistry.

1.7.3.2 Glutathione and Glutaredoxins

Cellular redox homeostasis is maintained by a network of redox proteins which act in a non-linear manner, and the role of a particular redox protein can only be fully appreciated when it is examined in the context of other cellular redox partners. The small tripeptide glutathione and the small protein thioredoxin share a similar function as cellular reductants in numerous cellular reactions. Glutathione functions to reduce disulphide bonds in the cytosol often in conjunction with glutaredoxin proteins. In eukaryotic systems glutathione can act as an electron donor for the enzyme glutathione peroxidase which has antioxidant functions within the cell. The enzyme glutathione reductase acts similarly to thioredoxin reductase by catalysing the reduction of oxidised glutathione at the expense of NADPH (Holmgren, 1985). Glutathione (L-γ-glutamyl-L-cysteinylglycine) is synthesised in the cell by the action of two enzymes, glutathione synthetase and γ-glutamylcysteine synthetase. Glutaredoxin is an 11 to 12 kDa redox active protein which unusually does not have a specific reductase that functions as an electron donor to the oxidised form of glutaredoxin. This enzyme is reduced through interaction either with glutathione and glutathione reductase, or with glutathione and PDI (Wells *et al.*, 1993). Glutaredoxin, encoded by the *grxA* gene, like many other thioredoxin superfamily members, was discovered indirectly as an activity which could donate electrons to ribonucleotide reductase when the acknowledged electron donator, thioredoxin, was removed from the cell (Holmgren, 1976). Genetic studies have revealed that glutathione itself is also not essential for normal *E. coli* cellular survival (Apontoweil and Berends, 1975; Fuchs and Warner, 1975; Greenberg and Demple, 1986), and genetic studies identified several more glutaredoxins present in the *E. coli* cytoplasm, glutaredoxin 2 (*grxB*) and glutaredoxin 3 (*grxC*) (Åslund *et al.*, 1994). More recently, sequence homology studies pointed to the existence of another glutaredoxin-like protein, NrdH, which unusually is reduced by thioredoxin reductase (Jordan *et al.*, 1997).

1.7.3.3 Protein Disulphide Isomerases

Cytoplasmic members of the thioredoxin superfamily such as thioredoxin and glutaredoxin catalyse reductive processes such as the transfer of electrons to various cellular enzymes and function in the reduction of disulphide bonds. Other members of the superfamily

function as catalysts of disulphide bond formation during the folding of proteins destined for secretion. These proteins are generally found not in the reducing cytoplasmic milieu but in oxidising cellular compartments such as the endoplasmic reticulum in eukaryotic cells and the bacterial periplasm. In the former, protein disulphide isomerase (PDI) catalyses this process and in the latter, the bacterial equivalents, DsbA and DsbB perform this vital role. PDI contains two thioredoxin-like active sites, both of which are biochemically functional. The active site of PDI, Cys-Gly-His-Cys, varies from thioredoxin only by substitution of His for Pro.

Most protein folding catalysts in contrast to those proteins that catalyse reductive processes, are strong oxidants and this is reflected in their high standard redox potentials (E_0'). Thioredoxin has one of the lowest redox potentials known for a redox protein, -270 mV (Krause *et al.*, 1991; Lin and Kim, 1989; Mössner *et al.*, 1998) and as a result is a highly efficient reductant. Glutaredoxin also has a low redox potential, from -233 mV to -198 mV (Åslund *et al.*, 1997). PDI, as a strong oxidant has a high redox potential, -147 mV to -175 mV (Lundström and Holmgren, 1993), and DsbA and DsbC are also quite oxidising, with redox potentials of -122 mV (Zapun *et al.*, 1993; Huber-Wunderlich and Glockshuber, 1998; Wunderlich and Glockshuber, 1993) and -130 mV (Zapun *et al.*, 1993) respectively. Two different enzymes participate in the catalysis of disulphide bond formation and isomerisation in the periplasm of *E. coli*, DsbA is the predominant dithiol oxidant which randomly introduces disulphide bonds into folding polypeptides (Zapun *et al.*, 1994; Wunderlich *et al.*, 1993), whereas, DsbC possesses disulphide isomerase activity (Zapun *et al.*, 1995; Missiakas *et al.*, 1994). DsbA is reoxidised by disulphide exchange with DsbB (Bardwell *et al.*, 1993; Guilhot *et al.*, 1995), which is located in the inner membrane and DsbA can also react directly with molecular oxygen (Bader *et al.*, 1998; Kobayashi *et al.*, 1997). The isomerisation activity of DsbC is maintained via reoxidation by an alternate membrane protein DsbD, which itself is kept reduced by cytoplasmic thioredoxin (Rietsch *et al.*, 1996; Rietsch *et al.*, 1997; Missiakas *et al.*, 1994). Mutagenesis studies have revealed that these two redox systems, DsbA/DsbB and DsbC/DsbD, function independently of each other (Zapun *et al.*, 1995; Rietsch *et al.*, 1997). Further Dsb enzymes have been identified, DsbE and DsbG, which have disulphide oxidoreductase roles in the cytoplasm (Fabianek *et al.*, 1998). Overall, recent investigations have highlighted that the efficiency with which the final catalyst of disulphide bond formation oxidises appears to be crucial for oxidative protein folding in the bacterial periplasm.

1.7.3.4 Peroxiredoxins

Peroxiredoxins act as antioxidants and catalyse the elimination of hydroperoxides with the help of reducing systems, such as the thioredoxin system (Chae *et al.*, 1994; Chae *et al.*, 1994). Peroxiredoxins are ubiquitous proteins and their sequences are conserved from bacteria to mammalian cells. Peroxiredoxins are flavoproteins and the first described member of this enzymatic group was AhpF from *S. typhimurium* (Poole and Ellis, 1996; Poole, 1996). These proteins are believed to have evolved from either thioredoxin reductase or an alternate common ancestor in order to function in the reactivation of cysteine-based peroxidases, such as alkylhydroperoxide reductase (AhpC), via reduction of active site disulphide bonds (Chae *et al.*, 1994; Chae *et al.*, 1994). AhpC homologues have been designated the peroxiredoxin family in the literature (Chae *et al.*, 1994; Kang *et al.*, 1998). AhpF proteins which function as NADH:peroxiredoxin oxidoreductases are designated peroxiredoxin reductases (PrxRs). As members of the thioredoxin superfamily, peroxiredoxins contain redox active cysteine residues, the C-X-X-C motif, however, they retain close homology to full-length bacterial thioredoxin reductases at the C-terminal end of the protein. An additional redox active C-X-X-C motif, within an N-terminal domain of approximately 200 amino acids, is attached to the thioredoxin reductase-like region of peroxiredoxin reductase (Hecht *et al.*, 1995; Bieger and Essen, 2000). Members of this protein family have thus far only been described in eubacteria, and a number of peroxiredoxin reductases have been implicated in oxidative stress defence (Storz *et al.*, 1989; Farr and Kogoma, 1991; Bsat *et al.*, 1996; Higuchi *et al.*, 1999; Rocha and Smith, 1999). Similarly to thioredoxin reductase, peroxiredoxin reductases catalyse electron transfer at the expense of FAD, pyridine nucleotide and redox active disulphide centres to catalyse the reduction of a redox active disulphide bond in a substrate protein.

Recently, a study has shown that a mutation which occurs at high frequencies in the *ahpC* gene, which encodes a peroxiredoxin in *E. coli*, can convert the AhpC protein from a peroxidase to a disulfide reductase. The mutation appears to be the result of a reversible expansion of a triplet nucleotide repeat sequence which leads to the addition of one amino acid to the AhpC protein. This expansion results in the mutational interconversion between the two activities. It is postulated that this interchange mechanism could provide and evolutionary advantage to the bacterium (Ritz *et al.*, 2001). Further investigations should

reveal if this interconversion of peroxiredoxin activity via triplet repeat expansion is utilised *in vivo* in other bacterial species

1.7.3.5 The Thioredoxin Superfamily: Specificity versus Redundancy

The availability of genomic sequence data for a wide variety of microorganisms has facilitated the identification of huge numbers of open reading frames (ORFs) in a particular bacterium. A challenge of the post-genomic era is a functional understanding of these ORFs, many of which lack homologues with known activities. Another feature becomes apparent upon inspection of such genomes, the presence of paralogues of ORFs which are believed to have originated from gene duplication events. These paralogues have been considered to represent functionally redundant members of a protein family, a type of cellular insurance, however evolution tends not to be an inherently wasteful process and to postulate that these ORFs serve no purpose other than as a back-up seems unsatisfactory. The thioredoxin family is a protein family that in most cells is represented by numerous thioredoxin-like proteins or proteins that contain a thioredoxin-like domain. In *E. coli* to date, there are two thioredoxins, three glutaredoxins and a novel thioredoxin-like protein with thioredoxin-like activity but glutaredoxin-like amino acid sequence (NrdH) (Potamitou *et al.*, 2002). Specific roles for these seemingly redundant proteins are beginning to be described and current thinking is that each of these proteins may have specialised cellular roles, at least under specific environmental conditions. Under normal environmental conditions, thioredoxins and glutaredoxins can act as backups to each other as shown by mutagenesis studies (Prinz *et al.*, 1997; Russel and Holmgren, 1988), also supporting the proposition of redundancy is a study showing that a lethal *E. coli* phenotype is only produced upon the removal of the cells three most efficient reductants, thioredoxin 1 and 2 and glutaredoxin 1 (Stewart *et al.*, 1998). However, it is becoming apparent that certain metabolic pathways or environmental conditions rely on the utilisation of only one of these reductant proteins, an example of this being *E. coli* thioredoxin 1 which has been shown to be the only protein capable of reducing the *E. coli* cellular enzyme methionine sulfoxide reductase (Stewart *et al.*, 1998). Another example is the formation of the reversible disulphide bond which activates the OxyR protein in response the hydrogen peroxide stress, cells lacking glutaredoxin 1 show a delay in the reduction of OxyR (Zheng *et al.*, 1998). Consequently, functional specificity of redox enzymes appears to be important for normal cell growth. It is postulated that this functional specificity may

imposed by particular redox potential or features of non-redox related substrate interactions.

Determination of the specialised roles of members of the thioredoxin superfamily may only be achieved following definition of the appropriate biological and physiological conditions which reveal functional specificity. The thioredoxin superfamily may represent a group of enzymes whose success is the result of a balance of both functional specificity and redundancy.

1.7.4 Redox Regulation of Transcription Factors by Thioredoxin

1.7.4.1 Eukaryotic Cells: Oxidative Stress and Thiol Redox State

The extracellular environment is predominantly oxidising and the intracellular reduced environment has previously been considered to be relatively constant unless a cell was undergoing an extreme stress response or damage. Redox homeostasis ensures that the redox balance within a cell remains relatively constant in the presence of an oxidising environment and allows numerous vital biological events within the cell to proceed normally, unperturbed by the effects of oxidation. Events include enzyme activation, DNA synthesis, cell cycle regulation and selective gene expression among others. While drastic oxidising conditions can have serious consequences for normal cellular metabolism, small variations in the amounts of ROS liberated from internal biochemical reactions, for example oxidative phosphorylation, can have a modulatory, not deleterious effect, on metabolism. This basal fluctuating level of cellular ROS is tightly controlled by antioxidant systems within the cell. If the antioxidant capacity of the cell is overwhelmed and redox homeostasis disrupted, oxidative injuries can rapidly be induced by free radicals resulting in various pathologies. In eukaryotic cells there are two major intracellular regulators of redox status, one is the cysteine-containing reducing tripeptide glutathione and the other is the small pleiotropic polypeptide thioredoxin. Glutathione is found ubiquitously in eukaryotic cells at a concentration of between 1 and 10 mM (Kosower and Kosower, 1978). The ratio of reduced glutathione to oxidised glutathione ensures that the intracellular milieu is highly reducing, as this ratio lies predominantly in favour of reduced glutathione with ratios of 30:1 up to 100:1 being reported (Hwang *et al.*, 1992). The thiol group present on the cysteine moiety of glutathione provides the reducing activity and the protein is postulated to play a role in several cellular reactions which are redox balance sensitive, such as protein folding (Hwang *et al.*, 1992), conversion of ribonucleotides to

deoxyribonucleotides (Holmgren, 1990) and the maintenance of reduced pools of cellular antioxidant molecules such as ascorbic acid and α-tocopherol (Winkler *et al.*, 1994). The thioredoxin/ thioredoxin reductase reducing system is also ubiquitous in eukaryotic cells with the amino acid sequences of the thioredoxin active site are highly conserved between eukaryotic and prokaryotic thioredoxins. Eukaryotic thioredoxin reductase has been purified as a 116-120 kDa homodimer from numerous mammalian sources (Luthman and Holmgren, 1982; Oblong *et al.*, 1993) and sequence analysis revealed a 26-60% sequence identity with prokaryotic thioredoxin reductase. Indeed, components of the thioredoxin system can be substituted for one another across prokaryotic and eukaryotic systems with differing rates of reducing efficiency (Oblong *et al.*, 1993; Jacquot *et al.*, 1990).

1.7.4.2 Oxidative Stress and Gene Expression

The transcription of certain genes has been recognised as being under the influence of the effects of oxidative stress. However, the exact mechanism by which oxidants from various sources and/or the variation in cellular redox state can modulate gene transcription in eukaryotic cells is not yet fully elucidated. The first example of gene activation by oxidative stress came from the investigation of prokaryotic regulatory proteins, SoxR and OxyR (Farr and Kogoma, 1991; Amabile-Cuevas and Demple, 1991). Since then, the transcription of various eukaryotic genes has been shown to be modulated by oxidative stress (Keyse and Tyrell, 1987; Lautier *et al.*, 1992). Moreover, oxidant-influenced gene transcription has been described for a diverse range of cellular genes, including those coding for elements of redox systems, antioxidant enzymes, cytokines, transcription factors, immune receptors and growth factors. Compared to the mechanisms of gene transcription operating in prokaryotic cells in response to the cellular redox state, the mechanisms operating in eukaryotic cells appear to be more complex. The contribution of a range of transcription factors in gene expression systems in eukaryotic cells represent an area of obvious complexity over bacteria and this aspect has been the subject of intense focus in recent studies. Transcription factors bind to the promoter regions of target genes and facilitate transcription via RNA polymerase II. Redox balance is postulated to influence two major steps in transcriptional activation, the first being the mechanism of activation of the transcription factor itself, which results in the transition of the transcription factor from the cytoplasm to the nucleus. Secondly, the binding of the transcription factor to the promoter region at the target gene 5′ end. A compelling piece of evidence to support the former supposition is that many transcription factors possess

cysteine residues with a redox sensitive thiol group in the DNA binding domain of the protein. These residues are frequently necessary for DNA binding site recognition and DNA binding may be inhibited through oxidation of the cysteine residue. In this regard the redox state of the nuclear compartment and the regulation of this state by redox enzymes is vital for correct transcription factor activity and hence gene expression. The presence of cysteine residues in transcription factor domains means that inter- or intra-molecular disulphide bond formation is possible which could have implications for the three-dimensional structure of the proteins and therefore the ability of the transcription factor to bind to DNA, or indeed assume its correct conformationally active state, as a dimer, for example. Finally, cysteine residues are an integral part of the 'zinc finger' structure which fits into the large DNA groove and through which certain transcription factors exert their effect. Therefore, through some or all of the above mechanisms of transcription activation, the expression of genes can be negatively or positively influenced by the thiol redox state of the cell or by the presence of discreet oxidant molecules.

1.7.4.3 Redox Regulation of NF-κB

Accumulating evidence has implicated various cytokines and cell adhesion molecules in inflammatory responses. Central to the inflammatory response is the accumulation of inflammatory cells in damaged tissue, for which expression of certain cell adhesion molecules such as, intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and TNF-α/IL-1 induced E-selectin (Sakurada *et al.*, 1996). Gene expression of these cytokines and cell adhesion molecules is under the control of a common transcription factor, nuclear factor κ-B (NF-κB), and IL-1 and TNF-α are known to stimulate inducible expression of these genes via a cellular signal transduction pathway leading to NF-κB activation (Yoshida *et al.*, 1999; Yoshida *et al.*, 1999; Alvaro-Gracia *et al.*, 1991; Baeuerle, 1991; Ledebur and Parks, 1995). NF-κB is a ubiquitous inducible transcription factor that is activated in various cell types in response to growth stimuli and stress in eukaryotic cells (Grimm and Baeuerle, 1993; Thanos and Maniatis, 1995). It was through the analysis of transcriptional activation mediated by the intronic κ light chain enhancer and the promoter located in the long terminal repeat of HIV-1 that a DNA sequence, GGAnnnTCC, denoted κB, which is recognised by the protein factor NF-κB, was revealed (Baeuerle and Baltimore, 1996). The most common form of NF-κB consists of a heterodimeric complex containing the p65/RelA subunits and the p50 subunits

(Baeuerle and Baltimore, 1996), which reside in a latent form in the cytoplasm due to an association with a family of cytoplasmic inhibitors, the most frequent form encountered being IκB-α (Baeuerle and Baltimore, 1996). Activation results in the disassociation of the NF-κB/IκB complex, releasing NF-κB dimers which are then free to undergo translocation to the nucleus. In the nucleus, the dimers can bind to cognate DNA sequences thereby activating transcription of those genes which possess the κB regulatory elements. Free IκB in the cytoplasm undergoes proteasomal degradation following release from the complex (Baeuerle and Baltimore, 1996). One of the most compelling lines of evidence for a role for oxidative stress in the activation of NF-κB is that this activation sequence can be initiated by a wide variety of conditions which result in the disruption of redox balance within the cell (Schreck *et al.*, 1992), and activation can be inhibited by compounds which have the ability to restore reducing conditions within the cell such as N-acetylcysteine (NAC) (Mihm *et al.*, 1995), and free radical neutralising agents (Schreck *et al.*, 1992).

Thus, in addition to activation by oxidative stress and chemical reductants, the redox-sensitive transcription factor NF-κB is also subject to activation by biological reductants such as thioredoxin. Thioredoxin appears to stimulate the signal transduction pathway leading to NF-κB activation at least at two independent steps (Okamoto *et al.*, 1997; Okamoto *et al.*, 1992; Hayashi *et al.*, 1993). Thioredoxin has been shown to act as co-stimulatory factor for the inflammatory response upon extracellular addition (Wakasugi *et al.*, 1987; Tagaya *et al.*, 1989; Schenk *et al.*, 1996) and, in addition, thioredoxin can augment DNA-binding by NF-κB at least *in vitro* (Okamoto *et al.*, 1997; Okamoto *et al.*, 1992; Hayashi *et al.*, 1993; Qin *et al.*, 1995). Evidence suggests that thioredoxin can upregulate DNA binding of NF-κB *in vitro* (Okamoto *et al.*, 1997; Hayashi *et al.*, 1993) and enhances κB dependent gene expression *in vivo* (Okamoto *et al.*, 1997; Okamoto *et al.*, 1992; Hayashi *et al.*, 1993). In the cytoplasm, thioredoxin can block degradation of IκB, whereas in the nucleus, thioredoxin enhances NF-κB transcriptional activities by enhancing its ability to bind to DNA (Hirota *et al.*, 1999). It has been demonstrated that thioredoxin can transiently co-migrate to the nuclear compartment where it can form a molecular complex with the DNA-binding loop of the NF-κB p50 subunit (Sakurada *et al.*, 1996), perhaps facilitating binding of the transcription factor to the DNA strand following reduction of a critical cysteine 62 residue (Matthews *et al.*, 1992). It was also demonstrated

that thioredoxin can also either have a stimulatory or inhibitory effect on the activation of NF-κB depending on cell type (Matthews *et al.*, 1992).

Activation of NF-κB in eukaryotic cells does not exclusively require endogenous thioredoxin, exogenous thioredoxin from a prokaryotic source has been reported to activate NF-κB in a mammalian cell line (Das, 2001). In this study, NF-κB was activated by addition of *E. coli* thioredoxin in a redox-dependent manner in A549 cells, and this activation was accompanied by the degradation of IκB in the cytosol. Moreover, activation of NF-κB was induced only by the reduced form of thioredoxin with the oxidised form of the protein having no effect. This observation may have implications in the inflammatory response to invading prokaryotes, as thioredoxin has been shown to be selectively secreted by bacteria *in vitro* (Kim *et al.*, 2002), perhaps as a means of modulating the host inflammatory cascade. Collectively, recent data suggests that thioredoxin acts in a signalling effector capacity to promote activation of the redox-sensitive transcription factor, NF-κB (Okamoto *et al.*, 1997; Okamoto *et al.*, 1992; Hayashi *et al.*, 1993). Hence, the cellular inflammatory response can be said to be subject to redox control, with NF-κB constituting a prominent target.

1.7.4.4 Additional Redox-Regulated Transcription Factors

Activator protein-1 (AP-1) is a transcription factor whose activation is a prerequisite to growth factor stimulated cell growth. Other stimuli which activate this transcription factor include protein kinase C (PKC) activators and modulators of redox intracellular homeostasis (Angel and Karin, 1991). This protein binds to the TPA response element (TRE) (5′- TGA[G/C]TCA -3′) which is located in the promoter region of a range of genes implicated in cell proliferation and tumour promotion (Lee *et al.*, 1987; Angel *et al.*, 1987). AP-1 is a dimeric protein which is comprised of the c-Jun and c-Fos proteins, and these proteins are products of the *c-jun* and *c-fos* oncogenes. The gene products form either the homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes and the interaction between Jun and Fos is via a 'leucine zipper' domain (Genz *et al.*, 1989; Turner and Tjian, 1989; Kouzarides and Ziff, 1988). Activation is of AP-1 is achieved upon the synthesis and transient accumulation of c-Fos and c-Jun proteins (Curran and Franza, 1988) and is regulated by complex mechanisms consisting of post-transcriptional events acting on pre-exisiting AP-1 molecules and transcriptional activation leading to increasing amounts of

AP-1-binding proteins. There is increasing evidence that the redox regulation of AP-1 transcription is important (Barnes and Adcock, 1998; Sasada *et al.*, 1996). AP-1 transcriptional activity is redox-sensitive with increased AP-1 expression induced by ROS (Singh and Aggarwal, 1995; Timblin *et al.*, 1998), and various antioxidants strongly activate the DNA binding and transactivation abilities of the AP-1 complex (Pahl and Baeuerle, 1994; Meyer *et al.*, 1993; Schenk *et al.*, 1994). The redox protein thioredoxin has been demonstrated to enhance the DNA-binding activity of Jun and Fos, with this process requiring other molecules such as the novel protein redox factor-1 (Ref-1) (Xanthoudakis and Curran, 1992; Xanthoudakis *et al.*, 1994). Ref-1 was identified in a cell-free system as a factor restoring AP-1 DNA binding. Studies have revealed that there is a direct active-site mediated association between Ref-1 and thioredoxin in the nucleus and that cysteine residues in the active site of thioredoxin are required for the potentiation of AP-1 activity (Hirota *et al.*, 1997). Ref-1 reduces a critical cysteine of c-Jun, and this reducing activity is recycled by the hydrogen donor capability of thioredoxin. In addition, redox modification of a conserved cysteine residue in the DNA binding domain of c-Jun may be another mechanism of controlling AP-1 DNA-binding (Abate *et al.*, 1990).

In addition to AP-1, p53 DNA-binding and transcriptional activities have been shown to be controlled by thiol-redox state, with four cysteines present in the DNA-binding domain of p53 being essential for activity of this factor (Hainaut and Milner, 1993; Verhaegh *et al.*, 1997). It has been shown that thioredoxin and Ref-1 enhance p53-dependent p21 activation for DNA repair (Ueno *et al.*, 1999). Thioredoxin dependent redox regulation of p53 activity indicates coupling of the p53-dependent repair mechanism and the oxidative stress response. Several other transcription factors appear to be dependent on the reactivity of critical cysteine residues in their DNA-binding domain (Bandyopadhyay and Gronostajski, 1994; Pognonec *et al.*, 1992; Abate *et al.*, 1990; Huang and Adamson, 1993; Makino *et al.*, 1999; Hayashi *et al.*, 1997) and the activity of these factors may also turn out to be thioredoxin-dependent. It seems that further examples of the mechanism of thiol-disulphide exchange by thioredoxin as a molecular on-off switch in gene control will undoubtedly emerge over time.

1.8 AIMS

Gastric colonisation by *H. pylori* results in a mucosal inflammatory response and is a risk factor for the development of peptic ulcer disease and gastric malignancy. However, the

exact mechanisms underlying inflammation and tissue damage resulting from infection with this bacterium have yet to be fully elucidated. *H. pylori* also elicits oxidative stress during colonisation of the gastric mucosa and this has consequences for both the pathogen and the host. Oxidative stress is known to cause damage to both DNA and membranes. In addition, oxidative stress can be responsible for the introduction of deleterious disulphide bonds into cytoplasmic proteins. The thiol-oxidoreductase thioredoxin reduces oxidised proteins via disulphide exchange, and is responsible for the maintenance of cellular redox homeostasis. Antioxidant systems such as the thioredoxin system are critical in the defence against reactive oxygen species generated by the oxidative burst of host polymorphonuclear leukocytes and macrophages.

Issues of thiol redox chemistry in *H. pylori* have only recently begun to be explored. The response of *H. pylori* to the challenge of oxidative stress within the gastric niche is critical to its success as a long term colonist and therefore the redox systems operating within the pathogen are of great interest. This presents study seeks to examine the redox system of *H. pylori* and to elucidate the contribution of the redox protein thioredoxin in particular to the challenge of oxidative stress. Characterisation of the functions of *H. pylori* thioredoxin should contribute to a better and more complete understanding of the mechanisms of *H. pylori*-induced gastric disease.

CHAPTER II
MATERIALS AND METHODS

CHAPTER II

Materials and Methods

2.1 CHEMICALS

Bovine serum albumin, acrylamide:bisacrylamide (29:1), ammonium persulphate, Nonidet-P40, leupeptin, PMSF, β-mercpatoethanol, PMA, EDTA, EGTA, ampicillin, kanamycin, chloramphenicol, sodium chloride, sodium hydroxide, magnesium chloride, potassium chloride, Tris(hydroxylmethyl)aminomethane (Tris), glycine, TEMED, Tween-20, dimethyl sulfoxide, Luria-Bertani Medium, Agar Select, IPTG, MTS, sodium dodecyl sulphate, iminodiacetic acid-Sepharose 6B, DTT (1,4-dithio-DL-threitol), ρ-aminobenzamidine-agarose were obtained from Sigma (Poole, Dorset, U.K. and St. Louis, M.O., U.S.A). Electrophoresis grade agarose, Brain Heart Infusion medium, RPMI culture medium, HBSS, were obtained from GIBCO BRL (Life Technologies, Scotland). Ethanol, methanol, glacial acetic acid, orthophosphoric acid, hydrochloric acid, acetone, glycerol and chloroform were obtained from BDH Ltd. (Poole, Dorset, U.K.). Poly(dI-dC) was from Pharmacia (Biosystems, Milton Keynes, U.K.). Cyanogen bromide activated Sepharose 4B resin was from Amersham (Amersham Biosciences, U.K.). IPGphor™ strips (code 17-6001-14) were from Amersham Biosciences, U.K) [γ-^{32}P]ATP (35 pmol, 3000 Ci/mol) was also from Amersham Pharmacia (Aylsebury, U.K.). Sterile Horse Serum and *H. pylori* selective DENT supplement (SR-0147) were from Oxoid (Basingstoke, Hampshire, U.K.) Difibrinated horse blood was from Biological Laboratories Europe Ltd. DEAE-52 and CM-52 chromatography media were obtained from Whatmann Ltd. (Maidstone, U.K.). Factor Xa was purchased from New England Biolabs (Hertfordshire, U.K.) All buffer reagents for SDS-PAGE were prepared in deionised water (Elga Prima reverse osmosis). Recombinant *H. pylori* Trx1 was received from Dr. Henry Windle, Department of Clinical Medicine, Trinity College Dublin.

2.2 ANTIBODIES AND OLIGONUCLEOTIDES

Polyclonal antibodies to I kappa B-α (IκB-α; sc-371), p38 MAP kinase (sc-535), phospho-specific p38 (sc-7975R), ERK (sc-4024), phospho-specific ERK (sc-7383) and AP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, C.A.) Polyclonal antibodies were raised in rabbit (BioResourse Unit, T.C.D.) against recombinant Trx1 and Trx2. For

electrophoretic mobility shift assays, NF-κB consensus oligonucleotide and AP-1 oligonucleotide were purchased from Promega (Promega Corp., Madison, WI.).

2.3 *HELICOBACTER PYLORI* CULTURE

2.3.1 *H. pylori* Strains and Growth Conditions

H. pylori reference strains NCTC11637 (R1) and NCTC11638 (R2) (both $VacA^+$ and $CagA^+$) were obtained from National Collection of Type Cultures, Colindale, U.K., and and SS1, a murine adapted strain ($VacA^+$ and $CagA^+$) (Lee *et al.*, 1997) was also used. Strains were maintained on Columbia Blood Agar plates (7% lysed horse blood) supplemented with DENT selective supplement (SR-0147) (Oxoid, Basingstoke, U.K.) and selective antibiotics where appropriate (**Fig. 1**). Plates were incubated in a microaerophilic humidified atmosphere generated within an anaerobic jar by Gas Generating Kits (BR-0038) (Oxoid, Basingstoke, Hampshire, U.K.) For liquid culture, strains were grown in Brain Heart Infusion Medium (GIBCOBRL, Life Technologies, Scotland) supplemented with 2% FCS (Xia *et al.*, 1993). Cultures were grown in 25 cm^2 cell culture flasks (Nunclon, Roskilde, Denmark) which had been equilibrated in a CO_2 atmosphere prior to flask sealing and incubation with constant shaking (150 rpm) for 24 to 36 h at 37°C in an orbital incubator (S1 50, Stuart Scientific, U.K.)

2.3.2 *H. pylori* Total Lysate Preparation

H. pylori cells were harvested in ice-cold sodium phosphate buffer (pH 7.4) from 3-4 day old Columbia Blood Agar plates. Cells were washed three times in ice-cold buffer and pelleted by centrifugation (8000 g, 10 min, 4°C) on each occasion. Pellets were then stored at –20°C until required. For total lysate preparation, previously frozen pellets were thawed on ice. These were resuspended in the appropriate ice-cold buffer by vortexing. Optionally, protease inhibitors were added (Roche Complete protease inhibitor, PMSF (1 mM), leupeptin (1 μg/ml)). The resuspended material was then lysed by sonication (6 x 30 sec pulses) in a Branson Sonifier Model 450, and centrifuged at 14,000 rpm for 25 min at 4°C. The supernatant was filter sterilised using a 0.45 μ low-protein binding filter (Acrodisc, Pall, U.K.) and stored at –20°C prior to protein estimation.

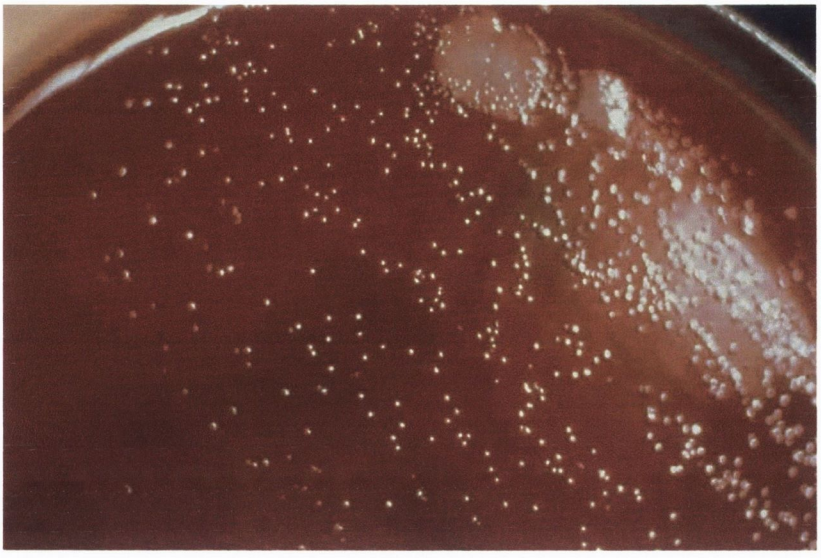


Fig. 2.1 *Helicobacter pylori* colony morphology. *H. pylori* colonies on Columbia blood agar after 72 hours growth. Colonies are circular, translucent in appearance and are approximately 2 mm in diameter.

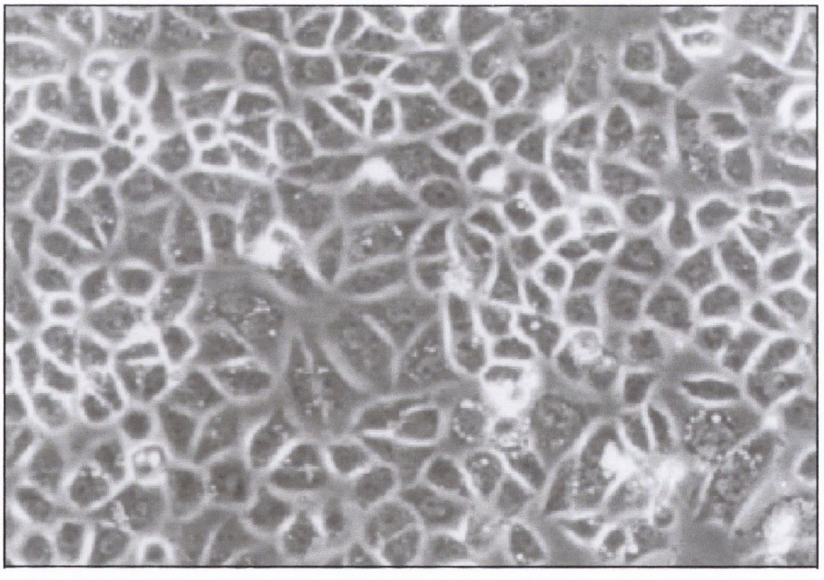


Fig. 2.2. Gastric epithelial cell morphology. Gastric epithelial cells (AGS cells) were grown in RPMI medium and photographed at 400 X magnification.

2.4 CELL CULTURE

2.4.1 Cell Line Source and Culture Reagents

The gastric epithelial cell line, AGS, was obtained from the European Collection of Animal Cell Cultures, ECACC (Porton Down, Salisbury, U.K.). This cell line was used for all experiments. This line is derived from human gastric adenocarcinoma (**Fig. 2**). RPMI 1640 medium, Hank's Balanced Salt Solution w/o calcium and magnesium, foetal calf serum (FCS), penicillin, streptomycin, L-glutamine and 10 X trypsin were purchased from GIBCO BRL (Life Technologies, Paisley, Scotland).

2.4.2 Cell Culture Maintenance

AGS cells were grown in RPMI medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (GIBCO BRL). Supplemented medium was stored at 4°C. A stock solution of the antibiotics was prepared [Appendix A] and stored in aliquots at –20°C until use. FCS was heat inactivated (56°C, 1 h) to inactivate complement and then aliquoted for storage at –20°C. Cells were routinely maintained in 75 cm^2 cell culture flasks (Nunclon, Roskilde, Denmark) at 37°C in 5% CO_2 and 95% air in a humidified incubator. Visual examination was undertaken using phase contrast microscopy. Cells were passaged upon reaching 80% confluence. For passage, cells were removed from the solid matrix of culture flasks by trypsinisation following aspiration of spent medium. Following two successive washes with HBSS medium containing Hepes buffer [Appendix 1] and centrifugation (1400 rpm, 5 min), cells were resuspended in RPMI for enumeration or seeding.

2.4.3 Cell Freezing and Revival

A cryopreservation solution was prepared containing 90% FCS and 10% DMSO. AGS cells were routinely harvested as for subculture, the supernatant was decanted and cells were resuspended in ice cold cryopreservative medium at 2 x 10^6 cells/ml. 1 ml of cell suspension was aliquoted into sterile cryovials (Sarstedt, Numebrech, Germany). The cryovials were then transferred to liquid nitrogen for long-term storage. For cell revival, cryovials removed from storage were quickly thawed by hand agitation. Cells were transferred to a Sterilin tube and washed with pre-warmed RPMI media. Gentle centrifugation (1000 rpm, 3 mins) was used to pellet the cells which were subsequently resuspended in RPMI (5 mls) supplemented with FCS to a final concentration of 20% and

transferred to a 25 cm^2 tissue culture flask and placed at 37°C, 5% CO_2 in a humidified incubator.

2.4.4 Cell Enumeration and Viability

Ethidium bromide/acridine orange (0.1% w/v) (EB/AO) (Lee *et al.*, 1975) was used to assess cell yield and viability. Using a modification of Hudson and Hay (1976), cells were counted on a Neubauer haemocytometer under ultraviolet (UV) light. Under UV excitation, live cells fluoresce green and non-viable cells stain orange. Enumerations were performed in duplicate and cells were routinely resuspended at a density of 5 x 10^5 cells/ml and used when less than 90% confluent.

2.4.5 Co-culture of Gastric Epithelial Cells with Recombinant Trx

AGS cells were removed from cell culture flasks by trypsinisation, counted (Section 2.4.4) and seeded in 6-well plates (Nunclon, Roskilde, Denmark) at a density of 5 x 10^5 cells/ml. At 90% confluency, cells were incubated with freshly reduced recombinant Trx at a range of concentrations. Trx was reduced with DTT (2 mM) for 1 hour at 37°C and desalted by spin chromatography (Section 2.11.5) prior to dilution in pre-warmed RPMI at the desired concentration. Incubation of AGS cells with culture media alone was included for each experiment.

2.4.6 MTT Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, Dorset, U.K.) is a tetrazolium salt which is incorporated into the mitochondria of viable cells. MTT dissolved in cell culture medium lacking phenol-red forms a yellow solution, however in the presence of viable cells isopropanol soluble purple formazan crystals are formed. The number of viable cells is directly proportional to the colour intensity formed and this intensity can be monitored spectrophotometrically (Denziot and Lang, 1986). AGS cells (2.5 x 10^4) were resuspended in 100 µl culture-medium and plated in a flat-bottomed micro-titre plate and incubated for 24 hours at 37°C and 5% CO_2. Cells were then incubated in the presence and absence of freshly reduced recombinant *H. pylori* Trx at a range of concentrations. Control wells containing medium alone were also included. 10 µl of MTT solution was then added to the wells and further incubated for between 1 and 3 hours. Following incubation, plates were removed from the incubator and 100 µl of MTT solubilisation solution (Sigma) was added to each well. Absorbance was measured at 490

nm using an ELISA plate reader (Labsystems Multiskanplus, Type 314, Finland). The optical density of the control well was subtracted from the average of the test results (n = 3), and viability was calculated for the range of Trx concentrations used.

2.5 CELL PROTEIN PREPARATION

2.5.1 Cell Fractionation and Preparation of Cytosolic Fractions

Spent media was removed, and cells were washed twice with ice-cold PBS [Appendix A]. Cells were subsequently harvested by scraping in ice-cold PBS (5 mls) and centrifuged at 1400 rpm for 5 min at 4°C. The pellet formed was resuspended in 1 ml ice-cold PBS and transferred to an Eppendorf tube. Tubes were centrifuged at 10,000 g for 10 min at 4°C, the supernatant discarded, and the pellet resuspended in 200 μl Buffer C [Appendix 1]. The sample was then sonicated three times for 10 s on ice in a Branson Sonifier, boiled for 5 min and centrifuged at 10,000 g for 2 min. The supernatant was retained for protein estimation and stored at –20°C until required.

2.5.2 Determination of Protein Concentration

The protein concentration of various cellular extracts was determined primarily by the method of Bradford (1976) using Bio-Rad Protein Assay Concentrate (code 500-0006, Bio-Rad, U.K.). Assay concentrate was freshly diluted 1:5 with distilled water and mixed well prior to each assay. A standard curve was prepared as follows. A BSA standard was prepared in the appropriate buffer to a final concentration of 20 mg/ml BSA, this was serially diluted to yield standards at 20, 10, 5, 2.5, 1.25, 0.312, 0.0625 and 0 μg/ml protein. However, for protein estimation in AGS nuclear extracts, samples were diluted 1:80 in Buffer D [Appendix 1] prior to addition of Bio-Rad dye concentrate. A stock of 1 mg/ml BSA in Buffer D was prepared and serially diluted in Buffer D to yield standards at 20, 10, 5, 2.5, 1.25, 0.312, 0.0625 and 0 μg/ml protein. The absorbance of protein solutions were read at 595 nm in duplicate in a Tecan Spectra Fluor Plus spectrophotometer. A specifically designed Excel macro averaged readings, constructed a standard curve and calculated the individual protein concentrations with reference to the standard curve automatically. In some instances, a protein standard of known concentration was included with unknown samples. A commercial BSA solution at a concentration of 2 mg/ml (BSA Protein Standard, Sigma, Poole, U.K.) was used to monitor accuracy and reproducibility of the Bradford method.

An alternate protein concentration assay system was required for protein samples which contained high levels of SDS as this chemical is known to interfere with Coomassie Blue dye binding. The Markwell protein assay (Markwell *et al.*, 1978) was used to estimate protein concentration of total cell lysates obtained from mammalian cells (Section 2.6.1). A standard curve was prepared as follows. A BSA standard was prepared in the appropriate buffer to a final concentration of 0.5 mg/ml BSA, this was serially diluted to yield standards at 250, 200, 150, 100, 50, 25 and 0 μg/ml protein. Samples were diluted 1:10, 1:20, 1:50 with distilled water to yield a final volume of 300 μl. To this 0.9 ml reagent C [Appendix 1] was added, vortexed, and incubated at room temperature for 10 min. 90 μl of reagent D [Appendix 1] was then added, vortexed and incubated at room temperature for 45 min. The absorbance of protein solutions were read at 660 nm in duplicate in a Pye Unicam 5625 spectrophotometer. Readings were averaged for standards and samples, a standard curve constructed and the protein concentrations were estimated with reference to the standard curve.

2.6 POLYACRYLAMIDE GEL ELECTROPHORESIS

2.6.1 Sample and Molecular Weight Standard Preparation

Following protein estimation, samples were generally aliquoted to yield 50 μg of protein. For protein concentration, this volume was diluted 1:5 with ice-cold acetone and incubated at –20°C for at least 45 min. The tubes were then centrifuged at 15,000 g for 4 min and the supernatant was aspirated and discarded. Any remaining acetone was allowed to evaporate at room temperature for approximately 15 min. Precipitated protein (50 μg) was resuspended in 5 to 20 μl 1X reducing or non-reducing sample buffer (diluted from 5 X stock) as required [Appendix A]. Protein samples and appropriate molecular weight standards were boiled at 100°C for 5 min and centrifuged briefly (30 s) to remove any insoluble solids.

2.6.2 SDS-PAGE and Gradient SDS-PAGE

An ATTO system was used for all SDS-PAGE gels (ATTO Corporation, Japan) and protein samples were electrophoresed using a Consort electrophoresis power supply unit. Proteins were separated on reducing gels prepared using a discontinuous buffer system as described by Laemmli (1970) and adapted by Sambrook *et al.* (1989). Resolving and stacking acrylamide gels were prepared to the required percentage acrylamide in the order indicated in **Tables 1 and 2**. The composition of the resolving gels for gradient SDS-

PAGE are shown in **Table 3**. APS and TEMED were added last with gentle swirling of the mixture. Electrophoresis was carried out at 25 mA per gel for approximately 1.5 h until the dye front had reached just above the gel base, at which stage electrophoresis was discontinued.

Component	10%	12.5%	15%	20%
Acryl/Bis 30%	6.66 ml	8.32 ml	10 ml	13.32 ml
Tris 1.5M	5.0 ml	5.0 ml	5.0 ml	5.0 ml
H_2O	8.32 ml	6.56 ml	4.89 ml	2.78 ml
APS 10%	100 µl	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl	10 µl

TABLE 1. Composition of resolving gels for SDS-PAGE.

Component	Amount
Acryl/Bis 30%	1.33 ml
Tris 1.0M	3.05 ml
H_2O	5.55 ml
APS 10%	50 µl
TEMED	10 µl

TABLE 2. Composition of stacking gel for SDS-PAGE.

Component	4%	5%	15%	20%	25%
Acryl/Bis30%	1.332 ml	1.665 ml	5 ml	6.66 ml	8.33 ml
Tris 1.5M	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
H_2O	6.16 ml	5.78 ml	2.44 ml	0.785 ml	0.62 ml
APS 10%	10 µl	10 µl	10 µl	10 µl	10 µl
TEMED	1 µl	1 µl	1 µl	1 µl	1 µl
Sucrose	-	-	1.5 g	1.5 g	1.5 g

TABLE 3. Composition of resolving gels for gradient SDS-PAGE.

2.6.3 Two-dimensional Gel Electrophoresis (2-D GE)

First dimension runs, i.e. isoelectric focusing, were performed using the IPGphor™ isoelectric focusing system (Amersham Pharmacia, U.K). Protein extracts were separated on 13 cm long immobiline drystrips (pH 3-10). Depending on the experiment, 50 to 150 μg of protein was solubilised in rehydration solution [Appendix A] and applied to the strips. Proteins were focused to equilibrium using the IPGphor™ Isoelectric Focusing System (Amersham Pharmacia Biotech). Second-dimension runs, i.e. separation of proteins according to molecular weight, were performed by SDS-PAGE on polyacrylamide gels (Section 2.6.2). IEF was performed as recommended by the manufacturer, Amersham. Isoelectric focusing was then carried out under the following conditions: rehydration at 60 V overnight for 10 h, followed by focusing at 500 V for 1 h, 1000 V for 1 h and finally 8000 V for 2-3 h. Following focusing, strips were stored at –20°C in a sealed 10 ml plastic pipette until required.

For second dimension electrophoresis, strips were equilibrated in approximately 10 ml of SDS Equilibration buffer [Appendix A] for 10 to 15 min with shaking. Care was taken to ensure that the gel side of the strip was facing upwards during the equilibration step. Strips were washed once briefly with 1 X Running buffer [Appendix A] and placed carefully onto pre-prepared SDS-PAGE gels (plastic backing of IPG strip flush with glass plate of the polyacrylamide gel). Second dimension gels were electrophoresed at 400 V, 30 mA for approximately 2.5 h.

2.6.4 Western Immunoblotting

Western immunoblotting was carried out using the semi-dry method for the transfer of electrophoresed proteins to immobilising membranes as described by Towbin (1979) and was performed using an ATTO semi-dry transfer system (ATTO Medical Supplies, Japan). During SDS-PAGE, polyvinylidene fluoride (PVDF) transfer membrane (0.45 μ, Pall Life Sciences) of dimensions 6.5 x 9 cm was briefly saturated with methanol for 10 – 15 s and then equilibrated in transfer buffer for approximately 20 – 30 min prior to semi-dry transfer. Whatmann 3mm filter paper cut to 6.5 x 9 cm was also saturated in transfer buffer prior to semi-dry blot sandwich construction, which was assembled in the order of cathode, filter paper, PVDF, acrylamide gel, filter paper and finally anode. Electrophoretic transfer was performed at 100 mA per gel for 60 min. Following semi-dry transfer, PVDF membrane was removed and processed for immunoblotting. The lane containing the

molecular weight marker was stained with Coomassie Blue R-250 followed by destaining in 50% methanol.

2.6.5 Immunoblot Detection and Development

Non-specific sites on the membrane following semi-dry transfer were blocked by incubation with freshly prepared PBS buffer containing 5% non-fat skimmed milk (Blocking solution) [Appendix A] for 1 h with gentle agitation on an orbital shaker (Stuart Scientific, U.K.) at room temperature. Blots were then washed twice with PBS and incubated with appropriate primary antibodies diluted (range 1:200 to 1:1000) in Primary and Secondary Antibody Diluent Solution [Appendix A] for 1 h with shaking in a sealed plastic bag. Following incubation with primary antibody, blots were washed several times over 30 min with 0.1% PBS-Tween Washing Solution [Appendix A]. Blots were then incubated with the relevant horse radish peroxidase-conjugated secondary antibody in Primary and Secondary Antibody Diluent Solution for 1 h with shaking in a sealed plastic bag. Following incubation with secondary antibody, blots were finally washed 4 to 5 times with 0.1% PBS-Tween Washing Solution over 30 min. Prior to development, blots were placed in PBS. Blots were stored in PBS at 4°C if development could not be undertaken immediately.

Detection of immunblots was performed using the enhanced chemiluminescence (ECL) method. Membranes were incubated for 1 min in a solution of iodophenol (400 μM), luminol (1.25 mM), and hydrogen peroxide (0.1% (v/v)) in 0.1 M Tris-HCl (pH 8.8) [Appendix 1]. The membrane was removed after 1 min and placed between acetate sheets which were then exposed to Kodak X-OMAT S film for the appropriate time period (range 30 s to 30 min). Exposed films were developed using an automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany).

2.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY

2.7.1 Preparation of Nuclear Fractions

Nuclear extracts were prepared as described by Osborn *et al.* (1989). Cells seeded in 6-well cell culture plates (1.5 ml volume) were treated as described in experimental procedure. Treatment termination was achieved by media removal and washing twice with ice-cold PBS [Appendix A]. All subsequent steps were carried out on ice with ice-cold buffers, which were prepared immediately prior to use. All centrifugation was carried out at 4°C.

Cells were pelleted by centrifugation and resuspended in 1 ml hypotonic Buffer A [Appendix A]. Following centrifugation (12,000 rpm, 10 min) the pellet was resuspended in 20 µl Buffer A containing 0.1% (v/v) Nonidet P-40 and incubated on ice for 10 min for cellular lysis. Material was further centrifuged (12,000 rpm, 10 min) and pellets were resuspended in 15 µl high salt buffer, Buffer C [Appendix 1], incubated on ice for 20 min and centrifuged again (12,000 rpm, 10 min). The supernatant was retained and 75 µl of Buffer D [Appendix 1] was added. Extracts were stored for up to six months at –70°C until required for protein estimation by Bradford assay.

2.7.2 Consensus Oligonucleotide Labelling

5'-end labelling of the 22 base pair oligonucleotide containing the NF-κB consensus sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') or the 21 base pair AP-1 (*c-jun*) consensus sequence (5'- CGC TTG ATG AGT CAG CCG GAA-3') (Promega Corp., WI, U.S.) was carried out according to the protocol of Sambrook *et al.* (1989). The reaction mixture was prepared in a sterile Eppendorf tube with sterile water to a final volume of 50 µl (**Table 4**).

Component	Amount	Final Volume
5'-ends of DNA probe(1-50 pmol)	5.7 µl	
T4 polynucleotide kinase (10-20U)	2.5 µl	
10X Kinase Buffer	5 µl	50 µl
[γ-^{32}P] ATP (3000 Ci/mmol, 10 mCi/ml, 50 pmol)	15 µl	
Sterile water	21.8 µl	

TABLE 4. Consensus olignucleotide labelling reaction components.

T4 polynucleotide kinase and kinase buffer were obtained from Pharmacia Biosystems, U.K. The reaction mixture was incubated in a pre-warmed Perspex box for 10 min at 37°C and the reaction was stopped by the addition of 2 µl of 0.5 M EDTA. To the reaction mixture, 50 µl of phenol:chloroform solution (1 part TE-saturated phenol and 1 part chloroform:isoamyl alcohol, (24:1 ratio) was added to facilitate DNA extraction. The mixture was vortexed for 1 min and centrifuged (13,000 g, 2 min). The upper aqueous layer was transferred to a fresh tube and 2 µl of 5 M NaCl was added. The tube was

vortexed and 100 µl of ethanol was added followed by incubation at –70°C for 30 min to precipitate the DNA. Following centrifugation at 13,000 g for 5 min, the supernatant was removed and the pellet dried in a vacuum dryer. Finally, the pellet was resuspended in 50 µl TE buffer [Appendix A]. Calculation of labelled oligonucleotide activity was achieved by counting 1 µl of this solution in 5 ml Ecosint using a Wallac 1409 DSA Liquid Scintillation Counter. 10,000 cpm of labelled oligonucleotide was used per EMSA reaction.

2.7.3 EMSA DNA-Protein Binding Reaction

The binding reaction was prepared for NF-κB and AP-1 activity detection by electrophoresis according to the protocol of Sen and Baltimore (1986). Nuclear extract protein (4 µg) was incubated with 10,000 cpm of ^{32}P-labelled NF-κB or AP-1 consensus oligonucleotides. The assay was conducted in 20 µl binding reaction in the presence of binding buffer [Appendix A] and 2 µg poly (dI-dC) as non-specific competitor. The reaction components were then incubated for 30 min at room temperature. The reaction was terminated by the addition of one-tenth volume of gel loading dye [Appendix A]. The samples were then ready for electrophoresis.

Component	Amount
Nuclear Extract	2-4 µg
10 X Binding Reaction Buffer	2 µl
[γ-^{32}P] ATP labelled probe	1 µl
Poly(dI-dC)	2 µl
Sterile water (to a final volume of 20 µl)	x µl

TABLE 5. DNA-Protein Binding Reaction.

2.7.4 Gel/Buffer Preparation and DNA-Protein Complex Electrophoresis

Nuclear extract proteins were separated on 5% polyacrylamide non-reducing 1-mm thick gels using the ATTO gel system (ATTO, Medical Supply Corporation, Japan). **Table 6** indicates the gel components and amounts. Distilled water, Accugel (29:1) (National Diagnostics, Kimberly Research, GA, U.S.) and 0.5 X TBE running buffer [Appendix A] were mixed carefully prior to the addition of nuclease free ammonium persulphate.

Polymerisation of the gel was achieved over 45 min at room temperature upon the addition of TEMED to the gel solution.

Component	Amount
Acrylamide Mix (40%)	3.125 ml
10X TBE Buffer	2.5 ml
Ammonium persulphate	50 mg
Distilled water	19.4 ml
TEMED	15 μl
DTT	5 μl

TABLE 6. EMSA Gel Solution Components.

The polymerised gels were pre-electrophoresed in 0.5 X TBE running buffer [Appendix 1] at 80 V for 30 min. Upon sample loading, the gels were then electrophoresed (Consort EUSS Unit) in the same buffer at 150 V for 2-3 h or until the dye front had migrated a sufficient distance. At this stage gels were removed from the gel moulds, transferred to filter paper, covered with cling-film and dried on an automatic gel drying unit. The filter paper was then exposed to Kodak X-OMAT S film for 24 - 48 h in a film cassette with intensifying screens at –70°C. Exposed Kodak X-OMAT S film was developed using a Curix 60 developing machine (AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany).

2.7.5 Positive Controls for NF-κB and AP-1 Activation.

Positive controls for both NF-κB and AP-1 activation were included in all EMSA experiments performed. HuT 78 is a T-cell lymphoma derived from the peripheral blood of a patient with Sezary syndrome. This cell line was obtained from the European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, U.K.) and is known to constitutively express NF-κB and therefore is suitable as a positive control for the activation of NF-κB. Nuclear extracts were prepared from the non-adherent HuT 78 cell line as described in Section 2.8.1 and the Bradford assay was used to estimate the amount of protein. 4 μg of this nuclear extract was run in parallel with samples in which the DNA-binding activity of NF-κB was being examined by EMSA. A positive control for the activation of AP-1 was

obtained as follows. Serum starved (3 h) AGS cells were exposed to deoxycholic acid (Sigma, Dorset, U.K.), a bile acid, at a concentration of 250 μM in RPMI for a period of 3 h. This compound is known to induce AP-1 activation at a concentration of 250 μM (Qiao *et al.*, 2000). Nuclear extracts were prepared as described in Section 2.7.1. and the Bradford assay was used to estimate the protein concentration of the extract. 4 μg of this nuclear extract was run in parallel with samples in which the DNA-binding activity of AP-1 was being examined by EMSA.

2.8 DNA MANIPULATION

2.8.1 Preparation of *H. pylori* Genomic DNA

H. pylori from 2 to 4 day old Columbia Blood Agar plates were harvested by scraping in ice-cold TE buffer (50 mM Tris, pH 8.0, 1 mM EDTA) and pelleted by centrifugation (7000 g, 4°C, 10 min). The pelleted material was washed and finally resuspended in 800 μl TE buffer. To this suspension 10 μl of 20% SDS plus 100 μl Proteinase K (10 mg/ml stock) was added and incubated for 1 h at 37°C. An equal volume of buffered phenol was then added, mixed gently and centrifuged for 10 min at 13,200 rpm at room temperature. The viscous layer which formed was transferred to a fresh tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added, the tube agitated and subjected to centrifugation at 13,200 rpm, room temperature, 10 min. Aqueous DNA was transferred to a fresh tube and 100 μl of 3 M sodium acetate was added followed by 600 μl of isopropanol. The tube was incubated at RT for 10 min. Precipitated DNA was then removed with a glass rod and placed in 500 μl TE buffer. The solution was subsequently treated with RNase and re-precipitated as above. Finally, the DNA was rinsed on a glass rod with 96% ethanol and resuspended in 200 μl TE buffer. *H. pylori* genomic DNA was stored in 50 μl aliquots at -80°C prior to use.

2.8.2 *E. coli* DNA Manipulation

Plasmids were purified using the QIAprep miniprep kit (Qiagen, Hilden, Germany). Restriction enzymes used were obtained from New England Biolabs (U.K) or Promega (U.K.).

2.8.3 Preparation of Competent *E. coli* Cells for Transformation

An overnight culture of the appropriate *E. coli* strain (DH5α / BL21) was grown in LB media at 37°C. The culture was diluted 1/50 with LB media and shaken vigorously (200 rpm) until mid-log phase was reached. The culture was chilled on ice to 0°C and subsequently centrifuged (5000 g, 4°C, 5 min). The resultant bacterial pellet was resuspended in 1/10 of the original volume of washing buffer [Appendix 1] and incubated on ice for 30 min. The material was then centrifuged for 5 min gently and the supernatant was removed. The pellet was then resuspended in 1/50 of the original volume of freezing buffer [Appendix 1] and dispensed into cryovials and frozen at – 80°C until use.

2.8.4 DNA Amplification by PCR

Polymerase Chain Reactions were performed in a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer, U.K.) with Taq DNA Polymerase (Boerhinger Mannheim, Germany). Primers were from Sigma-Genosys (U.K.) or Invitrogen (U.K.). For standard amplifications, typically, in each reaction approximately 100 ng of chromosomal DNA was mixed with 5 pmol of each specific primer in a reaction volume of 25 μl. Reactions were performed by denaturing DNA at 94°C for 2 min 15 s, annealing at 45°C for 30 s and extension at 72°C for 45 s. A total of 30 cycles were performed.

2.9 INSERTIONAL MUTAGENESIS

2.9.1 Construction of *H. pylori* Thioredoxin 2 Isogenic Mutants by Insertional Mutagenesis

The thioredoxin 2 gene (*trx2*) was inactivated in *H. pylori* reference strains NCTC11638 (R2) and NCTC11637 (R1) and the clinical isolate strain 1061 by gene disruption as described below. All plasmids and bacterial strains used for insertional mutagenesis of the *H. pylori trx2* gene are shown in **Table 1, Chapter 4**. Primers used for DNA amplification by PCR are listed in **Table 7**. Approximately 1-kb (959 bp) of sequence directly flanking both the upstream and downstream region of *trx2* from *H. pylori* was amplified by PCR with *Taq* polymerase using the primers N1458F and 1458R and cloned into the pGEM-T Easy vector (Promega). The resultant plasmid, pGEMT::T1458, was cleaved at the unique *Bst*X1 site within the *trx2* gene and the sticky ends were blunted with using Klenow enzyme treatment. The resistance cassette *aph*-A3 was amplified from the plasmid pJMK30 using primers, AphA3R and Kana-L. A directed ligation with the restriction fragment *Xba*I (1024 bp), containing the kanamycin resistance cassette and the vector

(pGEMT::T1458) resulted in the plasmid pGEMTrx2::kan in which the resistance cassette was inserted into the centre of the *trx2* gene. The plasmid was used for natural transformation of *H. pylori* strains NCTC11638 (R2), NCTC11637 (R1) and 1061. Characterisation of the mutants was undertaken by PCR analysis with primers derived from regions flanking the kanamycin cassette. Amplimers of the expected size were obtained following PCR analysis of the Trx2 mutants. PCR sequencing analysis was also undertaken and confirmed the gene disruption in the *H. pylori* strains. The resistance cassette *cat* was amplified from the plasmid pUCAT using primers, CatR and CatL. A directed ligation with the 1074 bp restriction fragment containing the chloramphenicol resistance cassette and the vector (pGEMT::T1458) resulted in the plasmid pGEMTrx2::cm in which the resistance cassette was inserted into the centre of the *trx2* gene. The plasmid was used for natural transformation of *H. pylori* strains NCTC11638 (R2), NCTC11637 (R1) and 1061. Characterisation of the mutants was undertaken by PCR analysis with primers derived from regions flanking the choramphenicol cassette

TABLE 7.

Oligonucleotides for insertional mutagenesis of trx2

Primer N1458F: 5′- ACC GCC AAG AAT ACA ACG AG - 3′
Primer 1458R : 5′- TCG CGT TAG CGG TTA GCA AT - 3′
Primer AphA3R : 5′- CTG GAT GAA TTG TTT TAG TAC - 3′
Primer Kana-L: 5′- TTA CCT ATC ACC TCA AAT GG - 3′
Primer CatR: 5′- TTA CAA GAC TTG CTG AAT AA - 3′
Primer CatL : 5′- GCA GCT GAA TTC TGC AGG TA - 3′

2.9.2 Natural Transformation of *H. pylori*

Natural transformation of *H. pylori* was performed essentially as described by Smeets *et al.* (2000). Briefly, transformation of *H. pylori* was achieved by the addition of 5 μg of plasmid DNA to a patch (approximately 1 cent-sized) of freshly grown *H. pylori* on Columbia Blood Agar plates. After incubation for 15 hours at 37°C under microaerobic conditions, the bacteria were transferred to selective Columbia Blood Agar plates

containing kanamycin (20 μg/ml) or chloramphenicol (20 μg/ml) and grown under microaerobic conditions for a further 5-7 days.

2.10 SITE-DIRECTED MUTAGENESIS

2.10.1 Construction of redox-active site mutated *H. pylori* Trxs

Mutations were introduced into *H. pylori* Trx2 coding gene (HP1458) by PCR-mediated site-directed mutagenesis, using an overlap extension approach. The template was the expression vector pPROK1-Trx2 (a gift from Drs. L. Poole and L. Baker, Wake Forest University, Madison, WI). Cys-28 and Cys-32 were replaced with a serine residues both individually and simultaneously. Degenerate and flanking primers used for DNA amplification by PCR are listed in **Table 8**. Amplification of mutagenic products from the pPROK1-Trx2 template was performed for 30 cycles at an denaturing temperature of 94°C, an extension temperature of 56°C and an annealing temperature of 72°C (Thermal Cycler, Perkin-Elmer), using *Pfu* Turbo polymerase (Stratagene, U.K.). Primers finaf1 and KFW96 yielded a PCR product of the expected size (200 bp) following amplification as did primers finaf2 and KFW97, which produced a 300 bp fragment following amplification by PCR. These two products were used as templates in a third PCR reaction to yield the complete mutagenic construct using the flanking primers KFW96 and KFW97 (400 bp fragment). This amplification was carried out using *Pfu* Turbo polymerase and was performed for 25 cycles at an denaturing temperature of 94°C, an extension temperature of 56°C and an annealing temperature of 72°C (Thermal Cycler, Perkin-Elmer). The pPROK1-Trx2 vector was cut using *Hind*III and *Eco*R1 restriction enzymes and the vector minus the Trx2 insert was gel extracted and purified (Qiagen Gel Extraction Kit, Qiagen, Hilden, Germany). The mutagenic insert was digested with *Hind*III and *Eco*R1 and ligated to the pPROK1 vector. This construct was used to transform *E. coli* DH5α. Plasmids were isolated from transformants selected on LB plates plus ampicillin (50 μg/ml) and the correct size of the mutagenic insert was verified by restriction digests with *Hind*III and *Eco*R1. 100 ng of plasmid DNA was sent for sequencing from 12 clones which were randomly selected from those shown to possess the correct size insert to determine the nature of the mutation in the *trx2* gene (DNA Sequencing Service, Dept. of Biochemistry, Cambridge University, U.K.).

TABLE 8.

Mutagenic and flanking oligonucleotides for site-directed mutagenesis of trx2

Primer finaf1: 5′- CTGGT**s**CCCGGATT**s**CAGAAAGATTGAGCCGATCATGG -3′(S)[a]
Primer finaf2: 5′- CTG**s**SAATCCGGG**s**ACCAGCTCGCCCCCACATTAAC -3′(AS)[b]
Primer KFW96: 5′- TCTGATTTAATCTGTATCAGG -3′(AS)
Primer KFW97: 5′- ATTCTGAAATGAGCTGTTG -3′(S)

[a]S, sense [b]AS, antisense **s**: degenerate base coding for serine or cysteine.

2.11 RECOMBINANT PROTEIN PURIFICATION

2.11.1 Expression and Purification of recombinant His-tagged *H. pylori* Trx2.

Transformants of *E. coli* BL21(DE3)pLysS with plasmid pET-16b (Novagen) containing the *trx2* gene were grown at 37°C in LB broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). *H. pylori* Trx was expressed as an NH_2-terminal decahistidine fusion protein in *E. coli*. The gene coding for Trx was amplified by PCR using Expand™ (Roche Molecular Biochemicals), using the amplification conditions recommended by the manufacturer. Under these conditions a single product was obtained and was cloned into the expression plasmid via the *Bam*H1 and *Nde*I restriction sites. The following primers were used: forward primer, 1458 F 5′- GAT CTC GCC ATA TGT CAG AAA TGA TTA ACG GGA AG - 3′ ; reverse primer, 1458 R 5′- CGC GGA TCC TCA CAA TAA CGC TTT TAG AGC GTC TTC - 3′. Overexpression of the fusion protein was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside at exponential phase. Incubation was continued at room temperature overnight. The induced cells were harvested by centrifugation (5000 g, 10 min, 4°C), washed once with 50 mM Tris-HCl (pH 7.5) and lysed by sonication in a Branson sonifier (6 x 30 sec). Sonicated material was centrifuged to remove debris (12,000 g, 25 min, 4°C) and filtered (0.45 μ) to clarify. The soluble fusion protein was purified by immobilised metal ion affinity chromatography (IMAC) on an Ni^{2+} column (3 ml) which was prepared and charged according to the manufacturer's instructions. The bound protein was eluted with 400 mM imidazole in 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. Typically, 10 –12 mg of recombinant Trx was obtained from I L of bacterial culture following IMAC purification.

2.11.2 Factor Xa Digestion and His Tag Removal

Factor Xa (Roche) was used at a ratio of 1:100 to 1:200 to cleave the His tag from recombinant His-tagged *H. pylori* Trx2. Prior to incubation with Factor Xa, recombinant protein was dialysed against Factor Xa buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 2 mM $CaCl_2$). The concentration of Trx2 at this stage was approximately 1-2 mg/ml. Incubation of the digestion reaction was carried out at room temperature for 12 to 14 h. After digestion, the His tag was removed by passing the reaction mix through a ρ-aminobenzamidine column (1 ml) at room temperature. At this stage any undigested material or contaminants from initial passage through the IMAC affinity column were removed by passing the Factor Xa-free material back through the IMAC affinity column.

2.11.3 Ion-Exchange Chromatography

E. coli strain BL21(DE3)pLysS cells containing the *H. pylori trx2* gene were grown (following inoculation from a fresh overnight starter culture) in 800 ml of Luria-Bertani medium (50 μg/ml ampicillin and 50 μg/ml chloramphenicol) to an optical density at 600 nm of 0.6 and were then induced at 37°C for 3 h by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) to a final concentration of 0.4 mM. Cells were harvested by centrifugation (5000 g, 4°C, 5 min) and pellets were stored at –20°C (several hours to overnight). Cellular lysis occurred upon thawing and pellet was resuspended in 1/10 volume of 5 mM potassium phosphate buffer (pH 7.0) and subjected to sonication (5 × 30 sec pulses with cooling on ice in a Branson Sonifier Model 450. Sonicated material was centrifuged at 13,000 g, 4°C for 25 min and supernatant was filtered (0.45 μ Pall Acrodisc low-protein binding filter) and dialysed overnight against 5 mM potassium phosphate buffer (pH 7.0) (2L x 3 changes at 4°C). The resultant supernatant was applied to a DEAE-cellulose column (Whatman Ltd., Maidstone, U.K.) pre-equilibrated with 5 mM potassium phosphate (pH 7.0) buffer (flow rate 0.6ml/min) and the non-binding fraction was collected and subsequently applied to a carboxymethylcellulose column (Whatman) pre-equilibrated with 5mM potassium phosphate buffer (pH 7.0) and eluted with a linear gradient of 5-60 mM potassium phosphate (Trx2 typically eluted within the gradient range of 20-30 mM potassium phosphate). Composition of the eluate was assessed by A_{280} and SDS-PAGE following elution and those fractions containing high levels of pure Trx2 were pooled and concentrated with an Amicon concentrator (molecular weight cut-off range 8000 kDa). Aliquots were subsequently stored at –20°C following the

addition of DTT to a final concentration of 2 mM. Typically a protein yield of 13-15 mg was obtained from 1 L of induced culture.

2.11.4 Thioredoxin activity assay

Thioredoxin activity was determined by the insulin precipitation assay described by Holmgren (1979). The standard assay mixture contained 0.1 M potassium phosphate (pH 7.0), 1 mM EDTA and 0.13 mM bovine insulin (Sigma, Poole, Dorset, U.K.) in the presence or absence of recombinant protein, and the reaction was initiated upon the addition of 1 mM dithiothreitol (DTT). An increase of the absorbance at 650 nm was monitored at 25°C in a Pye Unicam 5625 spectrophotometer. Prior to experiments Trx2 was fully reduced by incubation with 2 mM DTT at 37°C for 1 h. Following reduction, DTT was removed by spin chromatography.

2.11.5 Spin Chromatography

Excess salt (DTT) was separated from the recombinant protein preparation following reduction by the method of Penefsky (1977). Briefly, a 1ml syringe was tightly plugged with glass wool up to the 0.1ml mark and the syringe was then filled to capacity with Sephadex G-25 equilibrated in phosphate buffered saline (PBS, pH 7.5). The syringe was placed in test tube and centrifuged at 1600 g for 4 min. Following centrifugation the dry bed volume was approximately 0.8 to 1 ml. 0.1 ml of the appropriate protein buffer was then added to the dry Sephadex and the centrifuged as before. This equilibration process was repeated at least once more and then 0.1 ml of the protein solution containing salt was layered onto the dry Sephadex matrix and the syringe was placed in a clean teat tube and centrifuged at 1600 g for 4 min. The eluate containing the desalted protein was transferred from the test tube to a minifuge tube for experimental use. The salt remained trapped in the Sephadex column and was discarded.

2.11.6 Immuno-affinity purification of anti-Trx2 antibodies

Recombinant *H. pylori* Trx was dialysed against binding buffer (0.5 M Na_2PO_4, pH 7.5) to remove extraneous amino groups. A small amount of protein in buffer was retained to determine binding efficiency. Cyanogen bromide activated Sepharose was prepared according to the manufacturer's instructions (Sigma, Dorset, U.K.) and was added to the Trx. The slurry was mixed gently at 4°C overnight. The beads were then washed twice with 0.5 M Na_2PO_4 and a small sample of the wash was retained to determine binding

efficiency. The beads were washed with 1 M NaCl, 0.05 M Na_2PO_4, pH 7.5. 10 volumes of 100 mM ethanolamine (pH 7.5) was added to the beads and they were subsequently mixed at room temperature for 4 h or overnight at 4°C. Following this incubation step, the beads were washed twice with PBS. Merthiolate was added to a final concentration of 0.01% and beads were stored at 4°C until use. Approximately 1 ml of anti-Trx antiserum was diluted 1/10 with 10 mM Tris, pH 7.5 and the diluted antiserum was passed through an affinity column packed with the prepared Trx-coupled beads. The material was collected and passed though the affinity column 5 more times to ensure complete binding. The column was then washed with 10 bed volumes of 10 mM Tris (pH 7.5). The bound antibodies were eluted with 100 mM glycine (pH 2.5) and the eluate for the column was collected in tubes containing 1/10 volume of 1 M Tris (pH 8.0). Eluted antibodies were dialysed against PBS (pH 7.5), aliquoted and stored at –20°C. Protein estimation was carried out prior to freezing.

2.12 IDENTIFICATION OF TRX2 TARGETS; AFFINITY SELECTION AND THIOL-SPECIFIC LABELLING

2.12.1 Preparation of Sepharose 4B for ligand coupling

The desired amount of freeze-dried powder (1 g powder per 3.5 ml final gel volume) was weighed and suspended in 1 mM HCl. The resin was washed for 15 min with 1 mM HCl on a sintered glass filter.

2.12.2 Preparation of Trx-immobilised resin

The *H. pylori* recombinant Trx2 and Trx2 mutants, Trx2-C28S, Trx2-C31S and Trx2-C28S/C31S were overexpressed in *E. coli* and purified as described (Section 2.11.3). *H. pylori* Trx2 mutants in 100 mM sodium carbonate buffer (pH 9.5) containing 0.5 M NaCl were incubated for 1 h at room temperature with prepared Sepharose 4B resin with gentle agitation. Termination of the coupling reaction was achieved by centrifugation and the unreacted side chains of the resin were blocked by incubation with 50 mM Tris (pH 8.0) for 12 h at 4°C.

2.12.3 Collection of target proteins by immobilised Trx2 resin

H. pylori strain NCTC11638 total cellular lysate (5 mg protein) or AGS cell lysate (5 mg) was incubated with 1 ml of Trx2 immobilised resin (a total of 2 mg of Trx was immobilised) at room temperature for 1.5 h with gentle agitation. The resin was then

washed with 50 mM Tris (pH 8.0) and either 300 mM or 500 mM NaCl to remove non-specifically bound proteins. The washing was repeated until the absorbance of the wash buffer at 280 nm was almost zero (approx. 7 wash cycles). Finally, the resin was resuspended in 50mM Tris (pH 8.0), 300 mM or 500 mM NaCl, 10 mM DTT and incubated with gentle agitation at room temperature for 30 min. The eluted proteins were separated from the resin by centrifugation and analysed by SDS-PAGE.

2.12.4 Coomassie Blue G-250 staining (for Mass Spectrometry)

Following electrophoresis, SDS-PAGE gels were incubated in fixation solvent [Appendix A] overnight with shaking. Gels were washed 3 times in 2 L water with shaking and the incubated for 1 h in incubation solvent [Appendix A] with shaking. 0.025 % (w/v) Coomassie G-250 (Bio-Rad) was dissolved in 20 ml methanol (0.25 g/L incubation solvent). Coomassie G-250 solution was added to gels in the incubation solvent. Gels were then stained for 2 to 3 days after which time they were washed in water. Gels were further washed twice in 25 % (v/v) methanol and finally washed once more in water. Gels were stored (sealed) in water at 4°C.

2.12.5 Fluorescent labelling of proteins and identification of labelled proteins

Total cellular free thiols in *H. pylori* sonicates were conjugated with the thiol-specific fluorescent probe monobromobimane (mBrB; Molecular Probes, Oregon, U.S.). *H. pylori* extract (100 μg) was incubated for 1 h at 37°C in potassium phosphate buffer (0.1 M, pH 7.4) containing Trx (20 μg) NADPH (1.25 mM) and *E. coli* thioredoxin reductase (0.3 U, 3.3 μg) in a final volume of 50 μl. mBrB was added to a final concentration of 2.5 mM and the incubation was allowed proceed for an additional 20 min in the dark at room temperature. All samples were dialyzed against water for 1 h at room temperature prior to acetone precipitation and solubilisation of the precipitate in rehydration buffer (urea, 8M; CHAPS, 2%; bromophenol blue, 0.002%, DTT, 0.28%; IPG buffer, 0.5 %).

Trx1- and Trx2-reduced, mBrB-labelled *H. pylori* proteins were separated by 1- and 2-D electrophoresis (isoelectric focussing conditions, 20342 Vh), fixed in methanol (30% v/v)/acetic acid (10%, v/v), and fluorescent proteins were visualised by exposing gels to UV light and images were acquired on a Syngene gel documentation system. Proteins of interest incorporating the fluorophore thus identified were excised from the corresponding

Coomassie brilliant blue (R-250) stained 2-D gels and submitted for mass spectrometric analysis at the Taplin Biological Mass Spectrometry Facility, Harvard Medical School, Boston.

2.12.6 Reconstitution of recombinant proteins with the Trx system using a fluorescent labelling system.

Purified recombinant forms of Bcp and Tpx were reconstituted with the Trx system to demonstrate that mBrB could be incorporated into both proteins when reduced by Trx. In this case the assay mixture (final volume 50 μl), in 0.1 M potassium phosphate (pH 7.4), contained NADPH (1.25 mM), *E. coli* thioredoxin reductase (0.12 U), either *H. pylori* Trx1 or Trx2 (6 μg, 10 μM) and either *H. pylori* Tpx or Bcp. The mixture was incubated at 37°C for 20 min after which time mBrB was added to a final concentration of 2 mM and the incubation was allowed proceed for an additional 10 to 20 min in the dark at room temperature. Following precipitation with four volumes of ice-cold acetone the mixture of proteins was separated by SDS-PAGE (15% acrylamide) and mBrB incorporation into Trx, Bcp and Tpx was visualised by exposing the gels to UV light as described above.

CHAPTER III

MOLECULAR CLONING, OVEREXPRESSION, PURIFICATION AND CHARACTERISATION OF AN UNUSUAL *H. PYLORI* THIOREDOXIN

CHAPTER III

3.1 INTRODUCTION

3.1.1 Thiol-Disulphide Oxidoredutases

Aerobic life conditions based on the metabolism of molecular oxygen are inseparably associated with the generation of reactive oxygen species. These include hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($OH^{\cdot}$). The redox status of a cell remains tightly controlled and relatively constant unless cells are exposed to very extreme oxidising conditions. These reactive oxygen species are cytotoxic for the organism and also for invading microorganisms, therefore, cells have evolved highly sophisticated mechanisms in order to maintain redox homeostasis to counteract the excess of ROS produced in an aerobic environment. The evolution of a shield of efficient enzymatic defence mechanisms was necessary to counteract this oxidative challenge. However, if the oxidative defence mechanisms are overcome the resulting oxidative stress can result in damage to membranes, DNA and cellular proteins. The specific properties of proteins are defined by their three-dimensional conformation and disulfide bonds participate in maintenance of the stability and higher order structure of proteins. Although the spontaneous formation of disulfide bonds in a protein can occur *in vitro*, this process is many fold slower and substantially less effective than *in vivo* (Peters and Davidson, 1982). This observation suggests the existence of enzymes which are capable of catalysing the process of native disulfide bond formation *in vivo*. Thioredoxins (Trx) are small thiol proteins with a molecular mass of approximately 12 kDa and are present in all organisms, both prokaryotic and eukaryotic, studied to date (Holmgren, 1985). The enzyme uses the conserved motif Trp-Cys-Gly-Pro-Cys to either reduce disulphide bonds or oxidise sulphydryls in a variety of proteins, both intracellularly and extracellularly. The first recognised formal role of this protein was as a component of a cycle which shuttles hydrogen from NADPH, through the flavoenzyme thioredoxin reductase and Trx to the oxidised form of the enzyme ribonucleotide reductase, an enzyme essential for the synthesis of DNA. This cycle results in the generation of oxidised thioredoxin reductase. (Holmgren, 1989). As this enzymatic sequence progresses, the Trx active site cysteines undergo both disulphide bond reduction and oxidation as transient mixed disulphide bonds are formed during the catalytic process. It has become apparent that Trx is an enzyme which possesses a very broad specificity and the interaction of this enzyme for targets

which cross many diverse cellular functions has been described in the literature recently. Among the varied range of physiological processes in which the participation of this enzyme has been described include modulation of transcription factor DNA binding (Matthews *et al.*, 1992), hydrogen donation to reductive enzymes (Holmgren, 1989), refolding of disulphide containing proteins (Lundstrom *et al.*, 1990), chaperone activity (Kern *et al.*, 2003) and cell growth stimulation (Wakasugi *et al.*, 1990), and the response to oxidative stress (Nishinaka *et al.*, 2001).

3.1.2 *H. pylori* Thiol-Disulphide Redox Chemistry

Issues of thiol-disulphide redox chemistry have remained largely unexplored in *H. pylori* until recently. Unlike other microorganisms, such as *E. coli*, where the intricacies of thiol redox reactions and redox homeostasis have been more fully explored, the basis of the sensitivity of this microaerophilic pathogen to molecular oxygen is only beginning to be investigated in *H. pylori*. Although replete with enzymes to detoxify reactive oxygen species, including superoxide dismutase, catalase and peroxidases, *H. pylori* possesses only one of the two major intracellular disulphide reducing systems, the thioredoxin system. The other system, the glutaredoxin (Grx) system, in which electrons are transferred from NADPH to glutathione reductase, then to glutathione, and finally to one of several glutaredoxins, is absent (Tomb *et al.*, 1997). The genome sequencing project of *H. pylori* strain 26695 (TIGR) revealed the presence of two thioredoxin coding genes (HP0824 (Trx1); HP1458 (Trx2)) and two annotated thioredoxin reductases (TR, HP1164; TR, HP0825) (Tomb *et al.*, 1997). Recently, functional aspects of Trx have generated much attention in the literature. As an enzyme which can influence so many essential cellular processes and yet is highly conserved across so many species in structural terms, from prokaryotes to complex eukaryotic organisms, the modulation of redox activity is of great interest. Variation of the two central amino acids (X) of the redox active site (CXXC) determines the reducing power of the enzyme. In organisms containing different Trxs, it is a key question as to whether these isoproteins possess differential functions. In this chapter, Trx2 from *H. pylori*, which harbours the rare motif, CPDC, has been cloned, overexpressed and functionally characterised. Trx2 was found to be functional as a thiol-disulfide reductase and to be present in the cytosolic compartment of the bacterium. The recombinant enzyme was also overexpressed at sufficiently high purity to allow structural data to be obtained and utilised to begin construction, at a high resolution, of a three-dimensional structure map of this unusual redox enzyme.

3.2 OBJECTIVES

1. Sequence comparison of *H. pylori* Trx1 and Trx2.
2. To clone and overexpress *H. pylori* Trx2 in a soluble form.
3. To purify *H. pylori* recombinant Trx2 to homogeneity by affinity chromatographic techniques.
4. To determination the subcellular localisation of *H. pylori* Trx2.
5. To assess the thiol oxidoreductase activity of recombinant *H. pylori* Trx2.
6. To functionally characterise recombinant *H. pylori* Trx2.
7. To prepare recombinant *H. pylori* Trx2 in order to determine the high resolution three-dimensional structure of the protein by X-ray crystallography.

3.3 RESULTS

3.3.1 Initial cloning of the *H. pylori trx2* gene from *H. pylori*.

The gene encoding Trx2 (HP1458; TIGR Database Annotation (www.tigr.org)) was cloned from the genomic DNA of *H. pylori* NCTC11638. The complete coding region of the gene was amplified by PCR using forward and reverse primers deduced from the genome sequence (*H. pylori* strain 26695 Genome Sequence, TIGR (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=ghp). A single product was obtained following PCR. The product was blunt-ended and cloned into the pET16b cloning vector. Overexpression of the recombinant protein was achieved following transformation of the cloning host, competent *E. coli* DH5α. Both strands of construct were completely sequenced using the dideoxynucleotide chain termination method (Department of Biochemistry, University of Cambridge, U.K.). Following transformation of the expression host, competent *E. coli* BL21(DE3)pLysS, the protein was overexpressed after the addition of IPTG and purified using metal ion affinity chromatography over a nickel column (Ni^{2+}).

3.3.2 Sequence comparison of *H. pylori* Trx2 with other Trxs.

The nucleotide and deduced amino acid sequence of the *H. pylori trx2* gene are shown in **Fig. 3.1** *H. pylori trx2* encodes a protein of 104 amino acid residues and a predicted molecular weight of 11,700 with a theoretical pI value of 8.1. *H. pylori* Trx2 contains, near the amino terminus, the short and characteristic amino acid sequence -Trp-Cys-X-X-Cys-, which is highly conserved among classical Trxs and is present in members of the Trx superfamily. The sequence -Cys-X-X-Cys- functions as the active site of the thiol-disulfide

exchange of the protein. Unusually, the redox active site motif of Trx2 from *H. pylori* differs in the two central variant amino acids. While most members of the Trx family contain the consensus active site sequence -Cys-Gly-Pro-Cys-, the Trx2 protein from *H. pylori* contains the active site sequence -Cys-Pro-Asp-Cys- as shown (**Fig. 3.1**). In addition to the redox active site motif of Trx, other conserved positions are usually single amino acid positions spanning the protein. Although sequence similarities between different Trxs are commonly quite low, the three dimensional structures of this family of proteins is highly conserved, which facilitates structural comparisons between Trxs of highly divergent species, such as *E. coli* and human proteins.

Fig. 3.2 (A) shows an amino acid sequence alignment of a number of prokaryotic and eukaryotic Trxs with *E. coli* Trx as the reference molecule, as this protein constitutes the most thoroughly investigated Trx, in terms of structure and function. It is obvious that redox active site, CGPC, is highly conserved. *H. pylori* Trx1 shows the highest sequence identity to *E. coli* Trx (41%), with *H. pylori* Trx2 sharing just 27% identity with *E. coli* Trx. Interestingly, of all the amino acid sequences, both prokaryotic and eukaryotic, examined using global alignment, *H. pylori* Trx2 shows the least similarity to *E. coli* Trx. Even yeast and human Trxs appear to be more similar to *E. coli* Trx than *H. pylori* Trx2. Interestingly, *H. pylori* Trx2 has been shown to have the highest sequence identity with a Trx homologue from *Archaeoglobus fulgidus* (Klenk *et al.*, 1997). **Fig. 3.2 (B)** shows a paired alignment of the two Trxs, Trx1 (HP824) and Trx2 (HP1458), from *H. pylori*, which shows a relatively low level of primary sequence homology (31%) between the two proteins. The substitution of the redox active site, -Cys-Gly-Pro-Cys-, for -Cys-Pro-Asp-Cys- in *H. pylori* Trx2 will most likely alters the redox potential of the enzyme and hence may influence catalytic rate or substrate interaction efficacy. The isoelectric point (pI) of Trx2 from *H. pylori* is quite unique in having a calculated value of 8.1 (*H. pylori* strain 26695 Genome Sequence, TIGR (http://www.tigr.org/), most Trxs studied thus far are acidic proteins possessing pI values between 4.5 and 5. **Table 1** shows the Trx pI values from a selection of other prokaryotic Trxs. It is apparent that *H. pylori* Trx2 is unusual in terms of its relatively high pI value compared with other Trxs, this feature could prove influential during protein-protein interactions. **Table 2** shows the pI values for a range of oxidative stress management proteins from *H. pylori.* From this table, one can see that the theoretical pI for *H. pylori* Trx2 is very close to the experimental value obtained for Trx2 following 2-D gel electrophoresis (pI 7.7) (*Helicobacter pylori* Berlin 2-D Database;

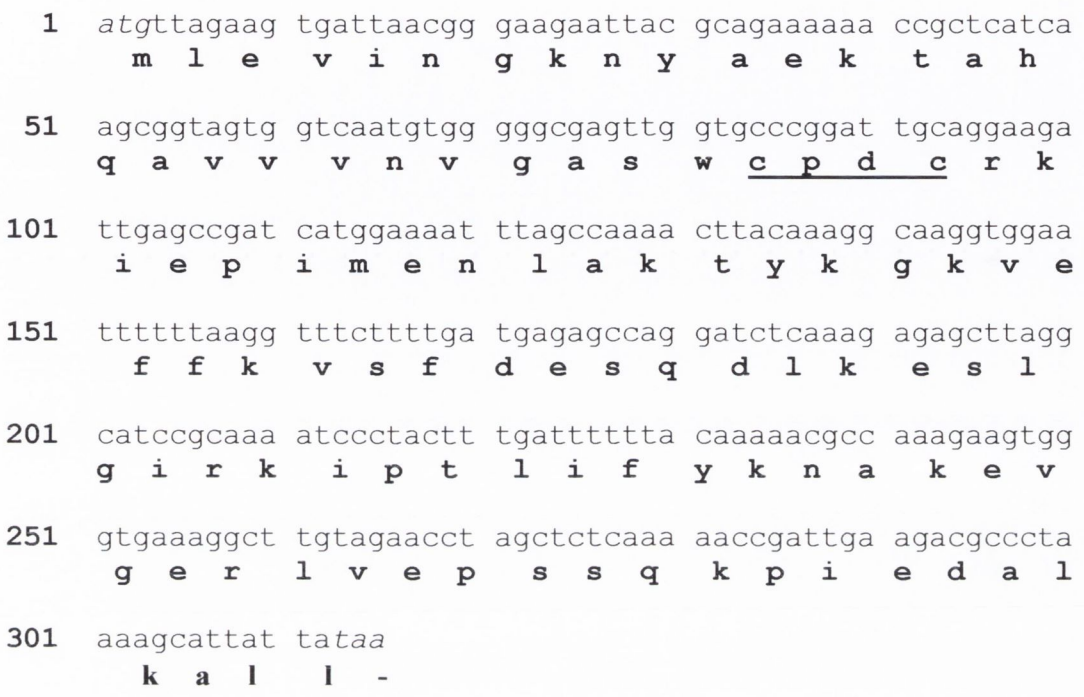


Fig. 3.1. Nucleotide sequence and deduced amino acid sequence of *H. pylori* strain NCTC11638 *trx2*. Nucleotides are numbered (*left margin*) beginning with the first base of the ATG initiation codon. Deduced amino acid residues are numbered shown beginning with the Leu immediately after the initiating methionine. The Trx2 unique redox active site (-Cys-Asp-Pro-Cys-) is underlined.

A

```
EC Trx     1MLHQQRNQHARLIPVELYMSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQG
HP Trx2    1MLEVINGKN----------YAEKTAH--------------QAVVVNVGASWCPDCRKIEPIMENLAKTYKG
HP Trx1    1MSH--------------------YIELTEENFESTIKK--GVALVDFWAPWCGPCKMLSPVIDELASEYEG
CJ Trx     1--------------------MGKYIELTSDNF---AQAKEGVALVDFWAPWCGPCRMLAPVIDELSNDFDG
BS Trx     1--------------------MAIVKATDQSFSAET--SEGVVLADFWAPWCGPCKMIAPVLEELDQEMGD
SC Trx     1MVTQFK---------------------TASEFDSAIAQ-DKLVVVDFYATWCGPCKMIAPMIEKFSEQYP-
human Trx  1MVKQIESKTA----------------FQEALDA---AGDKLVVVDFSATWCGPCKMIKPFFHSLSEKYSN
                                                          **  *    *

EC Trx     71 KLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNG-EVAATKVGALSKGQLKEFLDANLA---
HP Trx2    48 KVEFFKVSFDESQDLKESLGIRKIPTLIFYKNAKEVGERLVEPSSQKPIEDALKALL----...27%
HP Trx1    50 KAKICKVNTDEQEELSAKFGIRSIPTLLFTKDG-EVVHQLVGVQTKVALKEQLNKLLG---...41%
CJ Trx     49 KAKICKVNTDEQGDLAAEFGVRSIPTLIFFKNG-EVVDQLVGAQSKQAISDKLNSLL----...37%
BS Trx     49 KLKIVKIDVDENQETAGKYGVMSIPTLLVLKDG-EVVETSVGFKPKEALQELVNKHL----...39%
SC Trx     49 QADFYKLDVDELGDVAQKNEVSAMPTLLLFKNG-KEVAKVVGA-NPAAIKQAIAANA----...33%
human Trx  52 VIFL-EVDVDDCQDVASECEVKCMPTFQFFKKG-Q----KVGEFS-GANKEKLEATINELV...36%
                    *                ***    *          *
```

B

```
HP Trx1    1MSHYIELTE-ENFESTIKKGVALVDFWAPWCGPCKMLSPVIDELASEYEGKAKICKVNTD
HP Trx2    1---MLEVINGKNYAEKTAHQAVVVNVGASWCPDCRKIEPIMENLAKTYKGKVEFFKVSFD
                 *   *       *  * **  *   *   **   **   ** *

HP Trx1    60 EQEELSAKFGIRSIPTLLFTKDG-EVVHQLVGVQTKVALKEQLNKLLG
HP Trx2    58 ESQDLKESLGIRKIPTLIFYKNAKEVGERLVEPSSQKPIEDALKALL- 31%
                *   *    *** **** *   **   **          **
```

Fig. 3.2. Alignment of *H. pylori* Trx2 with other classical Trxs. **(A)** Global protein alignment against a reference sequence (BLOSUM 62). Amino acid sequences include Trx from *E. coli* (ECTrx), *H. pylori* (HPTrx1), *H. pylori* (HPTrx2), *C. jejuni* (CJTrx), *B. subtilis* (BSTrx), S. cerevisiae (SCTrx), and human Trx. The alignment is based on the amino acid sequence of *E. coli* Trx and was generated using an alignment tool from SciEdCentral. All amino acid numberings include the initiating methionine irrespective of the sequence of the mature protein. Residues with a high level of similarity are shown in blue and absolutely conserved residues are denoted with an asterisk. **(B)** Alignment of *H. pylori* Trx1 and Trx2. Identical residues are shown in blue and denoted with an asterisk.

TABLE 1. Trx pI values from other prokaryotic Trxs.

Prokaryotic Trx	pI value[1]
H. pylori Trx2	8.1
H. pylori Trx1	4.9
S. pneumoniae Trx	4.5
B. burgdorferi Trx	4.4
N. mengitidis Trx	4.4
H. influenzae Trx	4.8
M. tuberculosis Trx	4.6
V. cholerae Trx	4.5
A. fulgidus Trx	5.4
C. cresentus Trx	4.5
T. pallidum Trx	4.4
M. jannaschii Trx	4.9

[1] Source: CMR at TIGR (http://www.tigr.org)

TABLE 2. pI values for a range of oxidative stress management proteins from *H. pylori*.

Protein	Annotation (TIGR[1])	Theoretical pI[2]	2-DE pI[2]	Predicted mol. wt[2]
Trx2	HP1458	7.72	7.7	11 745
Trx1	HP0824	5.16	5.18	11 855
SodB	HP0389	5.77	5.57	24 618
KatA	HP0875	-	8.89	55 986
AhpC	HP1563	5.88	5.98	22 236
DsbC	HP0377	8.18	5.95	25 340
TR	HP0825	5.93	5.8	33 539
Tpx	HP0390	7.69	6.82	18 292

[1] TIGR: The Institute for Genomic Research (http://www.tigr.org)

[2] Source: http://www.mpiib-berlin.mpg.de/2D-PA

http://www.mpiib-berlin.mpg.de/2D-PAGE/EBP-PAGE/index.html). In addition to Trx2 from *H. pylori*, two other thioredoxins have been described which have usually high pI values. Trx from *Trypanosoma brucei brucei* has a predicted pI of 8.8 (Reckenfelderbaumer *et al.*, 2000) and a highly basic Trx III from the mitochondria of *Saccharomyces cerevisiae* has also been described (Pedrajas *et al.*, 1999). *H. pylori* is unusual amongst bacteria as it possesses a relatively high proportion of acidic proteins, which may reflect an adaptation to its unique environment.

3.3.3 Overexpression of the *H. pylori trx2* gene in *E. coli* and purification by immobilised metal ion affinity chromatography.

From 1 L of induced *E. coli* BL21 DE3 pLysS, approximately 10 mg of *H. pylori* Trx2 was purified as a NH_2-terminal His-tagged soluble protein. Analysis of fractions, collected from the metal ion (Ni^{2+}) affinity column, by SDS-polyacrylamide gel electrophoresis plus Coomassie R-250 staining revealed fractions containing pure protein. Recombinant Trx2 migrated as a single protein band with a apparent molecular mass of approximately 14 kDa, as expected (**Fig. 3.3**). Pure fractions were pooled and consisted of recombinant protein of a high level of purity as determined by SDS-PAGE.

3.3.4 Functional characterisation of *H. pylori* Trx2.

Cell lysates of three *H. pylori* reference strains were prepared. Total proteins from *H. pylori* reference strains NCTC11638, NCTC11637 and SS1, a murine adapted strain, were obtained by cellular lysis and solubilised by boiling in reducing SDS-polyacrylamide gel electrophoresis sample buffer. Polyclonal antibody raised in rabbits against recombinant *H. pylori* Trx2 and Trx1 protein was used for the immunodetection by Western Blotting of native *H. pylori* Trx in cell lysates. When examined by Western blotting, the anti-Trx2 antibodies reacted with the recombinant Trx2 as expected (M_r = ~14,200), however, these antibodies did not exhibit any immunological cross-reactivity with recombinant Trx1 (**Fig. 3.4, A, lane 3**). Anti-Trx2 antibodies did detect Trx2 in *H. pylori* showing it to be normally expressed by the bacterium (native M_r = 12,000). It was observed in the cytoplamic fraction (**Fig. 3.4, B**). Immunoblot analysis of the three reference strains of *H. pylori* showed the protein to be present in relatively similar amounts in the cytoplasmic fractions of strains NCTC11638 and NCTC11637, though there appears to be less protein present in the murine adapted strain, SS1 (band not visible in scanned blot, lane 1). In addition, immunoblot analysis of identical fractions of *H. pylori* with anti-Trx1 antibodies appears to

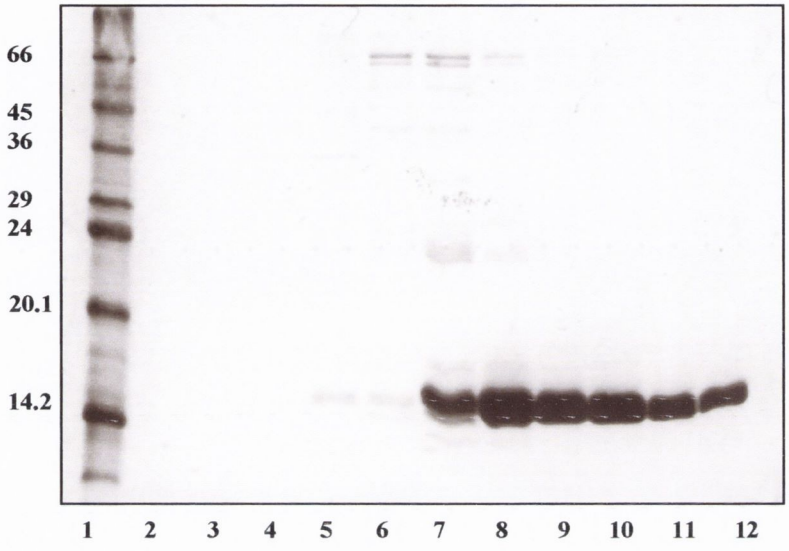


Fig. 3.3. SDS-PAGE analysis of purified *H. pylori* Trx2. Recombinant purified *H. pylori* Trx2 was analysed on a 15% polyacrylamide gel, in reducing sample buffer, as follows: lane 1, molecular weight markers; lanes 7 to 12, recombinant Trx2 following elution from an immobilised metal ion (Ni^{2+}) affinity column with 400 mM imidazole. Proteins were visualised by Coomassie R-250 staining. Fractions 9 to12 were pooled and protein estimation was carried out prior to storage at –20°C. Molecular masses (in kilodaltons) are indicated on the left.

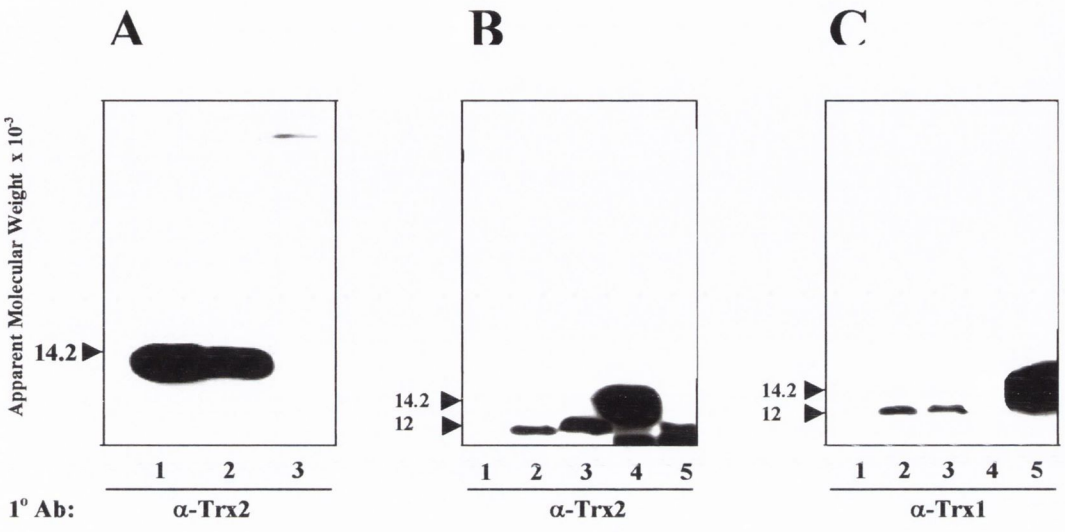


Fig. 3.4. Immunoblot analysis of recombinant *H. pylori* Trx2 and Trx1 and of cellular lysates of *H. pylori* reference strains. Recombinant *H. pylori* Trx and *H. pylori* cell lysates obtained form bacteria grown to stationary phase were precipitated with acetone and resolved by reducing SDS polyacrylamide gel electrophoresis and immunoblotted with anti-Trx1 and anti-Trx2 antisera where indicated. Panel A shows a Western blot of recombinant *H. pylori* Trx2, 20 μg and 10 μg, (lanes 1 and 2, respectively) and recombinant *H. pylori* Trx1, 20 μg (lane 3). Proteins were detected with affinity-purified anti-Trx2 antibodies (1:1000 dilution). Panels B and C show immunoblot analysis of cytoplasmic fraction of *H. pylori* strain SS1 (lane 1), cytoplasmic fraction of *H. pylori* strain NCTC 11637 (lane 2), cytoplasmic fraction of *H. pylori* strain NCTC11638 (lane 3), recombinant *H. pylori* Trx2 (+ His-tag) (lane 4), recombinant *H. pylori* Trx1 (+ His-tag) (lane 5) are detected using anti-Trx2 polyclonal antibodies (1:1000) (Panel B) and anti-Trx1 polyclonal antibodies (1:1000) (Panel C). The secondary antibody used was swine anti-rabbit IgG (Dako) (1:2000). Molecular weight markers are on the left side of each blot. All Western blots were developed using ECL.

reveal that the two Trx species, Trx1 and Trx2 are almost equally abundant within the cell (**Fig. 3.4, B and C**). However, 2-D proteomic analysis coupled with densitometry would be required to obtain a definitive percentage abundance value for these two proteins in *H. pylori* strains.

3.3.5 Enzymatic activity of recombinant *H. pylori* Trx2 protein.

The catalytic redox capacity of *H. pylori* Trx2 was determined using the insulin reduction assay. The two inter-chain disulfides of insulin can be reduced in the presence of Trx resulting in the generation of free A and B insulin chains. The rate of precipitation of the insoluble B chain was monitored spectrophotometrically as an increase in turbidity as described by Holmgren (1979). The standard assay mixture contained 0.1 M potassium phosphate (pH 7.3), 1 mM EDTA, 0.13 mM bovine insulin and Trx1 (14 μM) or Trx2 (14 μM), and the reaction was initiated by the addition of 1 mM dithiothreitol (DTT). The rate of change of the absorbance at 650 nm was monitored at 25°C (**Fig. 3.5**). Unexpectedly, the rates of precipitation for *H. pylori* Trx1 and Trx2 observed were quite dissimilar. *H. pylori* Trx1 (a His-tagged recombinant cloned in pET16b obtained from Dr. H. Windle, Dept. of Clinical Medicine, T.C.D.) efficiently catalysed the reduction of insulin as demonstrated by the rapid precipitation of insulin B chain after an initial time lag of approximately 6 to 8 minutes. These data indicate that the *H. pylori* Trx1 protein cloned and purified from *E. coli* is active as a disulphide reductase *in vitro*. However, *H. pylori* Trx2 under identical reaction conditions was ineffective in catalysing the reduction of insulin, with no activity evident until about 35 min, when the observed rate of reaction was slower than that of *H. pylori* Trx1. Moreover, the rate of reaction appeared to be only about as efficient as the small-molecule reductant, DTT, in the reduction of insulin. Therefore, removal of the His-tag from the recombinant protein was undertaken via digestion with Factor Xa.

3.3.6 Removal of recombinant Trx2 histidine-tag via Factor Xa digestion.

Removal of the His-tag from the purified recombinant material was achieved by digestion with Factor Xa (25 μg/mg protein) for 16 hours at room temperature. To terminate the reaction the Factor Xa was removed from the mixture by passage through a small (1 ml) benzamidine-agarose column followed by purification of the recombinant protein (minus His-tag) on a nickel column. Removal of the His-tag resulted in a 2-kDa decrease in the molecular mass of Trx (**Fig. 3.6**). Analysis of the reaction products was carried out by

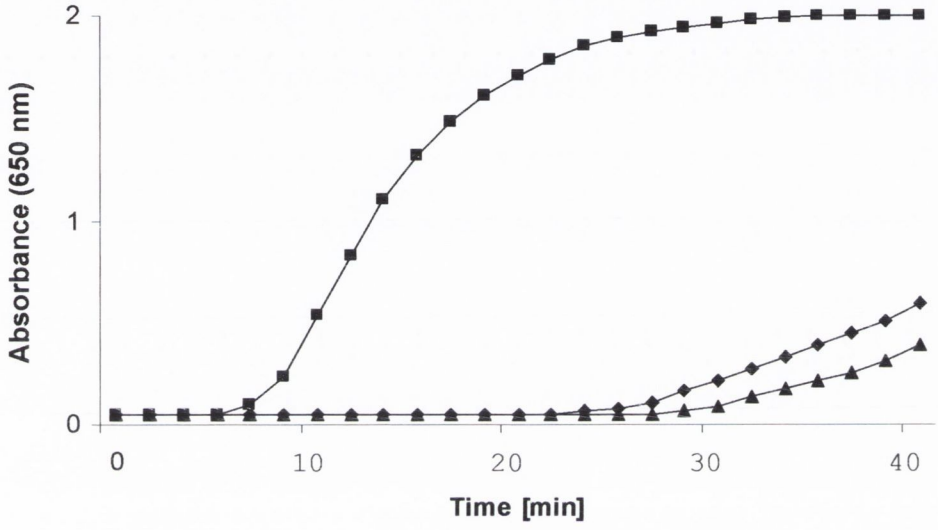


Fig. 3.5. Disulphide oxidoreductase activity of his-tagged recombinant *H. pylori* Trx2. The reduction of insulin catalysed by Trx2 and Trx1 from *H. pylori* was evaluated. The graph shows the time course of insulin reduction in the presence of either Trx1 from *H. pylori* (■, 5 μM) or Trx2 from *H. pylori* (◆, 5 μM). The time course of insulin reduction by the reductant, DTT, (▲, 1 mM) alone is also shown. The graph shown is representative of several independent experiments.

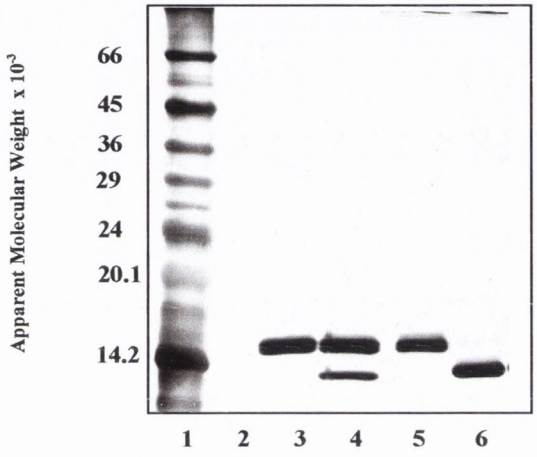


Fig. 3.6. SDS-PAGE analysis of *H. pylori* Trx2 and Trx1 following treatment with Factor Xa. Recombinant purified Trx2 and Trx1 treated with Factor Xa for 16 h at room temperature were analysed on a 15% polyacrylamide gel in reducing sample buffer. Lane 1, molecular weight markers; lane 3, Trx 2 untreated; lane 4 Trx2 treated; lane 5, Trx1 untreated; lane 6, Trx1 treated. Equivalent amounts of protein (10 μg) were loaded in all lanes. Proteins were visualised by silver staining. Molecular masses (in kilodaltons) are indicated on the left.

SDS-polyacrylamide gel electrophoresis. In the case of Trx1, His-tag cleavage was successful with 100% of the protein resolving as a single band on the gel at the expected molecular weight for the protein without the His-tag (**Fig. 3.6, lane 6**). In the case of Trx2, however, removal of the His-tag was achieved for approximately 15% of total recombinant protein present in the reaction (**Fig 3.6, lane 4**). In both reactions, equal amounts of Trx protein was present and the ratio of Factor Xa to Trx was the same in both cases. SDS-polyacrylamide gel electrophoresis revealed that approximately 85% of the protein was resistant to the action of the Factor Xa enzyme even over prolonged incubation periods and different reaction conditions and temperatures.

3.3.7 Overexpression and purification of recombinant *H. pylori* Trx2 in alternative expression host strains.

Following unsuccessful activity assays for Trx2, re-sequencing of the recombinant vector was undertaken to verify the correctness of the recombinant construct used for transformation of the expression host. Sequencing reaffirmed that the construct was correct and that the Factor Xa cleavage site was present. The construct was subsequently used to transform two alternate expression hosts, *E. coli* strain CAG597 and *E. coli* strain KS1000. Overexpression and purification of Trx2 was carried out in the same manner as for *E. coli* strain BL21, except that induction was carried out at a temperature of 30°C for the *E. coli* CAG597 strain. Activity analyses revealed that recombinant Trx2 purified from these alternate expression host strains was also deficient in reducing activity when examined by the insulin assay as almost identical activity data to those shown in **Fig. 3.5**, were obtained. As attempts to elucidate the reason for the apparent catalytic inactivity of the recombinant protein proved unsuccessful, use of a non-tagged recombinant protein for further investigations seemed a judicious approach. Fortuitously, production of a non-tagged recombinant *H. pylori* Trx2 was reported in the literature at this time (Baker *et al.*, 2001).

3.3.8 Overexpression of an alternate *H. pylori* Trx2 construct.

The pPROK1 vector (Clontech, Palo Alto, CA) containing the *H. pylori trx2* gene, pPROK1-Trx2, was obtained from Drs. Leslie Poole and Laura Baker (Wake Forest University, Madison, WI). The construct was used to transform *E. coli* BL21 DE3 pLysS using the heat-shock method of transformation. Expression of the recombinant protein was successfully induced by the addition of IPTG, and recombinant protein was expressed to levels of approximately 15% of total soluble protein.

3.3.9 Purification of recombinant *H. pylori* Trx2 using Ion Exchange Chromatography.

From 800 ml of induced *E. coli* BL21 DE3 pLysS, approximately 10 mg of recombinant *H. pylori* Trx2 was obtained and this was expressed exclusively as a soluble cytoplasmic protein. This product was purified as a non-tagged protein via a two-step purification procedure involving ion-exchange chromatography over DEAE-cellulose followed by CM-cellulose. Trx2 was released from the CM-cellulose column following addition of 20 mM salt. Analysis of fractions, collected from the final ion-exchange column, by SDS-polyacrylamide gel electrophoresis identified fractions containing pure protein represented by a single protein band with a molecular mass of approximately 12,000, as expected. These fractions were pooled and consisted of recombinant protein purified to homogeneity when the gel was visualised by silver staining (**Fig. 3.7**).

3.3.10 Disulphide oxidoreductase activity of non-tagged *H. pylori* recombinant Trx2.

The reduction of insulin by the non His-tagged *H. pylori* Trx2 was determined at pH 7.3 and the reaction was followed at an absorbance of 650 nm (**Fig. 3.8**). The rates of insulin precipitation were measured as $\triangle A_{650}$/min as before (**Section 3.3.5**). Once again, *H. pylori* Trx1 (a His-tagged recombinant cloned in pET16b obtained from Dr. H. Windle, Dept. of Clinical Medicine, T.C.D.) was included as a positive control for activity. Assay results revealed that the non-tagged version of *H. pylori* Trx2 was active as a disulphide reductase with insulin as a substrate. Interestingly, the rate of insulin reduction was not as efficient as that observed for Trx1. Trx2 appears to exhibit a lower turnover rate ($\triangle A_{650}$/min = 0.873) than Trx1 ($\triangle A_{650}$/min = 1.176) in the insulin reduction assay. With pure redox active, monomeric, recombinant *H. pylori* Trx2 obtained, structural studies were initiated.

3.3.11 Determination of *H. pylori* Trx2 three-dimensional structure.

Trx2 from *H. pylori* has been successfully crystallised and preliminary structural data has been obtained at a 2.4 Å resolution, following exposure of the crystals to X-rays at a syncotron radiation facility (**Table 3**). The following figures show a typical diffraction quality Trx2 crystal (**Fig. 3.9**) and preliminary X-ray diffraction pattern obtained from that crystal (**Fig. 3.10**). Crystallisation and structural studies were carried out by Dr. David A. R. Sanders of the X-Ray Crystallography Group, University of Saskatchewan, Saskatoon, Canada (Filson *et al.*, 2003). Purified recombinant *H. pylori* Trx2 (from this study) was crystallised by the sitting drop method of vapour diffusion crystallisation. Additional

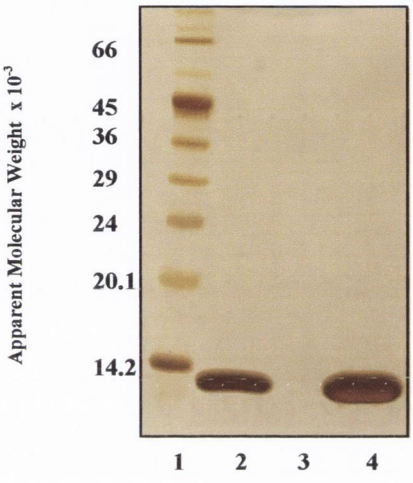


Fig. 3.7. SDS-PAGE analysis of purified *H. pylori* Trx2. Recombinant purified Trx2 from the expression vector pPROK1-*trx2* was analysed on a 15% polyacrylamide gel in reducing sample buffer as follows: lane 1, molecular weight markers; lane 2, recombinant Trx2 (2.5 μg); lane 4, recombinant Trx2 (5 μg). Proteins were visualised by silver staining. Molecular masses (in kilodaltons) are indicated on the left.

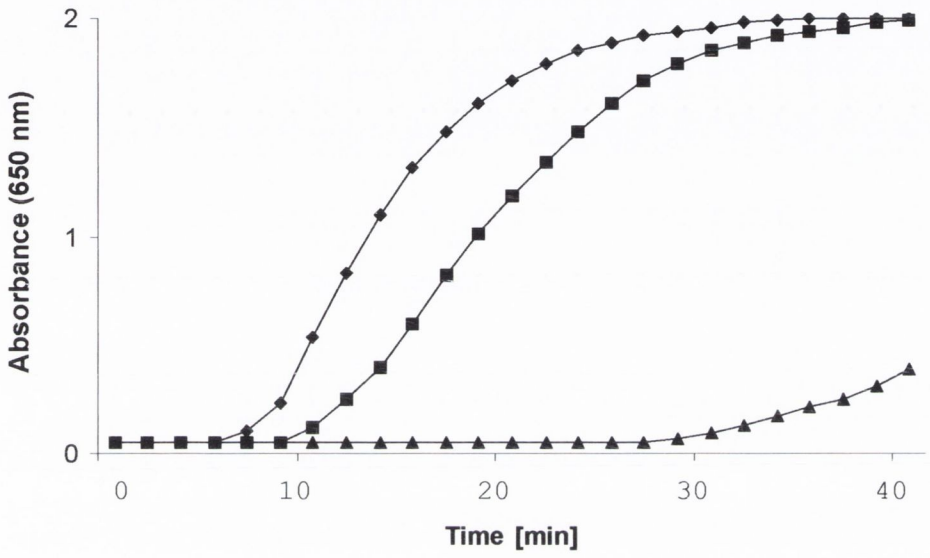


Fig. 3.8. Disulphide oxidoreductase activity of recombinant *H. pylori* Trx2. The reduction of insulin catalysed by Trx2 and Trx1 from *H. pylori* was assessed. The graph shows the time course of insulin reduction in the presence of either Trx2 from *H. pylori* (■, 5 μM) or Trx1 from *H. pylori* (◆, 5 μM). The time course of insulin reduction by the reductant, DTT (▲, 1 mM) alone is also shown. The graph shown is representative of several independent experiments

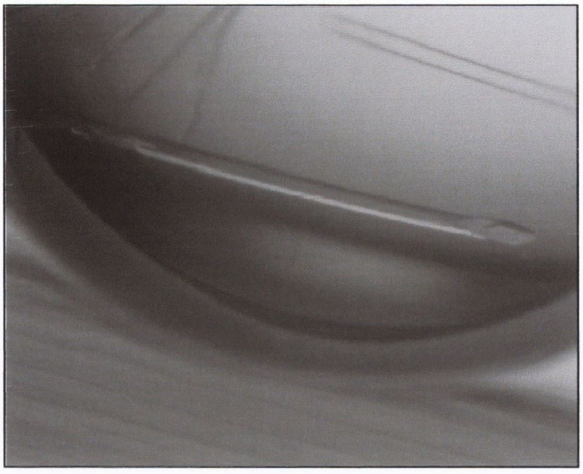


Fig. 3.9. Typical crystals of Trx2 from *H. pylori*. Rod shaped Trx2 crystals are depicted of dimensions 0.1 x 0.1 x 1-2 mm. Crystal conditions comprised sitting drops consisting 5 µl of 3 mg ml^{-1} protein (in 30 mM potassium phosphate pH 7.0, 10 mM DTT) plus 5 µl of well solution [30% PEG 6000, 0.1 M $(NH_4)_2SO_4$, 10 mM DTT]. Crystals were grown over 5 – 7 days (Dr. D. Sanders, University of Saskatchewan, Canada).

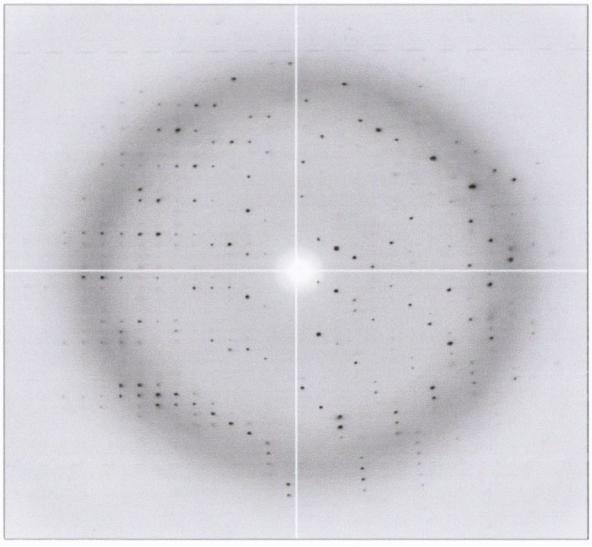


Fig. 3.10. The X-ray diffraction pattern obtained from crystals of *H. pylori* Trx2. The diffraction data set of Trx2 was obtained at a 2.4 Å resolution with 360° of data collected using the Mail-in data service of National Syncotron Light Source (NSLS), Brookhaven National Laboratory, NY. The data were collected on beamline X12C and initially processed on site and later reprocessed using MOSFLM (Leslie, 1992) (Dr. D. Sanders, University of Saskatchewan, Canada).

TABLE 3. Trx2 Crystal Data

Table 3: Data collection for Trx2
Values in parentheses refer to highest resolution shell (2.52-2.40)

Wavelength (Å)	1.00
Resolution (Å)	40.16 – 2.40
Space group	$P4_1$
Unit-cell parameters	a = b = 40.21,
	c = 64.65,
	$\alpha = \beta = \gamma = 90$
Total measurements	55405
Unique reflections	4079
V_M^* (Å^3 Da^{-1})	2.1
Solvent (%)	43 %
$I/\sigma(I)$	6.6 (2.3)
Average redundancy	13.6 (13.8)
Data completeness (%)	100 (100)
R_{merge}‡ (%)	7.3 (47.1)

* Assuming a monomer in the asymmetric unit.
‡ $R_{merge} = \Sigma\Sigma I(h)_j - I(h)/ \Sigma\Sigma I(h)_j$, where $I(h)$ is the the measured diffraction intensity and the summation includes all observations.

material has been prepared for crystallisation and on-going studies are focused on obtaining crystals which will yield higher resolution images to determine the complete three-dimensional structure.

3.4 DISCUSSION

The redox status that exists within a cell is tightly regulated and a relatively steady redox balance is usually maintained. This redox homeostasis is a feature of all viable cells and is vital for various cellular biological processes to occur, such as DNA synthesis, cell cycle regulation and transcriptional activation of certain genes (Powis *et al.*, 1995). Redox homeostasis is also responsible for the level of reactive oxygen species (ROS) that are present in the cell, and are by-products of biochemical reactions which exploit the mechanism of electron transfer. When the level of ROS exceeds the capacity of the cellular enzymatic mechanisms present to detoxify such products, deleterious effects occur culminating in cellular damage. Thiol-containing molecules are a vital arm of the antioxidant capacity of a cell. Trx is an efficient oxidoreductase which in conjunction with the dimeric flavoenzyme thioredoxin reductase, constitutes the exclusive regulator of intracellular redox status in the gastric pathogen, *H. pylori*. Trx is present in either a reduced or oxidised form and through the reversible oxidation of its active thiol, takes part in various redox reactions. This chapter sought to biochemically characterise Trx2 from *H. pylori* in order to gain an initial insight into its function in this important pathogen.

The first step towards the elucidation of the role of an enzyme functioning within a cellular redox network is the production and biochemical characterisation of a recombinant from of the protein. As this bacterium can be considered to possess the minimum components necessary to maintain the intracellular redox balance, the components of the Trx system should display minimal redundancy and have several unique functions. It is therefore of interest that one of the two Trxs coded for by the bacterial genome should be unusual in terms of its redox active site. Most Trx enzymes described to date across a broad range of prokaryotic and eukaryotic species possess the consensus redox active site -Cys-Gly-Pro-Cys- (Eklund *et al.*, 1991) however, in some species Trx deviates characteristically at the redox-active centre from this published consensus sequence. In *H. pylori* Trx2, this motif is substituted for -Cys-Pro-Asp-Cys-. A substitution such as this will alter the redox potential of the enzyme and may affect the efficiency with which the protein can function as a general oxidoreductase in particular redox environments. Trx2 from *H. pylori* is quite

unique in having a calculated pI value of 8.1, most Trxs studied thus far are acidic proteins possessing pI values between 4.5 and 5. The effect of pronounced charge differences between members of this enzyme family is not determined as yet but may be associated with unique functional interactions or localisation. In order to elucidate the particular biochemical and catalytic attributes of this unusual Trx, *H. pylori* Trx2 was cloned and overexpressed in a common *E. coli* expression strain. The protein was purified by immobilised metal ion chromatography using a nickel column and eluted with imidazole. As seen in **Fig. 3.3**, the protein was eluted from the column with minimal contaminating proteins.

Immunoblotting with polyclonal antibodies raised against *H. pylori* Trx1 and Trx2 reveal that both Trx1 and Trx2 are normally expressed in the *H. pylori* cytoplasm at least in three reference strains, NCTC11638, NCTC11637 and SS1 (**Fig. 3.4, Panel B and C**). The *H. pylori* thioredoxins do not exhibit immunochemical cross-reactivity when probed with polyclonal antisera raised against purified recombinant versions of both molecules (**Fig. 3.4, Panel A and B**) which is not very surprising given the low level of primary sequence homology between the two proteins. Seropositive bovine serum, obtained from animals hyperimmunized with *H. pylori* strain NCTC11638, does recognize Trx1, but not Trx2 (unpublished results). These immunological differences and significant primary structural differences within the redox active site motif (Trx1, -CGPC-; Trx2, -CPDC-), suggest that the Trxs are functionally dissimilar, perhaps an essential divergence in roles given the absence of the Grx system. To investigate whether the newly purified recombinant *H. pylori* Trx2 was functionally active, a classic assay to assess oxidoreductase activity, the insulin reduction assay, was used (Holmgren *et al.*, 1979). Reduced insulin B chain precipitates readily by aggregation. Dithiothreitol (DTT) reduces oxidised Trx faster than the direct reduction of insulin by approximately two orders of magnitude, whereas reduced Trx itself reduces insulin four orders of magnitude faster than DTT (Holmgren, 1979). The overall net effect then being that Trx catalyses the reduction of insulin by DTT. A simple method to determine the activity of Trx involves measuring the turbidity generated in a reaction mixture of insulin with DTT spectrophotometrically at 650 nm. The sensitivity of this convenient assay method is in the range of 100 pmol. When *H. pylori* recombinant Trx2 was used in this assay, it became apparent that the recombinant protein was unable to catalyse the reduction of insulin in the presence of DTT (**Fig. 3.5**). These data indicate that the capacity of recombinant Trx2 to act as a thiol-disulfide reductase *in vitro* was

compromised. In contrast to recombinant Trx1 which rapidly catalysed insulin reduction after a brief time lag, Trx2 showed little or no activity under the same conditions. Moreover, the insulin reducing activity of Trx2 on insulin was co-incident with that observed for DTT indicating that Trx2 was not catalytically reducing insulin. The probable reasons for the lack of redox activity are considered below.

Firstly, modification of the recombinant within the expression host was a possibility which may have accounted for the inactivity of Trx2. In order to rule out the possibility of the recombinant being inactivated through post-translational modification, it was necessary to transform two alternative *E. coli* expression hosts with the Trx2 construct. The two available hosts were both *E. coli* strain K12 derivatives, CAG597 and KS1000. CAG597 does not suppress the heat shock sigma factor *RpoH* and thus when incubated at 30°C becomes deficient in expression of heat shock proteins which can affect proteolysis of foreign proteins expressed in *E. coli*. *E. coli* strain KS1000 is deficient in the Tsp protease, a periplasmic protease, which can degrade cytoplasmically expressed proteins when cells are lysed to make a crude extract. Recombinant Trx2 was expressed in both hosts and subsequently purified by immobilised metal ion chromatography. However, the strategy of changing expression host did not appear to impact on the activity of the recombinant product. Assessment of activity using the insulin assay revealed that Trx2 produced from these two strains displayed similar low rates of insulin reduction activity compared to the recombinant *H. pylori* Trx2 produced from *E. coli* strain BL21.

The possibility that the His-tag interfered with the redox activity of Trx2 was also explored. Over the last decade the cloning of an affinity tag into a target proteins gene has revolutionised the purification of recombinant proteins as such tags have greatly facilitated protein purification. The most widely used affinity tag is the poly-histidine tag, usually comprising six or ten histidines (plus several additional amino acids) and positioned at the N- and/or C-terminal of the protein. However, it has been noted recently in the technical literature that this approach is not without drawbacks and in addition to the possibility of co-elution of metalloproteinases and oxidation of the eluted protein, the His-tag itself can possibly influence the behaviour of the recombinant protein (Ramage *et al.*, 2002). Instances where an N-terminal His-tag interfered with the refolding of a protein and the subsequent destabilisation of the same protein have been reported for a range of protein types including, a MAP kinase, a transcription factor and an intracellular binding protein.

In addition, an N-terminal His-tag as a cause of protein aggregation and prevention of ligand binding for a receptor tyrosine kinase has been documented (Ramage *et al.*, 2002). In such cases re-cloning of the protein to remove the His-tag and subsequent purification by conventional ion-exchange chromatography solved such problems. Following Factor Xa treatment, in contrast to recombinant *H. pylori* Trx1 from which there was complete cleavage of the His-tag from the recombinant, there was only limited dissociation of the tag in the order of about 15% of total recombinant *H. pylori* Trx2 protein (**Fig. 3.6**). This is indicative of the possibility of structural interference and/or protein misfolding due to the presence of the His-tag and hence the inability of the recombinant to catalyse the reduction of its substrate. As *H. pylori* Trx2 was being prepared with the ultimate intention of obtaining structural data, it was required that the recombinant meet the highest specifications for purity, homogeneity, and monodispersity at high concentrations, while importantly, still maintaining function. Therefore, use of a *H. pylori* Trx2 construct which resulted in the production of protein without the need for downstream purification with the aid of a His-tag was employed. This approach resulted in the production of a highly pure preparation of recombinant H. *pylori* Trx2, with no contaminants visible by silver staining. When the new recombinant was tested for oxidoreductase activity as determined by the reduction of insulin in the presence of DTT, it appeared to be active. Interestingly however, the Trx2 recombinant did not catalyse the reduction of the disulphide containing substrate insulin in the presence of DTT with the same efficiency as *H. pylori* Trx1 (**Fig. 3.8**). The inability of *H. pylori* Trx2 to act as a reductant of similar efficiency as Trx1 has been described in the literature. Baker *et al.* (2001) noted that the turnover rate for insulin reduction for Trx2 when assayed in the presence of *H. pylori* thioredoxin reductase was approximately 50% less than for Trx1 under the same experimental conditions. However, it was noted in the same study that both Trx2 and Trx1 reduced the small non-protein disulphide substrate DTNB with about the same catalytic parameters as those observed for Trx1 insulin reduction. This observation raises the possibility that the unique active site of Trx2 may influence the ability of the protein to participate in certain catalytic reactions and also influence the kinetics of substrate interaction for this protein. Hence, the unique redox active site of Trx2 may confer specificity of function within the bacterium.

Prerequisite to any study of a proteins three-dimensional structure is the need to produce recombinant protein of sufficiently high purity. This step marks the beginning of the investigation into the proteins function within the cell. The redox protein Trx has been

most intensively studied in *E. coli* for which the three dimensional structure was solved to 1.7 Å resolution in 1990 (Katti *et al.*, 1990). Since 1990, the 3-D structures of various Trxs from other species have also been resolved both in the reduced and oxidised state, therefore Trx represents a well characterised redox molecule. The active site sequence is conserved regardless of the lower overall sequence homology between the various Trxs. However, Trx from *H. pylori* has not been structurally characterised to date. It is reasonable to assume that Trx1 with its common redox active site will most likely conform structurally to many of the models already described in the literature, however Trx2 with its unique redox active site represents a possible departure in structural terms from other Trxs. There are a number of known structures for Trxs available through the Protein Data Bank (PDB, http://www.rcsb.org) including *E. coli*, *Chlamydomonas reinhardtii*, *Spinacia oleracea* and human Trxs. These structures can be used for molecular replacement that is vital for the elucidation of any new Trx structural model. Molecular replacement is a method of structure determination that operates on the assumption that similar proteins will have the same general structure, and can therefore be used to estimate the initial phases. In practice, proteins with greater than 30% identity (and a predicted common structure) are structurally determined using this technique. *H. pylori* Trx2 has the highest sequence identity with both *E. coli* and *C. reinhardtii* Trxs, though this is low at 30%. This places *H. pylori* Trx2 at the border of sequence identity that is likely to yield a solution by molecular replacement. However, given that Trxs are small proteins and the highly conserved nature of the thioredoxin fold, the possibility of molecular replacement providing a solution for marginally identical structures will be increased. As shown in this chapter, Trx2 from *H. pylori* is a classical Trx with several unusual features, including an atypical redox active site and unusually basic pI. The future resolution of the proteins three-dimensional structure will lead to a greater understanding of the role of this enzyme within *H. pylori*. A prerequisite in the understanding of an enzymes actions and potential target interactions flow from an understanding of three-dimensional structure. However, it is through determination of the redox potential of this protein and correlation of this information with potential target protein interactions that the most interesting facets of this second *H. pylori* Trx will most likely be achieved. This latter point is further considered in the general discussion.

In summary, the identification of a second Trx sequence in the *H. pylori* genome addressed the question as to whether the protein coded by this sequence was active as a thiol

disulphide oxidoreductase. The protein was overexpressed in *E. coli* cells and purified to homogeneity. This chapter demonstrates that the Trx2 gene codes for a protein with Trx activity as determined by the insulin reduction assay. Polyclonal antibodies were raised against the recombinant protein and Western blot analysis showed that the protein is normally expressed in *H. pylori* reference strains. Preliminary structural data has been obtained for this purified protein and is being utilised to construct, at a high resolution, a three-dimensional structure map of this unusual redox enzyme (Filson *et al.*, 2003). Determination of the redox potential of *H. pylori* Trx2 should provide an indication of the redox capacity of the molecule and facilitate elucidation of the *in vivo* functions of this enzyme. The following chapter describes the functional characterisation of Trx2 in *H. pylori in vitro*.

CHAPTER IV

THE ROLE OF THE THIOREDOXIN SYSTEM IN MEDIATING OXIDATIVE STRESS RESISTANCE IN *H. PYLORI*

CHAPTER IV

4.1 INTRODUCTION

4.1.1 Oxidative Stress in the Gastric Niche

Oxygen toxicity is a universal phenomenon and oxidative stress resistance is a fundamental property which enables a pathogen to survive the deleterious reactive oxygen species produced by the host organism. The pathogenesis of *H. pylori* is dependent on its persistence in the host gastric environment. This niche presents a formidable challenge to long-term colonisation, it presents extremely harsh conditions to a microorganism, acidity, constant movement in the form of gut wall peristalsis and also attack by the host immune system, by inflammatory infiltrates and phagocytic release of deleterious reactive oxygen species (Blaser, 1992; Marshall *et al.*, 1984; Nomura *et al.*, 1991; Parsonnet *et al.*, 1991). Hazards during transmission may result from exposure to the aerobic environment., therefore, mechanisms of detoxification of reactive oxygen species (ROS) are of particular interest in *H. pylori*. As a microaerophilic bacterium unable to tolerate atmospheric concentrations of oxygen, *H. pylori* is especially vulnerable to the detrimental effects of oxygen. However, the pathogen is equipped with enzymatic machinery to detoxify toxic oxygen derived products which it encounters in the gastric tract. In *H. pylori*, genes encoding catalase, a dimeric iron-cofactored superoxide dismutase and various putative peroxidases have been identified (Alm *et al.*, 1999; Odenbreit *et al.*, 1996; Pesci and Picket, 1994; Tomb *et al.*, 1997). Among the peroxidases, genes encoding an alkylhydroperoxide reductase, a thiol-specific peroxidase and a bacterioferritin comigratory protein have been identified (Doig *et al.*, 1999; McGee and Mobley, 1999). Thiol-dependent peroxidases depend on the presence of reducing equivalents within the cell for catalytic turnover and Trx provides reducing equivalents for thiol peroxidases, an enzymatic system which catalyses the reduction of hydrogen peroxide and organic peroxides (Jeong *et al.*, 2000). It is likely that *H. pylori* Trx-dependent peroxidases attenuate some of the damaging effects of the hosts' inflammatory response.

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4.1.2 Redox Homeostasis

Cellular production and removal of ROS necessitates the establishment of a critical balance within the cell. Therefore, in order to maintain this balance cells utilise a variety of antioxidant systems that effectively quench and neutralise these toxic by-products of

aerobic metabolism. Several studies have described the vital role played by sulphydryl groups (-SH) in response to oxidative stress and highlighted in particular the role of the thioredoxin (Trx) and glutaredoxin/glutathione (Grx) systems (Carmel-Harel and Storz, 2000). These systems play a major role in protecting cells from oxidative damage. *H. pylori* is significantly different from other prokaryotes in terms of deficiency in its complement of thiol-dependent redox active proteins. The bacterium is equipped with only one of the two major, almost ubiquitous, intracellular disulphide reducing systems, the Trx system and lacks the Grx system. As few cell types are deficient in the Grx system (Kanzok *et al.*, 2001), the regulation of thiol-mediated redox homeostasis is of interest in cells where significant deficiencies in redox biochemistry are distinguished. As a consequence of this deficiency it is probable that the redox metabolism of *H. pylori* is significantly different from other prokaryotes and that the redox protein Trx is important in cellular thiol-mediated oxidative defence for this pathogen.

The aim of this study was to obtain information on the specific *in vivo* role of the second unusual Trx of *H. pylori*, Trx2. To achieve this, mutants of the *H. pylori trx2* gene were constructed to evaluate the contribution of Trx2 to thiol-mediated oxidative stress management in the pathogen. Phenotypic analysis of the mutants reveal that *H. pylori* Trx2 has a role in oxidative stress resistance in the bacterium at least in response to certain kinds of oxidative stress, specifically oxidative stress generated by organic peroxides.

4.2 OBJECTIVES

1. To disrupt the *H. pylori trx2* gene by insertional mutagenesis.
2. To evaluate the growth characteristics of Trx2 mutant *H. pylori* strains.
3. To assess the response of the mutants to oxidative challenge *in vitro*.
4. To examine whether *H. pylori* Trx2 can function as a donor of reducing equivalents to other enzymes involved in the bacteriums' oxidative stress response.

4.3 RESULTS

4.3.1 Disruption of the *trx2* (HP1458) gene in *H. pylori* by insertional mutagenesis.

In order to determine the role of the HP1458 ORF in oxidative stress resistance, *H. pylori* mutants carrying a disrupted *trx2* gene were constructed by insertional mutagenesis. **Fig. 4.1 (A)** shows the HP1458 ORF analysed in this study and the strategy used for mutagenesis. Briefly, the HP1458 ORF cloned in pGEM-T Easy was disrupted by using

reverse primers in a PCR reaction and ligating a kanamycin or chloramphenicol cassette to the Klenow treated product to construct plasmids pTrxA2::kan/pTrxA2::cm respectively. *H. pylori* strains NCTC11638, NCTC11637 and clinical isolate strain 1061 were transformed with these plasmids by natural transformation (Smeets *et al.*, 2000) (**Table 1**). Following transformation, *H. pylori* cells were plated on Columbia Blood Agar containing kanamycin (20 μg/ml) or chloramphenical (20 μg/ml) and grown under standard microaerobic conditions. Kanamycin or chloramphenicol resistant colonies were isolated and passaged on the same medium. After passage, individual colonies were selected and screened for the mutation in the chromosomal DNA of the mutant. PCR analysis with primers flanking the insertion site but internal in the structural gene, was undertaken and PCR analysis demonstrated that the kanamycin and chloramphenicol cassettes were inserted by a double homologous recombination event into the chromosomal copy of *trx2* in all selected colonies from each strain background using mutant chromosomal DNA (**Fig. 4.1 (B)**). To additionally confirm the insertional mutation of the *trx2* gene, analysis of total cell lysates obtained from mutant and wild-type strains was carried out by immunoblotting. Immunoblotting using polyclonal antibodies raised in rabbits against Trx2 protein revealed the absence of Trx2 protein in the cell lysates for *H. pylori* strains where the trx2 gene was disrupted indicating that a full length gene product was not being transcribed (**Fig. 4.1 (B), panel B, lanes 2 and 4**). In contrast, Trx2 was readily identifiable in the lysate of the wild-type strains examined (**Fig. 4.1 (B), panel B, lanes 1 and 3**). All mutants grew normally on Columbia Blood Agar plates and the overall protein composition of the total cell lysate remained unchanged in the insertional mutants of strains NCTC11638 and NCTC11637 as assessed by SDS-PAGE and Coomassie Blue staining.

4.3.2 Growth characteristics of *H. pylori* Trx2 mutant strains.

The six *H. pylori* Trx2 insertion mutant strains (R2::kan, R2::cm; R1::kan, R1::cm; 1061::kan, 1061::cm) were grown on Columbia blood agar with the appropriate antibiotic selection (kanamycin 20 μg/ml, chloramphenicol 20 μg/ml) for several passages under standard microaerobic conditions. No difference in the growth rates was observed and colony formation was identical upon visual inspection for all strains. No single strain appeared to be growth compromised as a result of the absence of a functional Trx2 protein. A variation in a the cohesive property of the wild type versus the mutant *H. pylori* strains was apparent however. Liquid culture provided a medium in which to assess this physical property further and light microscopic examination (100 X magnification) revealed the

TABLE 1. *trx2* Mutagenesis; Plasmids and Bacterial Strains.

Strain/ Plasmid	Genotype/Characteristics	Source/Reference
Strain		
E. coli DH5α	ϕ80Δ*lac*Z ΔM15 Δ(*lac*ZYA-*arg*F) U169 *sup*E4 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1*rel*A *deo*R	Promega
H. pylori R2	Wild-type NCTC11638	NCTC[1]
H. pylori R1	Wild-type NCTC11637	NCTC
H. pylori 1061	Wild-type	A. van Vliet[2]
H. pylori R2::kan	R2 *trx2*::*aph-A3*	This study
H. pylori R2::cm	R2 *trx2*::*cat*	This study
H. pylori R1::kan	R1 *trx2*::*aph-A3*	This study
H. pylori R1::cm	R1 *trx2*::*cat*	This study
H. pylori 1061::kan	1061 *trx2*::*aph-A3*	This study
H. pylori 1061::cm	1061 *trx2*::*cat*	This study
Plasmid		
pGEM-T Easy	Cloning vector	Promega
pJMK30	pUC19 containing *aph-A3* cassette (Amp^r)	A. van Vliet[3]
pUCAT	pUC18 containing *cat* cassette from *Campylobacter coli*	A. van Vliet[3]
*Xba*I fragment	1.4 kb restriction fragment containing *aph-A3* from pJMK30	This study
pGEMT::T1458	pGEM-T Easy with 1024 bp *trx2* fragment inserted at *Eco*R1 site	This study
pGEMTrx2::kan	pGEMT:T1458 with *trx2* inactivated by insertion of *aph-A3* at unique *Bst*X1 site	This study
pGEMTrx2::cm	pGEMT:T1458 with *trx2* inactivated by insertion of *cat* cassette at unique *Bst*X1 site	This study

[1] National Collection of Type Cultures, Colindale, U.K.

[2] A. van Vliet, Dijkzigt Hospital, Rotterdam, The Netherlands/ Goodwin, A. *et al.* 1998. Mol. Microbiol. **28**: 383-393.

[3] A. van Vliet/ J.G. Kusters, Dijkzigt Hospital, Rotterdam, The Netherlands.

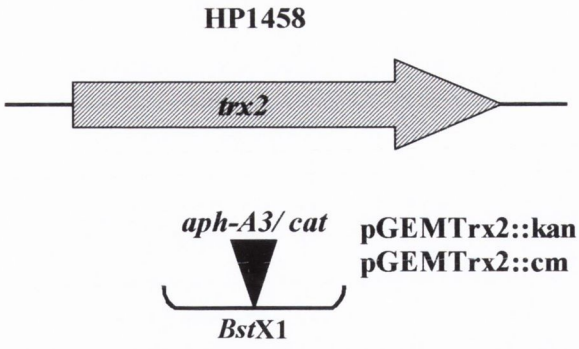


Fig. 4.1 (A). Mutagenesis of the *trx2* gene of *H. pylori*. Mutants were constructed by cloning kanamycin (*aph-A3*) or chloramphenicol (*cat*) resistance cassettes into the unique *Bst*X1 restriction site of the *trx2* gene in the insert DNA of the relevant plasmids (Table 1) followed by natural transformation of *H. pylori*.

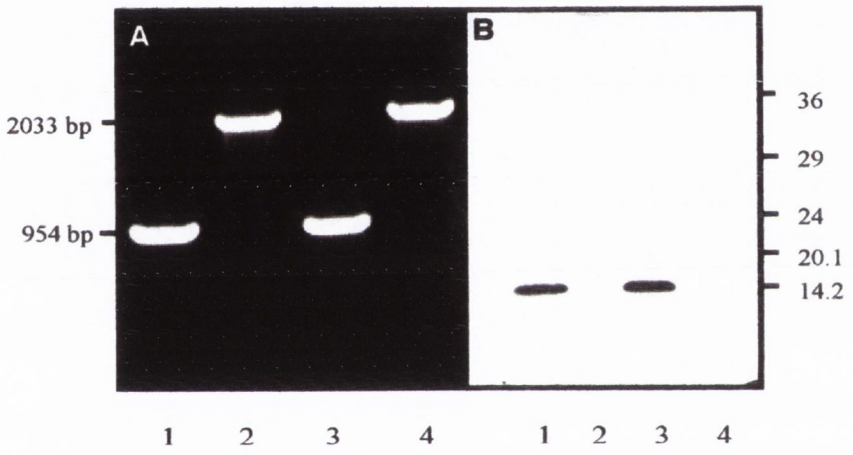


Fig. 4.1 (B). PCR and immunoblot verification of mutagenesis of the *trx2* gene in *H. pylori*. **(Panel A)** Agarose gel of PCR products obtained from the parental strain NCTC11638 (R2) (lane 1) and the Trx2 isogenic mutant (lane 2) genomic DNA, showing an increase in the size of the mutant PCR product due to insertion of the *aph-A3* resistance cassette. Also, the PCR products obtained from the parental strain 1061 (lane 3) and the Trx2 isogenic mutant (lane 4) genomic DNA, showing an increase in size of the mutant PCR product also due to insertion of the *aph-A3* resistance cassette. **(Panel B)** Immunoblot analysis using anti-Trx2 antibodies showing the presence of Trx2 in a total cell lysate (50 μg) of the parental *H. pylori* R2 strain (lane 1) and absence of the protein in the Trx2 isogenic mutant strain (lane 2). Also, the presence of Trx2 is confirmed in a total cell lysate of the parental *H. pylori* 1061 strain (lane 3) and absence of the protein shown in the Trx2 isogenic mutant strain (lane 4). Molecular weight markers are shown on the right.

Trx2 mutant strains to be less self-adhesive as judged by reduced clumping. To further assess the growth characteristics of the Trx2 mutant strains, culture in liquid medium was undertaken. **Fig. 4.2** shows the growth rates of two parental plus mutant strains in BHI with 2% FCS liquid batch cultures equilibrated in a microaerobic atmosphere, sealed and incubated with shaking at 37°C for the times indicated. All cultures were inoculated with bacteria from 3 day-old plate cultures and bacteria were resuspended to densities of 10^7 to 10^9 c.f.u. ml^{-1}. Results indicate that the Trx2 mutant *H. pylori* strains grew almost as well as the isogenic parental strains under the same growth conditions. No severe growth defect was noted due to the absence of the Trx2 protein in the strains modified by the insertion of either a kanamycin or chloramphenicol (not shown) resistance cassette into the *trx2* gene. Finally, the isolation of Trx2 deficient *H. pylori* indicates that Trx2 is not essential for viability of the bacterium at least under standard microaerobic culture conditions. Growth of the bacteria in liquid culture demonstrated that the growth rates of kanamycin and chloramphenicol insertional Trx2 mutants were identical (not shown).

4.3.3 Sensitivity of *H. pylori* strains to oxidative stress.

The inhibitory effect on the growth of *H. pylori* wild-type and isogenic Trx2 mutant strains exposed to a range of oxidants and other stress inducing chemical compounds was assessed on Columbia blood agar using disc-diffusion assays. Zones of growth inhibition were measured around filter paper discs containing amounts of different compounds. **Figs. 4.3 to 4.7** show the sensitivity of wild-type strains and Trx2 isogenic mutant strains to growth inhibition by a range of chemicals. The superoxide anion radical generating compound, menadione, and the thiol oxidant, diamide, were used to determine the sensitivity of *H. pylori* to redox cycling agents. Experiments showed that menadione had a deleterious effect on *H. pylori* growth at all concentrations tested. The Trx2 mutant strains did not display a higher level of sensitivity to these compounds than wild-type *H. pylori* in either the R2 (**Fig. 4.3, A**) or 1061 (**Fig. 4.3, B**) background. Diamide had a deleterious effect on *H. pylori* growth at elevated concentrations (> 10 mM), but again, the Trx2 mutants were not significantly more sensitive to its growth-inhibiting effects than the parental strains (**Fig. 4.4**). Moreover, both the parental and isogenic mutant 1061 strains were consistently more sensitive than the R2 strain to diamide stress,. Identical experiments carried out to assess the sensitivity of the various strains to ethanol revealed that this agent had no effect on the growth of any of the four strains tested in repeated assays. No zones of inhibition were noted indicating a lack of toxicity of ethanol (0.03% – 30%) to *H. pylori* growth,

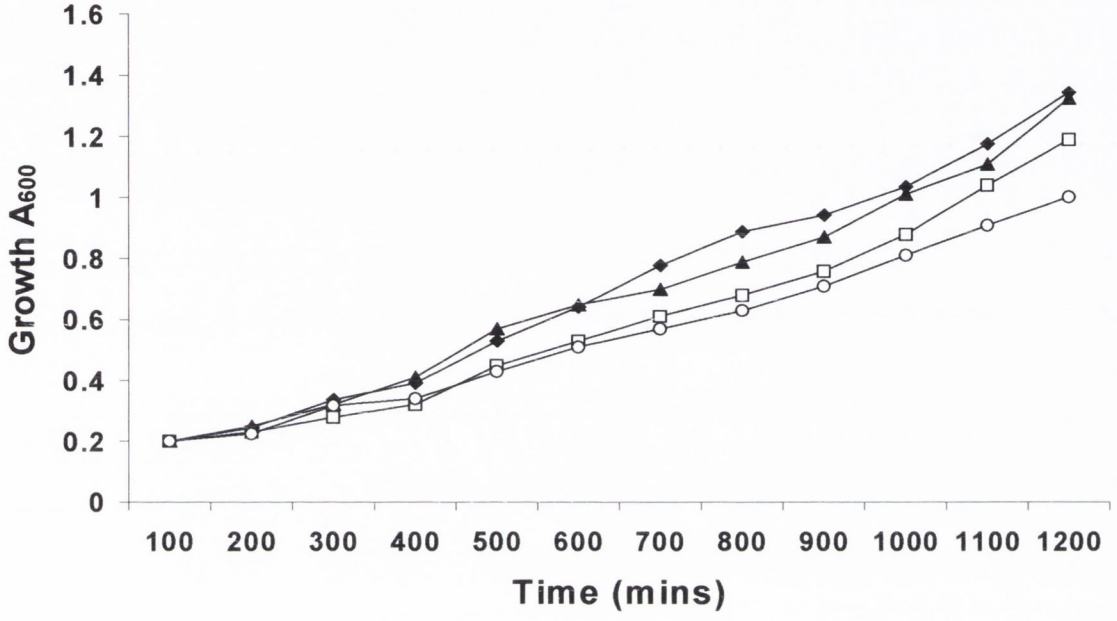


Fig. 4.2. Growth of *H. pylori* wild-type and isogenic Trx2 mutant strains in liquid culture. BHI/2% FCS medium was equilibrated in cell culture flasks in a microaerobic atmosphere. All strains were taken from 3 day old plates, resuspended to densities of 10^7-10^9 c.f.u. ml^{-1}, and diluted to a starting OD_{600} value of 0.2. Samples (1 ml) were removed at the times indicated and the OD_{600} was determined. (◆); *H. pylori* R2, (□); *H. pylori* R2::kan, (▲); *H. pylori* 1061, (○); *H. pylori* 1061::kan. The graph shown is representative of three independent experiments with similar results.

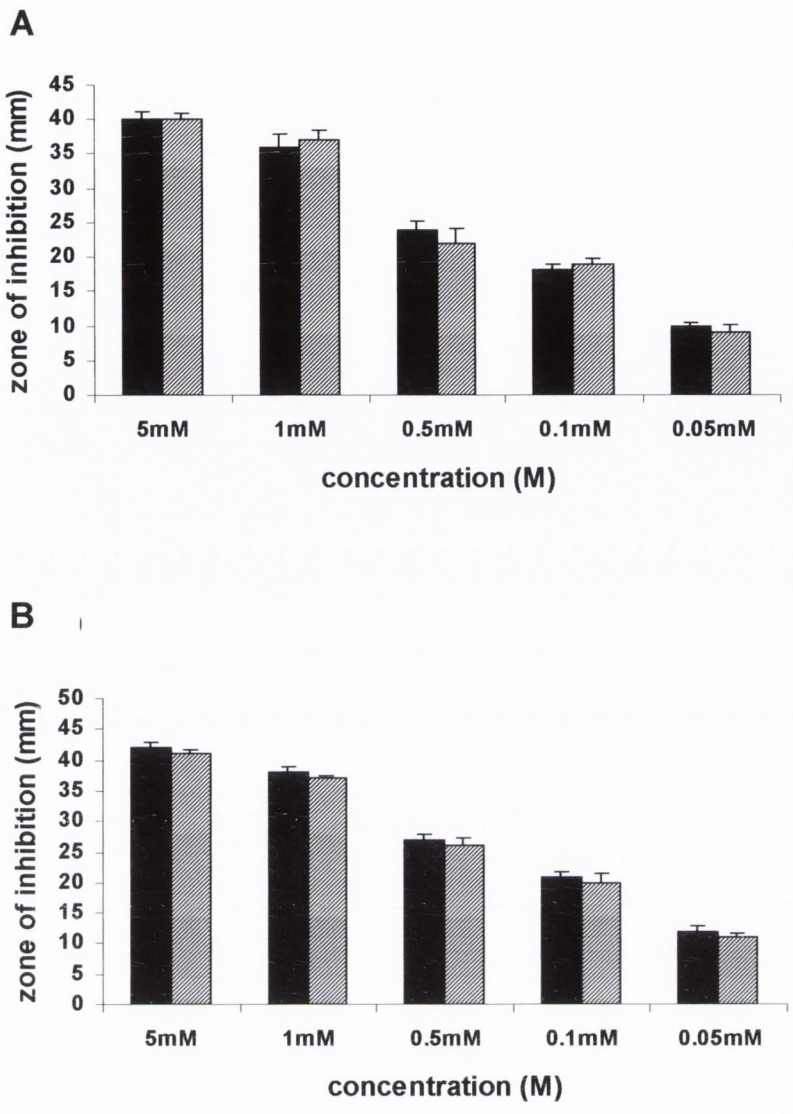


Fig. 4.3. Sensitivity of wild-type and mutant *H. pylori* strains to menadione. A: Comparison of the sensitivity of wild-type strain NCTC11638 (R2) (filled bars) and the isogenic Trx2 mutant strain (hatched bars) to menadione. **B:** Comparison of the sensitivity of wild-type strain 1061 (filled bars) and the isogenic Trx2 mutant strain (hatched bars) to menadione. Filter discs containing 5 μl of the compound in DMSO at the range of concentrations indicated were placed equidistant around a Columbia blood plate upon which the relevant strain had been spread. The histograms represent the mean of five replicate assays in which the diameters of growth inhibition were measured after 4 days incubation under the appropriate conditions. The histogram error bars represent the standard deviation of the means.

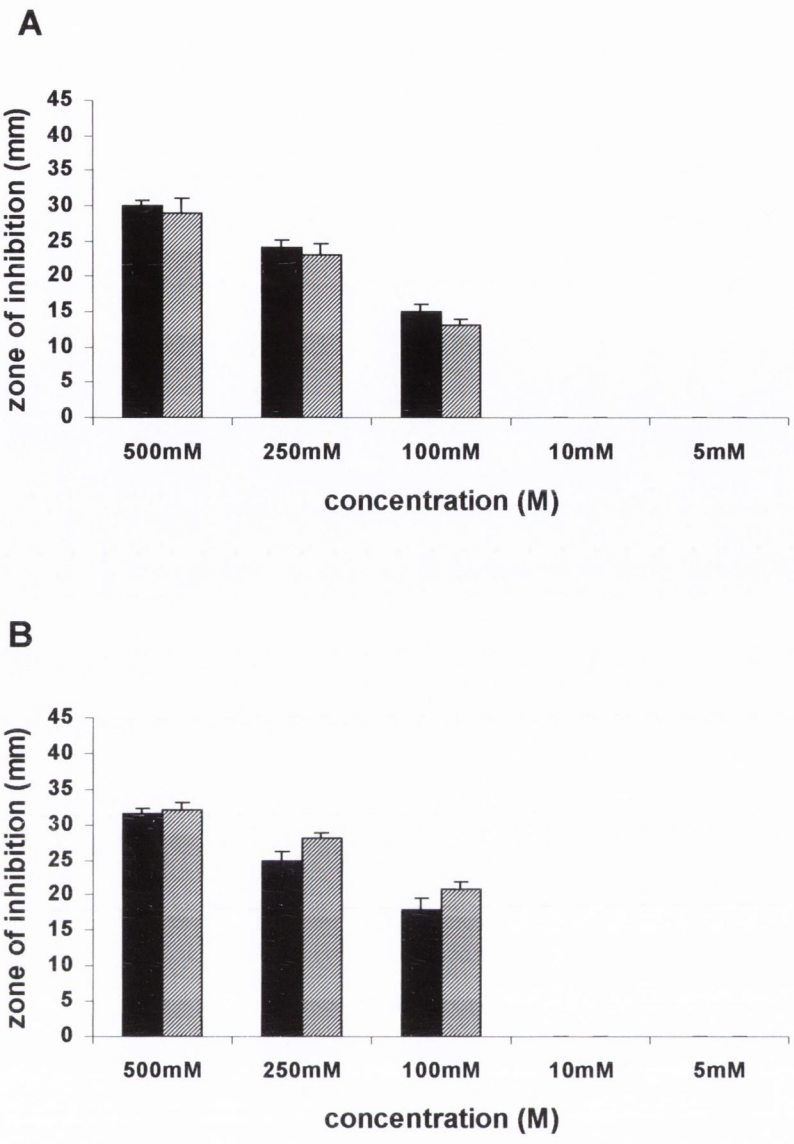


Fig. 4.4. Sensitivity of wild-type and mutant *H. pylori* strains to diamide. A: Comparison of the sensitivity of wild-type strain NCTC11638 (R2) (filled bars) and the isogenic Trx2 mutant strain (hatched bars) to diamide. **B:** Comparison of the sensitivity of wild-type strain 1061 (filled bars) and the isogenic Trx2 mutant strain (hatched bars) to diamide. Filter discs containing 5 µl of the compound in DMSO at the range of concentrations indicated were placed equidistant around a Columbia blood plate upon which the relevant strain had been spread. The histograms represent the mean of five replicate assays in which the diameters of growth inhibition were measured after 4 days incubation under the appropriate conditions. The histogram error bars represent the standard deviation of the means.

however, this apparent lack of toxicity may have been due to evaporation of ethanol during the incubation period.

Further assays were performed to evaluate the effect of the organic oxidants, cumene hydroperoxide (**Fig. 4.5**) and *tert*-butyl hydroperoxide (**Fig. 4.6**), on the growth of *H. pylori* wild-type and Trx2 isogenic mutant strains. The results demonstrated that Trx2 mutants were significantly more sensitive to cumene hydroperoxide compared to the respective parental strains (R2 and 1061) (**Fig. 4.5**). The oxidant *tert*-butyl hydroperoxide had a deleterious effect on *H. pylori* growth particularly at elevated concentrations, however again the Trx2 mutant strains were significantly more sensitive to the growth-inhibiting effects of this agent than the parental strains (**Fig. 4.6**). Overall, experiments revealed that the *H. pylori* Trx2 mutant strains were clearly more sensitive to both of the peroxides tested when compared with the wild-type strains. These data indicate a likely role for Trx2 in the detoxification of organic peroxides. Interestingly, a difference in the sensitivity of the strains to the two organic peroxides tested was also observed. Both of the strains were more sensitive to the oxidant stress imposed by the presence of *tert*-butyl hydroperoxide than cumene hydroperoxide as indicated by the increase in the zones of inhibition observed for *tert*-butyl hydroperoxide. Moreover, both the parental and isogenic mutant 1061 strains were consistently more sensitive than the R2 strain to peroxide stress.

Additionally, the effect of hydrogen peroxide on the growth of *H. pylori* wild-type and Trx2 mutant strains was assessed. As shown in **Fig. 4.7,** there is no growth inhibition by hydrogen peroxide in either of the wild-type strains, indicating that Trx2 is probably not directly involved in defence against oxidative stress imposed by hydrogen peroxide. The presence of a highly efficient and abundant hydrogen peroxide detoxifying enzyme, catalase, most likely fulfils this role. Interestingly, the wild-type 1061 strain displays some growth inhibition at the highest concentration of hydrogen peroxide (30% (v/v) solution) as indicated by the small zone of inhibition. The isogenic mutant strain, however, appears not to display any sensitivity to this oxidant as no growth inhibition was observed. This is in contrast to the response of the Trx2 mutants to cumene hydroperoxide and *tert*-butyl hydroperoxide, where lack of a functional Trx2 compromised growth. All disc-diffusion assays were performed with mutant Trx2 strains which were inactivated by insertion of a kanamycin resistance cassette. The experiments described above were also performed for chloramphenicol insertional mutants and very similar data were obtained (not shown).

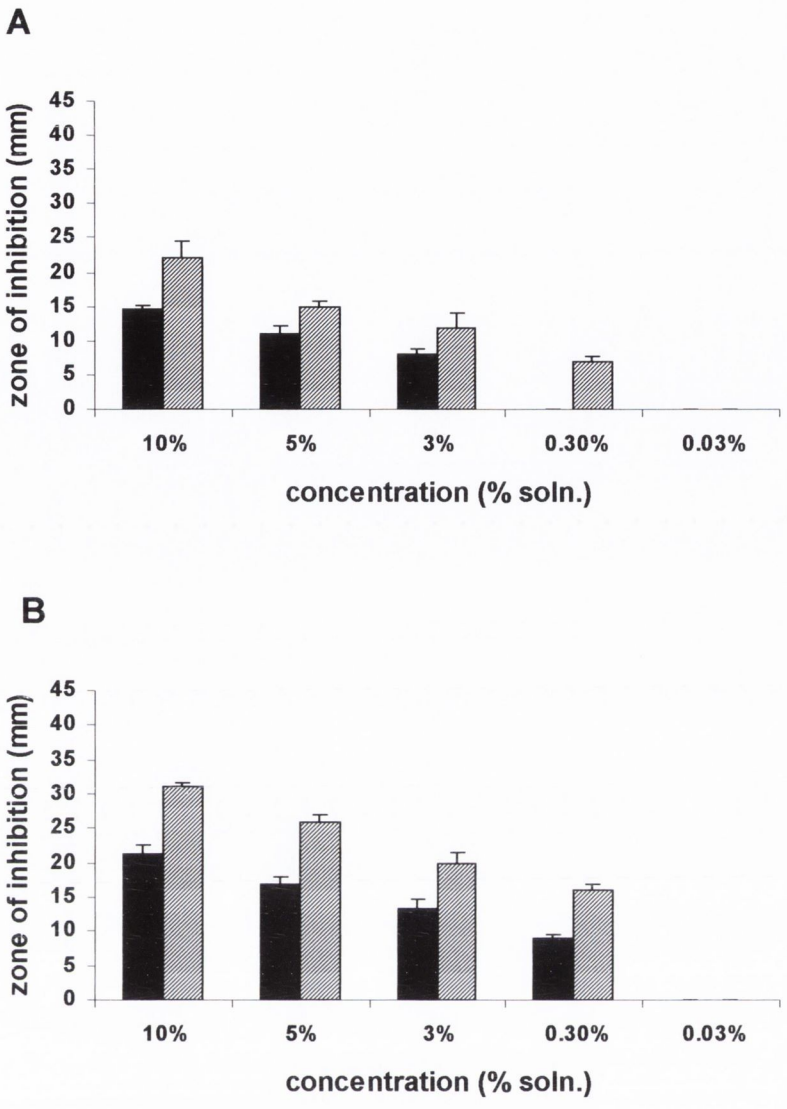


Fig. 4.5. Sensitivity of wild-type and mutant *H. pylori* strains to cumene hydroperoxide. A: Comparison of the sensitivity of wild-type strain NCTC11638 (R2) (filled bars) and the isogenic Trx2 mutant strain (hatched bars) to cumene hydroperoxide. **B:** Comparison of the sensitivity of wild-type strain 1061 (filled bars) and the isogenic Trx2 mutant strain (hatched bars) to cumene hydroperoxide. Filter discs containing 5 μl of the oxidant in DMSO at the range of concentrations indicated were placed equidistant around a Columbia blood plate upon which the relevant strain had been spread. The histograms represent the mean of five replicate assays in which the diameters of growth inhibition were measured after 4 days incubation under the appropriate conditions. The histogram error bars represent the standard deviation of the means.

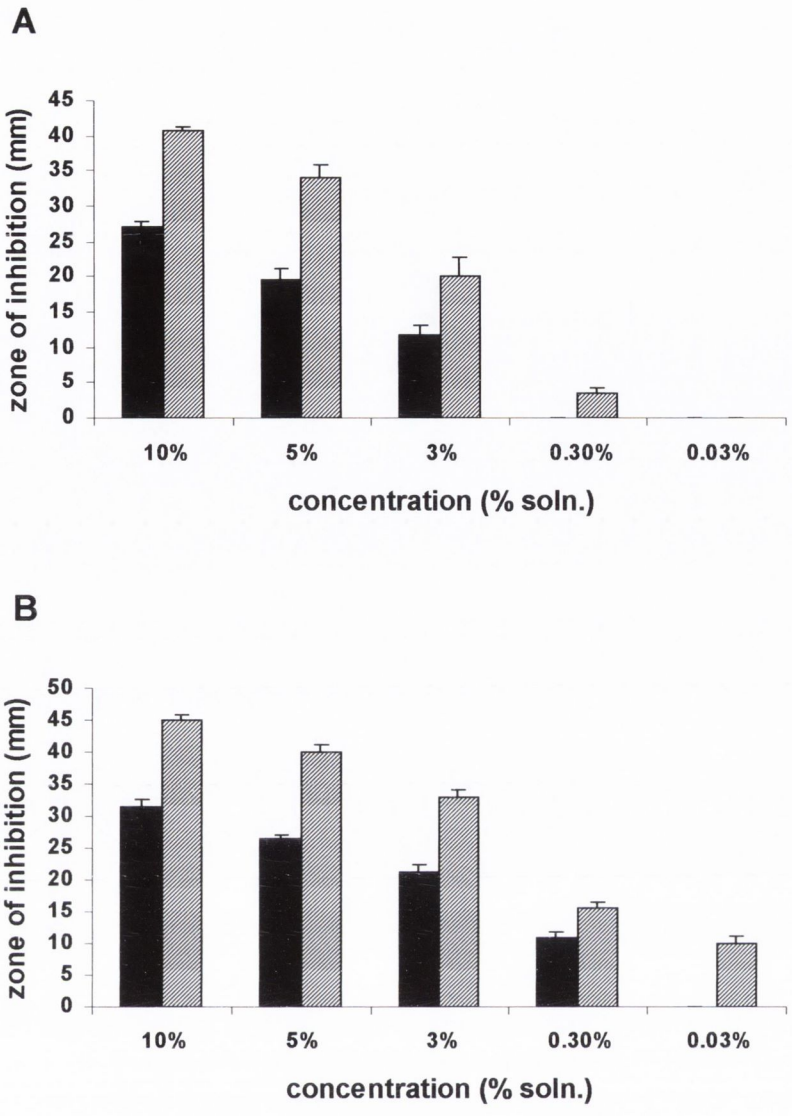


Fig. 4.6. Sensitivity of wild-type and mutant *H. pylori* strains to *tert*-butyl hydroperoxide. A: Comparison of the sensitivity of wild-type strain NCTC11638 (R2) (filled bars) and the isogenic Trx2 mutant strain (hatched bars) to *tert*-butyl hydroperoxide. **B:** Comparison of the sensitivity of wild-type strain 1061 (filled bars) and the isogenic Trx2 mutant strain (hatched bars) to *tert*-butyl hydroperoxide. Filter discs containing 5 µl of the oxidant in DMSO at the range of concentrations indicated were placed equidistant around a Columbia blood plate upon which the relevant strain had been spread. The histograms represent the mean of five replicate assays in which the diameters of growth inhibition were measured after 4 days incubation under the appropriate conditions. The histogram error bars represent the standard deviation of the means.

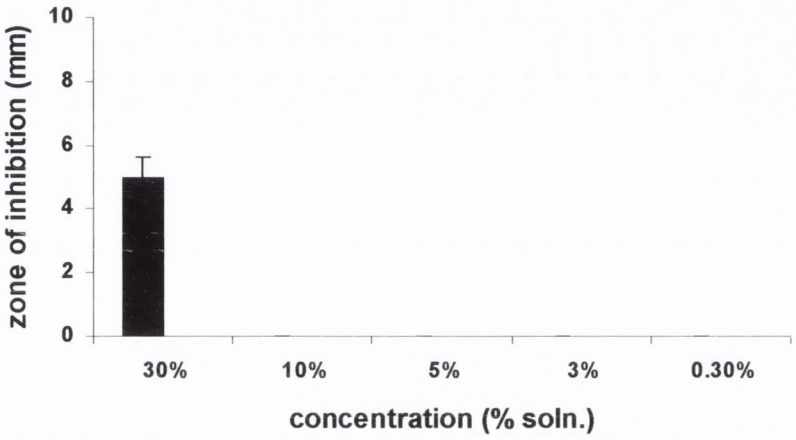


Fig. 4.7. Sensitivity of *H. pylori* strain 1061 to hydrogen peroxide. Sensitivity of wild-type strain 1061 (filled bar) to hydrogen peroxide. Filter discs containing 5 µl of H_2O_2 at the range of concentrations indicated were placed equidistant around a Columbia blood plate upon which the relevant strain had been spread. The histogram represents the mean of five replicate assays in which the diameters of growth inhibition were measured after 4 days incubation under the appropriate conditions. The histogram error bar represents the standard deviation of the means.

4.3.4 Disruption of *H. pylori trx2* and resistance to hydrogen peroxide in a selected *H. pylori* strain.

The observed resistance of the 1061 Trx2 mutant strain to a high concentration of hydrogen peroxide was unexpected (**Fig. 4.7**) and was therefore further evaluated by SDS-PAGE. SDS-PAGE analysis was performed on total cell lysates from both wild-type and mutant 1061 strains (**Fig. 4.8, A**). Initially, analysis revealed apparent upregulation of the enzyme catalase in the Trx2 mutant strain. Assumption of the identity of the upregulated protein as the enzyme catalase (HP0875; TIGR Annotation) was based on correlation of hydrogen peroxide resistance and the molecular weight of the upregulated protein (58,600 kDa). The possibility of the identity of the protein being the catalase-like protein, KatE (HP0485), was discounted as the molecular weight of this protein is too low at 35.7 kDa. This putative identification was confirmed following biochemical assays for catalase activity (**Fig. 4.8, B**). Catalase activity was determined with an oxygen electrode (Rank) used to measure oxygen production due to hydrogen peroxide decomposition. The assay was performed in PBS in the presence of 20 mM hydrogen peroxide and the reaction was initiated by the addition of *H. pylori* extract. 2-D SDS-PAGE analysis was performed on total cell lysates from both wild-type and mutant 1061 strains. Analysis revealed upregulation of catalase in the Trx2 mutant strain (**Fig. 4.9, A**). When lysate from the parental strain was examined, the spot corresponding to catalase was absent on the 2-D gel (**Fig. 4.9, B**). These data correlate with results obtained from 1-D SDS-PAGE analysis and catalase activity assays. Subsequent identification of catalase as the upregulated protein followed mass spectrometry analysis of the spot on a 2-D gel. (N-terminal sequencing, Protein and Nucleic Acid Chemistry Facility, Cambridge University, U.K.)

4.3.5 Identification of Trx interacting proteins in *H. pylori*.

In an attempt to identify electron acceptors for Trx in *H. pylori* an adaptation of the proteomic approach combined with fluorescent tagging using the thiol-specific probe monobromobimane (mBrB) described recently (Yano *et al.*, 2001), was used (**Fig. 4.10, A**). **Fig. 4.10, B** shows a 1-D SDS-PAGE profile of a sonicated preparation of whole *H. pylori* treated with mBrB following exposure of the gel to UV light. Total cellular free thiols in the *H. pylori* sonicate were conjugated with monobromobimane (mBrB) using a modification of the method described by Yano *et al.* (2001). *H. pylori* extract (100 μg) was incubated for 1 h at 37°C in potassium phosphate buffer (0.1 M, pH 7.4) containing Trx (20 μg) NADPH (1.25 mM) and *E. coli* thioredoxin reductase (0.3 U, 3.3 μg) in a final

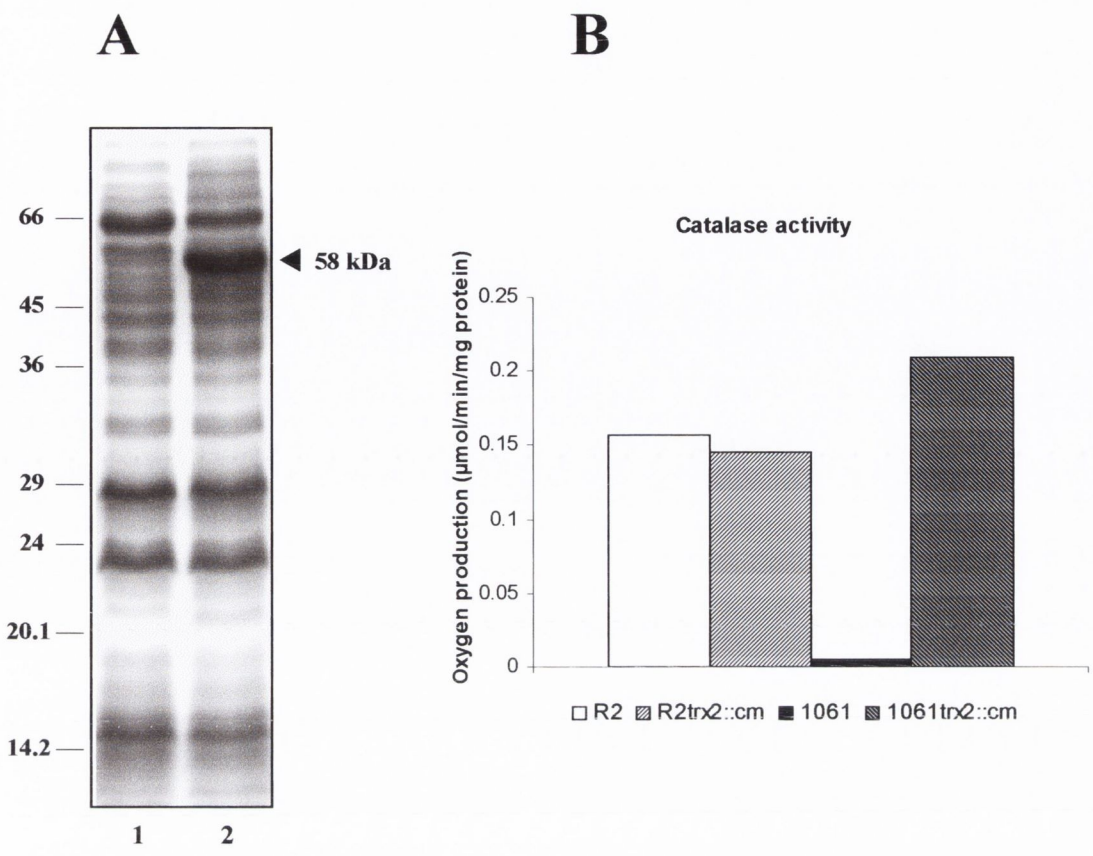


Fig. 4.8. SDS-PAGE analysis of *H. pylori* wild type strain 1061 and isogenic Trx2 mutant strain. A: Total cell lysates from both strains were analysed on a 15% polyacrylamide gel in reducing sample buffer as follows: lane 1, total cell protein from strain 1061 (protein mass 50 µg); lane 2, total cell protein from the isogenic Trx2 mutant strain (1061trx2::cm) (protein mass 50 µg). The filled arrow indicates the position of *H. pylori* catalase (58 kDa). Proteins were visualised by Coomassie staining. Molecular masses (in kilodaltons) are indicated on the left. **B:** Graph of catalase activity of *H. pylori* wild type strain R2 and isogenic Trx2 mutant strain and wild type strain 1061 and isogenic Trx2 mutant strain.

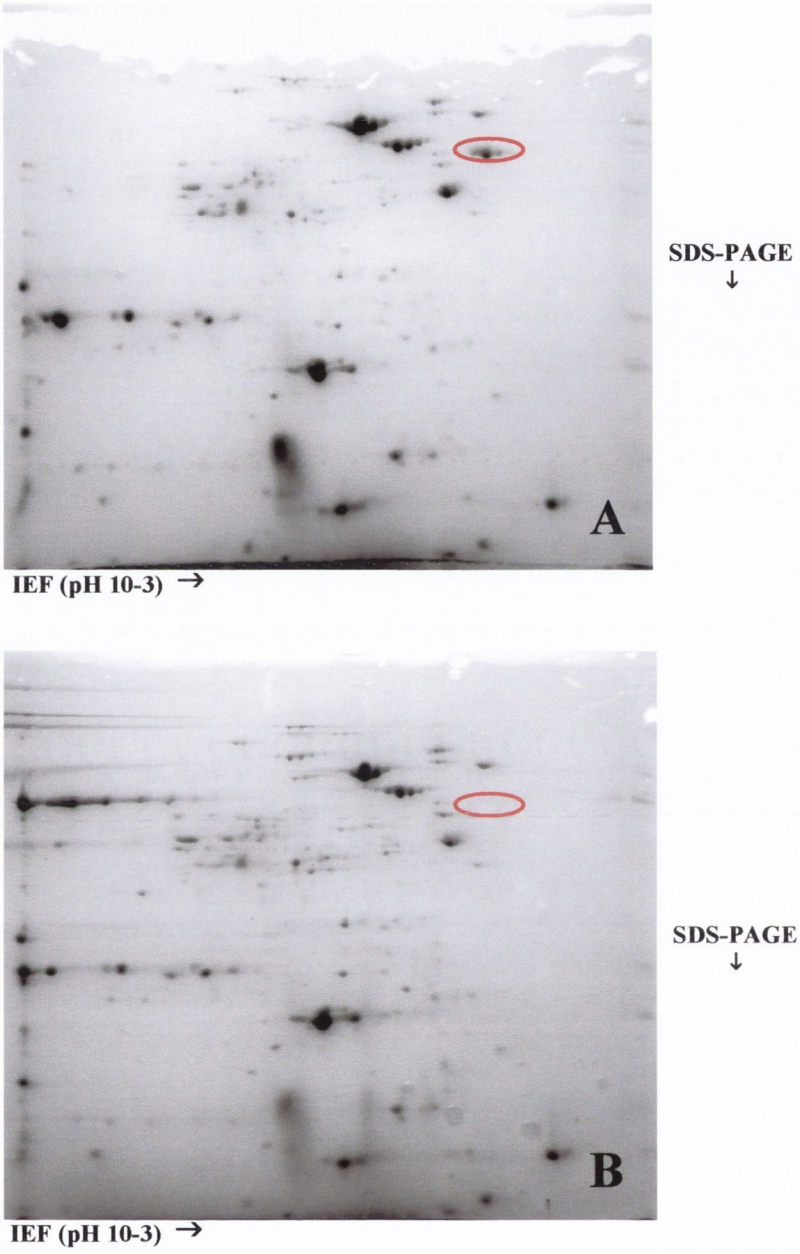


Fig. 4.9. Two-dimensional gel analysis of total proteins from *H. pylori* strain 1061 and isogenic Trx2 mutant strain. Bacteria were grown on Columbia blood agar and 50 μg of a clarified sonicate of *H. pylori* was separated by 2-D SDS-PAGE (IEF; pH 3 – 10). Proteins were visualised by staining with Coomassie Blue R-250. Panel A; isogenic Trx2 mutant strain. Panel B; parental strain. The position of *H. pylori* catalase (HP0875) is depicted with a red circle in panel A. The expected position of catalase in panel B is also depicted with a red circle.

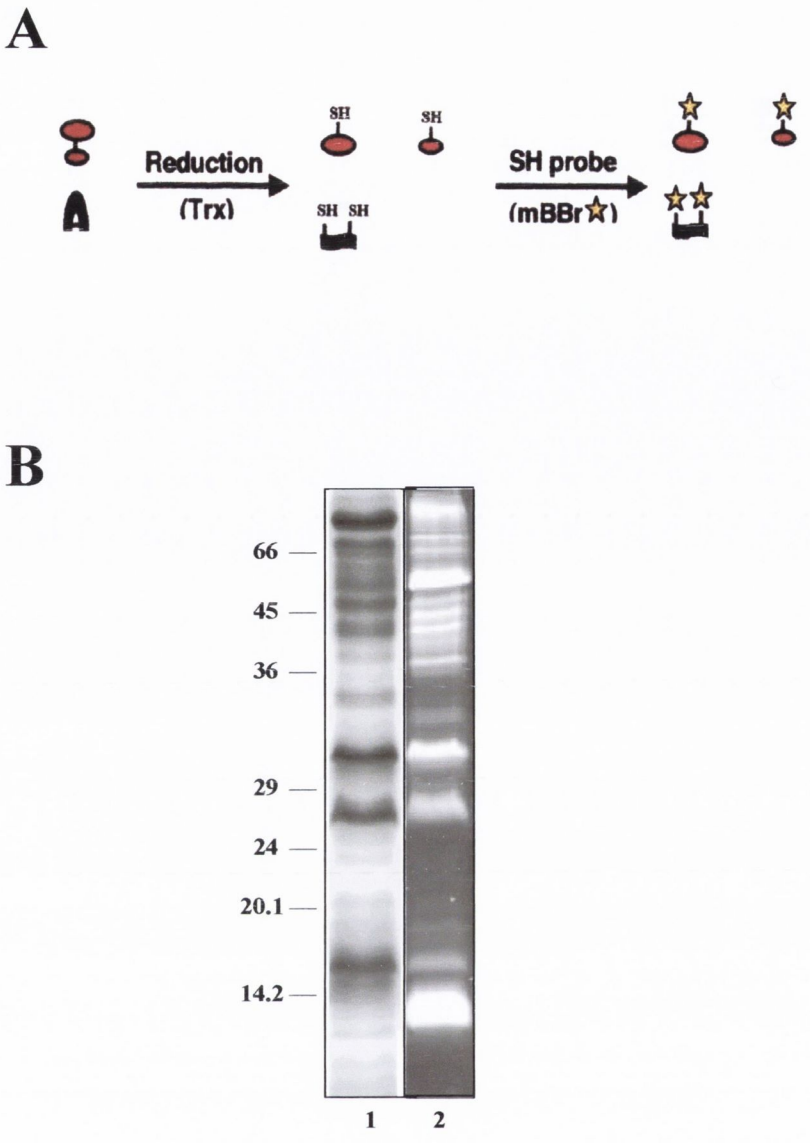


Fig. 4.10. Monobromobimane labelled total *H. pylori* proteins. **A:** Schematic of mBrB attachment to Trx-reduced proteins (reproduced from Yano *et al.*, 2001). mBrB labels sulphydryl groups newly exposed on target proteins following reduction by Trx and these sulphydryl groups are visualised as their fluorescent mBrB derivatives. **B:** SDS-PAGE analysis of *H. pylori* total proteins after Coomassie Blue staining (lane 1) or labelling with mBrB (lane 2). 50 μg of protein was loaded per lane and mBrB-labelled proteins were visualised under UV light at 362nm.

volume of 50 µl. Monobromobimane (Molecular Probes) was added to a final concentration of 2.5 mM and the incubation was allowed proceed for an additional 10 to 20 min in the dark at room temperature. SDS-PAGE analysis showed that many *H. pylori* proteins were labelled by the thiol fluorescent probe and that this approach could be applied to screen for potential electron acceptors for Trx2 and identify how Trxs are involved in the response to peroxide stress in *H. pylori*.

4.3.6 Trx-dependent reduction of *H. pylori* Thiol Peroxidase.

To validate putative *H. pylori* peroxiredoxin target substrates of *H. pylori* Trxs, the system was reconstituted in the case of Trx1/Trx2 and initially thiol peroxidase (Tpx), using recombinant versions of the proteins. Tpx and Trx1 were produced in recombinant form with a poly-His tag by overexpression in *E. coli*, using *H. pylori* strain NCTC11638 (R2) as a source of template DNA (Dr. H. Windle, Dept. of Clinical Medicine , T.C.D.). The results shown in **Fig. 4.11** demonstrate that when the dithiol form of Trx1 was reconstituted with recombinant Tpx in the presence of TR and NADPH, mBrB incorporation into Tpx occurred. In the absence of Trx1 but presence of NADPH and TR no change in the redox status of Tpx was evident (**Fig. 4.11, panel A, lane 1**). Interestingly, the dithiol form of Trx2 also provided reducing equivalents to Tpx as determined by mBrB incorporation (**Panel A, lane 3**). As expected, there was no change in the redox status of Tpx when it was incubated for the duration of the experiment in the absence of the Trx system composed of TR, NADPH and Trx1 or Trx2 (**Panel A, lane 4**). Therefore these data indicate that both Trx1 and Trx2 can behave as electron donors to Tpx. Moreover, it appears that Trx1 (**Panel A, lane 2**) may be very slightly more efficient at reducing Tpx than Trx2 (**Panel A, lane 3**). In both cases equivalent amounts of the two thioredoxins and Tpx were present in the reaction mixture (**Panel B**).

4.3.7 Trx-dependent reduction of Bacterioferritin Comigratory Protein.

To evaluate an additional potential *H. pylori* peroxiredoxin target substrate of *H. pylori* Trxs, the system was reconstituted in the case of Trx1/Trx2 and bacterioferritin comigratory protein (Bcp), using recombinant versions of the proteins. A recombinant form of *H. pylori* Bcp was generated (Dr. H. Windle, Dept. of Clinical Medicine , T.C.D.) and used to determine if it too was an electron acceptor for Trx1 and/or Trx2. The data shown in **Fig. 4.12, panel A** demonstrates that both recombinant Trx1 and Trx2 behaved as electron donors to recombinant Bcp in the presence of TR/NADPH (**Panel A, lanes 2**

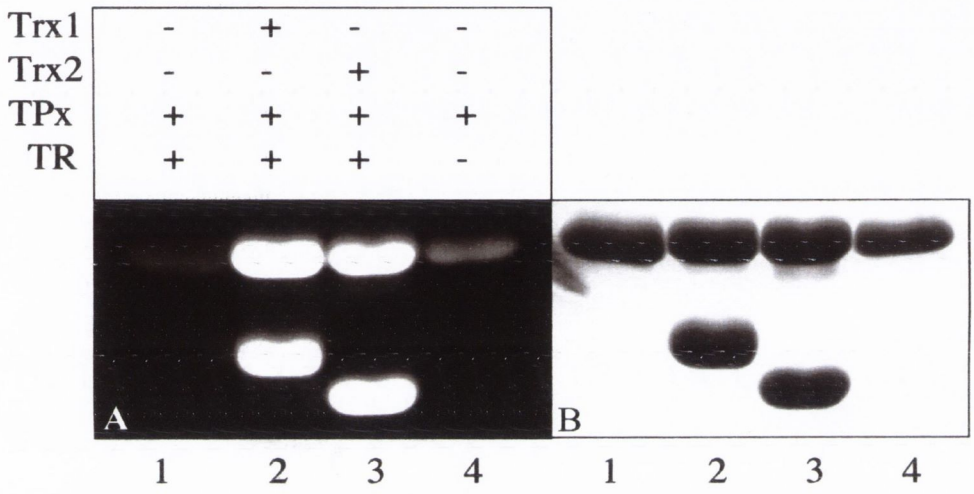


Fig. 4.11. Reconstitution of the Trx system with recombinant thiol peroxidase. Purified recombinant Tpx was incubated either in the presence of NADPH/thioredoxin reductase (TR) (lane 1), NAPDH/TR/Trx1 (lane 2), NADPH/TR/Trx2 (lane 3) or alone (lane 4) for 20 min and subsequently treated with mBrB for an additional 10 min. Samples were separated by SDS-PAGE and exposed to UV light (panel A) and subsequently stained with Coomassie Blue (panel B).

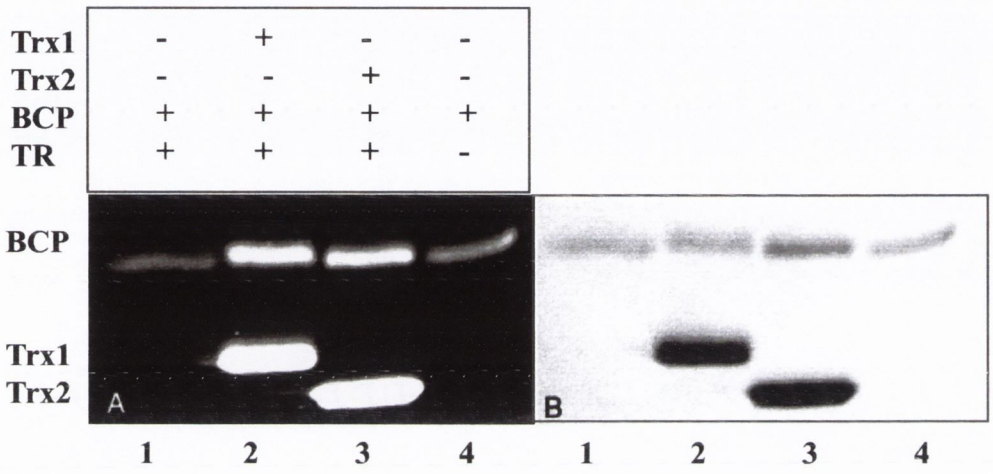


Fig. 4.12. Reconstitution of the Trx system with recombinant Bcp. Purified recombinant Bcp was incubated either in the presence of NADPH/thioredoxin reductase (TR) (lane 1), NAPDH/TR/Trx1 (lane 2), NADPH/TR/Trx2 (lane 3) or alone (lane 4) for 20 min and subsequently treated with mBrB for an additional 10 min. Samples were separated by SDS-PAGE and exposed to UV light (panel A) and subsequently stained with Coomassie blue (panel B).

and 3). As for Tpx, TR/NADPH had no effect on the redox status of BCP (**Panel A, lane 1**).

4.4 DISCUSSION

All organisms have evolved to cope with environmental stress conditions by the induction of a number of cellular responses. Such responses generally involve an increase in the expression of a subset of genes whose downstream protein products function in the elimination of toxic oxygen derived metabolites which the cell is encountering. Active oxygen species produced as aerobic metabolic by-products are extremely reactive and can result in widespread damage to cellular macromolecules such as DNA and proteins. The antioxidant systems critical to the defence of *H. pylori* against ROS generated by the oxidative burst from macrophages and polymorphonuclear leukocytes are necessary to allow the pathogen to establish chronic gastric infections and to combat the hosts' inflammatory responses. As a microaerophilic organism, *H. pylori* may be especially sensitive to the reactive forms of oxygen. The Trx system is the primary mediator of thiol-based redox metabolism in *H. pylori* and represents the sole intracellular disulphide reducing system. As oxidative stress results in the formation of deleterious disulphide bonds within cellular proteins, the Trx system is critical for the ability of *H. pylori* to survive the damaging effects of oxidative stress exposure. *H. pylori* contains two genes encoding Trxs (Trx1 (HP824); Trx2 (HP1458), TIGR). Trx1 contains the highly conserved redox active motif, -CGPC-, present in most Trxs examined to date, with few exceptions. Trx2 has an unusual redox active motif, -CPDC-, and a unusually high isoelectric point (pI 8.1) compared to the acidic Trx1 (pI 4.9), in fact most Trxs are acidic proteins. The difference in the active site motif for these proteins in a micro-organism recognised for its reduced genomic redundancy suggests that both Trxs may be functionally dissimilar. As the Grx system is absent within this pathogen, this functional divergence may be essential as Trx must fulfil additional roles which are normally associated with this absent reducing system.

In this chapter, a variety of oxidative stress inducing compounds were used to evaluate the oxidative stress resistance capacity of *H. pylori* strains lacking the *trx2* gene. The aim of this work was elucidation of the cellular role of *H. pylori* Trx2 and the further characterisation of the Trx system of this pathogen. The experimental approach adopted

revealed that mutation of the gene encoding Trx2 results in an increase in sensitivity to particular forms of oxidative stress.

Inactivation of the *trx2* gene was successfully achieved and provided a strain background where the putative function of Trx2 in oxidative stress defence could be tested. The *trx2* gene was inactivated by disruption due to insertion of either a kanamycin or chloramphenicol resistance cassette, and in both cases viable *H. pylori* colonies were obtained following natural transformation of three different *H. pylori* strains. Therefore the Trx2 protein is not essential for the viability of the bacterium at least under normal microaerobic growth conditions. That this redox protein is not essential suggests that there may be an element of redundancy in the Trx system of this bacterium. Indeed, Trx1 may be capable of interacting with Trx2 substrates within the cell, thus compensating for the loss of Trx2 reducing activity when the bacterium is cultured under certain environmental conditions. Investigation of an *H. pylori* Trx1 mutant and a Trx double mutant of *H. pylori* would be necessary to confirm this hypothesis. To identify possible subtle growth defects that may not have been obvious following culture on solid media due to the possible ROS sequestering effects of haem groups present in the blood component of the agar, the growth rates of wild-type and mutant strains were examined in liquid culture as described by Xia *et al.* (1993). Results revealed that there was a slight decrease in the growth rates of strains lacking a functional Trx2 protein under the incubation conditions established in sealed tissue culture flasks following equilibration in a 5% CO_2 atmosphere for several hours.

During the course of this work a study by Comtois *et al.* (2003) showed that the concentration of O_2 is important when assessing the response of the bacterium in terms of growth rate in liquid culture when either of the Trxs are mutated. This group used the technique of insertional inactivation of the relevant genes using a *cat* cassette or an aminoglycoside phosphotransferase gene derived from *Campylobacter coli*. This study revealed that in *H. pylori* strain 26695, the absence of Trx2 resulted in a severe growth deficiency when the bacterium was grown in a gas atmosphere of 10% (v/v) O_2, whereas, at lower concentrations of O_2 (2% (v/v) O_2) this effect was reversed and the growth rate recovered to meet and increase on that observed for the parental strain. The study by Comtois *et al.* further postulates that Trx2 is important for the normal microaerobic growth of *H. pylori*, with a deficiency of Trx2 resulting in an increased sensitivity to molecular oxygen. The protocol followed in this present study did not facilitate the stringent control

of molecular oxygen levels during strain growth and this, coupled with the use of a different strain background, may partly explain the difference in growth rates observed when compared with the data of Comtois *et al.* Due to use of a sealed system, oxygen concentrations may have dropped below those of a standard microaerobic environment during growth resulting in the apparent ability of the Trx2 mutant to grow as efficiently as the isogenic parental strain. Furthermore, double Trx mutants were isolated by Comtois *et al.* indicating that in fact neither Trx is required for viability. However, a component of the Trx system, a putative thioredoxin reductase (TR) appears to be essential for survival of the pathogen as shown by Chalker *et al.* (2001). This group used a comparative genomic approach and identified 45 ORFs which were subsequently screened using a rapid vector-free allelic replacement mutagenesis technique, and 33 were shown to be essential *in vitro*. All allelic replacement constructs were directly transformed into *H. pylori* strain 26695, and typically essential genes were so annotated following 3 independent control transformations in which no mutants were recovered. Mutagenesis of the thioredoxin reductase (TR) gene (ORF HP1164; TIGR Annotation) resulted in inability to recover viable *H. pylori* colonies following transformation with the appropriate construct, indicating that this gene is essential. *H. pylori* 26695 contains two genes annotated as TR, HP0825 and HP1164, however the two proteins are only 16% identical, with HP0825 more closely related to other TRs. HP1164 lacks the classical redox motif of other thioredoxin reductases and the authors postulate that this ORF may fulfil an alternative function within the bacterium. The study of Comtois *et al.*, complements this previous work as Chalker *et al.* did not highlight Trx 1 or 2 as essential genes using their comparative genomic approach.

Mechanisms for controlling oxidative stress appear to be unusually critical for cell viability in *H. pylori* and these studies suggest that other cellular reducing equivalents can replace the function of Trx as a reducing partner for the essential flavoenzyme TR. The critical role of oxidative stress management enzymes in *H. pylori* is highlighted in two recent studies. Olczak *et al.* (2003) investigated the ability of *H. pylori* strains with mutations in either *tpx* (encoding thiol-peroxidase) or *ahpC* (encoding alkylhydroperoxide reductase) to colonise mouse stomachs. The *tpx* strain was shown to only colonise 5% of inoculated mice. In addition, neither of two *ahpC* mutants in a murine-adapted strain (SS1) background (Olczak *et al.*, 2002) were able to colonise mouse stomachs, in contrast to a 78% colonisation rate with the parent strain. Also recently, Thompson *et al.* (2003) analysed the

global pattern of growth-phase dependent gene expression of *H. pylori* during *in vitro* culture was analysed by using a high-density DNA microarray. These workers found that certain genes were induced in the late-log to stationary phase, including many virulence related genes. Interestingly, it was observed that Trx2 was among four genes, categorised as energy metabolism genes, that was significantly induced during this growth phase. The authors postulate that this growth phase may correspond to the most virulent phase of growth of *H. pylori* and may be intimately related to its pathogenesis. As the genes adjacent to *trx2* are transcribed in the same direction as *trx2*, complementation of the *trx2* mutation is necessary to rule out polar disruption of these adjacent genes by insertion of resistance cassettes. However, as neither of the genes have been assigned functions in oxidative stress management it is unlikely that polar disruption is occurring and resulting in the observed phenotype.

Sensitivity to oxidants was measured by the widely used disc sensitivity assay. Sterile discs, to which a range of stress inducing agents had been applied, were placed on plates that had been previously streaked for confluent growth with wild-type or mutant cells. Zones of growth inhibition were measured around the discs after incubation. The distance was determined from the edge of the disc to the end of the clear zone in millimetres. This experimental approach revealed that the absence of a functional Trx2 protein in *H. pylori* resulted in a change in the sensitivity of the mutant Trx2 strain compared with the isogenic parental strain in response to organic peroxide induced stress. The response of the bacterium to the redox cycling agent, diamide, and the superoxide radical generator menadione, was similar for the mutant and wild-type strains. This suggests that in the absence of Trx2, *H. pylori* is capable of responding to disturbance of the redox balance of the cell induced by these agents in the same way as when this redox protein is present. This is shown by the similar zones of growth inhibition which are observed for both the wild-type and mutated strain. However, in the absence of a functional Trx2 protein the response of *H. pylori* to the organic peroxides, cumene hydroperoxide and *tert*-butyl hydroperoxide, is different when compared with the response of the wild-type bacterium. The Trx2 mutant is significantly more sensitive to the effects of cumene hydroperoxide-induced peroxide stress and even more highly sensitive to *tert*-butyl hydroperoxide-induced peroxide stress. These data indicate a role for Trx2 in organic peroxide detoxification. The above results were obtained from disk sensitivity assays only. The sensitivity of the Trx2 mutant to these types of stress was not examined in liquid culture experiments, where growth curves may

have given a more sensitive indication of the effects of these types of stress on bacterial viability over time. However, similar experiments in the literature have previously indicated that the stress response pattern obtained by disk sensitivity assays is generally mirrored closely in growth curve experiments.

Organic hydroperoxides formed following the attack of ROS can react with metals and metalloproteins leading to the production of secondary free radicals (Jiang *et al.*, 1992; Barr and Mason, 1995) which explains why organic peroxides have bactericidal activity (Akaike *et al.*, 1992). Alkylhydroperoxide reductase (AhpC) is frequently considered the main enzyme responsible for the conversion of organic peroxides to the corresponding alcohols in bacteria (Bsat *et al.*, 1996). AhpC is a thiol-dependent peroxidase that belongs to the peroxiredoxin family. Several studies have highlighted the important role played by AhpC in oxidative stress resistance in *H. pylori* (Baker *et al.*, 2001; Olczak *et al.*, 2002) and Baker *et al.* (2001) have shown that Trx1 not Trx2 is the source of electrons required for the catalytic activity of AhpC at least *in vitro*. Analysis of the effects of the *trx2* mutation suggest that *in vivo*, Trx2 participates in resistance to organic peroxide stress by a mechanism that may or may not involve AhpC as Trx2 could possibly act *in vivo* as an electron donor for this enzyme. Alternatively, Trx2 may have a role in the direct detoxification of peroxides, as is the case for *Mycobacterium tuberculosis* Trx (Zhang *et al.*, 1999). These workers demonstrated that *M. tuberculosis* Trx and TR do not serve as the source of reducing equivalents for the catalytic turnover of the *M. tuberculosis* AhpC but they directly reduce hydrogen peroxide and, to a lesser extent alkylhydroperoxides. It may be that both Trxs are involved in *H. pylori* peroxide stress resistance exerting their catalytic and/or electron donor capabilities on different enzymatic antioxidant components.

In certain systems, the loss of an oxidative stress resistant function has been reported to cause a concomitant increase in levels of an alternative cellular oxidative stress resistance function. Mutation of the *Bacillus subtilis* AhpCF was found to result in an elevation of expression from all regulon promoters, including those for *katA* and *ahpCF*, which are believed to promote hydrogen peroxide resistance (Bsat *et al.*, 1996), and a similar instance of compensation of antioxidant activities was observed in *Streptococcus mutans* (Yamamoto *et al.*, 2000). *H. pylori* may also be capable of modulating expression of its antioxidant-encoding genes in response to disturbance in the oxidative stress response incurred by mutation of a single oxidative stress resistance function. Olczak *et al.* (2002)

have shown that increased expression of an oxidative resistance factor appears to be a compensatory response used by oxidative stress deficient *H. pylori* to ensure that oxidative damage resistance is maintained. This study examined AhpC mutants of *H. pylori* and found that compensatory mutations occurred to circumvent the loss of AhpC from the bacterium. When the *trx2* gene was disrupted in *H. pylori* strain 1061, leading to loss of a functional Trx2 protein, it was observed that upregulation of a protein of an approximate molecular weight of 58 kDa occurred in the mutant *H. pylori* strain. A zone of growth inhibition was noted for the parental strain when exposed to high levels of hydrogen peroxide in the disc-diffusion assay, in contrast to the isogenic mutant strain which appeared to be totally resistant to the effects of hydrogen peroxide induced oxidative stress. As the appearance of the upregulated protein correlated with an apparent increase in resistance to hydrogen peroxide it was postulated that the protein may be the enzyme catalase.

Catalases dismutate hydrogen peroxide to water and molecular oxygen to avoid the formation of the toxic hydrogen radicals from hydrogen peroxide. Biochemical analysis of the mutant strain through a simple assay for catalase activity revealed that the mutant Trx2 strain possessed elevated catalase activity over the parental 1061 strain. It may be that loss of Trx2 from *H. pylori* results in the antioxidant capabilities of the bacterium perhaps on a regulatory level resulting in a change of gene expression of the *kat* gene, although this observation is a strain specific effect and appears to be related to passage of the strain over time. *H. pylori* strain 1061 appears to have certain metabolic and biochemical attributes that differentiate it from the reference strains (Dr. H. Windle; personal communication) so the importance of such strain specific effects, while interesting, must be treated with caution. The *H. pylori* reference strain 26695 is deficient in several virulence factors present in other *H. pylori* strains (Ilver *et al.*, 1998; McAtee *et al.*, 1998) and some studies have observed some subclones with very variable levels of catalase activity (Jungblut *et al.*, 2000). However, like other prokaryotes it is very likely that *H. pylori* is capable of modulating the expression of oxidative stress resistance factors in response to changes in the antioxidant enzyme levels in the cytoplasm.

It is highly probable that *H. pylori* Trxs interact with a wide number of cellular proteins. Trxs can act as electron donors to a number of enzymes involved in the detoxification of reactive oxygen species, including the AhpC family of peroxiredoxins (Jeong *et al.*, 2000).

H. pylori inhabits a gastric environment rich in phagocyte derived inflammatory infiltrates and reactive oxygen metabolites. In addition to the major enzymatic antioxidant proteins, SOD and catalase, *H. pylori* Trx-dependent peroxidases may attenuate some of the damaging effects of the hosts inflammatory response. Like the peroxiredoxins, such as AhpC, a second group of bacterial proteins, known as thiol peroxidases (Tpx) can use Trx to reduce harmful peroxides in the cell (Wan *et al.*, 1997; Zhou *et al.*, 1997). The *H. pylori* Tpx enzyme has been purified and has a Trx-linked peroxidase activity (Wan *et al.*, 1997). *H. pylori* Trx1 appears to be the sole *in vitro* electron donor for the peroxide detoxification enzyme AhpC (Baker *et al.*, 2001), however, it is possible that Trx2 may substitute for Trx1 *in vivo* for AhpC or other cellular detoxification enzymes, an obvious candidate being Tpx (HP0390; TIGR). Additionally, the unique redox active site of Trx2 may confer specificity of interaction that prevents redox interaction with certain detoxification enzymes but facilitates interaction with others. This study has shown Trx1 and Trx2 can behave as electron donors to Tpx. Trx2 may act as an electron donor for additional peroxiredoxins that contribute to the removal of ROS, *H. pylori* Bcp being a potential target (HP0136; TIGR). Bacterioferritin comigratory protein (Bcp) was initially described in *E. coli* as a molecule that comigrated with bacterioferritin (Neidhart *et al.*, 1983) and its sequence was found to have homology to some peroxiredoxin proteins and homologues have been described in higher eukaryotes. This study demonstrates that both recombinant Trx1 and Trx2 behaved as electron donors to recombinant Bcp in the presence of TR/NADPH. *H. pylori* Bcp isogenic mutants are only marginally more sensitive to oxidants compared to the parental wild type strain and it is postulated that Bcp is not a major contributor to general oxidative stress resistance but might have a specific role in the removal of specialised hydroperoxides (Comtois *et al.*, 2003).

From examination of the genome sequence of *H. pylori* it is clear that this pathogen possesses a wide range of enzymes which are capable of combating the deleterious effects of oxidative stress (Tomb *et al.*, 1997). However, targeted mutagenesis approaches which are necessary to define the exact roles of each of these enzymes have only been undertaken for a handful of these oxidative stress resistant candidates. In this study mutants of the *H. pylori trx2* gene were constructed to test whether Trx2 contributes to oxidative stress management in the *H. pylori*. Phenotypic analysis of the mutants reveal that *H. pylori* Trx2 has a role in oxidative stress resistance in the bacterium at least in response to certain kinds of oxidative stress, specifically oxidative stress generated by organic peroxides. Trx2 is

also capable of providing reducing equivalents to two cellular enzymes involved in the detoxification of ROS, Tpx and Bcp. The role of Trx2 in oxidative stress resistance remains less clear than the role of Trx1. Several studies have highlighted the role of Trx1 in oxidative stress defence in relation to its capability to provide reducing equivalents to the bacteriums three antioxidant peroxidase activities. However, this study revealed that Trx2 does have a role to play in oxidative stress resistance for this pathogen and supports the postulate that *H. pylori* Trxs have different functional roles within the bacterium.

CHAPTER V

H. PYLORI THIOREDOXIN AND HOST INFLAMMATION; REDOX-ASSOCIATED IMMUNOMODULATION

CHAPTER V

5.1 INTRODUCTION

5.1.1 The *H. pylori* Secretome

H. pylori colonises the overlying mucus layer of the gastric mucosal epithelium and infection induces chronic inflammation in the lamina propria. This induced local inflammation appears to be a result of transport of bacterial products, principally proinflammatory factors, into the mucosal tissue (Blaser, 1992). During infection, a local cellular infiltration with polymorphonuclear leukocytes, lymphocytes and macrophages occurs (Kirchner *et al.*, 1990). Despite this cellular immune response, and a potent humoral response, the bacterium is not eradicated and the infection persists. Although the mechanism(s) whereby *H. pylori* evades the immune response are not fully understood, it has been postulated that secretion of proteins by *H. pylori* may influence the gastric inflammatory response. Secreted proteins are of particular interest when considering the pathogenesis of *H. pylori* infection as the bacterium comes into adhesin-mediated direct contact with host tissue. Proteins which are selectively released by *H. pylori* in its microniche could interact with gastric epithelium to mediate host-pathogen interactions. Several proteins which are secreted by *H. pylori* have been previously identified including VacA (Schraw *et al.*, 1999). *H. pylori* thioredoxin (Trx) was also determined to be secreted from the bacterium by proteome analysis (Windle *et al.*, 2000), however, it was only recently that a global proteome analysis of secreted proteins of the pathogen has been undertaken (Bumann *et al.*, 2002). In this study twenty-six of thirty-three secreted proteins were identified, with both Trx1 and Trx2 among those identified. A further proteomic study was undertaken in which *H. pylori* proteins identified as being selectively enriched in growth medium were examined. Thirteen proteins were shown to be selectively released by the bacterium and not as a result of membrane shedding (Kim *et al.*, 2002). Trx2 was identified as one of these proteins.

5.1.2 Thioredoxin and Transcription Factors

Nuclear factor κB (NF-κB) or Rel proteins are members of a family of eukaryotic transcription factors which share functional similarities. In most cells, NF-κB complexes are usually found in the cytoplasm as inactive complexes with inhibitory IκB proteins (IκB-α, IκB-β) which mask their nuclear localisation signal. However, in some cell types

of the haematopoetic cell lineage, NF-κB complexes may be constitutively active in the nucleus (Baldwin, 1996). A characteristic of NF-κB is that many different agents can induce its DNA-binding activity, including many oxidants and reductants (Toledano and Leonard, 1991; Staal *et al.*, 1990). Accumulating evidence suggests that redox processes play a critical role in regulation the activation of certain transcription factors such as nuclear factor κB (NF-κB) (Schenk *et al.*, 1994). The redox-regulatory molecule Trx is a multifunctional reductant and is involved in the regulation of NF-κB DNA-binding activity. Trx has been shown to activate NF-κB in a cell free system and redox regulation of NF-κB activity has been reported to be exerted after the dissociation of IκB complex from NF-κB (Hayashi *et al.*, 1993). Other studies have shown that critical Cys62 in the p50 NF-κB subunit requires reduction by Trx for activation (Matthews *et al.*, 1992).

Redox processes also play a critical role in regulating the activation of other transcription factors such as activator protein-1 (AP-1) (Schenk *et al.*, 1994). The AP-1 family consists of homo- or heterodimers of members of the Jun and Fos families. All members possess a bZIP structural motif which consists of a leucine zipper dimerisation domain adjoining a basic region that can directly interact with DNA. AP-1 couples extracellular signals to gene expression associated with growth, differentiation and cellular stress. AP-1 transcriptional activity has been shown to be redox-sensitive with increased AP-1 expression induced by reactive oxygen species (Timblin *et al.*, 1998; Singh and Aggarwal, 1995). Paradoxically, the DNA-binding capacity of AP-1 is decreased by oxidation of critical cysteine residues (Cys-252 in c-Jun) and increased when such residues undergo reduction (Schenk *et al.*, 1994; Abate *et al.*, 1990). It has been demonstrated that transcription factor DNA-binding can be modulated by *E. coli* Trx (Das, 2001). Therefore, the aim of this study was to evaluate the effect of *H. pylori* Trx2 on transcription factor DNA-binding activity in gastric epithelial cells. This study revealed that recombinant *H. pylori* Trx is capable of modulating NF-κB and AP-1 transcription factor DNA-binding activity when AGS cells are exposed to reduced recombinant Trx2 protein. As Trx2 has been shown to be specifically secreted from the bacterium (Kim *et al.*, 2002), it may have a role to play in modulation of the host immune response. Secreted *H. pylori* Trx may act as a molecule which can dampen the host immune response and facilitate persistence, this paradigm is considered.

5.2 OBJECTIVES

1. To assess the effect of *H. pylori* Trx2 on transcription factor activation in gastric epithelial cells.
2. To evaluate the redox-dependence of such activity.
3. To explore upstream signalling events leading to any change in transcription factor activation patterns in gastric epithelial cells after exposure to Trx2.
4. To assess the effect, if any, of *H. pylori* Trx2 on gastric epithelial cell viability and proliferative capacity.

5.3 RESULTS

5.3.1 Effect of *H. pylori* Trx2 on IκB expression in gastric epithelial cells.

The level of the cytoplasmic inhibitor of NF-κB, IκB, in whole cell extracts of AGS cells was assessed following exposure of AGS cells to exogenous reduced recombinant *H. pylori* Trx. Incubation of AGS cells in RPMI medium containing FCS (10 % v/v) for 3 h with a freshly reduced preparation of recombinant *H. pylori* Trx2 and Trx1 (0 - 40 μg/ml; 0 - 2.8 μM) resulted in a biphasic dose-dependent change in the expression level of IκB (**Fig. 5.1**). The expression of IκB in AGS cells was increased in a dose dependent manner by exogenous *H. pylori* Trx2 (**Fig. 5.1, C**), with a sharp decrease in the expression level of IκB induced following exposure of AGS cells to concentrations of reduced Trx2 above 2.1 μM (30 μg/ml) (**Fig. 5.1, C; lane 6**). The expression of IκB in AGS cells was also increased in a dose dependent manner by reduced *H. pylori* Trx1. A sharp decrease in the expression level of IκB was observed when amounts of Trx1 in excess of 2.1 μM were used, however, the decrease was not as dramatic as that observed when elevated levels of Trx2 were used (**Fig. 5.1, D**). Immunodetection of a control protein such as ß-actin would have provided a control for the specific effect of Trx2 on IκB expression, however, the unvarying reproducibility of this experiment suggested that this observation was a specific effect. Based on these preliminary kinetic observations subsequent experiments related to IκB, NF-κB and AP-1 modulation by *H. pylori* Trx2 were performed using a fixed range of Trx2 concentrations (0 - 50 μg/ml; 0 - 3.5 μM) and an incubation time of 3 hours with this incubation time having yielded consistent results. These data indicated that Trx2 correlated with stabilisation of the IκB complex at a concentration of 2.1 μM and below. To assess whether stabilisation of IκB was occurring simultaneously with a decrease in NF-κB DNA-binding activity, the effect of Trx2 on this transcription factor was examined.

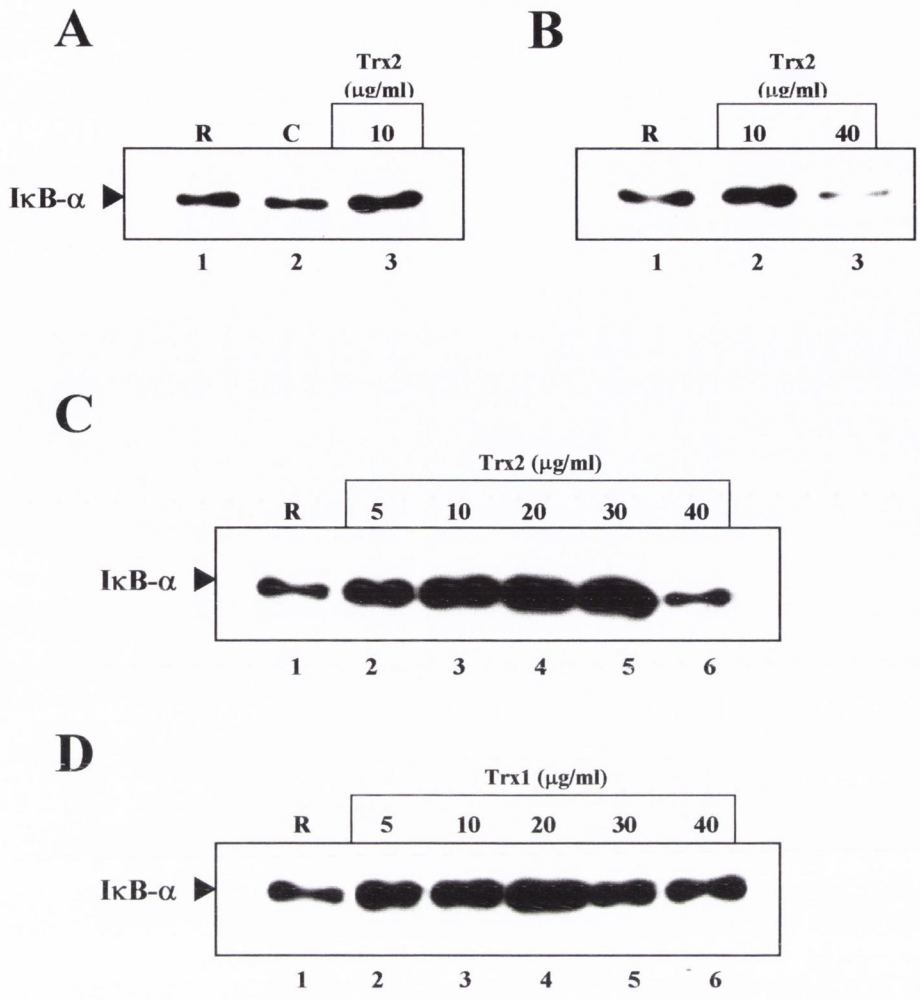


Fig. 5.1. The effect of *H. pylori* Trx2 on IκB-α expression in gastric epithelial cells. AGS cells were incubated with freshly reduced *H. pylori* Trx2 for 3 h. Proteins in total cell extracts were separated by polyacrylamide gel electrophoresis on 10% gels and transferred to PVDF membrane followed by Western blot analysis using anti-IκB-α antibody. The arrow indicates the position of the 37 kDa IκB-α band on Western blots (Panels A to D). Dose dependent effects of Trx2 on IκB-α expression in AGS cells (Panels A to C). The amount of Trx2 added to cells is shown above each panel. Unstimulated/Resting (R) cells are shown in lane 1 of each panel. A protein buffer control (C) is shown in Panel A, lane 2. Effect of *H. pylori* Trx1 on IκB-α expression in AGS cells (D). A representative blot of three independent experiments with similar results is shown for each panel.

5.3.2 Effect of *H. pylori* Trx on constitutive NF-κB in gastric epithelial cells.

The effect of *H. pylori* Trx on NF-κB modulation in gastric epithelial cells was assessed by electrophoretic mobility shift assay using oligonucleotide probes specific to the binding site of NF-κB. Incubation of AGS cells in RPMI medium containing FCS (10 % v/v) for 3 h with low amounts (≤ 10 μg/ml) Trx2 and Trx1 (5 - 30 μg/ml; 0.35 - 2.1 μM) resulted in inhibition of constitutively active NF-κB (**Fig. 5.2**). DNA-binding of NF-κB was inhibited by Trx2, with low amounts of Trx2 (0.35 μM) resulting in complete inhibition of NF-κB DNA binding activity (**Fig. 5.2, A**). In agreement with the IκB data, NF-κB DNA-binding activity was suppressed by Trx2, but the expected induction of NF-κB DNA-binding by elevated amounts of Trx2 (2.1 μM) was not apparent from the shift assay, as was seen with Trx1 (**Fig. 5.2, B**). However, activation of NF-κB in response to Trx1 exposure is quite weak and the inclusion of a positive control for activation such as treatment with 50 ng/ml PMA would have given a better indication of the efficacy of Trx1 to activate this transcription factor. These experiments demonstrate that recombinant *H. pylori* Trx2 is capable of modulating the DNA-binding activity of NF-κB in gastric epithelial cells, and interestingly, appears to have an inhibitory effect on NF-κB DNA-binding, at least at low concentrations. Maximal inhibition of NF-κB DNA-binding was observed at low Trx2 concentrations (5 - 20 μg/ml; 0.35 - 1.4 μM). Previous studies from our laboratory have shown that the maximal inhibition of either *H. pylori* TNF-α- or PMA-stimulated gastric epithelial cell NF-κB activation by *H. pylori* Trx1 was consistently observed at 0.7 - 1.4 μM Trx1 (Abdel-Latif, M.M., Ph.D. Thesis, 2002; O'Toole, D., MD Thesis, 2001). In addition, other studies carried out within our laboratory showed that pre-treatment of AGS cells with 0.7 μM (10 μg/ml) Trx1 for 2 h completely blocked PMA- (20 ng/ml) induced NF-κB activation (Abdel-Latif, M.M. *et al.*, Manuscript in preparation). Similar experiments carried out examining the effect of Trx2 on PMA- (20 ng/ml) induced activation of NF-κB did not reveal a consistent or reproducible effect similar to that observed for Trx1.

These data reveal that degradation of IκB in the cytoplasm of AGS cells appears to correlate with reduced NF-κB DNA-binding in the nucleus when cells are exposed to low concentrations of recombinant Trx2.

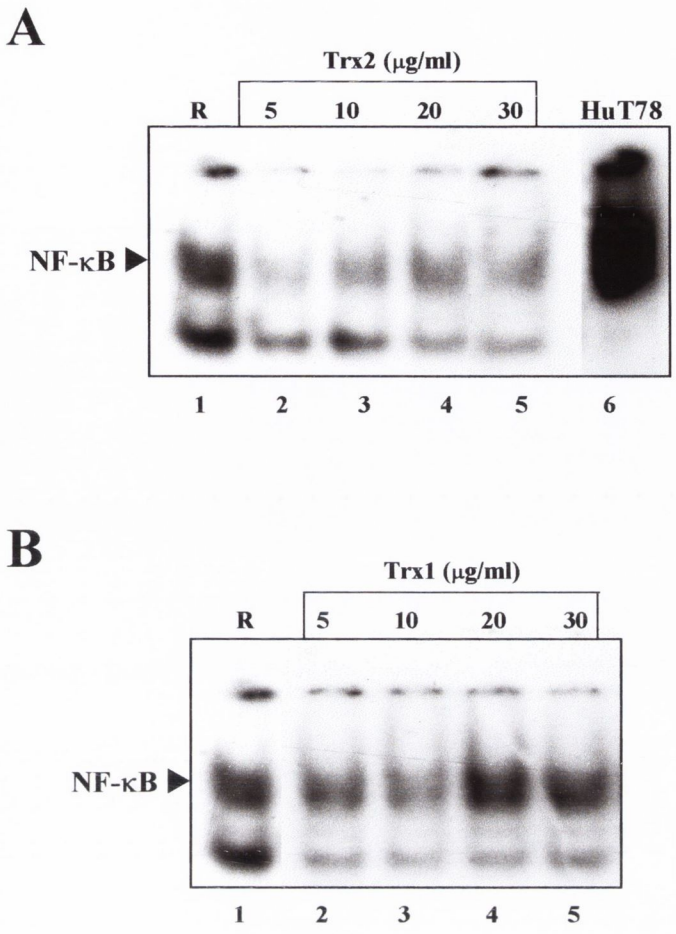


Fig. 5.2. Electrophoretic mobility shift assay analysis of the effect of *H. pylori* Trx on constitutive NF-κB DNA-binding activity in gastric epithelial cells. Concentration dependence of NF-κB activation in AGS cells by freshly reduced *H. pylori* Trx2 (5 μg/ml to 40 μg/ml; lanes 2 to 5) **(A),** and freshly reduced *H. pylori* Trx1 (5 μg/ml to 30 μg/ml; lanes 2 to 5) **(B)**. Nuclear extracts were prepared and analysed for NF-κB DNA-binding activity by EMSA. HuT 78 cells, a T-cell lymphoma derived from peripheral blood of a Sezary lymphoma patient, known to have constitutive high-level expression of NF-κB served as a positive control (lane 6, Panel A). Unstimulated/Resting (R) cells are shown in lane 1 of each panel. A solid arrow indicates the position of the induced NF-κB DNA-binding complex. A representative gel of two independent experiments with similar results is shown.

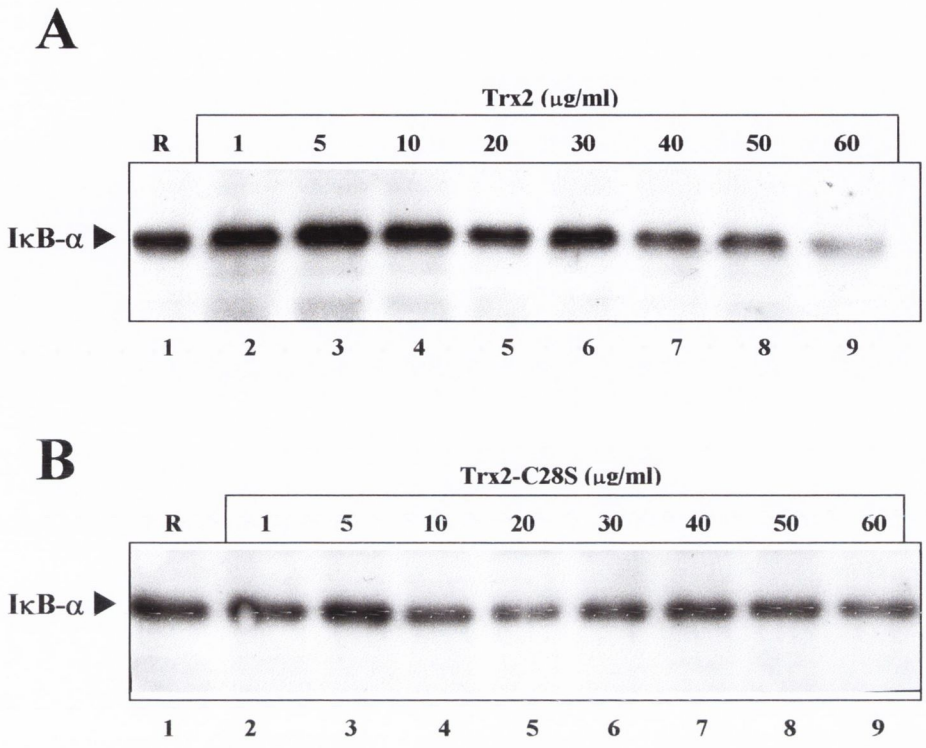


Fig. 5.3. Effect of redox-inactive *H. pylori* Trx2 on IκB expression in gastric epithelial cells. AGS cells were incubated with freshly reduced *H. pylori* Trx2-C28S and Trx2 for 3 h. Proteins in total cell extracts were separated by polyacrylamide gel electrophoresis on 10% gels and transferred to PVDF membrane followed by Western blot analysis using anti-IκB-α antibody. The arrow indicates the position of the 37 kDa IκB-α band on Western blots. **(A)** Dose dependent effect of Trx2 on IκB-α expression in AGS cells (lanes 2 to 9). **(B)** Dose dependent effect of *H. pylori* Trx2-C28S at increasing concentrations on IκB-α expression in AGS cells (lanes 2 to 9). Unstimulated/Resting (R) cells are shown in lane 1 of each panel. A representative blot of two independent experiments with similar results is shown in each panel.

5.3.3 Effect of redox-inactive *H. pylori* Trx2 on IκB expression in gastric epithelial cells.

H. pylori Trx2 site directed mutants were constructed by PCR-based mutagenesis techniques (Chapter VI, Section 6.3.3). In these mutants the Cys residues of the Trx2 redox active site, -Cys-Pro-Asp-Cys-, were mutated to Ser residues, creating the mutant Trx2-C28S. This recombinant protein was used to determine whether the ability of *H. pylori* Trx2 to modulate the expression of IκB in gastric epithelial cells was due to the redox activity of the active site. Expression IκB in whole cell extracts of AGS cells was assessed following exposure of AGS cells to *H. pylori* Trx2 and Trx2-C28S. Incubation of AGS cells in RPMI medium containing FCS (10 % v/v) for 3 h with Trx2 (0 - 60 μg/ml; 0 - 4.2 μM) (**Fig. 5.3, panel A**) resulted in an apparent decrease in the expression levels of IκB at higher concentrations of Trx2 as shown previously (**Fig. 5.1**) A decrease in expression levels of the inhibitor at concentrations of Trx2 above 2.1 μM was observed. When AGS cells were exposed to the mutant Trx2-C28S, there was no significant change in expression levels of IκB using the same concentrations of Trx2-C28S (**Fig. 5.3, panel B**). These data indicate that the redox activity of Trx2 may be important in the regulation of IκB and hence NF-κB DNA-binding activity. Redox regulation of other transcription factors such as AP-1 has been demonstrated in mammalian cells, therefore, the effect of Trx2 on c-fos protein expression and AP-1 DNA-binding activity in AGS cells was examined.

5.3.4 Effect of *H. pylori* Trx2 on AP-1 expression in gastric epithelial cells.

Expression of AP-1 protein (c-Fos), was assessed in nuclear extracts of AGS cells following exposure of AGS cells to Trx2. Incubation of AGS cells in RPMI medium containing FCS (10 % v/v) for 3 h with recombinant *H. pylori* Trx2 (5 - 40 μg/ml; 0.35 - 2.8 μM) resulted in a very small decrease in the level of c-Fos expression as determined by immunoblotting with c-Fos specific antibody (**Fig. 5.4**).

5.3.5 Effect of *H. pylori* Trx2 on AP-1 DNA-binding activity in gastric epithelial cells.

The effect of *H. pylori* Trx2 on AP-1 modulation in gastric epithelial cells was assessed by shift assay using oligonucleotide probes specific to the binding site of AP-1. Incubation of AGS cells with Trx2 (0 - 40 μg/ml; 0 - 2.8 μM) resulted in complete inhibition of the DNA-binding activity of AP-1 when the cells were treated with low amounts (0.35 μM)

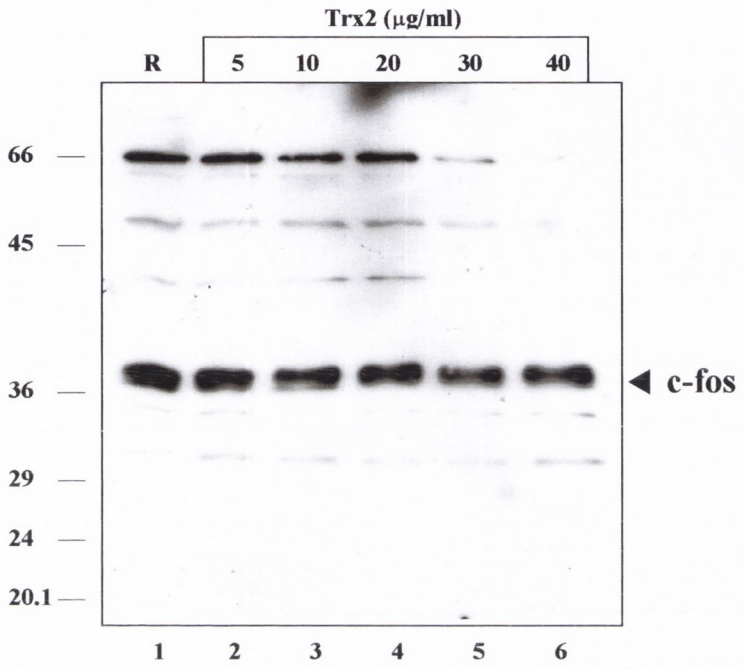


Fig. 5.4. The effect of *H. pylori* Trx2 on AP-1 (c-fos) protein expression in gastric epithelial cells. AGS cells were incubated with freshly reduced *H. pylori* Trx2 for 3 h. Proteins in total cell extracts were separated by polyacrylamide gel electrophoresis on 10% gels and transferred to PVDF membrane followed by Western blot analysis using anti-AP-1 (c-fos) antibody (1:1000). The arrow indicates the position of the 39 kDa AP-1 band on Western blots. Unstimulated/Resting (R) cells are shown in lane 1. Nuclear extracts were prepared and the dose dependent effect of Trx2 on AP-1 protein (c-fos) expression in AGS cells) examined. A representative blot of two independent experiments with similar results is shown.

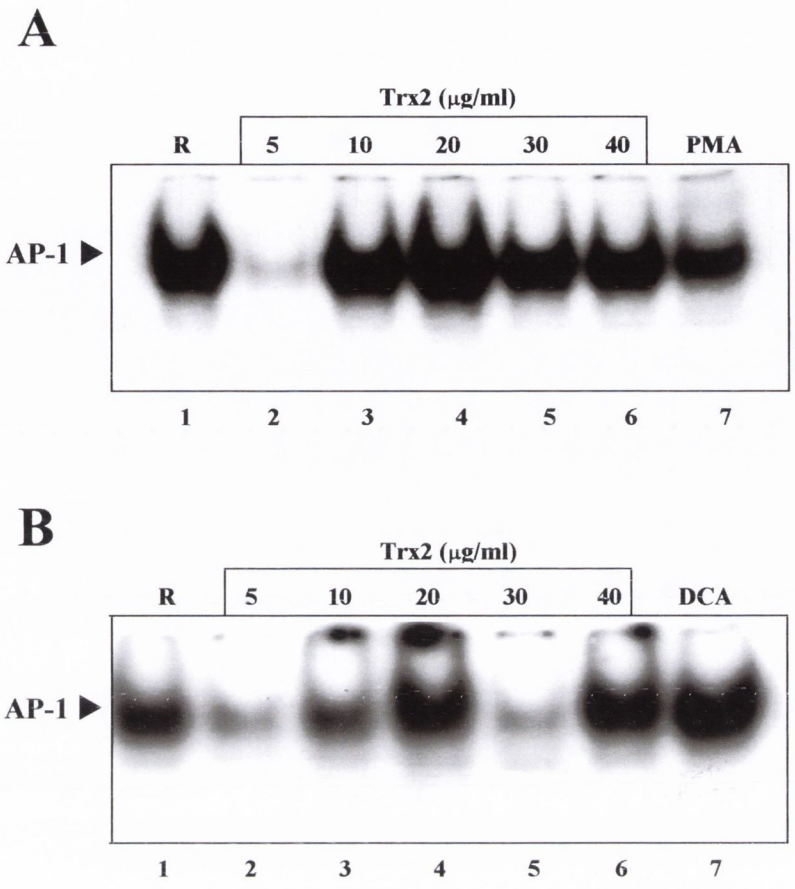


Fig. 5.5. Electrophoretic mobility shift assay analysis of the effect of *H. pylori* Trx2 on AP-1 DNA-binding activity in gastric epithelial cells. Concentration curve of AP-1 activation in AGS cells by freshly reduced *H. pylori* Trx2 (5 μg/ml to 40 μg/ml; lanes 2 to 6) **(A),** and freshly reduced *H. pylori* Trx2 (5 μg/ml to 30 μg/ml; lanes 2 to 6), **(B)**. Nuclear extracts were prepared and analysed for AP-1 DNA-binding activity by EMSA. Unstimulated/Resting (R) cells are shown in lane 1 of each panel. A solid arrow indicates the position of the induced AP-1 DNA-binding complex. In panel A, AGS cells treated with 50 ng/ml PMA (2 h) served as a positive control (lane 7). In panel B, HCT116 cells treated with 250 μM DCA (3 h) served as an additional positive control (lane 7). Representative gels of several independent experiments with similar results are shown.

Trx2 (**Fig. 5.5, A**). An increase in the DNA-binding of AP-1 was induced however by higher amounts of Trx2 (≥ 0.7 μM) and inhibited again at higher concentrations. The apparently complex response of the transcription factor AP-1 to the presence of recombinant *H. pylori* Trx2 was reproducible and consistent. These data reveal that *H. pylori* Trx is capable of modulating DNA-binding activity of the transcription factor AP-1 in gastric epithelial cells, and appears to have an inhibitory effect on the DNA-binding activity of AP-1 at a low concentration (5 μg/ml; 0.35 μM). As the dimeric sequence specific enhancer AP-1 becomes activated through phosphorylation of c-Jun by members of the stress activated MAPK family, the effect of Trx2 on MAPK expression in gastric epithelial cells was next investigated.

5.3.6 *H. pylori* Trx2 and MAP kinase signalling in gastric epithelial cells.

To assess the role of the MAP kinases, ERK1/2 and p38, in promoting AP-1 DNA-binding activity following incubation with Trx2, AGS cells were exposed to recombinant Trx2 for 3 hours. Subsequently, cell extracts were analysed for kinase activity by immunoblotting with phospho-specific MAP kinase antibodies. Cell extracts were separated by SDS-polyacrylamide gel electrophoresis, Western blotted and protein detected with antibodies that specifically recognised the phosphorylated form of ERK. Exposure of AGS cells to Trx2 resulted in ERK phosphorylation. Western blotting with anti-phospho ERK antibodies revealed increased phosphorylation after treatment with Trx2 for 3 hours (**Fig. 5.6, A; lanes 2 and 3**). To verify that equal amounts of ERK were present in all reactions, the blot was stripped and reprobed with an antibody that recognises the unphosphorylated form of ERK (**Fig 5.6, B**). Similar amounts of ERK were present in all lanes. Therefore, it appears that exposure of AGS cells to low concentrations of Trx2 activates MEK1/2 activity. Additionally, AGS cell extracts were separated by SDS-polyacrylamide gel electrophoresis, Western blotted and detected with antibodies that specifically bind the phosphorylated form of p38. Exposure of AGS cells to Trx2 did not induce p38 phosphorylation at the concentrations used (**Fig. 5.7, A**). To verify that equal amounts of p38 were present in all reactions, the blot was stripped and reprobed with an antibody that recognises the unphosphorylated form of p38 (**Fig 5.7, B**). Similar amounts of p38 were present in all lanes. Therefore, it appears that exposure of AGS cells to *H. pylori* Trx2 does not activate the p38 signalling pathway. An investigation of the effect of reduced recombinant *H. pylori* Trx2 on the JNK signalling pathway was undertaken, however, blot

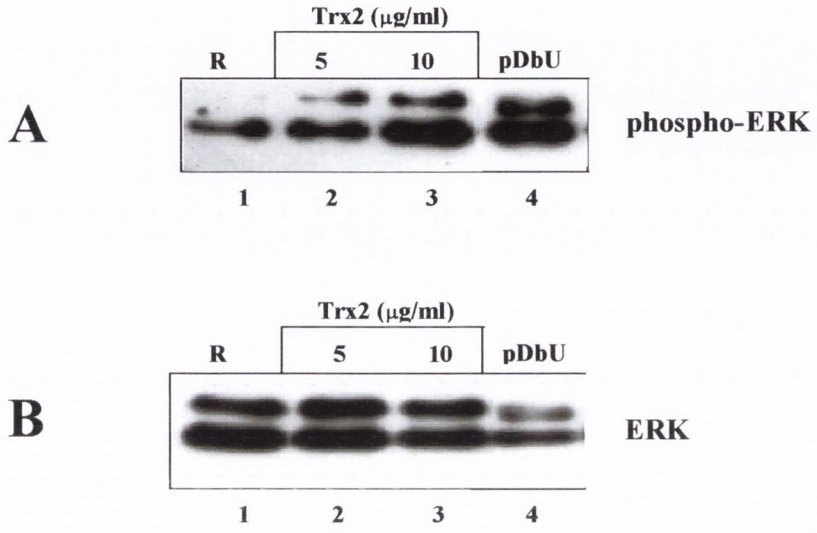


Fig. 5.6. The effect of *H. pylori* Trx2 on ERK activity in gastric epithelial cells. AGS cells were incubated with freshly reduced *H. pylori* Trx2 for 3 h. Proteins in total cell extracts were separated by polyacrylamide gel electrophoresis on 10% gels and transferred to PVDF membrane followed by Western blot analysis using anti-ERK (1:800) and anti-phospho ERK (1:200) antibodies. **(A)** Effect of *H. pylori* Trx2 (5 and 10 μg/ml) on phosphorylated ERK protein levels in AGS cells (lanes 2 and 3). **(B)** Effect of *H. pylori* Trx2 (5 and 10 μg/ml) on total ERK protein levels in AGS cells (lanes 2 and 3). Unstimulated/Resting (R) cells are shown in lane 1 of each panel. Lane 4 (Panels A and B) show treatment with the ERK/ p38 kinase pathway activator pDbU. Blots were probed with total ERK1/2 to demonstrate equal loading.

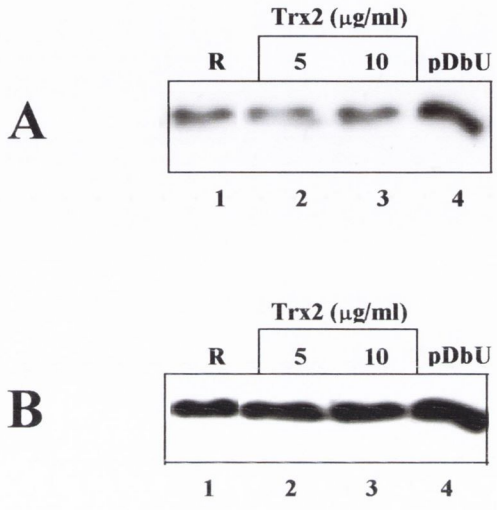


Fig. 5.7. The effect of *H. pylori* Trx2 on p38 activity in gastric epithelial cells. AGS cells were incubated with freshly reduced *H. pylori* Trx2 for 3 h. Proteins in total cell extracts were separated by polyacrylamide gel electrophoresis on 10% gels and transferred to PVDF membrane followed by Western blot analysis using anti-p38 (1:300) and anti-phospho p38 (1:200) antibodies. **(A)** Effect of *H. pylori* Trx2 (5 and 10 μg/ml) on phosphorylated p38 protein levels in AGS cells (lanes 2 and 3). **(B)** Effect of *H. pylori* Trx2 (5 and 10 μg/ml) on total p38 protein levels in AGS cells (lanes 2 and 3). Unstimulated/Resting (R) cells are shown in lane 1 of each panel. Lane 4 (Panels A and B) show the effect of treating cells with the ERK/ p38 kinase pathway activator pDbU. Blots were probed with total p38 to demonstrate equal loading.

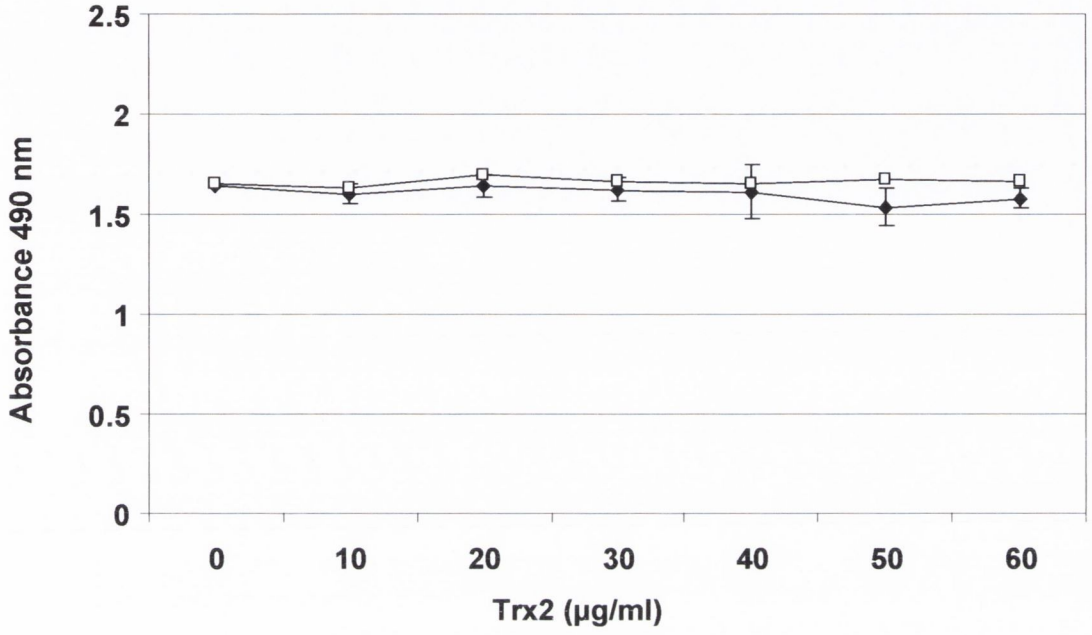


Fig. 5.8. Effect of *H. pylori* Trx2 on gastric epithelial cell viability. MTS assay showing the effect of increasing amounts of Trx2 (0 - 60 µg/ml; 0 - 4.2 µM) on AGS cells. Cells were exposed to freshly reduced recombinant *H. pylori* Trx2 for 3 h after which time viability was assessed. (◆); recombinant *H. pylori* Trx2. (□); cell culture media alone (RPMI) (control). The error bars represent the means ± SD of three independent experiments (each in duplicate).

quality was not adequate after repeated attempts to assess the effect of Trx2 on JNK activation.

5.3.7 Effect of *H. pylori* Trx2 on gastric epithelial cell viability.

The concentration range of recombinant *H. pylori* Trx2 which resulted in inhibition of NF-κB- and AP-1-DNA-binding activity had no deleterious effect on gastric epithelial cell viability as determined by the MTS assay. A colorimetric assay using (3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl) - 2 - (4-sulfophenyl) - 2H - Tetrazolium (MTS) is a reliable colorimetric assay for evaluating cell proliferation and hence cell viability. The assay revealed that there was no significant decrease in AGS cell viability following exposure to reduced recombinant *H. pylori* Trx2 over a concentration range of 5 - 60 μg/ml; 0.35 - 4.2 μM Trx2 (**Fig. 5.8**). AGS cells grown in RPMI media alone were used as the control for all MTS experiments.

5.4 DISCUSSION

A significant characteristic of *H. pylori* associated gastric disease is long-term persistence of the bacterium in the gastric mucosa despite the induction of a local and systemic immune response by the host. It is postulated that *H. pylori* may be capable of evading the host immune response, as clearance of the bacterium is not achieved. Alteration of host immune-mediated responses have been demonstrated previously for this bacterium. *H. pylori* has been shown to suppress the *in vitro* proliferative response of human mononuclear cells to mitogens and antigens (Benz *et al.*, 1993; Chmiela *et al.*, 1995; Knipp *et al.*, 1994; Knipp *et al.*, 1993) and such studies postulate that this may represent a means by which *H. pylori* might alter the host immune reaction. Most recently, it has been demonstrated that the *H. pylori* vacuolating cytotoxin (VacA) induces immune suppression (Gebert *et al.*, 2003). The authors found that VacA could efficiently block proliferation of T cells by inducing a G1/S cell cycle arrest and postulate that VacA-induced suppression of the local immune response could explain the chronicity of *H. pylori* infection. The ability of *H. pylori* proteins to directly modulate immune system components has been shown. Windle *et al.* (2000) demonstrated Trx-mediated catalytic reduction of host immunoglobulins and have suggested that this mechanism could facilitate immune evasion, as partial reduction of the immunoglobulin molecule can result in loss of biological activity (Rousseaux-Prevost *et al.*, 1984). Indeed, the authors have shown that all four IgG subclasses are subject to reduction by *H. pylori* Trx *in vitro*, and as *H. pylori*

Trx is one of thirteen proteins selectively released by the bacterium, one could hypothesise that *in vivo* extracellular Trx could function to incapacitate host antibody and hence aid in immune evasion.

Many transcription factors have been shown, or are postulated to be subject to redox-regulation by the redox enzyme Trx. This regulation results from either activation or inactivation dependent on Trx-catalysed reduction (Schreck *et al.*, 1992; Schenk *et al.*, 1994; Hirota *et al.*, 1997; Sen and Packer, 1996; Hirota *et al.*, 1999; Dalton *et al.*, 1999). The central role of NF-κB in the innate immune response suggests that pathogenic microorganisms have evolved mechanisms to interfere with the function of NF-κB or modulate NF-κB signal transduction. Trx is known to have dual and opposing roles with respect to regulation of NF-κB activity in certain cell types. Cytoplasmic Trx blocks IκB degradation but nuclear localised Trx promotes NF-κB transcriptional activity by enhancing its ability to bind DNA. Trx interferes with the degradation of IκB in the cytoplasm, while enhancing NF-κB transcriptional activity in the nucleus (Hirota *et al.*, 1999). Cytoplasmic Trx in higher eukaryotes is known to interfere with signalling through IKKs and prevents the degradation of IκB, thus inhibiting NF-κB activity (Powis and Montfort, 2001).

The importance of eukaryotic Trx as a secreted factor has been investigated and this enzyme has been shown to be capable of exerting immunomodulatory effects. Trx appears to be secreted from cells by a non-classical mechanism that has not yet been defined but has been shown not to be reliant on the presence of a signal peptide on the secreted protein (Rubartelli *et al.*, 1992). Human Trx was first identified as a secreted protein which upregulated the IL-2 receptor and had co-cytokine activity from HTLV-1 transformed T-lymphocytes. The secreted protein was initially called adult T-cell leukaemia derived factor (Wakasugi *et al.*, 1990) and was after a time shown to in fact be human Trx (Gasdaska *et al.*, 1994). Indeed, several reports have shown that a range of both neoplastic and normal cells secrete Trx, and this occurs when the cells are subjected to conditions of oxidative stress (Rubartelli *et al.*, 1995; Rubartelli *et al.*, 1992). Additionally, secreted Trx has been shown to have proinflammatory effects as it can ameliorate release of cytokines from cells such as monocytes and fibroblasts (Yoshida *et al.*, 1999; Schenk *et al.*, 1996). Furthermore, extracellular Trx has been shown to act as a chemotactic factor which can

potentiate migration of immune cells, including T-cells and neutrophils, with an efficacy level similar to the well characterised chemokine, IL-8 (Bertini *et al.*, 1999). Finally, a recent study has shown that a cloned truncated Trx (Trx80) is a novel mitogenic cytokine for human peripheral blood mononuclear cells (Pekkari *et al.*, 2000; Pekkari *et al.*, 2001). Therefore, it is clear that the redox enzyme Trx when secreted from eukaryotic cells is capable of modifying its microenvironment.

This preliminary investigation into the effects of *H. pylori* Trx2 was prompted by existing experimental data on *H. pylori* Trx1 and its effect on transcription factor DNA-binding from our laboratory. A three hour time-point was chosen to assess the effects of *H. pylori* Trx2 on IκB expression levels and NF-κB DNA-binding activity when the following observations were taken into account. Naumann *et al.* (1999) have investigated the effect of *H. pylori* infection on NF-κB DNA-binding activity. When AGS cells were analysed for NF-κB DNA-binding activity in response to colonisation by wild-type *H. pylori* strains, it was shown that enhanced binding of NF-κB in AGS cells occurred within 45 minutes (P12 strain) to 90 minutes (G27 strain) post-infection and that this DNA binding activity was further increased within 180 min for both strains. More recently, Forsyth-Ludwig and Naumann (2000) have demonstrated that AGS cells infected with *H. pylori* wild-type strains (P12 and PAI) and subjected to EMSA analysis exhibit increased NF-κB DNA-binding activity within 90 minutes, with further increased binding at 180 minutes. These workers also investigated whether NF-κB activation is sustained at later stages of infection (up to 6 hours) and demonstrated that maximal DNA-binding of NF-κB was observed between 90 and 180 minutes after infection and was not detectable later than 240 minutes after infection. In addition, experiments carried out within our laboratory (Abdel-Latif, M.M.; Ph.D. Thesis, 2002) confirmed the observation that maximal DNA-binding activity of NF-κB in *H. pylori* treated cells was apparent at 180 minutes with the reference *H. pylori* strain NCTC11638 and that *H. pylori* induced NF-κB in a dose and time dependent manner *in vitro* and *in vivo*. As time course experiments with *H. pylori* wild-type strains in AGS cells revealed that the DNA-binding activity of NF-κB was increased to its greatest extent at 3 hours, this time-point was chosen to assess the effect that *H. pylori* Trx2 might have on the DNA-binding activity of this transcription factor. When the effect of Trx1 pretreatment on *H. pylori*-stimulated AGS cells was assessed, it was observed that at a

concentration of 0.7 to 1.4 μM Trx1, inhibition of DNA-binding occurred when cells were pretreated for 2 to 4 hours prior to stimulation (Abdel-Latif, M.M., Ph.D. Thesis, 2002).

Recently, Das (2001) has shown that NF-κB is activated by the addition of exogenous *E. coli* Trx in a redox-dependent manner in A549 cells. The author demonstrated that this activation was accompanied by degradation of IκB in the cytosol and that the reduced form of Trx was necessary for the activation of NF-κB. It was further shown that *E. coli* reduced Trx activated the JNK pathway in this cell line through the initiating kinase, MEKK1. The finding that exogenous prokaryotic Trx when added to culture medium of a cell line modulates transcription factor DNA-binding activity is of interest. Moreover, Das (2001) demonstrated that the modulatory effect of exogenous *E. coli* Trx is not limited to transformed cell lines and that primary cultures exhibit similar behaviour, reflecting that this effect may occur *in vivo*. Results from this study suggest that *H. pylori* Trx2 is capable of modulating the DNA-binding activity of NF-κB at low physiologically relevant concentrations. Results also suggest that the modulatory properties of *H. pylori* Trx2 are most likely redox-dependent. Use of a redox active site recombinant *H. pylori* Trx2 mutant in which one of the active site Cys residues was mutated to a Ser residue, to assess the effect of the protein on IκB expression in the cytosol revealed that degradation of the IκB complex at higher Trx2 concentrations was not occurring in contrast to the degradation of IκB observed when cells were treated with wild-type recombinant Trx2. Therefore, *H. pylori* Trx2 might possibly function in reduction of a critical Cys residue on an upstream kinase resulting in IκB degradation and translocation of NF-κB to the cell nucleus when present in high concentrations.

Redox regulation of Jun and Fos molecules has been implicated and various antioxidants have been shown to strongly activate DNA-binding activity and transactivation abilities of AP-1 (Pahl and Baeuerle, 1994; Meyer *et al.*, 1993; Meyer *et al.*, 1994; Schenk *et al.*, 1994). AP-1 coordinately induces proinflammatory cytokine/chemokine gene expression together with NF-κB. Naumann *et al.* (1999) have investigated whether *H. pylori* infection induces the transcription factor AP-1 and have demonstrated that *H. pylori* wild type strains induce AP-1 DNA binding activity within 90 minutes. The DNA binding activity was induced to similar extents by both strains examined and moreover, this activity was further increased within 180 min, indicating that members of the c-Jun/c-Fos family were

activated. Similarly to NF-κB, time course experiments with *H. pylori* wild-type strains in AGS cells revealed that the DNA-binding activity of AP-1 was maximal at 3 hours, therefore, this time-point was chosen at which to assess the effect that *H. pylori* Trx2 might have on the DNA-binding activity of AP-1.

This preliminary study has shown that AP-1 DNA-binding is sensitive to the modulatory effects of recombinant *H. pylori* Trx2. *H. pylori* Trx2 appears to induce a complex pattern of AP-1 DNA-binding through an as yet undefined mechanism. It has been shown previously that transient overexpression of Trx can stimulate AP-1 dependent transcription (Hirota *et al.*, 1997; Meyer *et al.*, 1993; Gomez del Arco *et al.*, 1996). Hirota *et al.* (1997) described the molecular cascade of redox regulation of AP-1 mediated by Trx and Ref-1. Ref-1 was found to be an AP endonuclease (Xanthoudakis *et al.*, 1992; Xanthoudakis and Curran, 1992). They demonstrated that Trx can directly associate with Ref-1 in the nucleus and the requirement for cysteine residues in Trx active site for the potentiation of AP-1 activity. However, a more recent study has challenged this hypothesis and these workers have demonstrated that Ref-1 cysteine 64/65 is not required for redox regulation of AP-1 DNA-binding *in vivo* (Ordway *et al.*, 2003). It therefore appears that the mechanism by which Ref-1 regulates the redox-dependent activity of specific transcription factors is not as clear as previously thought with further work necessary to define the mechanisms of Ref-1 redox regulatory function. Karin *et al.* (1995) observed that while AP-1 DNA binding activity can be conveniently measured by EMSA, changes in AP-1 DNA-binding activity do not mirror the transcriptional activity of this complex factor, as firstly, several proteins can form complexes that bind to AP-1 sites. These proteins can vary considerably in their ability to activate transcription of target genes. Secondly, phosphorylation at specific sites enhances the transactivating potential of several AP-1 proteins, including c-Jun and c-Fos, without having any effect on their DNA-binding activities (Karin and Smeal, 1992; Deng and Karin, 1994).

The MAPK pathway has been implicated as a mechanism by which signals are transduced from the cell surface to the nucleus in response to a wide range of different stimuli including a wide variety of cytokines, chemical, physical or biological stresses, including oxidants. MAPKs participate in cellular response to these stimuli by further inducing the phosphorylation of other protein kinases and transcription factor subunits. AP-1 activity is regulated by three MAP kinase pathways, the ERK1/2 pathway, the SAP kinase/JNK

pathway and the p38 pathway (Whitmarsh and Davis, 1996) and regulation occurs at both the transcriptional level and at the posttranscriptional level. As all three MAP kinase cascades have been shown to mediate AP-1 induction in response to extracellular signals, this study sought to determine whether one of these pathways was activated in AGS cells following exposure to recombinant *H. pylori* Trx2. Cell extracts were analysed for kinase activity following treatment with Trx2 by immunoblotting with phospho-specific MAP kinase antibodies. Exposure of AGS cells to recombinant *H. pylori* Trx2 resulted in elevated levels of ERK phosphorylation, indicating possible activation of MEK1/2 kinases. The level of phosphorylation was higher at a Trx2 concentration of 0.7 μM when compared with the level of ERK phosphorylation observed for Trx2 at a concentration of 0.35 μM. The level of activation of ERK correlates with the EMSA data which shows that AP-1 DNA-binding activity is reduced at concentrations below 0.7 μM Trx, and increased at higher concentrations, which may be physiologically less relevant than lower concentrations of the protein. No change in expression of p38 in AGS cells was noted following treatment with Trx2. These results suggest that *H. pylori* Trx2 may activate ERK cascade but not the p38 kinase cascade in AGS cells. An investigation of the effect of reduced *H. pylori* Trx2 on the JNK signalling pathway was undertaken, however, blot quality was not adequate after repeated attempts to assess the effect of Trx2 on JNK activation. Finally, high concentrations of *H. pylori* Trx did not appear to affect the viability of gastric epithelial cells as determined by the MTT assay, therefore is unlikely that the effects of this enzyme observed are due to a toxic effect elicited by its exposure of the recombinant protein to AGS cells over the time period chosen (3 h).

This preliminary study has revealed that recombinant *H. pylori* Trx2 is capable of modulating the DNA-binding activity of mammalian transcription factors, NF-κB and AP-1 by as yet undefined mechanisms. In the case of NF-κB, one hypothesis is that Trx2 may enter gastric epithelial cells and interact with molecules upstream of IκB, resulting in the inhibition of IκB phosphorylation and degradation, as IκB expression in AGS cells is enhanced when low concentrations of the redox enzyme are present in the culture medium. Recombinant Trx from *E. coli* has been previously shown to modulate expression of IκB in a mammalian cell line (Das, 2001) The downstream effect of this inhibition of IκB degradation by Trx2 might be the observed inhibition of NF-κB DNA-binding activity. In addition, Trx2 appears to modulate AP-1 DNA-binding activity in a complex manner as

assessed by EMSA analysis. Preliminary immunoblot analysis suggests that this may be mediated through the ERK signalling pathway. Finally, at least in the case of NF-κB modulation by recombinant *H. pylori* Trx2, this seems to be dependent on the redox properties of the enzyme as use of a mutated active site, and therefore redox inactive, Trx2 recombinant (Trx2-C28S) failed to inhibit cytopasmic IκB degradation, as assessed by immunoblotting. A further possibility is that Trx may take part in the regulation of transcription factor activity via the redox state of the entire intracellular environment. Trx not only modifies proteins such as transcription factors, but also maintains intracellular environments in the reduced state. Exposure to exogenous Trx may cause perturbation of the intracellular redox state and consequently this change could cause redox modification of cysteine residues of proteins involved in the regulation of transcription factor activity.

Understanding the host cell response to the bacterium is important in understanding the pathogenesis of *H. pylori* infection. It is interesting to speculate that Trx which is selectively released by *H. pylori* may be responsible for modulation of the host microenvironment in a manner that may be beneficial to the pathogen. Even limited suppression of the host inflammatory response could be advantageous in terms of persistence. The most successful pathogen-host relationships could be considered those that do not result in overt disease in the host, perhaps in the case of *H. pylori*, specifically secreted proteins may play a role in establishing this physiological status. Identification of putative *H. pylori* Trx2 targets in gastric epithelial cells may help identify how this enzyme might possibly influence the host immune response during infection. The following chapter describes the isolation of *H. pylori* Trx2 interacting proteins in gastric epithelial cells and the bacterium itself.

CHAPTER VI

IDENTIFICATION OF INTERACTING PROTEINS OF *H. PYLORI* THIOREDOXIN 2

CHAPTER VI

6.1 INTRODUCTION

6.1.1 Thioredoxin; a pleiotropic molecule.

Thioredoxin (Trx) is an unusual protein which since its discovery it has been linked to a wide range of biochemical systems in both prokaryotic and eukaryotic cells. Its diverse physiological functions in different cell backgrounds are believed to have evolved from a ubiquitous fundamental reaction, disulphide reduction, to encompass a large range of specialised functions. Trx is most frequently associated with its highly conserved role as a donor of reducing equivalents to catalytic enzymes and in the maintenance of redox homeostasis, however, a number of specialised organism specific functions have been described in the literature. Trx is the only host *E. coli* protein required for filamentous phage assembly and export (Russel and Model, 1986; Feng *et al.*, 1999). Trx has been demonstrated to have a role in the chloroplast photosynthetic enzyme regulation by light (Buchanan, 1991) and in many organisms is necessary for the reduction of hydrogen peroxide facilitating oxidative stress management (Chae *et al.*, 1999), and the induction of apoptosis (Zhang *et al.*, 1997). Trx also has a role in redox regulation of transcription factors (Schenk *et al.*, 1994; Hirota *et al.*, 1999), and immunomodulation (Nakamura *et al.*, 1997; Bertini *et al.*, 1999) in mammalian cells. Many of these functions are dependent on the disulphide reductase activity of Trx, however, it is becoming apparent that Trx may have a redox-independent role to play in certain physiological systems, such as phage assembly. Also recently, *E. coli* Trx was shown to have chaperone properties (Kern *et al.*, 2003). Interestingly, the study demonstrates that the redox properties of Trx do not play a role in the described chaperone function.

Trxs belong to a wider group of disulphide reductases that either reduce or oxidise disulphide bonds. The catalytic efficiency of Trxs is based on the two redox-active cysteine residues that undergo a two-electron oxidation to form a mixed disulphide bond. Each cysteine of the proteins active site has a specific role to play, a nucleophilic attack by the thiolate of Cys32 (*E. coli* amino acid numbering) breaks the disulphide of the target protein, forming a disulphide bridge with the more electrophilic sulphur leaving the other Cys as a thiolate. This bridge is transitory and is subsequently broken by Cys35, resulting in a reduced target protein and Trx being released in the oxidised form (Kallis and

Holmgren, 1980; Krause and Holmgren, 1991; Gleason, 1992; Dyson *et al.*, 1997). The chemical basis of this catalytic mechanism remains to be fully elucidated however, and the contribution of invariant amino acids in the hydrophobic core of the molecule to catalytic rates remains to be determined. The reduced form of Trx reduces a disulphide bridge on a target protein, which may result in a conformational change in the target protein (Holmgren, 1995). This process of interaction, the formation of a Trx-target protein complex and subsequent reduction of the disulphide bridge on the target enzyme is broadly accepted (Holmgren, 1989; Holmgren, 1995). In contrast, the detailed mode of protein-protein interaction between target enzyme and Trx is not fully elucidated.

A combination of affinity chromatography and proteomics has been successfully applied to the search for Trx interacting proteins in several plant species, specifically within the chloroplasts of these species (Motohashi *et al.*, 2001; Balmer *et al.*, 2003). In this study this same approach has been applied to the search for Trx2-interacting proteins in *H. pylori.* As *H. pylori* Trx2 has been shown to be specifically released from the bacterium, the examination of putative interacting proteins in gastric epithelial cells was also undertaken in this study. This present study exploits the observation that reduced forms of Trx and potential interacting proteins form mixed disulphide intermediates during the reduction pathway (Holmgren, 1995).

6.2 OBJECTIVES

1. To exploit a thiol-specific fluorescent tag combined with proteomics to identify potential proteins reduced by *H. pylori* Trx2.
2. To replace the active site cysteine residues of *H. pylori* Trx2 with redox-inactive serine residues by site-directed mutagenesis.
3. To over-express in a soluble form and purify to homogeneity the *H. pylori* mutant Trx2 recombinant proteins.
4. To covalently couple mutant proteins to an affinity column to facilitate the formation of a transient heterodisulphide bond between Trx2 and potential *in vivo* targets from *H. pylori* and gastric epithelial cells
5. To identify these potential Trx2 targets by mass fingerprinting and begin to elucidate the redox cascade that exists within the gastric pathogen and identify potential interactions with gastric cell proteins.

6.3 RESULTS

6.3.1 Identification of *H. pylori* Trx interacting proteins via a proteomic approach using a fluorescent thiol-specific probe.

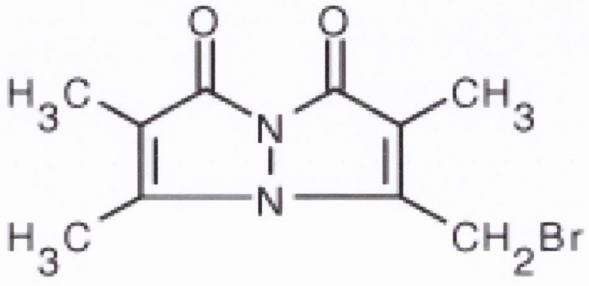
Soluble Trx-linked proteins from *H. pylori* were identified using a thiol-specific fluorescent probe, monobromobimane (mBrB; Molecular Probes) coupled with proteomic analysis in order to identify reduced species. Total cellular free thiols in *H. pylori* sonicates were conjugated with the thiol-specific probe essentially as described by Yano *et al.* (2001), as illustrated in **Fig. 6.1**. mBrB-labelled *H. pylori* proteins and Trx-reduced, mBrB-labelled *H. pylori* proteins were separated by 2-D SDS-polyacrylamide gel electrophoresis. Fluorescent derivatives were visualised by exposing the gels to UV light (362 nm). **Fig. 6.2** shows that many *H. pylori* proteins are labelled by the thiol-specific probe and this is expected as the majority of these molecules have sulphydryl groups that react with the fluorescent probe, so this represents the background fluorescence (**Panels A and B**). When *H. pylori* total cell lysate is compared (**Fig. 6.3, panel A**) with a *H. pylori* total cell lysate that has been treated with reduced Trx1 it is clear that there is an increase in the fluorescence intensity in a number of mBrB-conjugated species, indicating the exposure, via reduction by Trx1, of additional sulfhydryl groups (**Fig. 6.3, panel B**). The specificity of the incorporation of mBrB into Trx1-targeted proteins is demonstrated by the lack of increased fluorescence in proteins that do not possess disulfide bonds. An example of this is UreB or the B subunit of the urease enzyme, for which there is no apparent increase in fluorescence in Trx-reduced lysate (**Fig. 6.3, panel B; UreB**). UreB has two Lewis acid nickel ions and a reactive cysteine residue in the active site, with two other cysteine residues present in the protein (Kuhler *et al.*, 1995). These cysteines could participate in thiol-mediated disulphide bond formation, however if such bonds are formed it is most likely that they are not accessible to Trx, as treatment with Trx does not result in any difference in the fluorescence intensity of the protein. A careful analysis of the comparative gels indicates that at least 12 proteins are reduced after Trx treatment which cover a broad pI and molecular weight range.

When material treated with *H. pylori* Trx1 (**Fig. 6.4, panel A**) and Trx2 (**Fig. 6.4, panel B**) are compared it is also apparent that the two species of Trx show differential specificity with respect to the range of proteins reduced. This is particularly obvious for alkylhydroperoxide reductase, AhpC, which is preferentially reduced by Trx1 (**Fig. 6.4, panel A; 1a**) and not by Trx2 (**Fig. 6.4, panel B; 1b**). This observation is in good

A

Molecular Formula: $C_{10}H_{11}BrN_2O_2$

Molecular Weight: 271.11



B

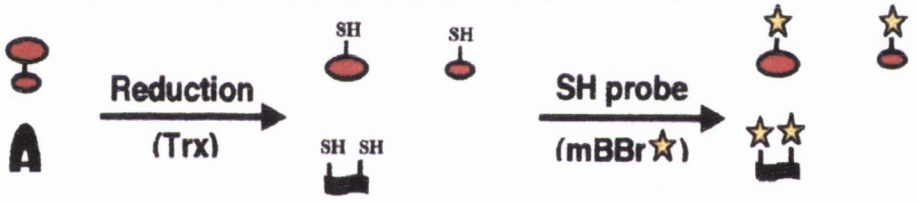


Fig. 6.1. Monobromobimane (mBrB) labelling of proteins. (A) Chemical structure of the thiol-fluorescent probe, mBrB (Molecular Probes). **(B)** Schematic of mBrB attachment to Trx-reduced proteins (reproduced from Yano *et al.*, 2001). mBrB labels sulphydryl groups newly exposed on target proteins following reduction by Trx and these sulphydryl groups are visualised as their fluorescent mBrB derivatives.

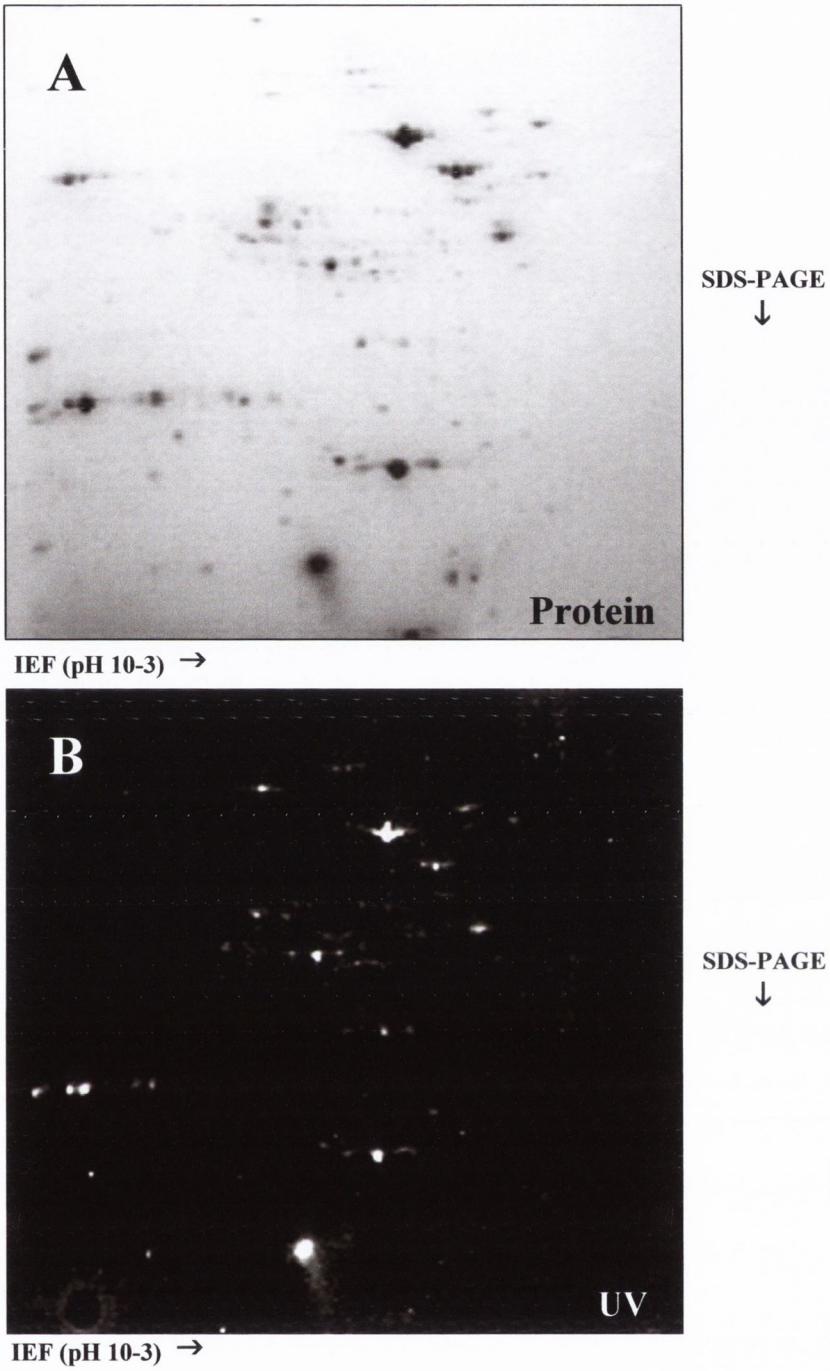


Fig. 6.2. Two-dimensional gel analysis of total proteins from *H. pylori* strain NCTC11638 labelled with the thiol fluorescent probe, mBrB. Bacteria were grown on Columbia blood agar and 50 μg of a clarified sonicate of *H. pylori* was derivatised with mBrB and separated by 2-D SDS-PAGE (IEF; pH 3 – 10). Proteins were visualised by staining with Coomassie blue R-250 in Panel A. Panel B shows the same gel visualised under UV light (362 nm).

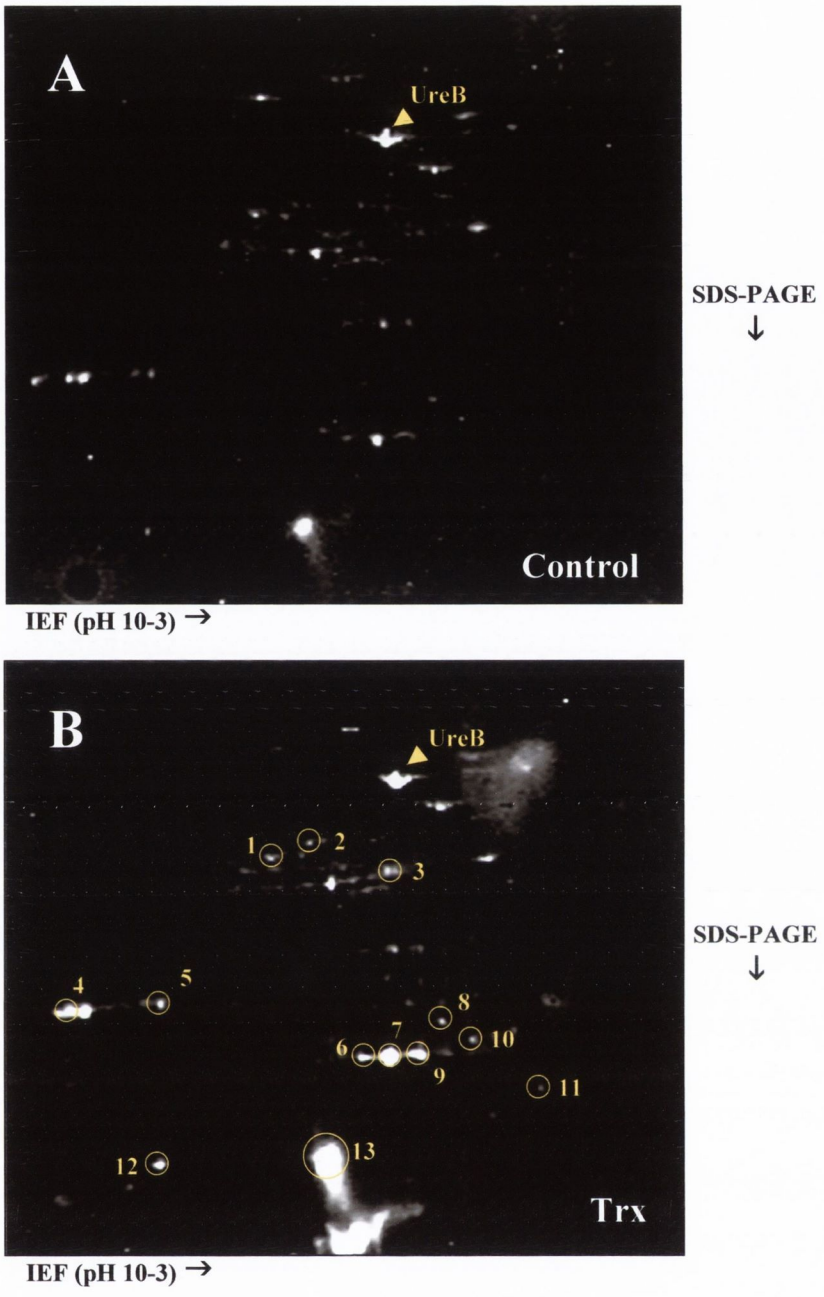


Fig. 6.3. Reduction of total proteins of *H. pylori* strain NCTC11638 by *H. pylori* Trx using the mBrB 2-D electrophoresis procedure. Bacteria were grown on Columbia blood agar and 50 μg of solubilised total cell protein were separated by 2-D SDS-PAGE (IEF; pH 3 – 10). Soluble proteins were either not reduced **(A)** or reduced by *H. pylori* Trx1 **(B)** for 1 h and deriviatised with mBrB. Gels were visualised under UV light at 362 nm. The numbered circles in panel B indicate proteins with increased fluorescence compared to the corresponding spots in the untreated preparation shown in panel A.

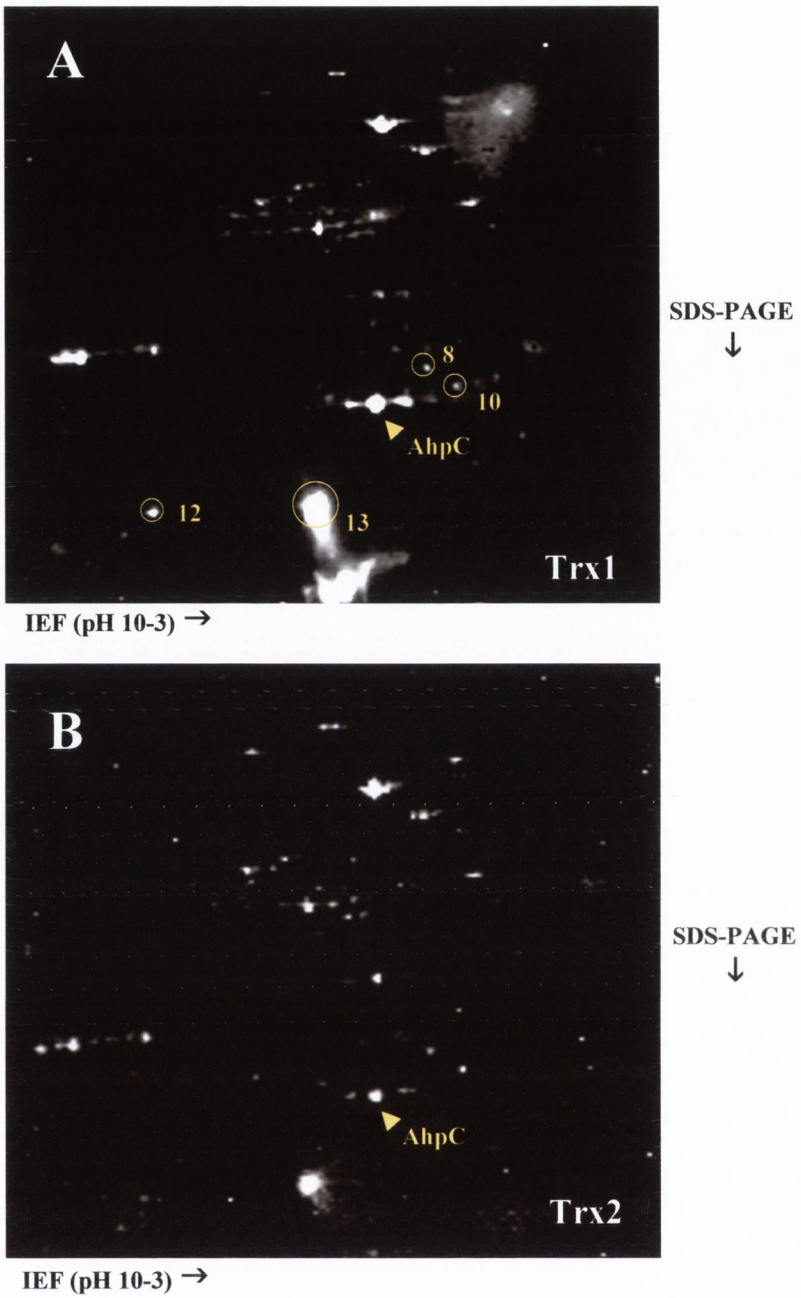


Fig. 6.4. Comparison of the profile of *H. pylori* proteins (NCTC11638) reduced by Trx1 and Trx2 using the mBrB 2-D electrophoresis procedure. Bacteria were grown on Columbia blood agar and 50 μg of solubilised total cell protein were separated by 2-D SDS-PAGE (IEF; pH 3 – 10). Soluble proteins were reduced with **(A)** *H. pylori* Trx1 and **(B)** *H. pylori* Trx2 for 1 h and derivatised with mBrB. Gels were visualised under UV light at 362 nm. Numbered proteins were identified by mass fing

Table 1. ***H. pylori*** **Trx-Interacting Proteins**

Protein	**Reduced by:**	**Mass**	**No. of Cys residues**
Elongation Factor P (2)	Trx1	20782	1
Thiol Peroxidase (3)	Trx1	18292	2
Succinyl CoA Transferase (1)	Trx1	22263	2

agreement with the literature as Baker *et al.* (2001) have reported previously that Trx1, but not Trx2 provides reducing equivalents to AhpC in *H. pylori*. Proteins identified by mass fingerprinting are shown in **Table 1**. Three proteins were identified as interacting proteins of Trx1 all of which harbour cysteine residues. These included thiol peroxidase, a member of the *H. pylori* peroxiredoxin family, elongation factor P, a translation factor with a role in protein synthesis and also succinyl CoA transferase. However, the most intensely fluorescent spot (designated spot 4 in **Fig. 6.4, panel A**) visible could not be identified by mass fingerprinting for unknown reasons. This species may be an artefact as prolonged de-staining of the gel resulted in disappearance of this spot, without disappearance of other proteins present. Use of fixed time-point in the experimental methodology may have resulted in some interacting species not being identified. For example, bacterioferritin comigratory protein (Bcp) which was previously demonstrated as a protein to which Trx donates reducing equivalents (Chapter IV), was not identified in this analysis. Additionally, several other spots, although fluorescent, have not yet been identified using this approach. Overall results show that *H. pylori* Trx, reduced with NADPH via TR, converted numerous proteins of *H. pylori* from disulphide to sulphydryl form as determined by incorporation of the thiol fluorescent probe, mBrB.

It is likely that these proteins represent potential target proteins of *H. pylori* Trx2, given that a recent report indicated that Trx2 is released from *H. pylori* as shown by 10-50 fold enrichment of the protein in the growth media. It was therefore of interest to survey whether or not Trx2 could reduce gastric cell proteins, therefore, this approach was extended to identify potential Trx2 interacting proteins from the gastric epithelial cell line, AGS.

6.3.2 Identification of Trx2-gastric epithelial cell (AGS) interacting proteins via a proteomic approach using a fluorescent thiol-specific probe.

Fig. 6.5 shows total proteins of AGS cells separated by 2-D SDS-PAGE. **Fig. 6.6** shows AGS cell proteins labelled by the thiol-specific probe, mBrB, following reduction by *H. pylori* Trx2 and separated by 2-D SDS-PAGE. AGS proteins identified by mass spectrometry analysis are shown in **Table 2**. When the amino acid sequence of the identified proteins are examined it is clear that in addition to nearly all of the proteins possessing numerous cysteine residues, two of the identified species, eukaryotic translation initiation factor 5A and the thyroid hormone binding protein precursor, harbour a CXXC

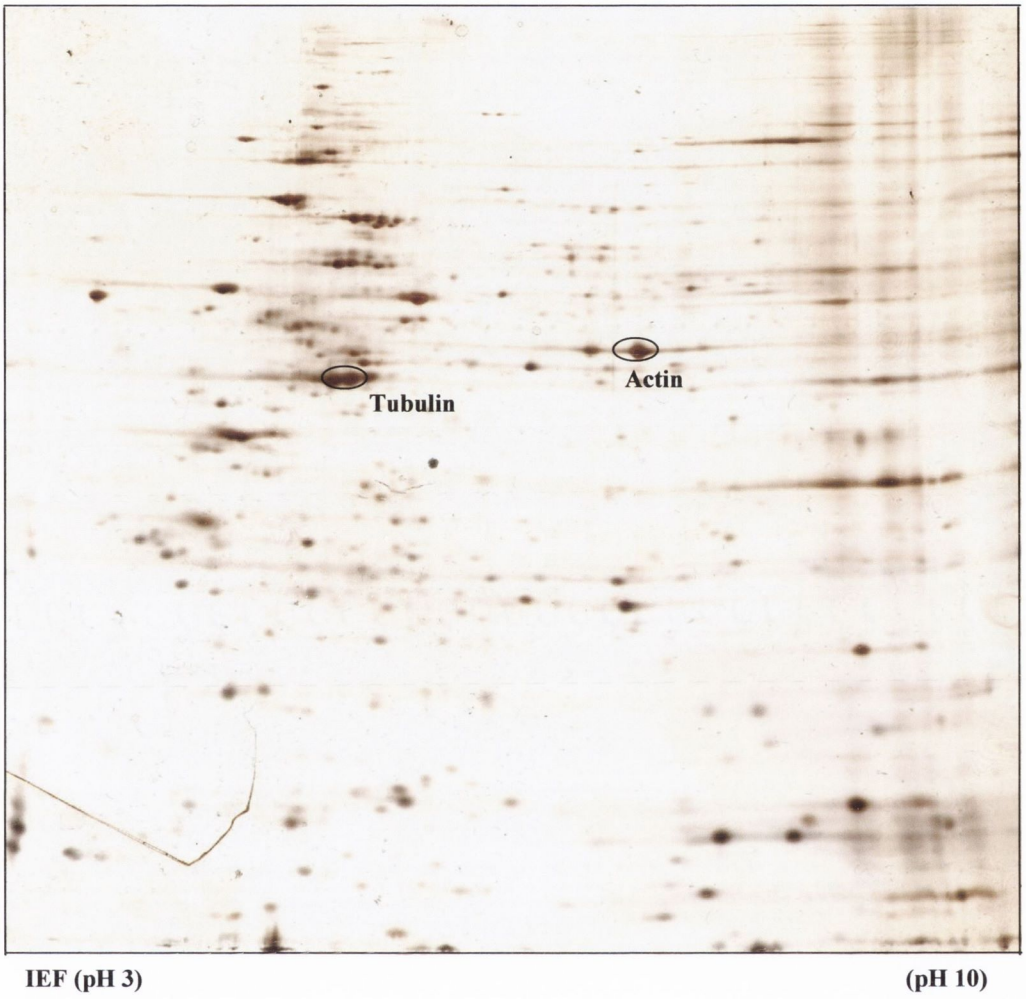


Fig. 6.5. 2-D gel electrophoretic analysis of gastric epithelial cell total proteins. AGS cells were grown to subconfluency in RPMI medium containing FCS (10 % v/v) were washed several times in ice-cold PBS and lysed by sonication. 50 μg of solubilised total cell protein was loaded for isoelectric focusing on Amersham IPG strips of a non-linear pH gradient of 3 to 10. Proteins separated on the 10-20% gradient SDS-PAGE gel were visualised by silver staining. The landmark proteins tubulin α-2 (37.7 kDa) and actin (41.3 kDa) are highlighted.

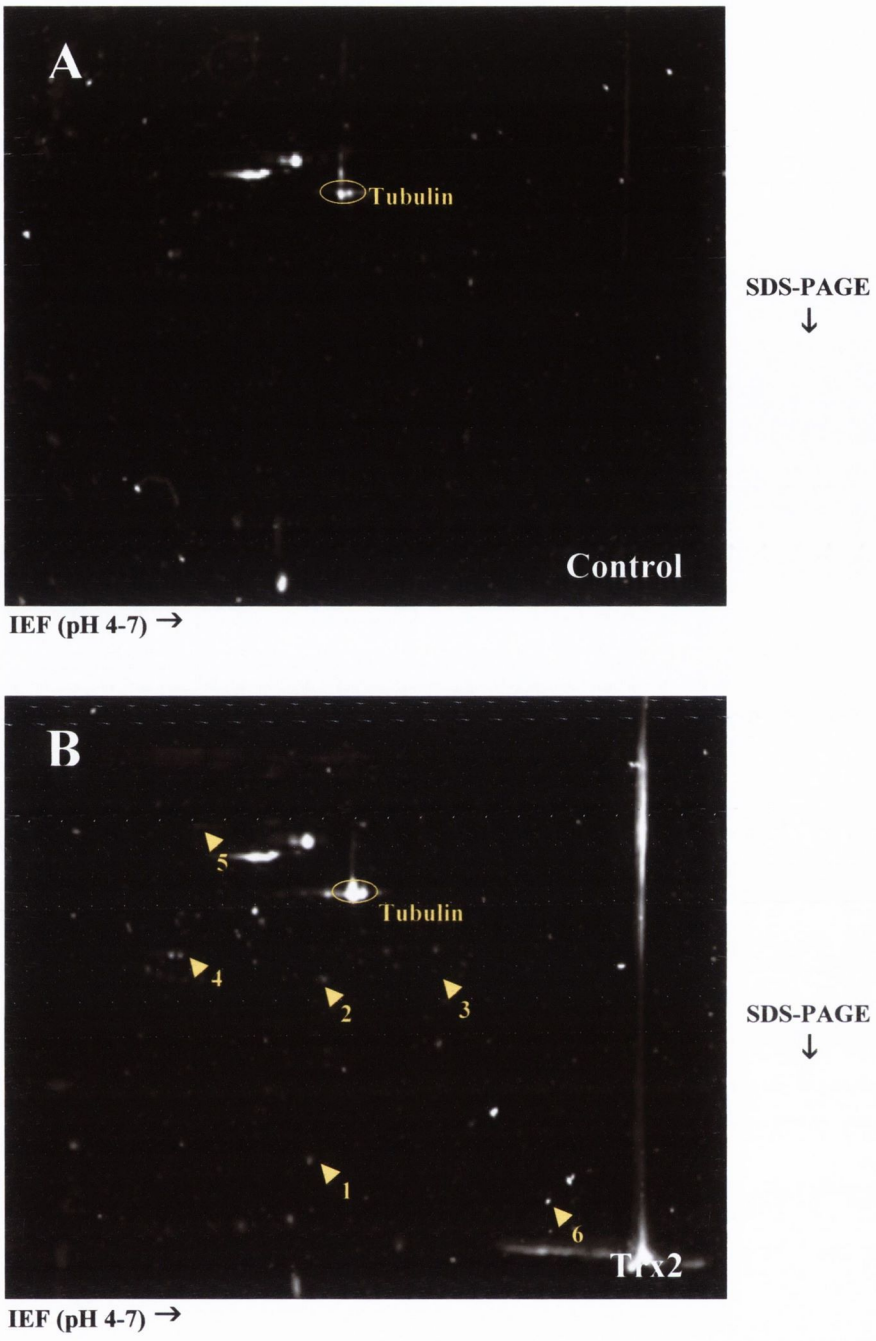


Fig. 6.6. Reduction of total proteins of gastric epithelial cells (AGS) by *H. pylori* Trx using the mBrB 2-D electrophoresis procedure. 50 μg of solubilised total cell protein from AGS cells was separated by 2-D SDS-PAGE (IEF; pH 4 – 7). Soluble proteins were either not reduced **(A)** or reduced by *H. pylori* Trx2 **(B)** for 1 h and deriviatised with mBrB. Proteins separated on 12.5% polyacrylamide gels were visualised under UV light at 362 nm. Numbered proteins were identified by mass fingerprinting analysis. The landmark protein, tubulin, is shown on the gel.

Table 2. Trx2-Gastric Epithelial Cell (AGS) Interacting Proteins

Protein	Reduced by:	Mass	Cys residues
Inorganic pyrophosphatase (3) Accession No.; AAH39462	Trx2	33095	6
Fatty acid binding protein 5 (psoriasis-assoociated); E-FABP **(6)** Accession No.; AAH19385	Trx2	15155	6
Eukaryotic translation initiation factor 5A (1) Accession No.; AAH32866	Trx2	17049	8 2 CXXC motifs
Laminin-binding Protein (4) Accession No.; BAA05738	Trx2	33464	-
Thyroid hormone binding protein precursor (5) Accession No.; AAA61169	Trx2	57023	7 2 CXXC motifs
Thioredoxin reductase (2) Accession No.; AAH18122	Trx2	34625	12

motif. The presence of this motif in these two proteins suggests that redox modulation of activity via Trx may be possible. The significance of these observations is considered further in the discussion.

While the described proteomic approach using the fluorescent thiol-specific probe, mBrB, was a partially functional approach for identifying Trx interacting proteins, certain limitations to the approach were apparent. For example, low abundance proteins are under-represented using standard 2-D analysis which imposes a sensitivity limit on this approach. Therefore certain protein-Trx interactions may have been missed. The approach does not enrich for low abundance proteins which may be reduced by Trx, and therefore may not have been identified. Technical limitations with the 2-D proteomic approach may have resulted in not all Trx interacting proteins being represented in the 2-D gel, particularly those with a very low or very high pI and hydrophobic proteins. Therefore, a more sensitive and specific approach to identifying Trx interacting proteins was also used in this study, which exploits the reported observation that the transient formation of mixed disulphide intermediates between Trx and target proteins can be stabilised by mutating one of the two cysteine residues present in the redox active site. The ability to stabilise Trx-substrate complexes using single cysteine mutants of Trx has been used successfully to 'affinity' purify cellular targets of Trx from cell extracts.

6.3.3 Construction of redox-active site mutants of *H. pylori* recombinant Trx2.

Mutagenesis of cloned *H. pylori trx2* was effected by a site-directed mutagenesis PCR approach. The two cysteine residues of the redox-active site at positions 28 and 31 of *H. pylori* Trx2 relative to the N-terminus were mutated to serine residues, both individually (C28S and C31S) and in combination (C28S/C31S) (**Table 3**). The correct nature of the mutation was verified by sequencing of selected clones with appropriate primers (Dept. of Biochemistry, Cambridge University, U.K.).

6.3.4 Overexpression and purification of redox active site mutants of *H. pylori* recombinant Trx2.

From 800 ml of induced *E. coli* BL21 DE3 pLysS, approximately 10 mg of recombinant *H. pylori* Trx2 was obtained for each active site mutant and this was expressed exclusively as a soluble cytoplasmic protein. These products were purified as non-tagged proteins via a two-step purification procedure involving ion-exchange chromatography over DEAE-

cellulose followed by CM-cellulose. Trx2 active site mutants were released from the CM-cellulose column following addition of 20 mM potassium phosphate. Analysis of fractions, collected from the final ion-exchange column, by SDS-polyacrylamide gel electrophoresis, identified fractions containing pure protein represented by a single protein band with a molecular mass of approximately 12,000, as expected. Pure fractions for each variant, Trx2-C28S, Trx2-C31S, Trx2-C28S/C31S, were pooled and consisted of recombinant protein purified to homogeneity and all of the recombinant proteins gave a single 12 kDa protein band as seen by silver staining of a 15% SDS-polyacrylamide gel (**Fig. 6.7**). Expression of the recombinant mutants did not effect either bacterial cell viability or levels of protein expression in comparison with wild-type recombinant Trx2.

6.3.5 Non-reducing SDS-PAGE analysis of *H. pylori* Trx2 redox active site mutants.

Fig. 6.8 shows *H. pylori* wild-type recombinant Trx2, and *H. pylori* recombinant active site mutants Trx2-C28S, Trx2-C31S and C28S/C31S which were solubilised in non-reducing sample buffer and analysed by SDS-PAGE on a 15% gel and then visualised by silver staining. The gel reveals that aggregation of the single mutants, Trx2-C28S and Trx2-C31S is occurring when no reducing agent is present upon protein solubilisation (**Fig. 6.8, lanes 3 and 4**), however, the majority of the protein for both recombinants is still resolving as a monomer. No higher molecular mass protein bands are visible on the gel for either the wild-type Trx2 recombinant or the double mutant, Trx2-C28S/C31S (**Fig. 6.8, lanes 2 and 5**).

6.3.6 Affinity trapping of *H. pylori* Trx2 interacting proteins.

Recombinant wild-type *H. pylori* Trx2 and recombinant Trx2 mutants, Trx2-C28S, Trx2-C31S, Trx2-C28S/C31S were coupled to cyanogen bromide-activated Sepharose 4B resin. **Figure 6.9 (B)** shows that Trx2 (wild-type and mutants) bound very efficiently to the activated resin following incubation in the appropriate buffer, with in excess of 90% of recombinant protein binding to the resin substrate. *H. pylori* strain NCTC11638 total cell lysate (5 mg) equilibrated in the appropriate buffer was applied to the resin. Following extensive washing of the column with a high salt buffer to remove non-covalently bound proteins, potential target proteins linked via newly formed heterodisulphide bonds were eluted with a high salt buffer containing 10 mM DTT, as demonstrated in the schematic in **Fig. 6.9 (A)**. The amount of protein collected following elution of columns containing bound wild-type *H. pylori* Trx2 and the double mutant Trx2-C28S/C31S was significantly

Table 3

Active site amino acids sequences of *H. pylori* Trx2 variants.

The underlined residues indicate the two active site cysteines that were mutated to serine residues in the respective mutant.

	23	35
Trx2	Val-Gly-Ala-Ser-Trp-Cys -Pro-Asp-Cys -Arg-Lys-Ile-Glu	
C28S	Val-Gly-Ala-Ser-Trp-**Ser** -Pro-Asp-Ser -Arg-Lys-Ile-Glu	
C31S	Val-Gly-Ala-Ser-Trp-Cys-Pro-Asp-**Ser** -Arg-Lys-Ile-Glu	
C28S/C31S	Val-Gly-Ala-Ser-Trp-**Ser** -Pro-Asp-**Ser** -Arg-Lys-Ile-Glu	

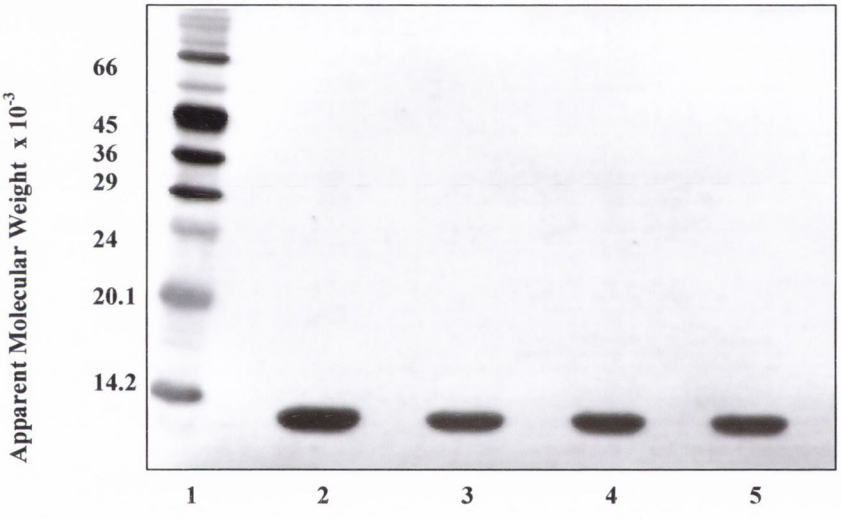


Fig. 6.7. SDS-PAGE analysis of *H. pylori* redox-active site mutant Trxs. Purified recombinant redox-active site mutant Trx2 proteins were analysed on a 15% polyacrylamide gel in reducing sample buffer as follows: lane 1, molecular weight markers (Low-Range Molecular Weight Standards; Sigma); lane 2, Trx2 wild-type; lane 3, Trx2 C28S; lane 4, Trx2 C31S; lane 5, Trx2 C28S/C31S. Proteins were visualised by silver staining. Molecular masses (in kilodaltons) are indicated on the left.

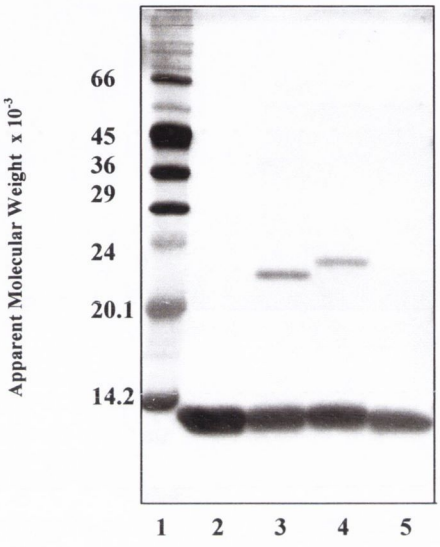


Fig. 6.8. Non-reducing SDS-PAGE analysis of wild-type recombinant *H. pylori* Trx2 and recombinant *H. pylori* Trx2 redox active site mutants. *H. pylori* wild-type recombinant Trx2 (lane 2), and *H. pylori* recombinant active site mutants Trx2-C28S (lane 3), Trx2-C31S (lane 4) and C28S/C31S (lane 5) were solubilised in non-reducing sample buffer and analysed by SDS-PAGE on a 15% gel. 10 μg of protein was loaded per lane and proteins were visualised by silver staining. Lane 1 shows molecular weight markers (Low-Range Molecular Weight Standards; Sigma).

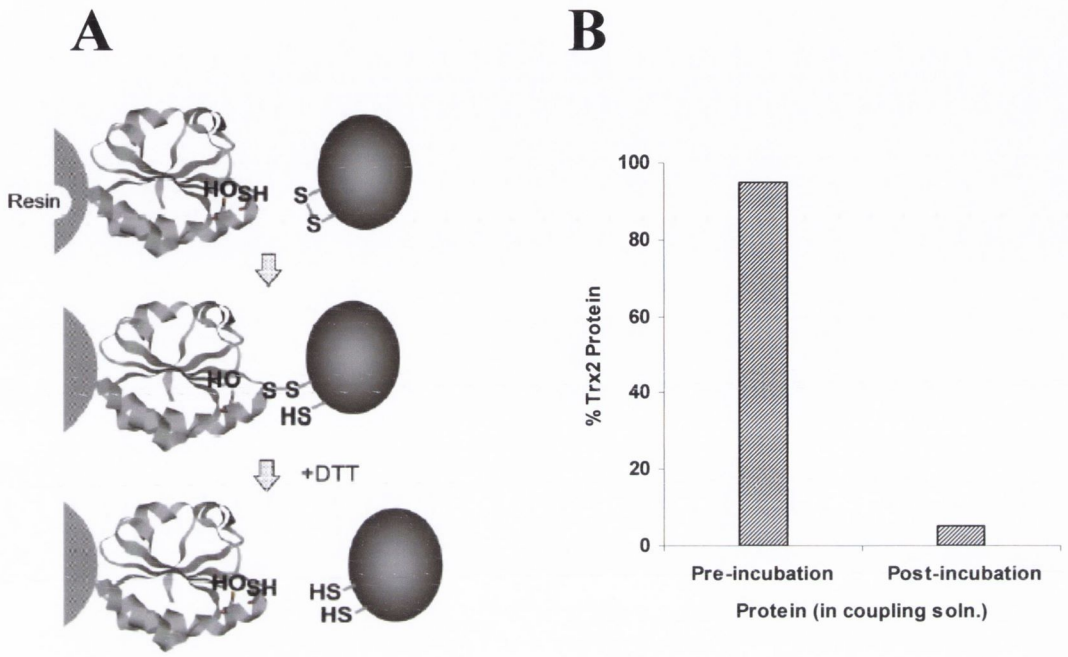


Fig. 6.9. Schematic of the process to capture target proteins using a Trx redox-active site mutant recombinant protein. (A) One of the internal cysteine residues at the *H. pylori* Trx2 active site was substituted with serine and immobilised on cyanogen bromide activated Sepharose. As shown in the third step of the schematic, the mixed disulphide intermediate formed between Trx and target should be disrupted by the addition of the reducing agent DTT, thus allowing elution of target proteins covalently attached to the resin. **(B)** Graph of the efficacy of Trx2 redox-active site mutant protein (Trx2-C28S) binding to the cyanogen bromide-activated Sepharose 4B resin. Similar coupling efficiencies were observed for wild-type and other mutant Trxs (Trx2-C31S, Trx2-C28S/C31S). Efficacy of binding was assessed spectrophotometrically at 280 nm.

less than that obtained from the Trx2-C28S and Trx2-C31S (not shown) mutant Trx containing columns (**Fig. 6.10**), approximately 20 to 30 μg compared with approximately 100 μg for both of the single mutants. A control experiment involving DTT elution of *H. pylori* total lysate proteins from a column of Sepharose not coupled to Trx2 revealed that a very low amount of protein was obtained from the resin (< 10 μg). Identical protein profiles, as assessed by SDS-polyacrylamide gel electrophoresis and silver staining, were obtained for eluted material from both of the single Trx2 mutant coupled columns (Trx2-C28S and Trx2-C31S) in several independent experiments, therefore all subsequent experiments were performed using the Trx2-C28S-coupled Sepharose affinity column. It is apparent that several proteins were visibly enriched in the eluate from the single mutant affinity column, when compared with eluates from 'control' columns (**Fig. 6.10**; lanes 4 and 6) even when eluates were assessed by standard 1-D SDS-PAGE. As small amounts of protein still remained bound to the columns after washing with 0.3 M NaCl (**Fig. 6.10**; lanes 1, 3 and 5), it was obvious that some non-specific interactions were resistant to the high salt wash used. Therefore, wash steps in subsequent experiments were carried out using a higher salt concentration (0.5 M NaCl) in order to further reduce such non-specific interactions.

6.3.7 Gradient SDS-PAGE analysis and identification of *H. pylori* Trx2 interacting proteins.

Gradient SDS-polyacrylamide gel analysis of material eluted from the Trx2-C28S column was performed in order to increase resolution of proteins which were enriched in the eluate from the Trx2-C28S column. Eluted material was acetone precipitated, separated on 10%-20% gradient gels and visualised by silver staining. A representative gel is shown in **Fig. 6.11**, where lane 2 shows the profile of total cellular proteins of *H. pylori* and lane 3 shows the final high salt wash (0.5 M NaCl) to remove non-covalently bound proteins from the affinity column. It is clear that in this final wash no proteins are present thus indicating that prior to elution with DTT only those proteins that are covalently bound to the mutant Trx2 resin are available for subsequent elution. In excess of 37 eluted protein species are visible on the silver-stained gel (**Fig. 6.11, lane 4**). Within these bands, mass spectrometry analysis of the four most prominent bands visible was carried out (Mass Spectrometry Facility, RCSI, Dublin) and the following proteins identified; (1) urease (HP0075; TIGR annotation), (2) EF-Tu, (HP1205), (3) urease α-subunit (HP0073), (4) Trx (HP1458).

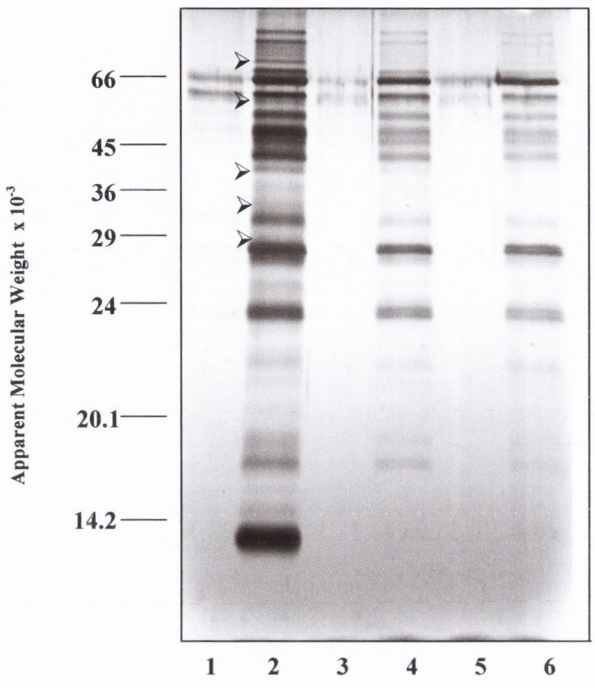
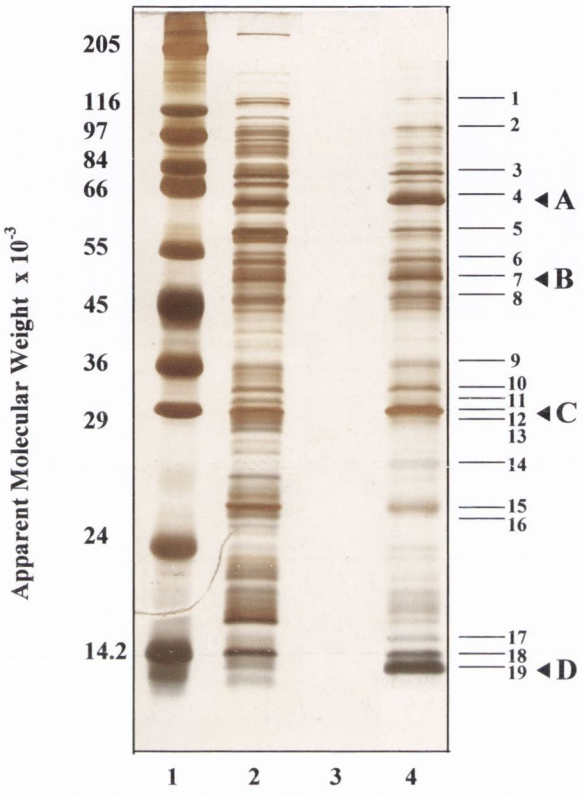


Fig. 6.10. Elution of proteins from *H. pylori* Trx2-immobilised resin with DTT. Proteins in *H. pylori* strain NCTC11638 total cell lysate bound to the affinity resin were eluted with 10 mM DTT. Lane 1, final NaCl wash (300 mM) eluate from the Trx2-C28S affinity column; lane 2, DTT-eluate from the Trx2-C28S affinity column; lane 3, final NaCl wash (300 mM) eluate from the Trx2-C28S/C31S affinity column; lane 4, DTT-eluate from the Trx2-C28S/C31S affinity column; lane 5, final NaCl wash (300 mM) eluate from the Trx2-WT affinity column; lane 6, DTT-eluate from the Trx2-WT affinity column. Equivalent volumes of DTT-eluted material were acetone-precipitated and loaded in lanes 2, 4 and 6. Molecular weight markers are shown on the left. Proteins were visualised by silver staining of the 15% SDS-PAGE gel. Arrow heads indicate those proteins which are visibly enriched in the eluate from the single mutant affinity column, when compared with eluates from 'control' columns (lanes 4 and 6).



Band	Predicted MW
1	127.1
2	104.9
3	84
4	64.04
5	58.93
6	54.17
7	50.83
8	46.67
9	36.35
10	32.68
11	29
12	28.52
13	27.63
14	26.53
15	25.89
16	25.16
17	17.47
18	14.34
19	12.14

Fig. 6.11. Elution of proteins from *H. pylori* Trx2-C28S-immobilised resin with DTT. Proteins in *H. pylori* strain NCTC11638 total cell lysate bound to the affinity resin were eluted with 10 mM DTT. Lane 1, broad range molecular weight markers (Sigma); lane 2, *H. pylori* total cell lysate prior to addition to a Trx2-C28S affinity column; lane 3, final NaCl wash (500 mM) eluate from the Trx2-C28S affinity column; lane 4, DTT-eluate from the Trx2-C28S affinity column. Equivalent volumes of DTT-eluted material were acetone-precipitated and loaded in lanes 2 to 4. Filled arrow heads (A-D) show the proteins that were identified by mass spectrometry (Section 6.3.7). Proteins were visualised by silver staining of the 10% - 20 % gradient SDS-PAGE gel.

6.3.8 2-D PAGE analysis of *H. pylori* Trx2-interacting proteins.

Isoelectric focusing and SDS-polyacrylamide gel electrophoresis were performed using the IPGphor system (Amersham). **Fig. 6.12** shows total cell proteins of *H. pylori* strain NCTC11638 following 2-D SDS-PAGE. **Fig. 6.13 (A)** shows *H. pylori* proteins eluted from the Trx2-C28S affinity column following addition of DTT and subjected to 2-D SDS-PAGE as described above. **Fig. 6.13 (B)** shows *H. pylori* proteins eluted from the Trx2-C28S/C31S affinity column following addition of DTT and subjected to 2-D SDS-PAGE as described above. Comparative analysis of the gels shows that a proportion of the proteins isolated as Trx2-interacting proteins are also isolated from a control column (Trx2-C28S/C31S affinity column) which would not be expected to form heterodisulphide bonds with proteins as there are no Cys residues in the double mutant. Of particular note, is the presence of the 60 kDa Chaperonin (GroEL protein; HP0010) at almost identical intensities in both **panel A** and the 'control' gel (**panel B**). This analysis clearly shows that non-redox linked interactions are occurring during the affinity trapping step, that are resistant to relatively high salt washes (0.5 M NaCl). Nevertheless, it is clear that several spots are recovered in panel A that are absent in the 'control' gel. These species are numbered in **Fig. 6.13, panel A**. Due to the low amount of protein present on the 2-D gels, staining to visualise proteins present was continued slightly beyond the recommended incubation time for the staining procedure, resulting in the resolution of some distortion lines on the gels.

As *H. pylori* Trx2 is reportedly a selectively secreted protein, it is conceivable that interactions between the Trx2 and host cell proteins occur. This was explored through stabilisation of Trx-substrate complexes from AGS cell lysates using single cysteine mutants of Trx2 as described for *H. pylori* Trx2 interacting proteins.

6.3.9 Affinity trapping of Trx2 interacting proteins in gastric epithelial cells.

Total cell lysates of AGS cells (5 mg) were applied to the wild-type Trx2 and Trx2-C28S affinity resins. Following extensive washing of the column with a high salt buffer (0.5 M NaCl) to remove non-covalently bound proteins, proteins covalently bound to the resin were eluted with a high salt buffer containing 10 mM DTT (**Fig. 6.14**). As described for *H. pylori* Trx2-interacting proteins in Section 6.3.6., the amount of AGS cell protein collected following elution of the column containing double mutant Trx2-C28S/C31S (**Fig. 6.14, lane 3**) was significantly less than that obtained from the Trx2-C28S (**Fig. 6.14, lane 2**)

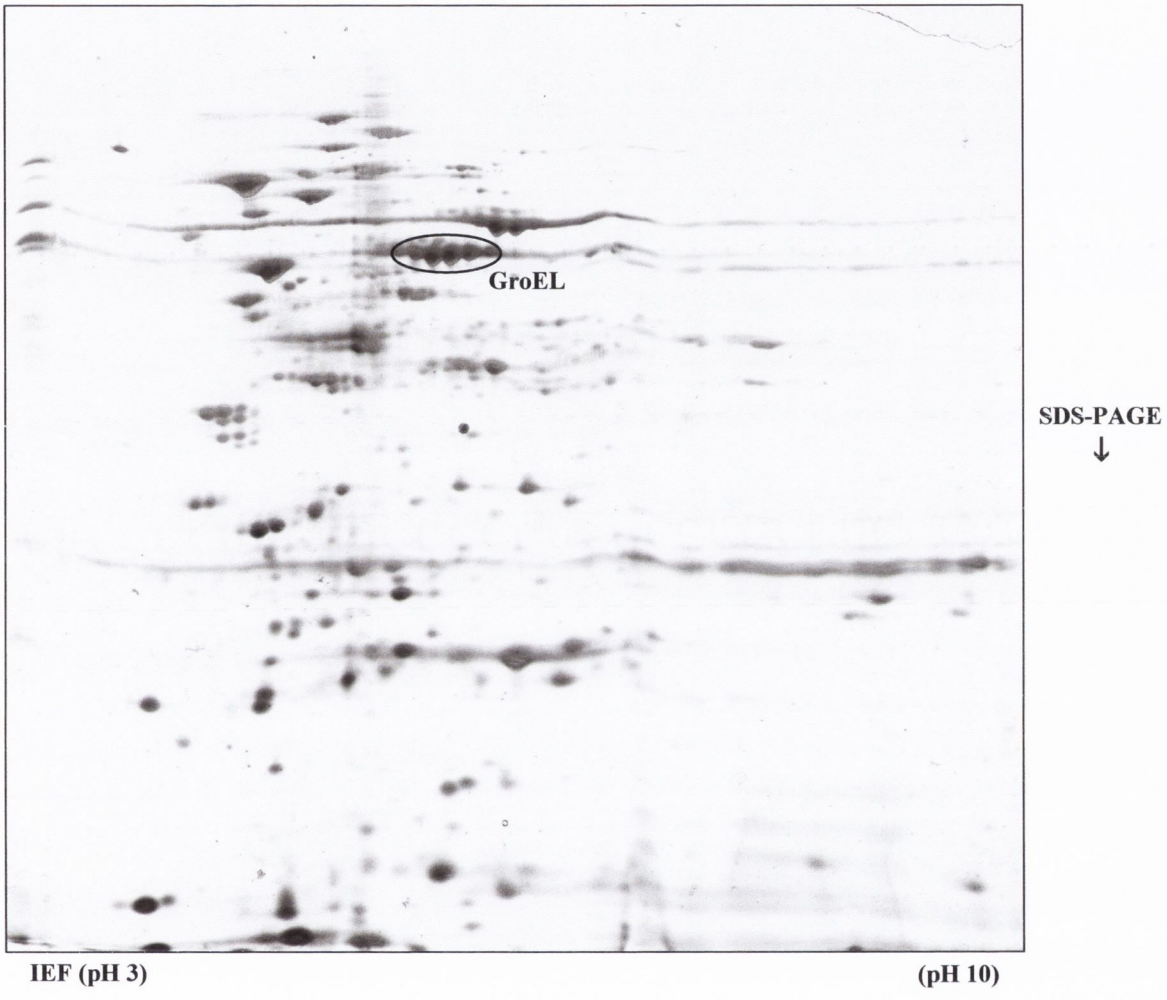


Fig. 6.12. 2-D gel eletrophoretic analysis of total *H. pylori* strain NCTC11638 cellular proteins. 50 μg of solubilised total cell protein was loaded for isoelectric focusing on IPG strips (pH 3-10). Proteins separated on the 10%-20% gradient SDS-PAGE gel were visualised by silver staining.

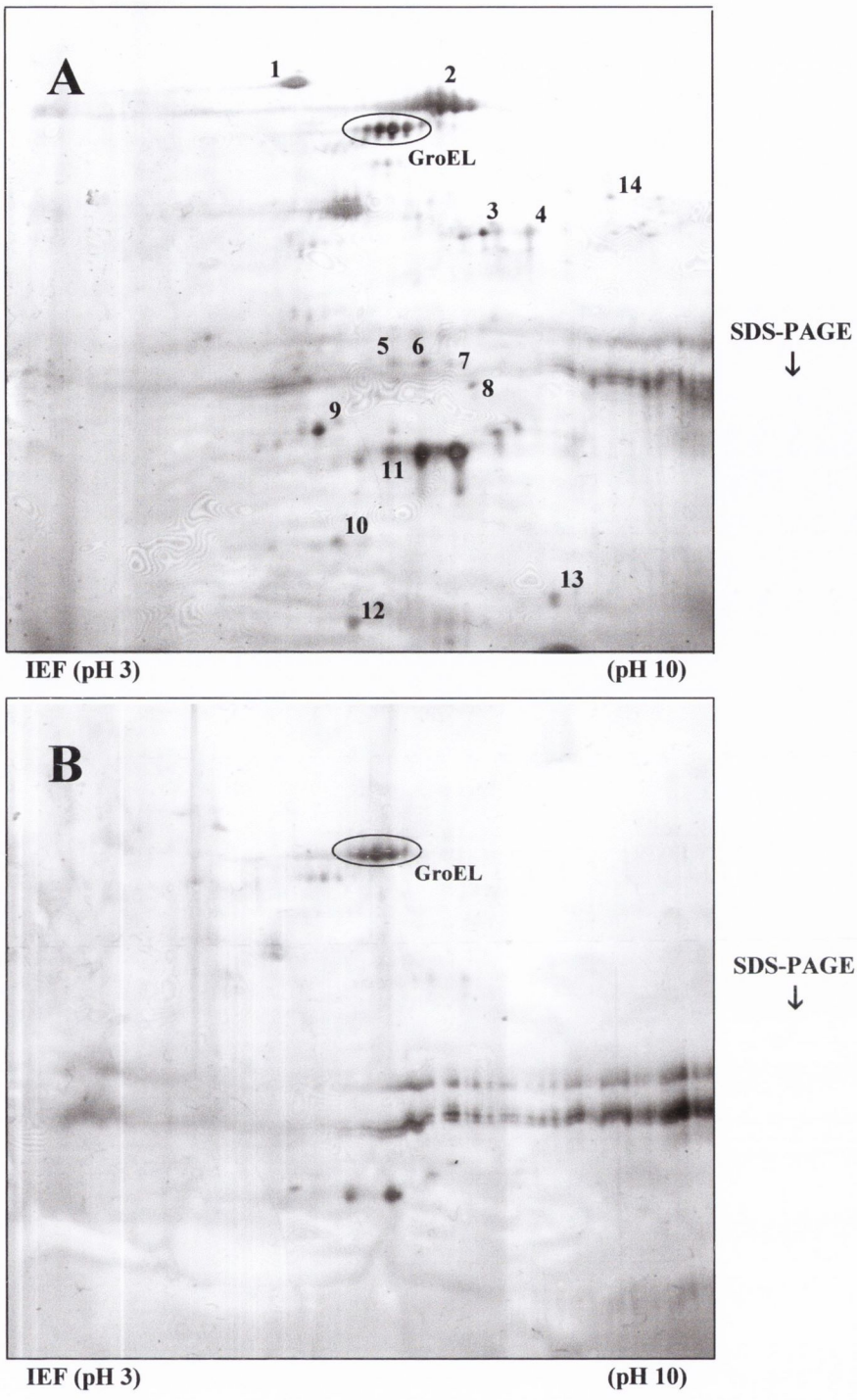


Fig. 6.13. 2-D gel electrophoretic analysis of *H. pylori* Trx2 targets. 50 μg of protein eluted from the Trx2-C28S resin were separated by isoelectric focusing on IPG strips (pH 3-10). **(A)** DTT-eluate from the Trx2-C28S affinity column. **(B)** DTT-eluate from the Trx2-C28S/C31S affinity column. Proteins separated on the 10%-20% gradient SDS-PAGE gel were visualised by silver staining. The numbers in panel A indicate the position of spots that are not present in panel B, indicating that these represent proteins that bind bound specifically to Trx2.

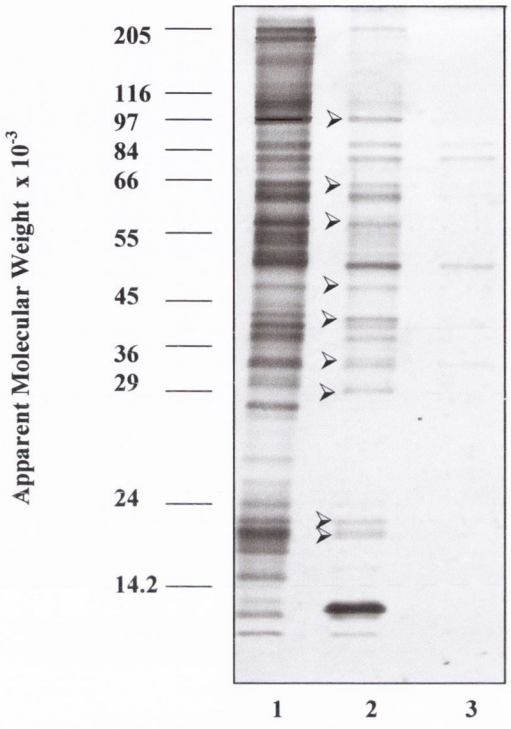
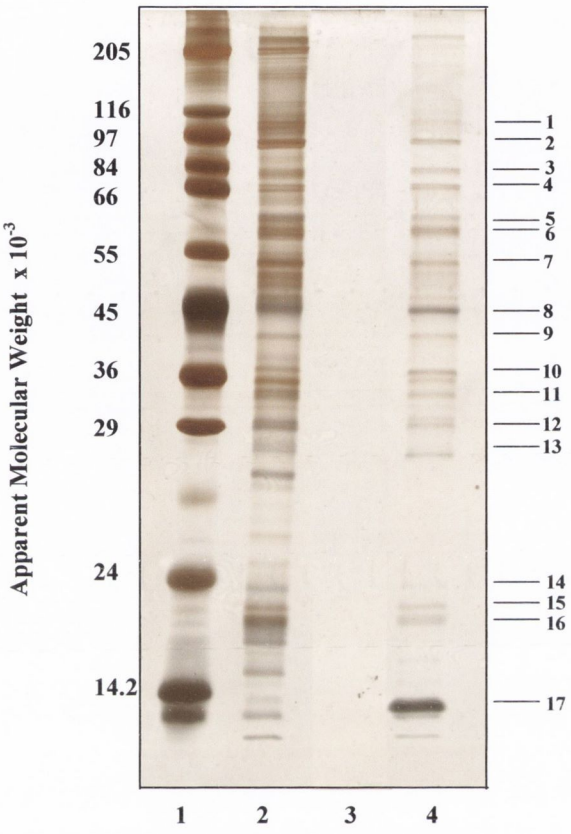


Fig. 6.14. Elution of gastric epithelial cell (AGS) proteins from Trx2-immobilised resin with DTT. Proteins in AGS total cell lysate bound to the affinity resin were eluted with 10 mM DTT. Lane 1, AGS total cell lysate; lane 2, DTT-eluate from the Trx2-C28S affinity column; lane 3, DTT-eluate from the Trx2-C28S/C31S affinity column. Equivalent volumes of DTT-eluted material were acetone-precipitated and loaded in lanes 1, 2 and 3. Molecular weight markers are shown on the left. Proteins were visualised by silver staining of the 10%-20% gradient SDS-PAGE gel. Arrow heads indicate those proteins which are visibly enriched in the eluate from the single mutant affinity column, when compared with eluate from the 'control' column (lane 3).



Band	Predicted MW
1	105.6
2	94.4
3	80
4	65.62
5	60.69
6	57.66
7	52.41
8	44.42
9	41.52
10	36
11	33.04
12	29.54
13	28.03
14	24.82
15	19.11
16	18.11
17	12.4

Fig. 6.15. Elution of AGS cell proteins from *H. pylori* Trx2-C28S affinity column with DTT. Lane 1, broad range molecular weight markers (Sigma); lane 2, total AGS lysate prior to addition to Trx2-C28S affinity column; lane 3, final NaCl wash (500 mM) eluate from Trx2-C28S affinity column; lane 4, DTT-eluate from Trx2-C28S affinity column. Equivalent volumes of DTT-eluted material were acetone-precipitated and loaded in lanes 2 to 4. Proteins separated on the 10-20% gradient SDS-PAGE gel were visualised by silver staining.

and Trx2-C31S mutant Trx containing column (not shown). As in Section 6.3.6., identical protein profiles were obtained for eluted AGS cell material from both the Trx2-C28S mutant coupled column and the Trx2-C31S mutant column (not shown) in several independent experiments. As before, a control experiment involving DTT elution of AGS total lysate proteins from a column containing non-Trx2 coupled activated Sepharose revealed that a very low amount of protein was obtained from the resin (< 10 μg).

6.3.10 Isolation of gastric epithelial cell-Trx2 interacting proteins.

Gradient SDS-polyacrylamide gel analysis of material eluted from the Trx2-C28S column was performed. Eluted material was acetone precipitated, separated on 10% - 20% gradient gels and visualised by silver staining. A representative gel is shown in **Fig. 6.15**, where lane 2 shows the profile of total cellular proteins from AGS cells and lane 3 shows the final high salt wash to remove non-covalently bound proteins from the affinity column. It is clear that in this final wash no proteins were present thus indicating that prior to elution with DTT only those proteins that were covalently bound to the mutant Trx2 resin were available for subsequent elution. In excess of 25 bands of eluted protein were visible on the silver-stained gel (**Fig. 6.15, lane 4**). When the predicted molecular weight of the protein eluted from the affinity column were determined and compared to the molecular weights of those proteins identified by mBrB analysis (**Section 6.3.2**), several of the bands were approximately in the 29 kDa to 36 kDa size region and the 17 kDa size region, into which inorganic pyrophosphatase, laminin binding protein, thioredoxin reductase and eukaryotic translation initiation factor 5A fall, respectively.

6.4 DISCUSSION

One of the factors limiting understanding of Trx function in *H. pylori* is the paucity of information regarding interacting proteins and enzymes. The function of *H. pylori* Trx has been explored in relation to the established process of oxidative stress management (Baker *et al.*, 2001; Comtois *et al.*, 2003; Chapter IV), however, the role of this redox enzyme in wider physiological processes in this pathogen has remained unexplored. This study describes the application of proteomic and mutagenesis approaches to begin to elucidate the spectrum of interacting proteins of *H. pylori* Trx2 *in vivo* and the redox cascade which operates within this bacterium. Trxs are capable of interacting with specific targets and it is becoming apparent that it is not only the nature of the redox active site which is fundamental in the determination of Trx-protein interactions but also the structure and

surface topology of individual Trxs. Until recently studies of the enzymatic mechanism of Trx have tended to focus on the reductive capabilities of the protein, however, this step follows Trx-protein complex formation, the establishment of which is slower than reduction (Schwarz *et al.*, 1997). The physiological importance of the two forms of Trx in the *H. pylori* has not been fully elucidated.

The fluorescence tagging approach initially described by Yano *et al.* (2001) using the thiol-specific fluorescent probe, monobromobimane, to label sulphydryl groups newly exposed on proteins following reduction by Trx was utilised in this study. The original study described the approach as applied to *Arachis hypogaea L.* (peanut) seeds in order to elucidate the role of Trx in heterotropic processes and resulted in the isolation of 20 proteins and identification of 5 proteins targeted by Trx *h* in this species. The extension of this application to *H. pylori* proteins was undertaken in this study. Three interacting proteins of *H. pylori* Trx2 have thus far been identified using the mBrB approach, including, thiol peroxidase, elongation factor P and succinyl CoA transferase with one other target refractory to identification by mass spectrometry analysis.

In addition, this approach was applied to AGS cells, in order to determine if *H. pylori* Trx can interact with host cell proteins, an event which could influence pathogenesis. Six putative interacting targets of Trx2 have been identified in AGS cells, including inorganic pyrophosphatase, fatty acid binding protein 5, eukaryotic translation initiation factor 5A, laminin binding protein, thyroid hormone binding protein precursor and thioredoxin reductase. When the amino acid sequence of the identified proteins are examined it is clear that in addition to nearly all of the proteins possessing numerous cysteine residues, two of the identified species, eukaryotic translation initiation factor 5A and the thyroid hormone binding protein precursor, harbour a CXXC motif. For eukaryotic translation initiation factor 5A, these two motifs may cooperate to form a zinc finger structure which facilitates binding to RNA (Lopez-Ribera *et al.*, 1997), for the thyroid hormone binding protein precursor, interaction with Trx may be necessary for modulation of activity of the protein as has been described for related proteins (Lennon, 1992; Tasanen *et al.*, 1988). Application of the thiol-specific probe, mBrB, combined with proteomics and enzyme assays, was used to identify 23 Trx targets in the starchy endosperm of *Triticum aestivum* cv. Butte (wheat), of which twelve targets were not know to be previously Trx-linked, but functional in diverse areas such as metabolism, protein storage, degradation and oxidative

stress (Wong *et al.*, 2003). Most recently, Maeda *et al.* (2003) have described an improvement of the established method for identification of Trx-targeted proteins based on 2-D PAGE and fluorescence labelling of sulphydryl groups. These workers have used a highly sensitive Cy5 maleimide dye and large format 2-D gels, resulting in a 10-fold increase in the observed number of labelled protein spots. Use of this improved dye could be advantageous for identification of additional targets not resolved by methods currently in use.

Trxs from *H. pylori* can interact with a number of different proteins, both from the host and within their own system (Windle *et al.*, 2000), therefore it is important to accurately determine the relative abilities of these proteins to interact with Trx. The interaction of Trx with target proteins is a transient one and the redox mechanism is based on reversible oxidation of two cysteine thiol groups to a disulphide accompanied by the transfer of two electrons and two protons. The net result is the covalent interconversion of a dithiol and a disulphide and mechanisms underlying this reaction are well understood. The transient formation of mixed disulphide intermediates between Trx and interacting proteins can be stabilised by mutating one of the two cysteine residues present in the redox active site. The ability to stabilise Trx-substrate complexes using single cysteine mutants of Trx has been exploited successfully to affinity purify cellular targets of Trx from cell extracts.

In this study three mutant forms of *H. pylori* Trx2 were generated in which two active site cysteines were mutated via site-directed mutagenesis to serine residues individually (C28S, C31S), and in combination (C28S/C31S). Sequencing verified the nature of the individual mutations. Recombinant wild-type Trx2 and recombinant Trx2 active site mutants were purified by ion-exchange chromatography and the purity of the proteins assessed by SDS-PAGE. Interestingly, when subjected to non-reducing SDS-PAGE analysis, Trx active site mutants in which the Cys residues have been individually mutated to Ser residues appear to form higher molecular weight aggregates. Neither the wild-type Trx2 recombinant or the double Trx2 mutant form such aggregates under non-reducing conditions. Therefore, formation of intermolecular disulphide bonds between redox active site mutant monomers may be possible. Results suggest that in the oxidised state, Trx2 monomers are capable of forming aggregates when either of the Cys residues are substituted for Ser in the active site. Removal of the Cys31 residue for Trx2-C28S or the Cys28 residue for Trx2-C31S may facilitate intermolecular disulphide bond formation, leading to the aggregation of

monomers. Complete oxidation of the Trx2 single mutants would most likely result in an increase in the amount of aggregate observed following SDS-PAGE analysis. Further biochemical analyses and circular dichroism (CD) spectral studies will show whether *H. pylori* Trx2-C28S, C31S, C28S/C31S are capable of interacting with *H. pylori* TR and hence whether substitutions of the active site Cys residues has had any effect on the secondary structure of the molecule, though this is unlikely given the nature of the amino acid substitution.

To gain insight into the cellular processes regulated by *H. pylori* Trx2, an approach combining the techniques of affinity chromatography and proteomics was undertaken. This combined process takes advantage of the chemical mechanism by which Trx reduces a specific regulatory disulphide (Brandes *et al.*, 1996; Balmer and Schürmann, 2001; Verdoucq *et al.*, 1999), which requires that a transient heterodisulphide bond is formed between Trx and target prior to complete reduction of the targeted disulphide. The formation of mixed-disulphide intermediates between Trx and a target was initially studied in relation to *E. coli* Trx (Holmgren, 1989) and this phenomenon was then investigated in more detail when studies to elucidate the structure of the intermediate state were initiated (Qin *et al.*, 1994; Jeng *et al.*, 1998). These studies used Trx with a mutated Cys to Ser residue in the active site. It was demonstrated that this normally transient heterodisulphide bond or intermediate state is stabilised when Trx lacks one of the critical cysteine residues of the redox active site (Motohashi *et al.*, 2001). When this stabilisation event occurs, thereby covalently linking the target protein to Trx, it is only through addition of the reductant, DTT, that the target trapped in the intermediate state is released. This affinity approach has been successfully applied to investigation of proteins targeted by (spinach) chloroplast Trx (Motohashi *et al.*, 2001). The combined affinity/proteomics approach was also successfully used to investigate the regulatory functions of chloroplast Trxs (Balmer *et al.*, 2002). Both studies have led to the identification of approximately 35 previously unrecognised potential Trx target proteins functional in a spectrum of processes, some not previously known to be linked to this redox protein.

The four most abundant proteins which bound to the Trx2-C28S affinity column from *H. pylori* NCTC11638 were identified by mass spectrometry. These were urease (HP0075), urease α-subunit (HP0073), elongation factor Tu (EF-Tu; HP1205) and Trx. These proteins are some of the most prevalent proteins present in *H. pylori* total cell lysates when

analysed by SDS-PAGE and additionally have not previously been linked to Trx. These proteins are not known to require reduction of a disulphide bond for activity, a step which would be dependent on the catalytic action of Trx, however, all of these proteins do contain cysteine residues. The urease α-subunit possesses one Cys residue and the urease β-subunit possesses seven Cys residues, however, modulation of the activity of this enzyme has not been linked to a requirement for reduction of disulphide bonds by Trx in the literature thus far. Elongation factor Tu has a well established role in translation and EF-Tu from *H. pylori* possesses two Cys residues. The presence of these Cys residues in these proteins has exploited for design of a purification protocol for the enzymes based on thiol-Sepharose chromatography (Caldas *et al.*, 1998). Efficient purification of EF-Tu and EF-G from *E. coli* was achieved and the authors suggested that properties of the reactive thiols of elongation factors may be of importance. It was also reported by Richarme (1998) that *E. coli* EF-Tu, like Trx, catalyses protein disulphide formation, reduction and isomerisation of RNase. It was observed that EF-Tu forms intramolecular disulphides upon oxidation and that this can result in the appearance of multimeric forms of the protein.

Therefore, the presence of these proteins in the lysate from the affinity columns may be due to non-specific binding to the affinity resin. Indeed, binding of these proteins to the resin could be due to the high concentration of Trx immobilised on the Sepharose matrix, a factor that could result in retention of proteins that normally would not appear to be targeted by Trx (Balmer *et al.*, 2003). Identification of proteins eluted from the Trx2-C28S/C31S affinity column will allow any non-specific proteins to be excluded from a definitive list of interacting proteins, and it is clear from 2-D analysis of proteins from a 'control' column that there are several such non-specific proteins which can be identified. It is obvious that many *H. pylori* proteins are capable of interacting with Trx2 owing to their capability to bind to the Trx2 resin and it is likely that many of the interacting proteins contain cysteines and may be members of processes not previously known to be redox-regulated in *H. pylori*.

Secreted proteins are of particular interest when considering the pathogenesis of *H. pylori* infection considering the close proximity of the bacterium to host tissue. However, proteins which are selectively released by *H. pylori* in its microniche are free to interact with gastric epithelium and have the potential to mediate host-pathogen interactions. As *H. pylori* Trx2 has been identified as one of thirteen proteins selectively released by the bacterium (Kim *et*

al., 2002) and has been shown to be capable of modulating the activity of certain epithelial cell proteins at least *in vitro* (Chapter V; Abdel-Latif, M.M., Ph.D., 2002), the identification of interacting targets in epithelial cells would be of interest. Catalytic reduction of disulphide bonds in host proteins could result in the modulation of activity of factors involved in host-pathogen interactions and the inflammatory response. In this study at least 25 potential target proteins from AGS cells were resolved. It conceivable that these proteins might influence physiological processes and pathways that may come into play during infection and possibly modulate the outcome of the host immune response to the pathogen.

Non-redox associated activities of Trxs are currently being investigated. Recently, chaperone activity has been reported for a prokaryotic Trx and identification of this type of activity associated with Trx may represent a plausible explanation for some of the apparent non-specific binding observed in this study. Chaperones are a class of polypeptide binding proteins that have been implicated in protein folding, protein renaturation, protein degradation and protein targeting to membranes and also in control of protein-protein interactions (Hartl and Hayer-Hartl, 2002). It is known that Trx is capable of interacting with a wide range of proteins (Arner and Holmgren, 2000) and also is employed to increase the solubility of certain fusion proteins (Kapust and Waugh, 1999). *E. coli* Trx has been shown to have chaperone properties (Kern *et al.*, 2003). The authors postulate that the redox properties of Trx do not play a role in chaperone function, as Trx redox active site mutants participate in the refolding of an experimental protein, citrate synthase. It is possible that this chaperone property is not unique to *E. coli* Trx but may be a feature of many other Trxs, perhaps including Trx from *H. pylori*. Preliminary data from our laboratory indicates that *H. pylori* Trx1 has chaperone-like activity (Janice Walshe, M.Sc. Project (2003), T.C.D.). In view of recent evidence it seems that Trx should not be considered simply as a high capacity hydrogen donor for reductive enzymes, but as a regulator of protein function that can operate in various physiological processes within the cell by virtue of a facile redox switch.

In summary, this study sees the initiation of the challenge to elucidate additional Trx-linked processes of *H. pylori*. The Trx system constitutes the only thiol-dependent reduction-oxidation system present in *H. pylori* and therefore the targets of components of this system are of interest. Two different but complementary approaches were utilised in

this study to isolate Trx-interacting targets both from *H. pylori* and gastric epithelial cells. The results of these experiments show that Trx can interact with various proteins, some of which do not have an obvious oxidative stress resistance function. Use of recombinant *H. pylori* Trx1 redox active site mutants in the affinity/proteomics approach should help elucidate any functional redundancy and indeed functional specificity of *H. pylori* Trxs. In uncovering the identity of all proteins targeted by Trxs in *H. pylori*, the function of both Trxs in wider physiological processes within the bacterial cell should become apparent. Acquisition of three dimensional structural data on *H. pylori* Trxs and their target substrates will provide the basis for a detailed mechanistic understanding of these interactions. In addition to identifying potential new Trx targets, the approach utilised in this study should permit construction of a metabolic framework to understand pathways and processes in addition to the redox cascade which may be of importance in virulence and long-term persistence of *H. pylori*.

CHAPTER VII

GENERAL DISCUSSION

CHAPTER VII

General Discussion

7.1 The thioredoxin system of *H. pylori*

With the realisation that the human gastrointestinal tract is not simply an inert barrier to the external environment, an appreciation of the large, diverse and clinically relevant relationship between eukaryotic and prokaryotic cells has been established. Our understanding of host-microbe biology is still in its infancy, however, the influence of host-microbe interactions on human health constitutes an expanding focus of gastrointestinal research. It is clear moreover, that the relationship between host and microbe is critical and that a breakdown in this balance results in the manifestation of disease. Infection of the upper gastrointestinal tract with the gastric pathogen *H. pylori* causes a spectrum of conditions ranging from asymptomatic infection to active chronic gastritis. Infection is also a predisposing factor for the development of both peptic ulcer disease and gastric adenocarcinoma (Cover and Blaser, 1996; Walker and Crabtree, 1998). The bacterium colonises the human gastrointestinal tract and adheres closely to the luminal surface of the gastric epithelium *in vivo* (Hessey *et al.*, 1990). *H. pylori* colonises the stomachs of an estimated half of all humans and maintains residency for the remainder of the lifespan of the host, causing non-symptomatic gastritis (Mobley *et al.*, 2001). Only a subset of hosts go on to develop symptoms of infection which may lead to more serious gastric disease, however, the environmental, host or microbial factors that influence the outcome of infection are as yet not clearly defined. It is clear that *H. pylori* infection represents an example of dynamic co-evolution between human and bacterium which, if dissected, could provide a valuable insight into a spectrum of other pathogen-host relationships which also result in human disease. The frequency and persistence of *H. pylori* infection worldwide is notable and the factors directing infection of the host towards benign co-existence or gastric pathology are of fundamental interest.

In bacteria the cytoplasm is maintained as a reducing environment and this environment prevents inappropriate disulphide bond formation in cytoplasmic proteins, in addition to preventing oxidative inactivation of active site thiols, both of which can result from exposure to oxidative stress. Management of host-generated oxidative stress by *H. pylori* is vital to successful infection and survival of the bacterium. Oxidative stress results in

damage to genetic material, membranes and proteins, therefore, *H. pylori* possess a battery of enzymes to combat reactive oxygen metabolites. Two common response features to oxidative stress include the restoration of redox homeostasis and elimination of the harmful oxidant. Several studies have described the vital role played by sulphydryl groups (-SH) in response to oxidative stress and the thioredoxin (Trx) and glutaredoxin (Grx) systems play a major role in protecting cells from oxidative damage. *H. pylori* differs from other prokaryotes in terms of deficiency in its complement of thiol-dependent redox active proteins. The bacterium is equipped with only one of the two major, almost ubiquitous, intracellular disulphide reducing systems, the Trx system and lacks the Grx system. As oxidative stress results in the formation of deleterious disulphide bonds within cellular proteins, the Trx system is critical for the ability of *H. pylori* to survive the damaging effects of oxidative stress exposure.

The Trx system of *H. pylori* was first characterised by Windle *et al.* (2000) where the redox activity of Trx was characterised using several mammalian proteins of different architecture but all containing disulphide bonds. *H. pylori* Trx was shown to efficiently reduce insulin, human immunoglobulins, and soluble mucin. *H. pylori* was also found to behave as a stress response element as broth grown bacteria secreted Trx in response to chemical, environmental and biological stresses. This study suggested for the first time that *H. pylori* Trx might assist the bacterium during the colonisation process, a role disparate from intracellular redox homeostasis maintenance. *H. pylori* contains two Trxs, one of which, Trx2, has an active site not usually observed in other Trxs from other prokaryotic species. Additionally, Trx2 has an atypical pI (8.1), being more acidic than normal Trxs, which generally have pIs of between 4.5 and 5. These properties combine to make Trx2 an interesting protein for analysis with respect to function. A component of the Trx system in *H. pylori*, thioredoxin reductase (TR), has been shown to be essential for cell survival by systemic allelic replacement mutagenesis (Chalker *et al.*, 2001), however and somewhat intriguingly, *H. pylori trx1/trx2* double mutants are viable (Comtois *et al.*, 2003). This raises the question as to what cellular protein(s) can replace Trx as a donor of reducing equivalents to various cytoplasmic enzymes that require this reduction step to fulfil their catalytic cycle. It appears that TR is capable of interacting with an alternate enzyme which can substitute for Trx when both functional Trx proteins are disrupted following mutagenesis.

7.2 *H. pylori* thioredoxin; a role in oxidative stress management.

Toxicity caused by molecular oxygen appears to be a universal occurrence and the molecular basis of this toxicity has been the focus of much research over the past decade (Imlay, 2003). Antioxidant systems critical to defence of *H. pylori* against ROS generated by the oxidative burst from macrophages and polymorphonuclear leukocytes are key to the ability of the pathogen to establish chronic gastric infection and to combat the hosts inflammatory response. As a microaerophilic organism, *H. pylori* may be especially sensitive to the reactive forms of oxygen, and this oxygen sensitivity is likely to be quite important as *H. pylori* is an organism that must confront the oxidative attack of the host immune system. Membranes and cytoplasmic proteins are susceptible to oxidative damage and a recent study has revealed that the host effectively induces lethal and premutagenic oxidative DNA adducts on the *H. pylori* genome (O'Rourke *et al.*, 2003). O'Rourke *et al.* showed that the environment in the host during the colonisation process can generate oxidative DNA lesions that can affect the survival and genetic information of *H. pylori*. In this study the *H. pylori trx2* gene was disrupted by insertional mutagenesis to test whether Trx2 contributes to oxidative stress management in the *H. pylori*. Phenotypic analysis of the mutants reveal that *H. pylori* Trx2 has a specialised role in oxidative stress resistance in the bacterium, specifically oxidative stress generated by organic peroxides. Trx2 was also found to be capable of providing reducing equivalents to two cellular enzymes involved in the detoxification of ROS, thiol peroxidase and bacterioferritin co-migratory protein. The role of Trx2 in oxidative stress resistance remains less clear than the role of Trx1 (Baker *et al.*, 2001; Comtois *et al.*, 2003), however, this study revealed for the first time that Trx2 may have a particular role in management of peroxide stress in the pathogen. This may be mediated through a cellular detoxification enzyme which requires specific interaction with Trx2 to complete its catalytic cycle. These data support the postulate that *H. pylori* Trxs have different functional roles within the bacterium.

7.3 The host immune response; effect of exogenous *H. pylori* thioredoxin.

Pathogenesis entails not only the differential expression of bacterial genes and action of bacterial virulence factors, but also the response of the host. Colonisation results in dynamic and complex molecular crosstalk between pathogen and host, and host-bacterium relationships can be broadly defined as pathogenic, commensal or symbiotic. The relationship between *H. pylori* and the human host is complex and appears not to be based on a symbiotic formula, as there are no as yet specifically proven benefits to the host

resulting from the presence of the bacterium. Damage to host tissues arises from a sustained inflammatory response to those strains which carry a specific genetic complement of virulence factors. Therefore, the response of the host immune system to *H. pylori* is of interest. Secreted bacterial factors have been shown to be important for host-pathogen interactions and Trx2 has been recently shown to be specifically released (10-50 fold enrichment in the growth medium) from *H. pylori* (Kim *et al.*, 2002). This finding raises the possibility that these proteins a could somehow modulate or influence host responses to infection. Although *H. pylori* is notoriously prone to lysis and thus considered 'leaky', the observed selective enrichment of these proteins in the culture medium argues against such a non-specific mechanism to account for their appearance there, as shown by careful proteomic analysis of the *H. pylori* 'secretome' (Kim *et al.*, 2002). The function of these molecules in the extracellular domain has not yet been determined . Nor is it known if these proteins are released specifically *in vivo*. Additionally, there would be a requirement for an extracellular source of reducing power (TR/NADPH) for the secreted Trx if it was to participate in redox-dependent activity. *E. coli* Trx can readily use mammalian TR to form a functional Trx system (Arner *et al.*, 1999). Although it is not yet known if *H. pylori* Trxs can do the same, this could be readily determined. Nor is it known if the pathogen's Trx would have access to mammalian gastric TR (Hansson *et al.*, 1988) or NADPH (Saru *et al.*, 1975). It is known that *H. pylori* Trx1 can catalytically reduce human immunoglobulins and mucin (Windle *et al.*, 2000). In addition, preliminary studies demonstrate that *H. pylori* Trx1 can reversibly modulate the tight junction integrity of polarised transporting epithelial monolayers using the T84 colonic cell line model (Terrés, A.M., unpublished observations). Other studies demonstrate that Trx1 has anti-inflammatory properties as judged by its ability to attenuate NF-κB DNA-binding activity in a gastric cell line (Abdel-Latif, M.M., Ph.D. Thesis, 2002). Together, these data reveal that *H. pylori* Trx1 is capable of participating in redox reactions in mammalian proteins, at least *in vitro*.

Redox-regulation of transcription factor activation has been shown to occur in a variety of mammalian cell lines and may represent a means by which the immune system could be subject to modulation by bacterial factors, in the case of *H. pylori*, modulation by secreted Trx. This study has shown that *H. pylori* Trx2 is capable of modulating the DNA-binding activity of mammalian transcription factors, NF-κB and AP-1 in the gastric epithelial cell line, AGS, by an as yet undefined mechanism, most likely mediated through the proteins

action as a thiol oxidoreductase. This cell line was used exclusively for experiments investigating the influence of Trx2 on immunomodulation as it is a well characterised cell line which has been used extensively in the literature in investigations examining the effect of *H. pylori* on cell signalling in the gastric environment. The studies undertaken in our laboratory, as mentioned previously, which demonstrate that Trx1 has anti-inflammatory properties as judged by its ability to attenuate NF-κB DNA-binding activity in AGS cells (Abdel-Latif, M.M., Ph.D. Thesis, 2002) were very reproducible and this cell line was used for similar experiments with Trx2 for this reason. Use of an alternate gastric cell line in similar experiments would provide further evidence of the ability of this redox protein to modulate the activity of factors involved in the immune response. Moreover, use of non-malignant primary gastric cells would be necessary to assess the ability of both Trx1 and Trx2 to influence the immune response in the gastric environment. The pathway of *H. pylori* Trx2 induced modulation of NF-κB DNA-binding activity is not clear and preliminary investigations in this study suggest that Trx2 may interact with IκB to influence NF-κB DNA-binding activity. However, investigations carried out with single cells types, whether primary or transformed, cannot mimic the complexity of the intracellular milieu where signalling between cells of different cell lineages, modulates the response of individuals cells to an invading microbe or indeed secreted bacterial factors. Future investigation of the contribution of *H. pylori* Trx2 to immune response modulation within the host must be examined with this caveat in mind.

7.4 Interacting proteins of *H. pylori* thioredoxin.

As few cell types are deficient in the glutathione/glutaredoxin system, regulation of thiol-mediated redox homeostasis is of interest in cells/organisms where significant deficiencies in redox biochemistry are noted. As the Trx system constitutes that only thiol-dependent reduction-oxidation system present in *H. pylori*, interacting proteins of components of this system are of interest. An approach which exploits a thiol-specific fluorescent tag combined with proteomics to flag proteins reduced by *H. pylori* Trx2 *in vivo* was used to isolate potential interacting proteins of *H. pylori* Trx2 in *H. pylori* and in gastric epithelial cells. A more sensitive and specific approach was also used in this study, which exploits the observation that the transient formation of mixed disulphide intermediates between Trx and target proteins can be stabilised by mutating one of the two cysteine residues present in the redox active site. The ability to stabilise Trx-substrate complexes using single cysteine

mutants of Trx has been exploited successfully to affinity purify cellular targets of Trx from various cell extracts. In this study three mutant forms of *H. pylori* Trx2 were generated in which two active site cysteines were mutated via site-directed mutagenesis to serine residues individually (C28S, C31S), and in combination (C28S/C31S) and used to stabilise Trx-substrate complexes from *H. pylori* lysates. This affinity approach has been successfully applied in investigation of proteins targeted by (spinach) chloroplast Trx (Motohashi *et al.*, 2001). The combined affinity/proteomics approach was also successfully used to investigate the regulatory functions of chloroplast Trxs (Balmer *et al.*, 2002). Both studies have led to the identification of approximately 35 previously unrecognised potential Trx target proteins functional in a spectrum of processes, some not previously known to be linked to this redox protein. As much can be inferred about a proteins function by the proteins with which it associates, its cellular location and any changes in these parameters introduced by stimuli, identification of the spectrum of *H. pylori* Trx2 interacting proteins is of great interest.

7.5 FUTURE PROSPECTS

The data presented in this thesis indicates a role for *H. pylori* Trx2 in peroxide stress management. It also reveals that *H. pylori* Trx2 is capable of modulating the DNA-binding activity of mammalian transcription factors involved in the host inflammatory response. Furthermore, *H. pylori* Trx2 is capable of interacting with many target proteins both within the bacterium and gastric epithelial cells. These data all highlight the 'usefulness' and diversity of function of this truly pleiotropic redox enzyme. However, further work undertaken to expand knowledge of the functions of this enzyme itself and within the context of the redox cascade within *H. pylori* could be envisaged. A number of experimental avenues present themselves at this stage, some complementary to the approaches undertaken in this thesis, and others representing a departure from described approaches. These future directions are considered.

Insertional mutagenesis of the *H. pylori trx2* gene and phenotypic analysis of the resulting mutant *H. pylori* strains revealed that absence of a functional Trx2 enzyme and resulted in a notable increase in the sensitivity of the strains to stress induced by the organic peroxides, cumene hydroperoxide and *tert*-butyl hydroperoxide. Complementation of the *trx2* mutation is necessary to rule out polar disruption of adjacent genes by insertion of a resistance cassette, which may have resulted in the observed phenotype following exposure

to peroxide stress. A *H. pylori* Trx1 insertional mutant would be a valuable way of delineating the specificity and redundancy of the two species of Trx in *H. pylori*. Although it is possible that Trx2 has a specific role in the response to organic hydroperoxides, use of a Trx1 mutant in the same assay system is necessary to confirm that this enzyme does not influence the response of Trx2 to organic hydroperoxide-induced stress. Construction of a *trx1/trx2* double *H. pylori* mutant would represent another valuable mutagenesis investigation. Construction of this mutant has been previously reported (Comtois *et al.*, 2003), however, a detailed investigation of the response of this mutant to certain categories of stress, for example, oxidative stress, disulphide stress and others was not undertaken. Construction of this *H. pylori* genotype and a broad phenotypic analysis would be valuable to ascertain if a functional Trx is required for management of other specific types of stress, disulphide stress being of particular interest.

Superoxidase dismutase-deficient mutants have an impaired ability to colonise the mouse stomach, showing that the bacterium is exposed to the toxic effects of oxygen-derived products during infection (Seyler *et al.*, 2001). Olczak *et al.* (2003) investigated the ability of *H. pylori* strains with mutations in either *tpx* (encoding thiol-peroxidase) or *ahpC* (encoding alkylhydroperoxide reductase) to colonise mouse stomachs. The *tpx* strain was shown to only colonise 5% of inoculated mice. In addition, neither of two *ahpC* mutants in a murine-adapted strain, SS1, background (Olczak *et al.*, 2002) were able to colonise mouse stomachs, in contrast to a 78% colonisation rate with the parent strain. As *H. pylori* Trx2 appears to be required for resistance to peroxide stress, it would be interesting to test whether a *H. pylori trx2* mutant strain is capable of colonising a mouse stomach. Expanding this approach to test the colonisation efficacy of *H. pylori trx1* mutants and *H. pylori trx1/trx2* double mutants in the same animal model would also undoubtedly yield interesting results and help to establish if the Trx system has a formal role in colonisation and infection persistence *in vivo*.

H. pylori Trxs interact with a wide spectrum of proteins within the cell and one way of exploring the biochemical functions of Trx in *H. pylori* is to identify interacting proteins. Construction of redox active site *H. pylori* Trx1 mutants would be useful for assessing the functional specificity of both *H. pylori* Trxs. A Trx1 Cys-Ser single mutant could be used in the same manner as mutant Trx2 in an affinity chromatography/proteomics approach to isolate Trx1 interacting proteins within *H. pylori* and additionally within human gastric

epithelial cells. A comparison of the target spectrum for each redox protein would give valuable insight into the redox cascade operating within the bacterium. Furthermore, use of a recombinant *H. pylori* Trx1 redox active site mutant in a affinity/proteomics approach should help elucidate any functional redundancy of *H. pylori* Trxs in relation to both targets and cellular processes. Plasmid constructs for redox active site mutants of *H. pylori* Trx2 in an appropriate shuttle vector could be used to complement Trx2-deficient *H. pylori* strains to probe for activities not directed by the redox function of the enzyme. Such a strain could be used to assess the function of the protein in non-redox related cellular processes, such as chaperone function.

Most studies investigating the bacterial response to oxidative stress have focused on the response of the bacterium to a spectrum of agents which induce oxidative stress, such as hydrogen peroxide, organic hydroperoxide and superoxide-induced stress, however, other more subtle categories of oxidative stress have not received such attention. Recently, DNA macroarray and proteome analysis was used to analyse the response of *Bacillus subtilis* to disulphide stress induced by diamide, a specific oxidant for thiols (Leichert *et al.*, 2003). The authors found that the response to disulphide stress was a complex combination of different regulatory networks and that redox-sensing cysteines appeared to play a key role in different signalling pathways sensing various different kinds of stress, including heat and toxic elements. In addition, it seemed that the global response of *B. subtilis* to disulphide stress showed a close relationship to hydrogen peroxide-induced oxidative stress and paraquat-induced stress. Transcriptome experiments showed that *B. subtilis* Trx and TR were induced over time following exposure to disulphide stress. A similar approach applied to *H. pylori* and its response to disulphide stress could yield valuable information regarding the role of redox-sensing cysteine containing proteins in the pathogen and could give an overall view *H. pylori* regulatory mechanisms triggered by disruption of redox homeostasis within the bacterial cell.

Variations in the architecture of the active site have been shown to be critical for determining the redox potentials for Trx (Chivers *et al.*, 1997; Takahashi and Creighton, 1996; Krause *et al.*, 1991) and an understanding of the redox potential for this enzyme will be critical for characterising the redox partners of Trx2 in *H. pylori*. The reaction between Trx and thioredoxin reductase (TR) can be used to calculate the redox half-reaction potential for Trx molecules. An assay examining the ability of Trx to act as a substrate for

TR which allows determination of the equilibrium potentials of oxidised and reduced Trx and NADP(H) will facilitate the calculation of redox potentials using the following formula:

$$E_o'(Trx) = E_o'(NADP) + RT/nF \ln ([NADP][Trx\text{-}(SH)_2]/[NADPH][Trx\text{-}S_2])$$

Calculation of the redox potential of Trx2 will also shed light on the function of the unique active site residues, Pro and Asp, which replace the consensus active site residues, Gly and Pro, found in most other Trx molecules. Perhaps the unique redox active site of *H. pylori* Trx2 serves to facilitate interaction with a different range of proteins than Trx1. Structural data currently being accumulated should be informative as to the validity of this hypothesis. Variation of the consensus active site residues may cause subtle structural rearrangement in the vicinity of the active site of the enzyme and therefore allow Trx2 to form complexes with specific cellular proteins. Moreover, the unique active site may be less oxidising than the consensus *E. coli* Trx (- 270 mV) and this might influence the catalytic efficiency of Trx2 when it encounters certain interacting proteins *in vivo*. Additional insight into the function of *H. pylori* Trx2 could be gained by expanded site-directed mutagenesis experiments. Mutation of the active site residues, Pro and Asp, to the consensus active site residues, Gly and Pro, simultaneously, would provide a way to assess whether the biochemical interactions of this protein are subject to influence of the redox potential of the enzyme. Replacement of these residues with the consensus residues would highlight whether *H. pylori* Trx2 and Trx1 are functionally interchangeable, or if subtle structural changes due to the nature of the redox active site amino acids are responsible for directing certain substrate interactions. As *H. pylori* Trx2 is a small molecule, NMR based structural investigations would be possible and relatively straightforward. These additional data which would provide the structure of oxidised/reduced Trx2 in solution would be complementary to the crystallographic structural investigations of this protein recently published (Filson *et al.*, 2003) and currently underway with collaborators. Circular dichroism (CD) spectral studies could show whether *H. pylori* Trx2-C/28S, C31S, C28S/C31S are capable of interacting with *H. pylori* TR.

Analysis of complete genome sequence of *H. pylori* led to the prediction that the bacterium has a relatively limited capacity for gene regulation (Alm *et al.*, 1999; Berg *et al.*, 1997). *H. pylori* possesses just three sigma factors and global regulators, such as RpoS, OxyR and SoxRS, are absent (Alm *et al.*, 1999; Berg *et al.*, 1997; Tomb *et al.*, 1997). One of the few

global regulators present in *H. pylori* is the Fur protein, with the most intensively studied function of this enzyme in iron transport control, however Fur also has a role in urease expression modulation in response to nickel and in acid (Escolar *et al.*, 1999; Bijlsma *et al.*, 2002; Bereswill *et al.*, 2000; Delany *et al.*, 2001; van Vliet *et al.*, 2001). Fur is often found to be involved not only in autoregulation in response to iron, but also in autoregulation in response to other environmental factors. The Trx system may be a part of an oxidative stress operon functioning within the pathogen, however, there is not yet any information on how distinct oxidative stresses might differentially regulate the expression of components of the Trx system. In *E. coli*, the expression of Trx2 (*trxC*) is regulated by the transcriptional activator OxyR in response to oxidative stress (Ritz *et al.*, 2000). Oxidised OxyR binds directly to the *trxC* promoter and induces its expression in response to elevated hydrogen peroxide levels or the disruption of one or several of the cytoplasmic redox pathways. Recently, the importance of disulphide bond formation was underscored by observations that the activation of the important prokaryotic oxidative stress responsive transcriptional activator, OxyR, was dependent on this regulatory event (Zheng *et al.*, 1999), thus providing the first well characterised example of oxidative stress response activation by disulphide bond formation. Activation of OxyR induces peroxidase expression and oxidant clearance from the cytosol.

An interesting future study could investigate if *H. pylori* Trx2 is subject to any genetic controls which may represent a paired down version of genetic control exhibited by other enteric bacteria, perhaps reflecting the genetic conservation imposed by possession of a small genome. It may be that Trx2 deferentially regulated than Trx1 in response the stress and may explain why this study has shown that in the absence of a functional Trx2 protein, but with a functional Trx1 protein, the bacterium exhibits sensitivity to organic peroxide induced stress. Further investigation of *H. pylori* Fur-mediated gene regulation, identification of further members of the Fur regulon coupled with definition of binding sites of Fur in the promoters of the regulon may help understand in more detail how the bacterium's response to oxidative stress in regulated.

During the course of this study two important and related papers were published, both of which complement the work described in this thesis (Baker *et al.*, 2001; Comtois *et al.*, 2003). Both groups have revealed that the Trx system of *H. pylori* is of importance in oxidative stress management, with both Trx species appearing to exhibit both redundancy

and specificity when analysed in the context of oxidative stress resistance and electron donor capacity to other antioxidant enzymes which require formation of a disulphide bond to complete their catalytic cycles. This study, which focused on the unusual Trx2 enzyme revealed that it contributes to oxidative stress management in the bacterium and also is capable of interacting with a wide variety of proteins within the bacterium itself and also with proteins from a gastric epithelial cell line. Further physicochemical and functional characterisation of Trx2 will be enhanced with completion of the high resolution three-dimensional structural model (Filson *et al.*, 2003) using Trx2 protein purified during the course of this study.

As the thiol metabolism of *H. pylori* differs to that described in many other prokaryotic species, components of the Trx system may represent putative targets for the development of novel anti-*H. pylori* therapies. With the convergence of proteomics and genomics on *H. pylori*, many of the uncertainties and idiosyncrasies characteristic of *H. pylori* pathogenicity currently unresolved, may soon be dissected and understood. The genomic and proteomic revolution will undoubtedly serve to elucidate the dynamic process of *H. pylori*/host co-evolution and consequently contribute to the understanding of a wider spectrum of bacterium-host relationships that currently result in damage to the host.

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Appendix A

Reagents and Buffers

Cell Culture Reagents

Culture Cocktail

L-Glutamine (20 mM)	100 ml
Penicillin (100 U/ml) /Streptomycin (10 μg/ml)	100 ml

Stored in 10 ml aliquots at –20°C. One aliquot used per 500 ml medium.

RPMI Complete Medium

RPMI 1640	500 ml
Heat inactivated FCS	50 ml
Culture Cocktail	10 ml

Hanks Balanced Salt Solution (HBSS) Washing Solution

Hanks Balanced Salt Solution	500 ml
1M Hepes Buffer	10 ml

Ethidium Bromide (EB) Stock

Ethidium Bromide	100 mg
PBS	20 ml

Acridine Orange (AO) Stock

Acridine Orange	20 mg
PBS	20 ml

EB/AO Working Solution

Ethidium Bromide Stock	4 ml
Acridine Orange Stock	4 ml
Distilled Water	100 ml

Cell Culture Cryopreservative Medium

FCS	9 ml
Dimethylsulfoxide	1 ml

10 x Phosphate Buffered Saline (PBS)

$Na_2HPO_4.2H_2O$ (8 mM)	14.24 g
KH_2PO4 (1.5 mM)	2.04 g
NaCl (137 mM)	80.0 g
KCl (2.7 mM)	2.0 g

Adjust pH to 7.4 and made up to 1 L final volume.

1% Nonidet P-40

PBS	100 ml
Nonidet P-40	1 ml

Protease Inhibitors

PMSF	1 M
Leupeptin	2 mg/ml
Roche Complete® protease inhibitor	

Prepared as per manufacturers' instructions (1 tablet per 2 mls distilled water).

Bradford Reagent

Coomassie Brilliant Blue G	100 mg
96% Ethanol	50 ml
0.85% orthophosphoric acid	100 ml

Made up to 1 L final volume with distilled water and filtered prior to use.

Markwell Assay – Reagent A

$NaCO_3$	2 % (w/v)
NaOH	0.4 % (w/v)
Potassium/sodium Tartrate	0.6 % (w/v)
SDS	1 % (w/v)

Prepare 200 ml and store at room teperature.

Markwell Assay – Reagent B

$CuSO_4.5H_2O$	4 % (w/v)

Markwell Assay – Reagent C

1 volume reagent A + 100 volumes reagent C

Prepare fresh immediately prior to use.

Markwell Assay – Reagent C

Folins Reagent – dilute 1/3 with distilled water

5X Reducing Sample Buffer

Glycerol	5 ml
β-mercaptoethanol	6.25 ml
20 % SDS	5 ml
1.0 M Tris pH 6.8	1.25 ml
0.2 % Bromophenol Blue	0.3 ml

Make up to 25 ml with distilled water.

Resolving Gel Buffer

Tris base	18.165 g
Distilled water	100 ml

Adjust pH to 8.8 with conc. HCl.

Stacking Gel Buffer

Tris base	12.11 g
Distilled water	100 ml

Adjust pH to 6.8 with conc. HCl.

10% Ammonium Persulphate (APS)

APS	50 mg
Distilled water	1 ml

Prepared fresh prior to use.

Water-Saturated Butanol

n-Butanol	50 ml
Distilled water	50 ml

Solution was mixed vigorously and upper phase used.

10X Running Buffer

Tris base	30 g
Glycine	114 g
SDS	5.0 g
Distilled water	1000 ml

Diluted 1:10 prior to use.

Transfer Buffer

Tris base	2.9 g
Glycine	1.45 g
SDS	0.185 g
Methanol	100 ml

Make up to 500ml with distilled water.

Coomassie Blue R-250 Gel Stain

Coomassie Brilliant Blue R-250	0.5 g
Methanol	200 ml
Glacial acetic acid	35 ml
Distilled water	265 ml

Coomassie Blue G-250 Gel Stain (for Mass Spectrometry)

Fixation Solvent

50 % Methanol	500 ml
2 % Phosphoric Acid (85 %)	23.5 ml
Distilled water	480 ml

Incubation Solvent

34 % Methanol	340 ml
2 % Phosphoric Acid (85 %)	23.5 ml
17 % Ammonium Sulphate	170 g
Distilled water	640 ml

Destain Solution

Methanol	400 ml
Glacial acetic acid	70 ml
Distilled water	530 ml

Immunoblotting

Blocking Solution

Skimmed dried milk powder (Marvel)	5 g
PBS	100 ml

0.1% PBS-Tween Washing Solution

Tween 20	1 ml
PBS	1000 ml

Primary and Secondary Antibody Diluent Solution

Skimmed dried milk powder (Marvel)	5 g
PBS	100 ml

Sodium Azide 0.05%

Sodium azide (10%)	2 μl
Distilled water	1 ml

Amido Black Protein Detection

Amido Black	0.5 % (w/v)
Isopropanol	25 % (v/v)
Acetic Acid	10 % (v/v)

Staining was for 2 min and destaining was done by several washes in deionised water.

Ponceau S Protein Detection

Ponceau S	0.2 % (w/v)
TCA	(3 % (v/v)

Staining was carried out until bands were visible and destaining was done by several washes in deionised water.

Stripping Buffer

Tris base pH 6.8 (62.5 mM)	6.25 ml
β-mercaptoethanol (100 mM)	0.68 ml
SDS (2%)	20 ml

Make up to 100 ml final volume with distilled water.

Enhanced Chemiluminescence

Developing Solution

Iodophenol	4 mg
Luminol (Sodium Salt)	12 mg
DMSO	0.5 ml
0.1 M Tris pH 8.8	50 ml
H_2O_2	18 µl

Cytosolic Preparation Extract

Cell Lysis Buffer – Buffer C

Tris base (20 mM)	to 50 ml
SDS (1%)	0.5 g
NaCl (150 mM)	7.5 ml
EDTA (1 mM)	100 µl
EGTA (1 mM)	0.5 ml
PMSF (0.5 mM)	2.5 µl
Leupeptin (10 µg/ml)	25 µl

Nuclear Extract Preparation

Extraction Buffers – All stocks should be sterile.

Buffer A	**Stock Soln.**	**Volume**
Magnesium chloride (1.5 mM)	1 M	15 µl
Potassium chloride (10 mM)	1 M	100 µl
Hepes buffer, pH 7.9 (10 mM)	1 M	100 µl
PMSF	1 M	5 µl
DTT	1 M	5 µl

Make up to 10 ml with sterile water.

Buffer C	**Stock Soln.**	**Volume**
Magnesium chloride (1.5 mM)	1 M	15 µl
Sodium chloride (420 mM)	5 M	100 µl
Hepes buffer, pH 7.9 (20 mM)	1 M	100 µl
PMSF (0.5 mM)	0.1 M	5 µl
EDTA (0.2 mM)	0.5 M	4 µl
25% Glycerol		2.5 ml

Make up to 10 ml with sterile water.

Buffer D	**Stock Soln.**	**Volume**
Potassium chloride (50 mM)	1 M	400 µl
DTT (0.2 mM)	5 M	2 µl
Hepes buffer, pH 7.9 (10 mM)	1 M	100 µl
PMSF (0.5 mM)	0.1 M	20 µl
EDTA (0.2 mM)	0.5 M	1.6 µl
20% Glycerol		800 µl

Make up to 10 ml with sterile water.

Electrophoretic Mobility Shift Assay

10 X Tris Borate EDTA buffer (TBE)

Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 ml

Make up to 1L with distilled water and autoclave.

0.5 X TBE Running Buffer

10X Tris Borate EDTA buffer	50 ml
Distilled H_2O	450 ml

TE Buffer (1 X)

Tris base (pH 8.0)	10 mM
EDTA	1 mM

Binding Reaction Buffer (10 X)	**Stock Soln.**	**Volume**
Tris pH 7.5 (100 mM)	1 M	50 µl
NaCl (1 M)	5 M	100 µl
Glycerol (40%)		200 µl
EDTA (10 mM)	0.5 M	10 µl
DTT (50 mM)	1 M	25 µl
Nuclease free BSA (1 mg/ml)	10 mg/ml	50 µl

Make up to 500 µl with sterile water.

Prokaryotic Cloning

Luria-Bertani (LB) Broth Medium

Gibco® LB Powder	37 g
Distilled water	1000 ml

Antibiotic Selection

Ampicillin	100 mg/ml
Kanamycin	30 mg/ml

Prepared in distilled water, sterile filtered (0.2 µ), aliquoted and stored at –20°C.

Chloramphenicol	50 mg/ml

Prepared in ethanol, aliquoted and stored at –20°C.

Plasmid Screening Solutions

Solution II

NaOH (1 M)	1 ml
SDS (10%)	0.5 ml

Sterile water	3.5 ml

Prepare fresh.

Lysis Solution

Solution II	4 ml
Gel loading buffer	0.9 ml
Sterile water	1.1 ml

Solution III

Formic acid (1.8 M), Potassium Acetate (3 M)	3 µl

Competent Cell Preparation Buffers:

Washing Buffer

1M Tris (pH 8.0)	10 ml
1M $CaCl_2$	50 ml
1M $MgCl_2$	10 ml
Distilled water	930ml

Freezing Buffer

Glycerol	15 ml
1M Tris (pH 8.0)	1 ml
1M $CaCl_2$	5 ml
1M $MgCl_2$	1ml
Distilled water	78 ml

Agarose Gel Electrophoresis

TAE Buffer (10X)

Tris base	24.2 g
Glacial Acetic Acid	5.71 ml
EDTA, pH 8.0 (0.5 M)	20 ml

TAE Running Buffer

TAE	50 ml

Distilled water	450 ml

Gel Loading Dye (6 X)

Xylene cyanol (0.25%)	0.125 g
Bromophenol Blue (0.25%)	0.125 g
Glycerol (30%)	15 g

Make up to a final volume of 50ml with distilled water.

2-D Gel Electrophoresis

Rehydration Stock Solution

Urea (8 M)	12 g
CHAPS (2 % w/v)	0.5 g
Bromophenol Blue	trace

Make up to a final volume of 25 ml with double distilled water. Store in 1 ml aliquots at –20°C. Before use add 2.8 mg DTT and 5 µl IPG buffer (Amersham) per 1 ml aliquot of rehydration stock solution.

SDS Equilibration Buffer	**Final Conc.**	**Amount**
Tris-HCl (1.5 M, pH 8.8)	50 mM	6.7 ml
Urea	6 M	72.07 g
Glycerol (87 % v/v)	30 % (v/v)	69 ml
SDS	2 % (v/v)	4.0 g
Bromophenol Blue	trace	trace

Make up to a final volume of 25 ml with double distilled water. Store in 20 ml aliquots at –20°C. Before use add 100 mg DTT per 10 ml SDS equilibration buffer.

H. pylori Growth Media

Cryogenic Preservative Freezing medium

Brain Heart Infusion Media	1 ml
Glycerol	20 % (v/v)
Horse Serum	7 % (v/v)

Columbia Blood Agar Medium

Columbia Agar base	4.1 g
Sterile Horse Blood	7 ml
DENT Supplement	400 µl/100 ml broth

Appendix B

Publications

Henry J. Windle, **Áine Fox**, Déirdre Ní Eidhin and Dermot Kelleher. 2000. "The Thioredoxin System of *Helicobacter pylori*."
Journal of Biological Chemistry, Vol. **275**, No.7, pp. 5081 – 5089.

Heather Filson[§], **Áine Fox**[§], Dermot Kelleher, Henry J. Windle and David A. Sanders. 2003. "Crystallisation and Preliminary Structure Analysis of an Unusual Thioredoxin from *Helicobacter pylori*."
Acta Crystallographica, Biological Crystallography, Vol. **D59**, pp. 1280 - 1282.
§ authors of equal contribution

Henry J. Windle, **Áine Fox**, Ana Terrés and Dermot Kelleher. 2003. "Cloning, overexpression and characterisation of thiol peroxidase and bacterioferritin comigratory protein from *Helicobacter pylori*: thioredoxin 1 but not thioredoxin 2 targets "
(Manuscript submitted to Journal of Bacteriology, May 2003)

Presentations

Poster Presentation: "The *Helicobacter pylori* Redox Network: Analysis of a Redox Pathway via Modulation of Thioredoxin Affinity for Target Proteins *in vitro*."
Áine Fox, Henry J. Windle, Kenneth Whelan and Dermot Kelleher.
Digestive Disease Week 2003, American Gastroenterology Association, Orlando, Florida. 17^{th} – 22^{nd} May 2003.

Poster Presentation: "Analysis of a *Helicobacter pylori* Thioredoxin 2 Mutant in Response to Oxidative Stress"
Áine Fox, Henry J. Windle and Dermot Kelleher.
4^{th} International Workshop on Campylobacter, Helicobacter and Related Organisms, Germany. 2001.

Poster Presentation: "*Helicobacter pylori* Thioredoxin and the Oxidative Stress Response"
Áine Fox, Henry J. Windle and Dermot Kelleher.
Irish Society of Gastroenterology, Spring Meeting. 2002.

Poster Presentation: "The *Helicobacter pylori* Redox Network: Identification of Target Substrates of the Redox Protein Thioredoxin."
Áine Fox, Henry J. Windle, Kenneth Whelan and Dermot Kelleher.
Irish Society of Gastroenterology, Summer Meeting. 2003.