The University Of Sheffield.

Pushing the envelope: periplasmic networks of the zoonotic pathogen *Campylobacter jejuni*

A thesis submitted in part fulfilment for the degree of

Doctor of Philosophy

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Abstract

Campylobacter jejuni is a Gram-negative, pathogenic bacterium which is commensal in a large portion of chicken flocks and causes disease in humans through the consumption of contaminated poultry: the leading cause of gastroenteritis worldwide. *C. jejuni* is an epsilon-proteobacterium, far distinct from other canonical pathogens such as *E. coli* or *Salmonella spp.*, and as such presents a unique challenge in unravelling the molecular mechanisms which make *C. jejuni* such a successful pathogen worldwide. This alternate format thesis presents two published papers and a third chapter of unpublished work, presented in manuscript format. The work presented covers various aspects of the cell envelope, including a genetic linkage study focused on the periplasmic formate dehydrogenase complex and a novel nucleotide salvage pathway, the periplasmic chaperone network of *C. jejuni*, and the periplasmic methionine sulfoxide reductase system. The results described herein further our knowledge of *Campylobacter* metabolism and pathogenesis, and have promising implications for the control of this unique pathogen.

Acknowledgments and advice

The First Truth is that all life is suffering, pain, and misery. The Second Truth is that this suffering is caused by selfish craving and personal desire. The Third Truth is that this selfish craving can be overcome. The Fourth Truth is that the way to overcome this misery is through gaining publications with an impact factor of greater than 4.

In regards to my PhD I can only say that it has been the best time of my life, then again I grew up on a council estate and was an ugly teenager, so perhaps that's not saying much after all. For anyone considering a PhD I would urge them to do so as you will not have the same freedom of thought in any other career: the one true liberation from life.

For those doing a PhD, tough times lie ahead. You don't really know enough to do things right the first time and chances are you'll do it wrong the second time anyway. You must accept this, remain calm, and move on. There are always new things to discover and you should remind yourself often that you are incredibly lucky to be doing what you are. Persistence is key: it will all come together in the end, though it may not feel like it now.

Who do I thank: of course Dave for his constant guidance and insight, without which I don't know where I would be. But perhaps more importantly his tolerance of my transient brushes with insanity: I suspect most others would have booted me out early doors. On that note, I also thank the NHS for their tasty drugs which kept me on the level long enough to get an assay in then and again.

I also thank all Kelly lab members past and present, whether I liked you or not: you have all had an impact on my way of working and have taught me a great deal. I think our lab is unique in that we are (mostly) all friends, rather than just colleagues, which does more for productivity than any screaming supervisor ever would. Undoubtedly, Dave is the best mentor, to quote Dr. Clarkson, *in the world*, and the success of his students is testament to that.

See you on the other side,

Aidan

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Chapter 1: General Introduction

1.1 *Campylobacter jejuni*

1.1.1 Importance

C. jejuni is one of the leading causes of food-borne gastroenteritis worldwide. It is widely considered a commensal in chicken flocks and is able to colonise humans as an opportunistic pathogen, normally through the consumption of under-cooked or otherwise contaminated poultry. Despite being a microaerophile, *C. jejuni* persists through numerous environments with various oxygen tensions, from the fully aerobic atmosphere of the abattoir to the near-anaerobic gut lumen of the host. Although generally acute and self-limiting in humans, the economic cost of *Campylobacter* infections in the UK alone is predicted to be between £50-100m per annum (Tam and O'Brien 2016), and can in rare cases trigger harmful sequelae including Guillain-Barré syndrome and reactive arthritis (Esan *et al*. 2017). Antibiotic resistance is a developing issue with *Campylobacter*, with increased resistance to macrolides, such as erythromycin, and fluoroquinolones, such as ciprofloxacin, rising significantly in recent years (Post *et al*. 2017, Tang *et al.* 2017). As such, understanding the metabolism, virulence and organisation of *Campylobacter* at a molecular level is crucial to design and implement new preventions.

1.1.2 Prevalence

Although most commonly associated with poultry, *Campylobacter* is also prevalent in both wild and domestic birds, pigs, cattle and sheep (Cha *et al*. 2017, Denis *et al*. 2016, Sanad *et al*. 2014, Waldenström *et al*. 2002). As well as providing additional transmission routes to humans, *Campylobacter* infection in livestock induces inflammation, causing a reduction in both wellbeing and yield, and, particularly in sheep and cattle, can result in enzoonotic abortion (Campero *et al*. 2005, Sanad *et al*. 2014). Nonetheless, contaminated chicken remains the major cause of *C. jejuni* transmission to humans, with European Food Safety Authority (EFSA) surveys indicating that 50-80% of all human cases of campylobacteriosis originate from chicken, and an average of 71.2% of broiler flocks in the EU were *Campylobacter* positive in 2008 (EFSA 2010). More recently in the UK, the Food Standards Agency (FSA) compiled a report of *Campylobacter* prevalence across all major supermarkets and found that an average of 72.8% of chicken skin was contaminated, with an average of 6.9% of all packaging also testing positive (FSA 2015).

1.1.3 Physiology

C. jejuni is a Gram-negative bacterium with a single chromosome, with some subspecies able to harbour plasmids conferring antibiotic resistance and other traits. The genome of *C. jejuni* is relatively small, around 1.6 MBp or 1700 genes, compared to *E. coli* or *S. aureus* with 4.4 and 2.8 MBp, respectively. However, as an epsilon-proteobacterium, quite distinct from most well studied bacteria, its small genome encodes many uncharacterised proteins with often little to no known homology to give clues as to function.

Campylobacters are naturally highly competent, resulting in exceptional genetic diversity and rapid spread of antimicrobial resistance. Natural competence has been attributed to a number of factors, but most specifically the Cts system, namely the nucleoside triphosphate binding proteins CtsE and CtsP, which are similar to components of Type II secretion systems from other Gram-negatives that are essential for transformation efficiency in *C. jejuni* (Beauchamp *et al*. 2015). Natural transformation can occur even under conditions where growth is highly restricted, requiring only an active electron transport chain and effective translation, supporting the idea that *C. jejuni* can adapt to its environment and persist even when growth is not immediately viable, explaining its ability to survive transition between numerous environments (Vegge *et al*. 2012).

Further to this concept, *C. jejuni* is capable of entering a viable but non-culturable state (VBNC) in response to extreme stress conditions, akin to spore formation in Gram-positives, permitting survival until the environment becomes more conducive to growth (Jackson *et al*. 2009). The existence of the VBNC state coupled with the fastidious culturing conditions required for *C. jejuni* growth *in vitro* likely contributes to a gross underestimate of its prevalence. Indeed, there is substantial literature and expanding research focused on improved detection methods for *C. jejuni* as high-throughput culturability is so poor (Kim *et al*. 2016, Haas *et al*. 2017, Nachamkin and Nguyen 2017, Rodgers *et al*. 2017).

1.2 Virulence and pathogenesis

1.2.1 Campylobacteriosis

The main route of *Campylobacter* infection in humans is through the consumption of undercooked chicken, or cross-contamination of food prepared on the same surface. The second highest route of transmission is unpasteurised milk, but can also rarely arise from animal-to-human or human-to-human transmission (Modi *et al*. 2015). The symptoms of campylobacteriosis are primarily diarrhoea, often haemorrhagic, and abdominal pain, but can also include nausea, vomiting, fever and muscle pain. Symptoms generally last about a week, with severity declining after the first 24-48hrs, though there is a 20% chance of

relapse (Blaser 1997). A systematic review of campylobacteriosis incubation time determined a mean range of 2.5 to 4.3 days, with children showing much shorter incubation periods compared to adults (Awofisayo-Okuyelu *et al*. 2017). It is well known that while infection with *Campylobacter* does elicit an antibody response it does not protect from reinfection with the same strain, so under endemic conditions serial reinfection can occur, though the incubation time may be increased and reduce to risk of infection after exposure (Awofisayo-Okuyelu *et al*. 2017, Black *et al*. 1988, Russell *et al*. 1990, Strid *et al*. 2001). IgA and IgG antibodies raised by infection can trigger sequelae including Guillain-Garré syndrome, reactive arthritis and inflammatory bowel disease, with 34-49%, 44-62% and 23-40% of respective cases associated with *Campylobacter* infection (Zautner *et al*. 2014).

1.2.2 Cytolethal distending toxin

Cytolethal distending toxin (CDT) is a genotoxin utilised by a number of pathogens, including *C. jejuni*, that attacks host DNA to introduce double stranded breaks, inducing senescence and apoptosis. CDT is composed of three subunits; CdtA and CdtC bind to cholesterol rich lipid rafts on the cell surface and permit CdtB, the active type-1 deoxyribonuclease, to permeate into the cell via clathrin-dependent endocytosis (Figure 1). In the cytosol CdtB translocates across the golgi and endoplasmic reticulum by hijacking host retrograde trafficking pathways. CdtB is equipped with a nuclear localisation signal in its amino acid sequence that transports it into the nuclease. Here, CdtB introduces double stranded DNA breaks into the host genome which ultimately results in cell cycle arrest and cell death (Boesze-Battaglia *et al*. 2009, Lara-Tejero and Galán, 2000).

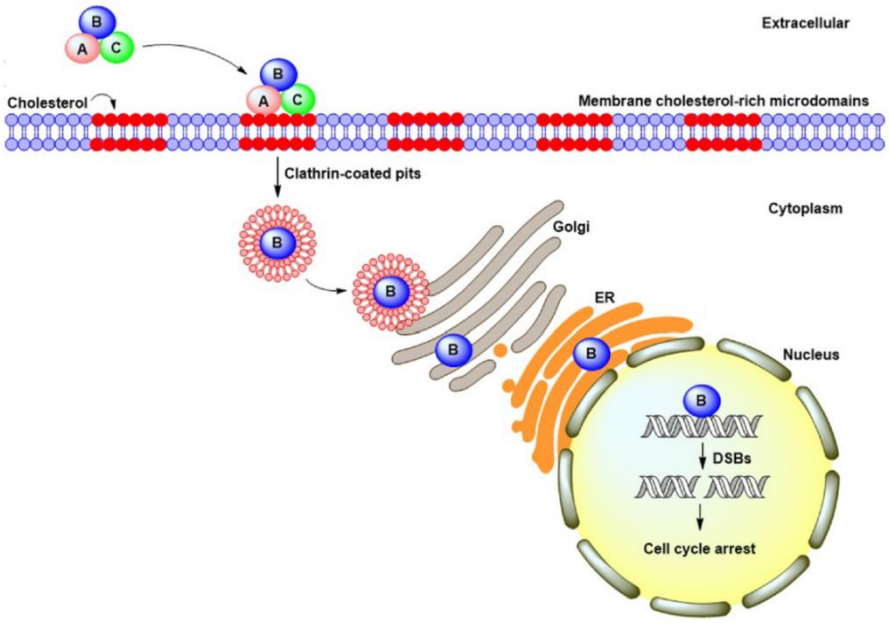

Figure 1: CDT cellular intoxication pathway (Lai *et al*. 2016).

1.2.3 Outer membrane vesicles

Like most Gram-negative pathogens, *C. jejuni* utilises outer membrane vesicles (OMVs) to deliver virulence factors to the environment and host cells. Historically there have been numerous models of OMV biogenesis, but recently a model based on a retrograde phospholipid transport system has been proposed which is highly conserved in Gram-negatives and for the first time provides an adequate explanation of regulation (Figure 2) (Roier *et al*. 2016). The retrograde phospholipid transport system was identified previously and characterised as maintaining outer membrane lipid asymmetry (Mla system) (Malinverni and Silhavy 2009). The system comprises an outer membrane (OM) associated protein (MlaA or VacJ), a periplasmic lipid binding protein (MlaC) and an inner membrane ABC-type transporter complex (MlaBDEF). Putatively, MlaA extracts aberrant phospholipids (PLs) from the outer leaflet of the OM, passes them to MlaC which chaperones the lipids across the periplasm to the MlaBDEF complex, where they are presumably recycled into the inner membrane. In terms of OMV biogenesis, suppression of the Mla system leads to an accumulation of OM PL bilayers, which induces spontaneous budding of vesicles from the membrane (Roier *et al*. 2016). Thus, OMV production can be actively regulated by controlling OM PL bilayer content via the Mla system.

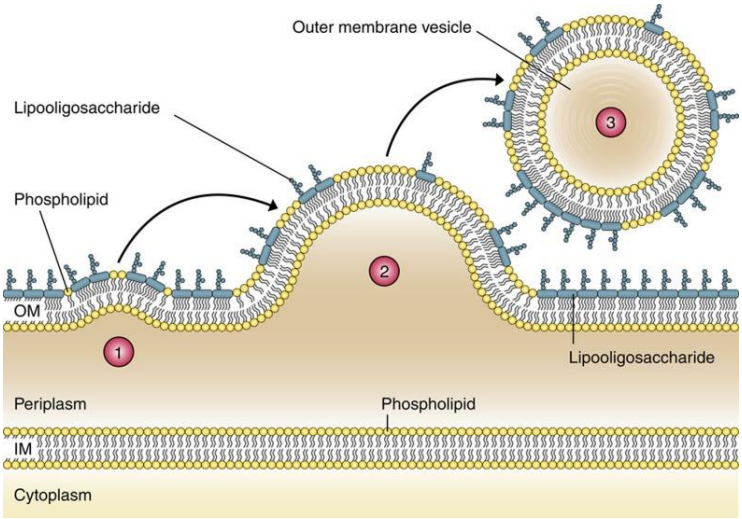

Figure 2: A new model of OMV formation in Gram-negative bacteria (Roier *et al*. 2016).

CDT delivery is almost certainly via OMVs: all three subunits have been detected in OMV's and the majority of extracellular CDT is strongly associated with OMV's (Lindmark *et al*. 2009). Indeed, isolated OMV's exert cytotoxic effects on human cell lines similar to that of CDT, suggesting they are present and biologically active, although the induction of host interleukin-8 (IL-8) has been demonstrated to be CDT-independent (Lindmark *et al*. 2009, Elmi *et al*. 2012).

Two independent studies on the proteome of *C. jejuni* OMV's have been conducted, identifying 151 and 134 vesicular proteins, respectively (Elmi *et al*. 2012, Jang *et al*. 2014). CDT was detected in both studies, along with numerous *N*-linked glycoproteins. *N*-linked glycoproteins are located in the periplasm, and so are not exposed on the cell surface, but within OMV's they can be delivered directly to the target, avoiding immune detection during transit (Elmi *et al*. 2012). *N*-linked glycosylation may also play a role in OMV protein selection, as when the *C. jejuni N*-linked glycosylation pathway is expressed in *E. coli*, *N*-linked glycoproteins are identified within *E. coli* OMV's (Fisher *et al*. 2011). However, how specific proteins are targeted to OMV's is still largely unknown.

Isolated OMV's are equally cytotoxic to Caco-2 intestinal epithelial cells (IECs) as whole live *C*. *jejuni* cells in 24hr incubation experiments. Similarly, isolated OMV's and whole *C. jejuni* cells induced the same level of immune response from IECs, as measured by IL-8, IL-6, TNF-α and hBD-3 excretion levels. Taken together the results suggest the vast majority of *C. jejuni*'s cytotoxicity and induction of host immune response is due to OMV's (Elmi *et al*. 2012). Indeed, *in vivo* infection experiments with the model larvae *Galleria mellonella* demonstrated that isolated OMV's caused a similar level of killing as live *C. jejuni* cells, highlighting their importance in pathogenesis (Elmi *et al*. 2012). More recently it has been demonstrated that *C. jejuni* OMV's contain an abundance of proteases which are able to degrade host E-cadherin and occludin, increasing the levels of bacterial adhesion and invasion into T84 IEC's (Elmi *et al*. 2016).

1.2.4 Flagellum

The flagella complex in *C .jejuni* is composed of an inner membrane (IM) type-3 secretion system (T3SS) associated with C and MS rings which form the motor, periplasmic proximal and distal rods, an outer membrane (OM) L-ring and the extracellular hook and filament (Figure 3). As with the *N*-linked glycoproteins of OMVs discussed above, *O*-linked glycosylation of the structural filament proteins FlaA and FlaB is crucial for host immune evasion, as phase variation and selective polymorphisms are abundant in both the *N*-linked and *O*-linked glycosylation loci (Szymanski and Wren 2005). The accessory proteins FliS and FliW are responsible for chaperoning FlaAB through the T3SS to polymerise the filament, where FliW binds the N-terminal domain and FliS the C-terminal domain (Radomska *et al*. 2017). FliS has higher specificity for glycosylated FlaAB, ensuring that the glycosylated species are abundant in the filament. FliW binds independent of glycosylation state and interacts with the post-transcriptional regulator CsrA, acting as a sensor of cytoplasmic FlaAB concentration, providing a mechanism of regulating FlaAB translation (Radomska *et al*. 2017). Furthermore, construction of the MS and C rings by the T3SS forms

a cytoplasmic complex which interacts with FlgS of the FlgRS two-component system, activating σ^{54}-dependent flagellar gene expression, providing a regulatory checkpoint in flagellar biogenesis (Boll and Hendrixson 2013).

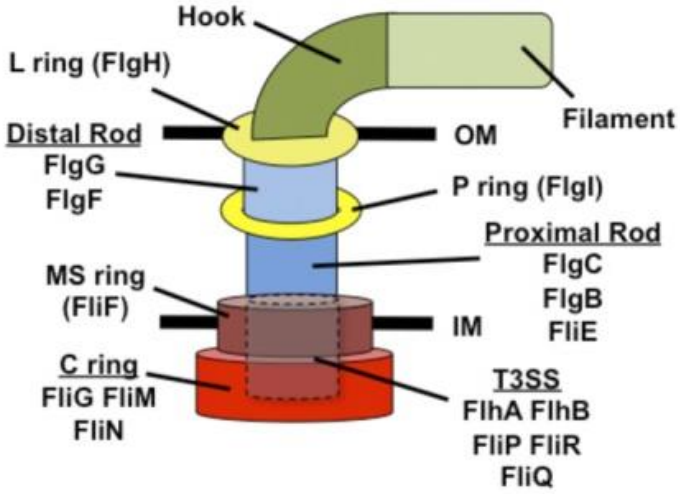

Figure 3: Organisation of the C. jejuni flagellum (Boll and Hendrixson 2013).

In addition to motility, the flagella functions to excrete proteins into the environment, including the *Campylobacter* invasion antigens (Cia proteins). Excretion of Cia proteins is dependent on a functional flagella complex and an environmental stimulus such as bile salts (Konkel *et al*. 2004, Rivera-Amill *et al*. 2001). CiaC has been shown to be excreted from the flagellum and delivered to the cytosol of host cells, where it induces cell membrane ruffling through the alteration of cytoskeletal arrangement, presumably assisting internalisation (Neal-McKinney and Konkel 2012). CiaD is also delivered to the cytosol of the host cell via the flagella and is required for host cell invasion by activating the MAP kinase signalling pathway, leading to IL-8 excretion and bacterial internalisation into *Campylobacter*-containing vacuoles (CCV) (Samuelson *et al*. 2013). Once internalised, secretion of CiaI prevents association of the CCV with Cathepsin D, a marker for lysosomal degradation, enhancing intracellular survival (Buelow *et al* 2011).

1.2.5 Cell surface and capsule

C. jejuni binding to host cell fibronectin is mediated by the OM, surface exposed CadF protein which is required for efficient adhesion and invasion (Konkel *et al*. 1997, Krause-Gruszczynska *et al*. 2007). CadF binding to the host cell surface triggers activation of the host GTPase's Rac1 and Cdc42, both of which are required for *C. jejuni* internalisation, most likely by modifying actin structure (Krause-Gruszczynska *et al*. 2007). CadF is thought to activate Rac1 and Cdc42 by hijacking the fibronectin → integrin beta1 → FAK → DOCK180/Tiam-1 signalling cascade of the host to induce GTPase activity

(Boehm *et al*. 2011). A second, more recently discovered fibronectin binding lipoprotein from *C. jejuni*, FlpA, has also been implicated in host cell adhesion and GTPase activation by β1-integrin mediated Erk1/2 phosphorylation (Larson *et al*. 2013). PEB1 is often referring to as the major cell binding factor, and has been shown to have a dominant role in cell adhesion and invasion, yet more recently a clear role in aspartate and glutamate utilisation as *in vivo* carbon sources has been shown, highlighting the interesting multifunctional nature of this important protein (Leon-Kempis *et al*. 2006, Pei *et al*. 1998). A number of other adhesion promoting surface proteins have been identified in *C. jejuni*, including CapA and JlpA, but to date no function has been assigned (Ashgar *et al*. 2007, Kawai *et al*. 2012). It is likely *C. jejuni* has a number of cell surface exposed proteins which can promote host adhesion and trigger signalling cascades which facilitate internalisation.

Capsule polysaccharide (CPS) is unique to enteric pathogens and is a promising target for vaccines, as a CPS vaccine administered to New World monkeys provided 100% protection against disease, an infection model shown to closely mimic human campylobacteriosis (Jones *et* al. 2006, Monteiro *et al*. 2009). There is also a strong link between CPS-positive strains of *C. jejuni* and the development of Guillain-Barré syndrome, possibly of greater importance even than sialylated lipo-oligosaccharides (LOS), which have a well-established connection with Guillain-Barré syndrome, yet are also frequently detected in uncomplicated enteritis cases (Heikema *et al*. 2015). As with most enteric Gram-negatives, *C. jejuni* does not synthesis full length lipo-polysaccharide (LPS) but instead a shorter LOS lacking the O-chain. CPS is structurally independent of LOS, anchored to the membrane by a dipalmitoyl-glycerophosphate lipid anchor (Corcoran *et al*. 2006). *C. jejuni* CPS is primarily composed of complex heptoses and *O*-methyl phosphoramidate (MeOPN), the combination and linkage of which varies significantly between CPS types (Karlyshev *et al*. 2005). The large group of *kps* genes are responsible for CPS synthesis, assembly and transport, involving 20-40 ORF's. While genes for capsule assembly and transport are reasonably conserved, the polysaccharide synthesis genes are highly variable, both in the number of ORF's and sequence, responsible for the variation in CPS structures between strains, which is a strong driver of immune evasion (Poly *et al*. 2011). Furthermore, phase-variable GC homopolymeric tracts are found in *kpsM* and the MeOPN transferase *cj1402c*, allowing for CPS synthesis and MeOPN modifications, respectively, to be turned on and off to further increase diversity within the same serotype (Bacon *et al*.2001, Peguegnat *et al*. 2017). Indeed, MeOPN modification is required for some bacteriophage recognition, and CPS interferes with type VI secretion and host interaction, indicating that the ability to turn CPS synthesis and modification off at certain points of infection is important for efficient colonisation (Bleumink-Pluym *et* al. 2013, Rubinchik *et* al. 2014, Sørensen *et al*. 2011).

While there are a few examples of direct regulation of CPS by host interaction, independent of phase variation, the mechanisms are yet to be elucidated (Corcionivoschi *et al*. 2009, Reid *et al*. 2008).

1.3 Metabolism

1.3.1 Microaerophily

Campylobacter is a member of the epsilon-proteobacteria, more closely related to deep-sea vent bacteria than other canonical pathogens such as *E. coli* or *Salmonella*. *Campylobacter* is a true microaerophile: it cannot act as a facultative anaerobe or aerobe, it requires some oxygen but less than atmospheric, generally 5-10% O_2 in the gas atmosphere for efficient *in vitro* growth. The mucosal layer of the gut lumen in which the majority of *C. jejuni* proliferate has a steep oxygen gradient, meaning *in vivo* growth is most likely oxygen-limited. Most *Campylobacter* strains lack the pathways for carbohydrate fermentation, such as glycolysis, preventing growth at complete anaerobiosis (Vegge *et al*. 2016). Metabolism is centred on the oxidation of amino-acids and organic acids as carbon sources and alternative electron donors/acceptors to oxygen, but ultimately is still oxygen-dependent for growth.

The central metabolism enzymes pyruvate oxidoreductase (POR) and oxoglutarate oxidoreductase (OOR) of the citric acid cycle (CAC) are highly oxygen sensitive, due to labile iron-sulfur clusters, and thought to be essential for growth, partially explaining *C. jejuni*'s oxygen sensitivity (Kendall *et al*. 2014). Indeed, the POR and OOR enzymes of *C. jejuni* are normally found in obligate anaerobes and they reduce flavodoxin (FldA) to direct electron flow to an unusual Complex I, or Nuo complex, which utilises reduced FldA rather than NADH as in conventional aerobes (Weerakoon and Olson 2008) (Figure 4). Electron flow from the menaquinone (MQ) pool can proceed to two terminal oxidases, the cytochrome *c* oxidase CcoNOQP and *bd*-like quinol oxidase CioAB. CcoNOQP has far higher affinity for oxygen than CioAB (K_d's of ~40 nM and ~8µM, respectively) and a *ccoN* null mutant demonstrates complete attenuation in a chicken colonisation model, while a *cioA* mutant displays no attenuation, suggesting CcoNOQP is the major terminal complex for oxygen-dependent respiration in *C. jejuni* (Jackson *et al*. 2007, Weingarten *et al*. 2008). Furthermore, null mutants in the alternative electron acceptor reductase complexes nitrate reductase (NapA), nitrite reductase (NrfA), or DMSO/TMAO reductase show minimal attenuation in chicken colonisation, supporting the role of oxygen as the most important electron acceptor in the host (Weingarten *et al*. 2008).

Finally, an absolute requirement for at least trace oxygen can be explained by the single NrdAB-type ribonucleotide reductase in *C. jejuni*, which requires oxygen to mediate DNA synthesis, without which replication is impossible (Sellars *et al*. 2002). Therefore, in summary, *C. jejuni* encodes systems for the utilisation of a number of alternative electron acceptors to oxygen for efficient energy conservation and proliferation under oxygen-limited conditions, likely to be the most common *in vivo* environment, yet oxygen is still the most energetically preferable substrate and is ultimately required for DNA synthesis and replication, explaining *C. jejuni's* strictly microaerophilic nature.

1.3.2 The citric acid cycle

The citric acid cycle (CAC) of *C. jejuni* is largely similar to the canonical CAC of model bacteria such as *E. coli*, with the exception of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, which are replaced with pyruvate oxidoreductase (POR) and oxoglutarate oxidoreductase (OOR), respectively (Figure 4). As mentioned above, POR and OOR reduce flavodoxin (FldA), rather than NAD, to direct electron flow to Complex I (Nuo complex). As such, the Nuo complex of *C. jejuni* lacks NADH binding subunits and instead has two unique subunits, Cj1574 and Cj1575, which are thought to interact with FldA (Weerakoon and Olson 2008). At the time of writing, unpublished work in our lab has shown that purified FldA does mediate both pyruvate and 2-oxoglutarate oxidation, and purification of Cj1574 and Cj1575, termed NuoX and NuoY, respectively, is underway with the goal of demonstrating an interaction with FldA. While POR and OOR are believed to be essential, Yahara *et al.* 2017 demonstrated that a null mutant in *fumC*, responsible for the hydration of fumarate to malate, was viable, surmising that under nutrient replete conditions anaplerotic pathways suffice to power the cell, even with an incomplete CAC, demonstrating *C. jejuni's* flexible metabolism.

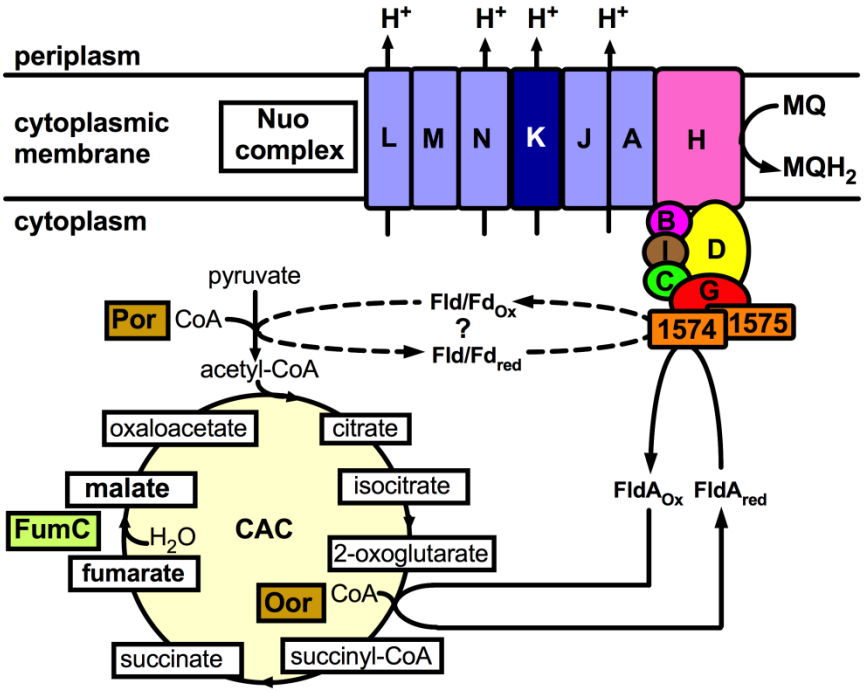

Figure 4: The CAC cycle of C. jejuni (Yahara *et al*. 2017).

Production of acetyl-CoA from pyruvate is a critical step in central metabolism, with pyruvate ultimately becoming the carbon source for all CAC intermediates and endogenously synthesised amino and fatty acids (Gao *et al.* 2017). Interestingly, Gao *et al*. were able to show by ^{13}C isotopologue profiling that serine, long used as an additive to growth medium for *C jejuni*, is the major carbon source for the cell *in vivo*. Exogenous serine is transported into the cell by the transporter SdaC, then deaminated to pyruvate by SdaA (Velayudhan *et al*. 2004), which enters the CAC via acetyl-CoA. Glutamate and aspartate are imported via the Peb transporter system (Leon-Kempis *et al*. 2006) and also contribute to the CAC by conversion to 2-oxoglutarate and fumarate/oxaloacetate, respectively, by the transaminase AspB and the aspartate ammonia lyase AspA (Guccione *et al*. 2008). .

C. jejuni is capnophilic, requiring high CO_2 levels for optimum growth (Bolton and Coates, 1983). Bicarbonate, formed by hydration of CO_2, is required for the activity of various carboxylases, including pyruvate carboxylase-dependent oxaloacetate synthesis. *C. jejuni* encodes the beta-class carbonic anhydrase CanB which is required for adequate bicarbonate availability at reduced CO_2 levels, as the spontaneous hydration rate of CO_2 is insufficiently low (Al-Haideri *et al*. 2016). A *canB* mutant was unable to grow on serine, pyruvate or lactate as the sole carbon source under CO_2 replete conditions, indicating bicarbonate availability is essential for growth on C3 compounds which are converted to pyruvate before carboxylation to oxaloacetate (Al-Haideri *et al*. 2016). Therefore CO_2 can be considered equally important to central metabolism and amino acid utilisation as oxygen.

1.3.3 Electron donors and acceptors

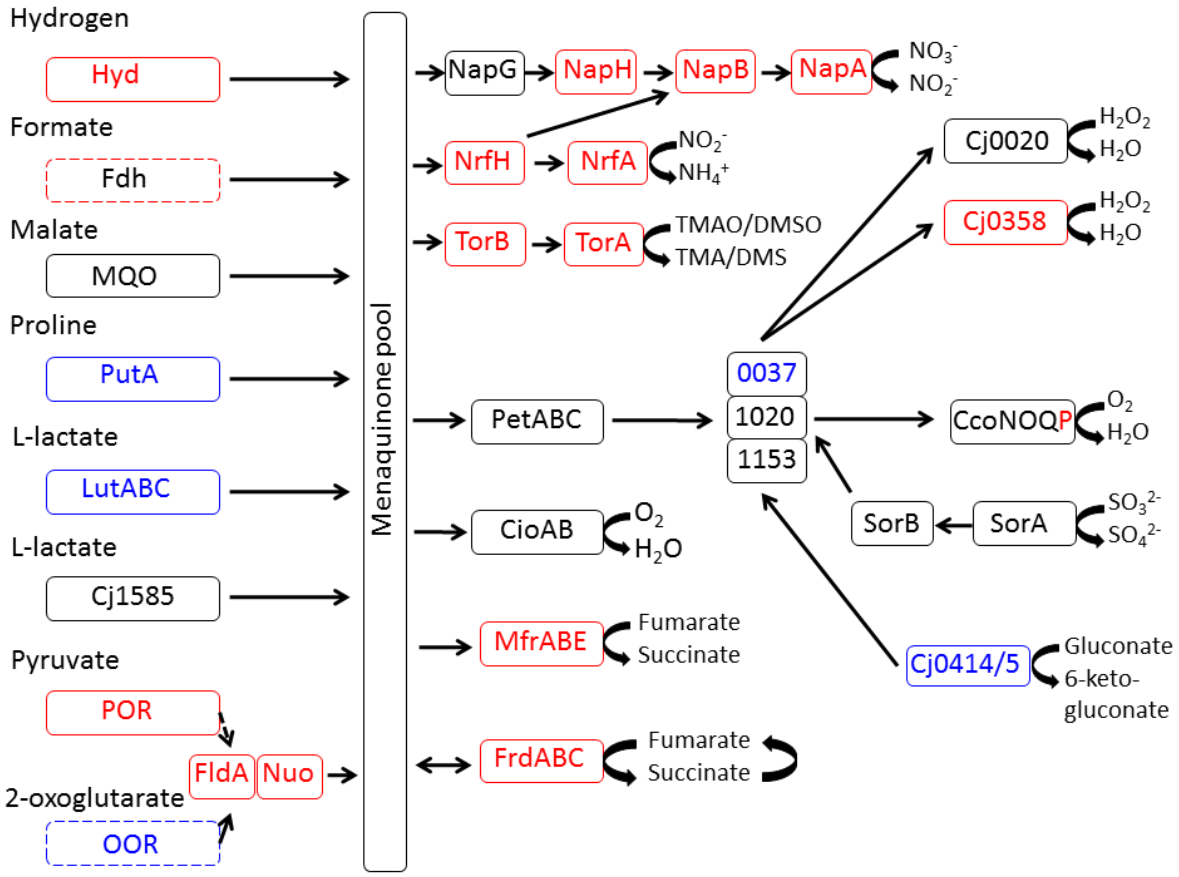

Figure 5: Electron donors and acceptors of *C. jejuni*. Pathways highlighted in red are upregulated during oxygen limitation, those in blue are more highly expressed in the presence of oxygen (Guccione *et al.* 2017).

C. jejuni uses a plethora of alternate electron donors, and other acceptors to oxygen, summarised in Figure 5. The majority of electron flow through the ETC is via the hydrogen carrier menaquinone (MQ). While *E. coli* utilises either menaquinone or ubiquinone for anaerobic or aerobic metabolism, respectively, *C. jejuni* relies solely on MQ, which is synthesised via a distinct 6-amino-6-deoxyadeno-futalosine pathway, yielding MQ with a C_{30} prenyl side chain (Li *et al*. 2011).

The CAC donates electrons to the MQ pool via the oxidoreductases POR and OOR, via FldA and the Nuo complex (see sections 1.3.1 and 1.3.2). Hydrogen is readily available in the host gut and is utilised by the hydrogenase complex HydABCD to generate electrons for the MQ pool and establish a strong membrane potential when oxygen is limited (van der Stel *et al*. 2017). HydB, the catalytic subunit, has a FeNi active site which is dependent on the specific nickel uptake operon *nikZYXWV* (Howlett *et al*. 2012). Formate dehydrogenase (FDH) has a catalytic subunit, FdhA, with a molybdenum or tungsten–pterin cofactor and an

active site selenocysteine (SeC), the only SeC residue in the *C. jejuni* proteome (Smart *et al*. 2009). Oxidation of formate generates both electrons for the MQ pool and protons which can be utilised to establish a membrane potential. Like *hyd* mutants, *fdh* mutants show little attenuation in a chick colonisation model, despite hydrogen and formate being the obvious major electron donor alternatives to oxygen due to their abundance in the caecum (Weerakoon *et al*. 2009). However, a double *hyd/fdh* mutant was severely impaired in chick colonisation, leading to the conclusion that the two complexes are partially redundant for *in vivo* survival, but do indeed represent the major alternative electron source for respiration (Weerakoon *et al*. 2009).

Other alternate electron donors are organic acids, including malate and lactate, which are utilised by the dehydrogenase MDH and oxidoreductase MQO, and oxidase LutABC, respectively (Velayudhan and Kelly 2002, Thomas *et al*. 2011). While serine is the preferred amino acid for energy conservation via pyruvate oxidation (see section 1.3.2), proline directly contributes electrons to the MQ pool. Proline is taken up by the Na^+ symporter PutP and oxidised to glutamate by the dehydrogenase PutA via an FAD cofactor which redoxes with the MQ pool (Tanner 2008).

Molecular oxygen is the most preferable terminal electron acceptor as it has the highest energy potential (midpoint redox potential, Em, of +840 mV). Oxygen is utilised by the oxidase complexes, CioAB and CcoNOQP, where the Cco complex is considered the major terminal oxidase, receiving electrons through high potential periplasmic *c*-type cytochromes, while the Cio complex oxidises MQH_2 directly with a much weaker affinity for oxygen (Jackson *et al*. 2007, Weingarten *et al*. 2008). Reduced MQ can donate electrons to PetABC, a cytochrome *bc*-type complex, which in turn reduces periplasmic *c*-type cytochromes which donate electrons to the Cco complex, as well as the periplasmic di-haem cytochrome *c* peroxidases Cj0358 and Cj0020, which putatively degrade hydrogen peroxide produced by the ETC to water. The *c*-type cytochrome pool feeding the oxidase complex can also be reduced independent of the MQ pool by the sulphite and gluconate dehydrogenases, SorAB and Cj0414/5, respectively.

After oxygen, nitrate is the next energetically favourable (Em, +420 mV) and is present at micromolar concentrations in the host gut, where it can be reduced to nitrite by the nitrate reductase NapABHG (Potter *et al*. 2001, Pittman *et al*. 2007). The periplasmic location of Nap makes it ideally suited to scavenge these relatively low nitrate concentrations. The nitrite reductase NrfAH is available to reduce the potentially toxic nitrite produced by Nap to yield ammonia. There is some evidence that Nap and Nrf may form a periplasmic complex in *E. coli*, which as well as maximising energy conservation may also

prevent nitrite leakage and reduce nitrosative stress (Potter *et al*. 2001). Nrf also plays a role in defence from the host immune system, by reducing nitric oxide to ammonia, providing an example of respiratory detoxification (Poock *et al*. 2002, Pittman *et al*. 2007).

TMAO is found in the gut as a waste product from other microbiota, such as *Acinetobacter,* which convert diet derived carnitine to TMAO, or directly from fish, and so presents a plausible electron donor in the host niche of *C. jejuni*. The dual TMAO/DMSO reductase TorAB consists of the molybdoreductase TorA and cognate monoheme *c*-type cytochrome TorB and has been shown by quantitative ^{1}H NMR to reduce TMAO and DMSO to TMA and DMS, respectively (Sellars *et al*. 2002).

Fumarate and succinate can be derived from the environment by the C4-dicarboxylate transporter family, or fumarate can be produced endogenously from aspartate by AspA (Guccione *et* al. 2008, Wösten *et al*. 2017). The cytoplasmic facing FrdABC complex is the sole succinate dehydrogenase, a central step in the CAC, but is also able to reduce fumarate (Weingarten *et al*. 2009). *C. jejuni* also encodes a periplasmic fumarate reductase unique to epsilon bacteria, MfrABE, which can also reduce the fumarate analogues mesaconate and crotonate. Mfr is highly oxygen repressed, with expression massively increased following the switch to oxygen limitation, as experienced *in vivo* (Guccione *et al*. 2010). The expression of Mfr is also strongly repressed by nitrate, via the RacRS system, as fumarate is much less energetically favourable (Em, +30 mV) (van der Stel *et al*. 2015). The periplasmic location of Mfr means it does not rely on fumarate import and so can rapidly adapt the cell to fumarate utilisation under oxygen limited conditions when nitrate is limited.

1.4 Stress response

1.4.1 Host defences

The innate immune system is the first line of defence against enteric infection, mobilising macrophages and neutrophils to engulf and destroy invading bacteria, their primary weapon being reactive oxygen species (ROS) and reactive nitrogen species (RNS). NADPH oxidase reduces molecular oxygen to superoxide anions in a so-called superoxide burst in response to phagocytised bacteria (Pan *et al*. 2009). Superoxide is dismutated to hydrogen peroxide (H_2O_2) which can freely diffuse across bacterial membranes and oxidise a number of targets, including engaging in Fenton chemistry with iron sulfur clusters. H_2O_2 can also be utilised by the myeloperoxidase of neutrophils to generate the potent oxidative species hypochlorous acid (Klebanoff *et al*. 2013). Nitric oxide (NO) synthase is also induced

by phagocytised bacteria, producing NO from L-arginine (Bogdan 2015). NO can itself react with metals, particularly iron, or combine with superoxide to generate peroxynitrite ($OONO^-$), itself a strong oxidant.

Infection of mammalian cells with *C. jejuni* triggers a large increase in nitric oxide synthase and NADPH oxidase expression, both of which are capable of killing engulfed cells (Corcionivoschi *et al*. 2012, Iovine *et al*. 2008). As such, *C. jejuni* has a vast array of defences against oxidative and nitrosative stress, discussed below.

1.4.2 Oxidative stress response

Superoxide anions can be neutralised by the single cytoplasmic superoxide dismutase SodB, creating O_2 and H_2O_2. *sodB* mutants display enhanced sensitivity towards superoxide and indirect sensitivity towards H_2O_2 due to superoxide mediated Fenton chemistry, and decreased intracellular survival (Novik *et* al. 2010, Palyada *et al*. 2009).

H_2O_2 derived from either the host or *C. jejuni*'s own superoxide dismutase must be detoxified to limit Fenton chemistry and other oxidative processes. Catalase (KatA) is the primary detoxifier of H_2O_2, a tetramer with each subunit containing a haem *b* prosthetic group (Grant and Park 1995). The haem groups catalyse a two-step reaction to form O_2 and two molecules of H_2O from two molecules of H_2O_2. *katA* mutants display no catalase activity and are attenuated for intracellular survival, though this phenotype was recoverable in macrophages by addition of an NADPH oxidase inhibitor, confirming intracellular ROS-mediated killing of *C. jejuni* (Day *et al*. 2000, Palyada *et al*. 2009). A gene downstream of *katA*, *cj1386*, encodes a haem *b* binding protein which interacts with KatA (Flint and Stintzi 2015). Cj1386 does not have catalase activity, but is required for haem trafficking to KatA and optimal catalase activity (Flint and Stintzi 2012). As with *katA* mutants, a *cj1386* mutant was significantly attenuated in a chick colonisation model (Flint and Stintzi 2012).

In addition to catalase, *C*. *jejuni* possesses three peroxiredoxins: alkyl hydroperoxide reductase (AhpC), thiol peroxidase (Tpx) and bacterioferritin comigratory protein (BCP). These peroxiredoxins reduce H_2O_2 to H_2O by an active site cysteine which oxidises to sulfenic acid, or further to form a disulphide bridge if a second cysteine is present. In either case, the oxidised cysteines are returned to their reduced state by an oxidoreductase (Nelson *et al*. 2011). Single mutants in either *ahpC*, *tpx* or *bcp* show little or no enhanced sensitivity to H_2O_2, while a double *tpx bcp* mutant was significantly sensitive, suggesting some redundancy between the peroxiredoxins and catalase (Atack *et al*. 2008, Baillon *et al*. 1999).

A major generator of ROS is via the iron-mediated Fenton reaction, so many bacteria have mechanisms to sequester intracellular iron to prevent Fenton chemistry. The DNA-binding protein from starved cells (Dps) forms a dodecamer which is capable of binding 500 iron atoms per monomer in the Fe^{3+} form, which is oxidised from free Fe^{2+} by ferroxidase activity of Dps (Sanchuki *et al*. 2015). Furthermore, Dps can bind DNA to protect it from oxidative damage by blocking access to Fe^{2+} and H_2O_2 (Ceci *et al*. 2004).

Two periplasmic *c*-type cytochrome peroxidases (Ccp), Cj0020 and Cj0358, are expressed by *C. jejuni* which have haem-dependent peroxidase activity (Bingham-Ramos *et al*. 2008). However, mutants in either Ccp do not display enhanced H_2O_2 sensitivity, yet do show slight attenuation in a chick colonisation model, leaving the role of *C. jejuni* Ccp's in ROS defence unclear (Bingham-Ramos *et al*. 2008, Flint *et al*. 2014).

Methionine residues in mature proteins are susceptible to oxidation to methionine sulfoxide (MetSO), particularly by the reactive chlorine species hypochlorous acid, produced by myeloperoxidase of neutrophils (Trivedi *et al*. 2015). Oxidation of methionine to either the R- or S- isomer of MetSO can induce conformational changes which inactivate proteins, yet some evidence is emerging that MetSO may be actively introduced as a post-translational modification to regulate enzyme activity (Drazic and Winter 2014). *C*. *jejuni* encodes two structurally unrelated cytoplasmic methionine sulfoxide reductases (Msr), MsrA and MsrB, which reduce the S- and R- isomers of MetSO, respectively (Atack and Kelly 2008). While single mutants in either *msrA* or *msrB* were only slightly attenuated, a double *msrAB* mutant displayed a severe growth defect and significantly increased sensitivity to oxidative and nitrosative stress, highlighting the importance of this repair system in *C. jejuni* (Atack and Kelly 2008). More recently, a well conserved periplasmic Msr system has been identified in Gram-negative bacteria and is present in *C. jejuni* (see chapter 4) (Gennaris *et al*. 2015).

1.4.3 Nitrosative stress response

NO produced by nitric oxide synthase can generate oxidative species which are dealt with by the systems described above. NO itself can be directly detoxified by the single domain haemoglobin Cgb by consuming NO via sacrificial oxidation of its haem, which is then reduced by an as yet unidentified NADH-dependent reductase system (Tinajero-Trejo and Shepherd 2013). A second, truncated haemoglobin, Ctb, shows no direct NO detoxifying activity, but has been shown to bind both O_2 and NO under normoxic and hypoxic conditions, respectively, which is thought to influence *cgb* expression via the nitrosative stress response regulator, NssR (Smith *et al*. 2011). However, there is evidence that the Cgb Ctb system is ineffective under oxygen-limited conditions, as found in the host, which

puts their physiological importance for NO detoxification under question (Avila-Ramirez *et al*. 2013).

NrfA is a periplasmic nitrite reductase which reduces nitrite produced by the nitrate reductase NapA into ammonia (see section 1.3.3). NrfA has been shown to play a prominent role in defence against nitrosative stress, both by directly consuming NO and by reduction of NO-derived nitrite which could otherwise form the oxidative species nitrogen dioxide (Pittman *et al*. 2007). Interestingly, *cgb* is induced by nitrite and nitrate, suggesting growth with nitrite results in nitrosative stress. This was confirmed with *cgb* and *nssR* mutants, which were unable to grow in the presence of nitrite, while a *nrfA* mutant still maintained slow growth (Pittman *et al*. 2007). Taken together, Cgb and NrfA have distinct yet co-dependent roles in detoxifying NO and defending against nitrosative stress.

1.4.4 Stress response regulators

Regulator Protein	Gene	Function
PerR	*cj0322*	Iron-dependent oxidative stress defence
Fur	*cj0400*	Iron homeostasis, acid survival and oxidative stress defence
CosR	*cj0355c*	Oxidative stress defence and multidrug resistance
CmeR	*cj0368c*	Multidrug efflux pump regulation
LysR	*cj1000*	Metabolism and oxidative stress defence
RrpAB	*cj1556*	Oxidative and aerobic stress defence

Table 1: Primary stress response regulators of *C. jejuni*.

C. jejuni lacks many regulatory systems common to other Gram-negatives, such as the well characterised stress response regulators SoxR, OxyR and FNR, instead utilising a number of unique regulatory proteins (Table 1) (Constantinidou *et al*. 2006, Seo *et al*. 2015). Two iron-sensing regulators, PerR and Fur, play a major role in *C. jejuni* gene regulation, using iron-limitation as a signal for entering the host and activating Fenton chemistry defence systems in response to excess iron. PerR represses the major peroxidases, including *katA* and *ahpC*, under iron-limited conditions (Butcher *et al*. 2015). PerR binds and regulates its own promoter, and iron suppression of PerR binding provides an autoregulatory mechanism to control PerR levels (Kim *et al*. 2011). Fur (ferric uptake regulator) activates oxidative stress resistance genes, including *katA*, under iron-replete conditions, to combat

host defences. Fur also regulates iron uptake and storage systems, an important function in preventing free iron engaging in Fenton chemistry. PerR and Fur are tightly linked functionally, often competing for binding at the same promoter, such as *katA*, under conflicting conditions, providing a flexible response to iron signalling and oxidative stress (Butcher *et al*. 2015). Fur has also been shown to regulate acid-stress responses by modulating gene expression involved in cell envelope biogenesis and motility, which seems to cross-protect against oxidative stress by inducing catalase expression, as the affinity between Fur and the *katA* promoter is reduced at lowered pH (Askoura *et al*. 2016).

CosR is an Omp-R type oxidative stress sensor which regulates close to 100 genes including *katA*, *sodB* and *ahpC* (Hwang *et al*. 2011, Hwang *et al*. 2012). *cosR* is an essential gene in *C. jejuni*, limiting its functional characterisation, however utilisation of antisense peptide nucleic acids (PNAs) to supress *cosR* has allowed transcriptome analysis (Hwang *et al*. 2011, Hwang *et al*. 2012). *cosR* expression is reduced in response to oxidative stress, causing upregulation of its repressed targets, such as *katA* (Garénaux *et al*. 2008). A potential mechanism for oxidative stress sensing by CosR has been identified where a single cysteine (C218), sensitive to oxidation, alters DNA-binding affinity and liberates CosR from target transcripts (Grinnage-Pulley *et al*. 2016). Interestingly, CosR has been shown to repress the multidrug efflux pump CmeABC, considered crucial for antimicrobial resistance in *C. jejuni* (Hwang *et al*. 2012). *cmeABC* is also repressed by its cognate regulator CmeR, which binds bile salts as a signal of antimicrobial barrage, causing a conformational change and release from the *cmeABC* promoter (Lei *et al*. 2011). The dual repression of *cmeABC* by CmeR and CosR allows differential expression to suit various environmental conditions (Grinnage-Pulley *et al*. 2016).

C. jejuni encodes a single LysR-type regulator, *cj1000*, which appears to control O_2 consumption by modulation of respiratory enzymes (Dufour *et al*. 2013). The Cj1000 regulon was determined by microarray and included many common oxidative stress targets common with PerR and Fur, as well as oxygen-limited respiratory enzymes, including *mfrA*. The sulphite oxidase *sorA* is positively regulated by Cj1000, and O_2 consumption in the *cj1000* mutant with sulphite as electron donor was impaired, leading the authors to suggest sulphite as a possible ligand for Cj1000, though to date no further research on the regulatory mechanism of Cj1000 has been published (Dufour *et al*. 2013).

C. jejuni encodes two MarR-type transcriptional regulators, RrpA and RrpB, involved in resistance to oxidative and aerobic stress (Gundogdu *et al*. 2011). Both RrpA and RrpB have been shown to bind DNA and mutation of the respective genes results in increased sensitivity to oxidative stress, attenuated aerobic survival and reduced virulence in the

Galleria mellonella infection model (Gundogdu *et al*. 2011, Gundogdu *et al*. 2015). Microarray analysis of the *rrpB* mutant showed positive regulation of *katA*, *perR*, *ahpC* and *dps*, and negative regulation of *sodB* (Gundogdu *et al*. 2011). RT-PCR confirmed reduced *katA* expression in both single *rrpA* and *rrpB* mutants, but interestingly not in the double *rrpAB* mutant, which also failed to display increased sensitivity to H_2O_2, suggesting coordination between RrpA and RrpB regulons which is disrupted in single mutants (Gundogdu *et al*. 2015).

Taken together it is clear that *C. jejuni* has a highly divergent, overlapping regulatory system for responding to oxidative stress which involves a number of non-canonical transcriptional regulators. For instance, *katA* is a member of the regulons of PerR, Fur, CosR and RrpAB, demonstrating *C. jejuni*'s flexibility in responding to environmental stimuli which likely contributes to its effectiveness as an invasive pathogen.

References

Al-Haideri H, White MA and Kelly DJ (2016). Major contribution of the type II beta carbonic anhydrase CanB (Cj0237) to the capnophilic growth phenotype of *Campylobacter jejuni*. *Environ Microbiol*. 18(2): 721-35.

Ashgar SS, Oldfield NJ, Wooldridge KG, Jones MA, Irving GJ, Turner DP and Ala'Aldeen DA (2007). CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. *J Bacteriol*. 189(5): 1856-65.

Askoura M, Sarvan S, Couture JF and Stintzi A (2016). The *Campylobacter jejuni* Ferric Uptake Regulator Promotes Acid Survival and Cross-Protection against Oxidative Stress. *Infect Immun*. 84(5): 1287-300.

Atack JM, Harvey P, Jones MA and Kelly DJ (2008). The *Campylobacter jejuni* thiol peroxidases Tpx and Bcp both contribute to aerotolerance and peroxide-mediated stress resistance but have distinct substrate specificities. J *Bacteriol*. 190(15): 5279-90.

Atack JM and Kelly DJ (2008). Contribution of the stereospecific methionine sulphoxide reductases MsrA and MsrB to oxidative and nitrosative stress resistance in the food-borne pathogen *Campylobacter jejuni*. *Microbiology*. 154(8): 2219-30.

Avila-Ramirez C, Tinajero-Trejo M, Davidge KS, Monk CE, Kelly DJ and Poole RK (2013). Do globins in microaerophilic *Campylobacter jejuni* confer nitrosative stress tolerance under oxygen limitation? *Antioxid Redox Signal*. 18(4): 424-31.

Awofisayo-Okuyelu A, Hall I, Adak G, Hawker JI, Abbott S and McCarthy N (2017). A systematic review and meta-analysis on the incubation period of Campylobacteriosis. *Epidemiol Infect*. 145(11): 2241-2253.

Bacon DJ, Szymanski CM, Burr DH, Silver RP, Alm RA and Guerry P (2001). A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. *Mol Microbiol*. 40(3): 769-77.

Baillon ML, van Vliet AH, Ketley JM, Constantinidou C and Penn CW (1999). An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. *J Bacteriol*. 181(16): 4798-804.

Beauchamp JM, Erfurt RS and DiRita VJ (2015). Characterization and localization of the *Campylobacter jejuni* transformation system proteins CtsE, CtsP, and CtsX. *J Bacteriol*. 197(3): 636-45.

Bingham-Ramos LK and Hendrixson DR (2008). Characterization of two putative cytochrome *c* peroxidases of *Campylobacter jejuni* involved in promoting commensal colonization of poultry. *Infect Immun*. 76(3): 1105-14.

Black RE, Levine MM, Clements ML, Hughes TP and Blaser MJ (1988). Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis*. 157(3): 472-9.

Blaser MJ (1997). Epidemiologic and clinical features *of Campylobacter jejuni* infections. *The Journal of Infectious Diseases* 176: S103–5.

Bleumink-Pluym NM, van Alphen LB, Bouwman LI, Wösten MM and van Putten JP (2013). Identification of a functional type VI secretion system in *Campylobacter jejuni* conferring capsule polysaccharide sensitive cytotoxicity*. PLoS Pathog*. 9(5):e1003393.

Boehm M, Krause-Gruszczynska M, Rohde M, Tegtmeyer N, Takahashi S, Oyarzabal OA and Backert S (2011). Major host factors involved in epithelial cell invasion of *Campylobacter jejuni*: role of fibronectin, integrin beta1, FAK, Tiam-1, and DOCK180 in activating Rho GTPase Rac1. *Front Cell Infect Microbiol.* 1: 17.

Boesze-Battaglia K, Brown A, Walker L, Besack D, Zekavat A, Wrenn S, Krummenacher C and Shenker BJ (2009). Cytolethal distending toxin-induced cell cycle arrest of lymphocytes is dependent upon recognition and binding to cholesterol. *J Biol Chem.* 284(16): 10650-8.

Bogdan C (2015). Nitric oxide synthase in innate and adaptive immunity: an update. *Trends Immunol.* 36(3): 161-78.

Boll JM and Hendrixson DR (2013). A regulatory checkpoint during flagellar biogenesis in *Campylobacter jejuni* initiates signal transduction to activate transcription of flagellar genes.

Bolton FJ and Coates D (1983). A study of the oxygen and carbon dioxide requirements of thermophilic campylobacters. *J Clin Pathol*. 36(7): 829-34. *MBio.* 4(5): e00432-13.

Buelow DR, Christensen JE, Neal-McKinney JM and Konkel ME (2011). *Campylobacter jejuni* survival within human epithelial cells is enhanced by the secreted protein CiaI. *Mol Microbiol*. 80(5): 1296-312.

Butcher J, Handley RA, van Vliet AH and Stintzi A (2015). Refined analysis of the *Campylobacter jejuni* iron-dependent/independent Fur- and PerR-transcriptomes. *BMC Genomics*. 16: 498.

Campero CM, Anderson ML, Walker RL, Blanchard PC, Barbano L, Chiu P, Martínez A, Combessies G, Bardon JC and Cordeviola J (2005). Immunohistochemical identification of *Campylobacter fetus* in natural cases of bovine and ovine abortions. *J Vet Med B Infect Dis Vet Public Health*. 52(3): 138-41.

Ceci P, Cellai S, Falvo E, Rivetti C, Rossi GL and Chiancone E (2004). DNA condensation and self-aggregation of *Escherichia coli* Dps are coupled phenomena related to the properties of the N-terminus. *Nucleic Acids Res*. 32(19): 5935-44.

Cha W, Mosci RE, Wengert SL, Venegas Vargas C, Rust SR, Bartlett PC, Grooms DL and Manning SD (2017). Comparing the Genetic Diversity and Antimicrobial Resistance Profiles of *Campylobacter jejuni* Recovered from Cattle and Humans. *Front Microbiol*. 8: 818.

Constantinidou C, Hobman JL, Griffiths L, Patel MD, Penn CW, Cole JA and Overton TW (2006). A reassessment of the FNR regulon and transcriptomic analysis of the effects of nitrate, nitrite, NarXL, and NarQP as *Escherichia coli* K12 adapts from aerobic to anaerobic growth. *J Biol Chem*. 281(8): 4802-15.

Corcionivoschi N, Clyne M, Lyons A, Elmi A, Gundogdu O, Wren BW, Dorrell N, Karlyshev AV and Bourke B (2009). *Campylobacter jejuni* cocultured with epithelial cells reduces surface capsular polysaccharide expression. *Infect Immun*. 77(5): 1959-67.

Corcionivoschi N, Alvarez LA, Sharp TH, Strengert M, Alemka A, Mantell J, Verkade P, Knaus UG and Bourke B (2012). Mucosal reactive oxygen species decrease virulence by disrupting *Campylobacter jejuni* phosphotyrosine signaling. *Cell Host Microbe*. 12(1): 47-59.

Corcoran AT, Annuk H and Moran AP (2006). The structure of the lipid anchor of *Campylobacter jejuni* polysaccharide. *FEMS Microbiol Lett*. 257(2): 228-35.

Day WA, Sajecki JL, Pitts TM and Joens LA (2000). Role of catalase in *Campylobacter jejuni* intracellular survival. *Infect Immun*. 68(11): 6337-45.

Denis M, Nagard B, Rose V, Bourgoin K, Cutimbo M and Kerouanton A (2017). No Clear Differences between Organic or Conventional Pig Farms in the Genetic Diversity or Virulence of *Campylobacter coli* Isolates. *Front Microbiol.* 8: 1016.

Drazic A and Winter J (2014). The physiological role of reversible methionine oxidation. *Biochim Biophys Acta.* 1844(8): 1367-82.

Dufour V, Li J, Flint A, Rosenfeld E, Rivoal K, Georgeault S, Alazzam B, Ermel G, Stintzi A, Bonnaure-Mallet M and Baysse C (2013). Inactivation of the LysR regulator Cj1000 of *Campylobacter jejuni* affects host colonization and respiration. *Microbiology*. 159(6): 1165-78.

EFSA (2010). Scientific opinion on quantification of the risk posed by broiler meat to human camplyobacteriosis in the EU. *EFSA Journal* 8: 1–89. Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008 Part A: Campylobacter and Salmonella prevalence estimates. *EFSA Journal* 8: 1–99.

Elmi A, Watson E, Sandu P, Gundogdu O, Mills DC, Inglis NF, Manson E, Imrie L, Bajaj-Elliott M, Wren BW, Smith DG and Dorrell N (2012). *Campylobacter jejuni* outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect Immun*. 80(12): 4089-98.

Elmi A, Nasher F, Jagatia H, Gundogdu O, Bajaj-Elliott M, Wren B and Dorrell N (2016). *Campylobacter jejuni* outer membrane vesicle-associated proteolytic activity promotes

bacterial invasion by mediating cleavage of intestinal epithelial cell E-cadherin and occludin. *Cell Microbiol*. 18(4): 561-72.

Esan OB, Pearce M, Hecke O, Roberts N, Collins D, Violato M, McCarthy N, Perera R and Fanshawea TR (2017). Factors Associated with Sequelae of Campylobacter and Non-typhoidal Salmonella Infections: A Systematic Review. *EBioMedicine* 15: 100–111.

Fisher AC, Haitjema CH, Guarino C, Çelik E, Endicott CE, Reading CA, Merritt JH, Ptak AC, Zhang S and DeLisa MP (2011). Production of secretory and extracellular N-linked glycoproteins in *Escherichia coli*. *Appl Environ Microbiol.* 77(3): 871-81.

Flint A and Stintzi A (2015). Cj1386, an atypical hemin-binding protein, mediates hemin trafficking to KatA in *Campylobacter jejuni*. *J Bacteriol*. 197(5): 1002-11.

Flint A, Sun YQ, Butcher J, Stahl M, Huang H and Stintzi A (2014). Phenotypic screening of a targeted mutant library reveals *Campylobacter jejuni* defenses against oxidative stress. *Infect Immun*. 82(6): 2266-75.

Flint A, Sun YQ and Stintzi A (2012). Cj1386 is an ankyrin-containing protein involved in heme trafficking to catalase in *Campylobacter jejuni*. *J Bacteriol*. 194(2): 334-45.

FSA (2015). A microbiological survey of *Campylobacter* contamination in fresh whole UK produced chilled chickens at retail sale – an interim report to cover Quarters 1 & 2 (https://www.food.gov.uk/science/microbiology/campylobacterevidenceprogramme/retail-survey)

Gao B, Vorwerk H, Huber C, Lara-Tejero M, Mohr J, Goodman AL, Eisenreich W, Galán JE and Hofreuter D (2017). Metabolic and fitness determinants for in vitro growth and intestinal colonization of the bacterial pathogen *Campylobacter jejuni*. *PLoS Biol*. 15(5): e2001390.

Gennaris A, Ezraty B, Henry C, Agrebi R, Vergnes A, Oheix E, Bos J, Leverrier P, Espinosa L, Szewczyk J, Vertommen D, Iranzo O, Collet JF and Barras F (2015). Repairing oxidized proteins in the bacterial envelope using respiratory chain electrons. *Nature*. 528(7582): 409-412.

Garénaux A, Guillou S, Ermel G, Wren B, Federighi M and Ritz M (2008). Role of the Cj1371 periplasmic protein and the Cj0355c two-component regulator in the *Campylobacter jejuni* NCTC 11168 response to oxidative stress caused by paraquat. *Res Microbiol*. 159(9-10): 718-26.

Grant KA and Park SF (1995). Molecular characterization of katA from *Campylobacter jejuni* and generation of a catalase-deficient mutant of *Campylobacter coli* by interspecific allelic exchange. *Microbiology.* 141(6): 1369-76.

Grinnage-Pulley T, Mu Y, Dai L and Zhang Q (2016). Dual Repression of the Multidrug Efflux Pump CmeABC by CosR and CmeR in *Campylobacter jejuni*. *Front Microbiol*. 7: 1097.

Guccione E, Leon-Kempis Mdel R, Pearson BM, Hitchin E, Mulholland F, van Diemen PM, Stevens MP and Kelly DJ (2008). Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol Microbiol*. 69(1): 77-93.

Guccione E, Hitchcock A, Hall SJ, Mulholland F, Shearer N, van Vliet AH and Kelly DJ (2010). Reduction of fumarate, mesaconate and crotonate by Mfr, a novel oxygen-regulated periplasmic reductase in *Campylobacter jejuni*. *Environ Microbiol* 12(3): 576-91.

Guccione EJ, Kendall JJ, Hitchcock A, Garg N, White MA, Mulholland F, Poole RK and Kelly DJ (2017). Transcriptome and proteome dynamics in chemostat culture reveal how *Campylobacter jejuni* modulates metabolism, stress responses and virulence factors upon changes in oxygen availability. *Environ Microbiol*. 19(10): 4326-4348.

Gundogdu O, da Silva DT, Mohammad B, Elmi A, Mills DC, Wren BW and Dorrell N (2015). The *Campylobacter jejuni* MarR-like transcriptional regulators RrpA and RrpB both influence bacterial responses to oxidative and aerobic stresses. *Front Microbiol*. 6: 724.

Gundogdu O, Mills DC, Elmi A, Martin MJ, Wren BW and Dorrell N (2011). The *Campylobacter jejuni* transcriptional regulator Cj1556 plays a role in the oxidative and aerobic stress response and is important for bacterial survival in vivo. *J Bacteriol*. 193(16): 4238-49.

Haas K, Overesch G and Kuhnert P (2017). A Quantitative Real-Time PCR Approach for Assessing *Campylobacter jejuni* and *Campylobacter coli* Colonization in Broiler Herds*. J Food Prot*. 80(4): 604-608.

Heikema AP, Islam Z, Horst-Kreft D, Huizinga R, Jacobs BC, Wagenaar JA, Poly F, Guerry P, van Belkum A, Parker CT and Endtz HP (2015). *Campylobacter jejuni* capsular genotypes are related to Guillain-Barré syndrome. *Clin Microbiol Infect*. 21(9): 852.

Howlett RM, Hughes BM, Hitchcock A and Kelly DJ (2012). Hydrogenase activity in the foodborne pathogen *Campylobacter jejuni* depends upon a novel ABC-type nickel transporter (NikZYXWV) and is SlyD-independent. *Microbiology* 158(6): 1645-55.

Hwang S, Kim M, Ryu S and Jeon B (2011). Regulation of oxidative stress response by CosR, an essential response regulator in *Campylobacter jejuni*. *PLoS One*. 6(7): e22300.

Hwang S, Zhang Q, Ryu S and Jeon B (2012). Transcriptional regulation of the CmeABC multidrug efflux pump and the KatA catalase by CosR in *Campylobacter jejuni. J Bacteriol*. 194(24): 6883-91.

Iovine NM, Pursnani S, Voldman A, Wasserman G, Blaser MJ and Weinrauch Y (2008). Reactive nitrogen species contribute to innate host defense against *Campylobacter jejuni*. *Infect Immun*. 76(3): 986-93.

Jackson RJ, Elvers KT, Lee LJ, Gidley MD, Wainwright LM, Lightfoot J, Park SF and Poole RK (2007). Oxygen reactivity of both respiratory oxidases in *Campylobacter jejuni*: the cydAB genes encode a cyanide-resistant, low-affinity oxidase that is not of the cytochrome bd type. *J Bacteriol*. 189(5): 1604-15.

Jackson DN, Davis B, Tirado SM, Duggal M, van Frankenhuyzen JK, Deaville D, Wijesinghe MA, Tessaro M and Trevors JT (2009). Survival mechanisms and culturability of *Campylobacter jejuni* under stress conditions. *Antonie Van Leeuwenhoek*. 96(4): 377-94.

Jang KS, Sweredoski MJ, Graham RL, Hess S and Clemons WM (2014). Comprehensive proteomic profiling of outer membrane vesicles from *Campylobacter jejuni*. *J Proteomics*. 98: 90-8.

Jones FR, Baqar S, Gozalo A, Nunez G, Espinoza N, Reyes SM, Salazar M, Meza R, Porter CK and Walz SE (2006). New World monkey *Aotus nancymae* as a model for *Campylobacter jejuni* infection and immunity. *Infect Immun*. 74(1): 790-3.

Karlyshev AV, Champion OL, Churcher C, Brisson JR, Jarrell HC, Gilbert M, Brochu D, St Michael F, Li J, Wakarchuk WW, Goodhead I, Sanders M, Stevens K, White B, Parkhill J, Wren BW and Szymanski CM (2005). Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. *Mol Microbiol.* 55(1): 90-103.

Kawai F, Paek S, Choi KJ, Prouty M, Kanipes MI, Guerry P and Yeo HJ (2012). Crystal structure of JlpA, a surface-exposed lipoprotein adhesin of *Campylobacter jejuni*. *J Struct Biol*. 177(2): 583-8.

Kendall JJ, Barrero-Tobon AM, Hendrixson DR and Kelly DJ (2014). Hemerythrins in the microaerophilic bacterium *Campylobacter jejuni* help protect key iron-sulphur cluster enzymes from oxidative damage. *Environ Microbiol*. 16(4): 1105-21.

Kim M, Hwang S, Ryu S and Jeon B (2011). Regulation of *perR* expression by iron and PerR in *Campylobacter jejuni*. *J Bacteriol*. 193(22): 6171-8.

Kim J, Oh E, Banting GS, Braithwaite S, Chui L, Ashbolt NJ, Neumann NF and Jeon B (2016). An Improved Culture Method for Selective Isolation of *Campylobacter jejuni* from Wastewater. *Front Microbiol*. 26;7: 1345.

Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC and Nauseef WM (2013). Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J Leukoc Biol.* 93(2): 185-98.

Konkel ME, Garvis SG, Tipton SL, Anderson DE and Cieplak W (1997). Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol Microbiol*. 24(5): 953-63.

Konkel ME, Klena JD, Rivera-Amill V, Monteville MR, Biswas D, Raphael B and Mickelson J (2004). Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J Bacteriol.* 186(11): 3296-303.

Krause-Gruszczynska M, van Alphen LB, Oyarzabal OA, Alter T, Hänel I, Schliephake A, König W, van Putten JP, Konkel ME and Backert S (2007). Expression patterns and role of the CadF protein in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiol Lett*. 274(1): 9-16.

Lai CK, Chen YA, Lin CJ, Lin HJ, Kao MC, Huang MZ, Lin YH, Chiang-Ni C, Chen CJ, Lo UG, Lin LC, Lin H, Hsieh JT and Lai CH (2016). Molecular Mechanisms and Potential

Clinical Applications of *Campylobacter jejuni* Cytolethal Distending Toxin. *Front Cell Infect Microbiol*. 6: 9.

Lara-Tejero M and Galán JE (2000). A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. *Science*. 290(5490): 354-7.

Larson CL, Samuelson DR, Eucker TP, O'Loughlin JL and Konkel ME (2013). The fibronectin-binding motif within FlpA facilitates *Campylobacter jejuni* adherence to host cell and activation of host cell signaling. *Emerg Microbes Infect*. 2(10): e65.

Lei HT, Shen Z, Surana P, Routh MD, Su CC, Zhang Q and Yu EW (2011). Crystal structures of CmeR-bile acid complexes from *Campylobacter jejuni*. *Protein Sci*. 20(4): 712-23.

Leon-Kempis Mdel R, Guccione E, Mulholland F, Williamson MP and Kelly DJ (2006). The *Campylobacter jejuni* PEB1a adhesin is an aspartate/glutamate-binding protein of an ABC transporter essential for microaerobic growth on dicarboxylic amino acids. *Mol Microbiol.* 60(5): 1262-75.

Lindmark B, Rompikuntal PK, Vaitkevicius K, Song T, Mizunoe Y, Uhlin BE, Guerry P and Wai SN (2009). Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from *Campylobacter jejuni*. *BMC Microbiol*. 9: 220.

Li X, Apel D, Gaynor EC and Tanner ME (2011). 5'-methylthioadenosine nucleosidase is implicated in playing a key role in a modified futalosine pathway for menaquinone biosynthesis in *Campylobacter jejuni*. *J Biol Chem*. 286(22): 19392-8.

Malinverni JC and Silhavy TJ (2009). An ABC transport system that maintains lipid asymmetry in the gram-negative outer membrane. *Proc Natl Acad Sci U S A*. 106(19): 8009-14.

Modi S, Brahmbhatt MN, Chatur YA and Nayak JB (2015). Prevalence of *Campylobacter* species in milk and milk products, their virulence gene profile and anti-bio gram. *Vet World*. 8(1): 1-8.

Monteiro MA, Baqar S, Hall ER, Chen YH, Porter CK, Bentzel DE, Applebee L and Guerry P (2009). Capsule polysaccharide conjugate vaccine against diarrheal disease caused by *Campylobacter jejuni*. *Infect Immun*. 77(3): 1128-36.

Nachamkin I and Nguyen P (2017). Isolation of *Campylobacter* Species from Stool Samples by Use of a Filtration Method: Assessment from a United States-Based Population. *J Clin Microbiol*. 55(7): 2204-2207.

Neal-McKinney JM and Konkel ME (2012). The *Campylobacter jejuni* CiaC virulence protein is secreted from the flagellum and delivered to the cytosol of host cells. *Front Cell Infect Microbiol.* 2: 31.

Nelson KJ, Knutson ST, Soito L, Klomsiri C, Poole LB and Fetrow JS (2011). Analysis of the peroxiredoxin family: using active-site structure and sequence information for global classification and residue analysis. *Proteins*. 79(3): 947-64.

Novik V, Hofreuter D and Galán JE (2010). Identification of *Campylobacter jejuni* genes involved in its interaction with epithelial cells. *Infect Immun* 78(8): 3540-53.

Palyada K, Sun YQ, Flint A, Butcher J, Naikare H and Stintzi A (2009). Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. *BMC Genomics* 10: 481.

Pan L, Zhang X, Song K, Tang B, Cai W, Wu X, Rupp RA and Xu J (2009). Real-time imaging of autofluorescence NAD(P)H in single human neutrophils. *Appl Opt.* 48(6): 1042-6.

Pequegnat B, Laird RM, Ewing CP, Hill CL, Omari E, Poly F, Monteiro MA and Guerry P (2017). Phase-Variable Changes in the Position of O-Methyl Phosphoramidate Modifications on the Polysaccharide Capsule of *Campylobacter jejuni* Modulate Serum Resistance. *J Bacteriol*. 199(14).

Pei Z, Burucoa C, Grignon B, Baqar S, Huang XZ, Kopecko DJ, Bourgeois AL, Fauchere JL and Blaser MJ (1998). Mutation in the peb1A locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. *Infect Immun.* 66(3): 938-43.

Pittman MS, Elvers KT, Lee L, Jones MA, Poole RK, Park SF and Kelly DJ (2007). Growth of *Campylobacter jejuni* on nitrate and nitrite: electron transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress. *Mol Microbiol*. 63(2): 575-90.

Poly F, Serichatalergs O, Schulman M, Ju J, Cates CN, Kanipes M, Mason C and Guerry P (2011). Discrimination of major capsular types of *Campylobacter jejuni* by multiplex PCR. *J Clin Microbiol*. 49(5): 1750-7.

Poock SR, Leach ER, Moir JW, Cole JA and Richardson DJ (2002). Respiratory detoxification of nitric oxide by the cytochrome c nitrite reductase of *Escherichia coli*. *J Biol Chem*. 277(26): 23664-9.

Post A, Martiny D, Waterschoot N, Hallin M, Maniewski U, Bottieau E, Van Esbroeck M, Vlieghe E, Ombelet S, Vandenberg O and Jacobs J (2017). Antibiotic susceptibility profiles among *Campylobacter* isolates obtained from international travelers between 2007 and 2014. *Eur J Clin Microbiol Infect Dis.* doi: 10.1007/s10096-017-3032-6.

Potter L, Angove H, Richardson D and Cole J (2001). Nitrate reduction in the periplasm of gram-negative bacteria. *Adv Microb Physiol*. 45: 51-112.

Radomska KA, Wösten MMSM, Ordoñez SR, Wagenaar JA and van Putten JPM (2017). Importance of *Campylobacter jejuni* FliS and FliW in Flagella Biogenesis and Flagellin Secretion*. Front Microbiol*. 8: 1060.

Reid AN, Pandey R, Palyada K, Naikare H and Stintzi A (2008). Identification of *Campylobacter jejuni* genes involved in the response to acidic pH and stomach transit. *Appl Environ Microbiol*. 74(5): 1583-97.

Rivera-Amill V, Kim BJ, Seshu J and Konkel ME. Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. *J Infect Dis*. 183(11): 1607-16.

Rodgers JD, Simpkin E, Lee R, Clifton-Hadley FA and Vidal AB (2017). Sensitivity of Direct Culture, Enrichment and PCR for Detection of *Campylobacter jejuni* and *C. coli* in Broiler Flocks at Slaughter. *Zoonoses Public Health*. 64(4): 262-271.

Rubinchik S, Seddon AM and Karlyshev AV (2014). A negative effect of *Campylobacter* capsule on bacterial interaction with an analogue of a host cell receptor*. BMC Microbiol*. 14: 141.

Russell RG, Sarmiento JI, Fox J and Panigrahi P (1990). Evidence of reinfection with multiple strains of *Campylobacter jejuni* and *Campylobacter coli* in *Macaca nemestrina* housed under hyperendemic conditions*. Infection and Immunity*. 58(7): 2149-55.

Samuelson DR, Eucker TP, Bell JA, Dybas L, Mansfield LS and Konkel ME (2013). The *Campylobacter jejuni* CiaD effector protein activates MAP kinase signaling pathways and is required for the development of disease. *Cell Commun Signal*. 11: 79.

Sanad YM, Jung K, Kashoma I, Zhang X, Kassem II, Saif YM and Rajashekara G (2014). Insights into potential pathogenesis mechanisms associated with *Campylobacter jejuni*-induced abortion in ewes. *BMC Vet Res*. 10: 274.

Sanchuki HB, Valdameri G, Moure VR, Oliveira MA, Pedrosa FO, Souza EM, Korolik V and Huergo LF (2015). Purification of the *Campylobacter jejuni* Dps protein assisted by its high melting temperature. *Protein Expr Purif*. 111: 105-10.

Sellars MJ, Hall SJ and Kelly DJ (2002). Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen*. J Bacteriol*. 184(15): 4187-96.

Seo SW, Kim D, Szubin R and Palsson BO (2015). Genome-wide Reconstruction of OxyR and SoxRS Transcriptional Regulatory Networks under Oxidative Stress in *Escherichia coli* K-12 MG1655*. Cell Rep* 12(8): 1289-99.

Smart JP, Cliff MJ and Kelly DJ (2009). A role for tungsten in the biology of *Campylobacter jejuni*: tungstate stimulates formate dehydrogenase activity and is transported via an ultra-high affinity ABC system distinct from the molybdate transporter. *Mol Microbiol*. 74(3): 742-57.

Smith HK, Shepherd M, Monk C, Green J and Poole RK (2011). The NO-responsive hemoglobins of *Campylobacter jejuni*: concerted responses of two globins to NO and evidence in vitro for globin regulation by the transcription factor NssR. *Nitric Oxide*. 25(2): 234-41.

Sørensen MC, van Alphen LB, Harboe A, Li J, Christensen BB, Szymanski CM and Brøndsted L (2011). Bacteriophage F336 recognizes the capsular phosphoramidate modification of *Campylobacter jejuni* NCTC11168. *J Bacteriol*. 193(23): 6742-9.

Strid MA, Engberg J, Larsen LB, Begtrup K, Mølbak K and Krogfelt KA (2001). Antibody Responses to *Campylobacter* Infections Determined by an Enzyme-Linked Immunosorbent Assay: 2-Year Follow-Up Study of 210 Patients. *Clinical and Diagnostic Laboratory Immunology*. 8(2): 314-319.

Szymanski CM and Wren BW (2005). Protein glycosylation in bacterial mucosal pathogens. *Nat Rev Microbiol.* 3(3): 225-37.

Tam CC and O'Brien SJ (2016). Economic Cost of *Campylobacter*, Norovirus and Rotavirus Disease in the United Kingdom. *PLoS ONE* 11(2): e0138526

Tanner JJ (2008). Structural biology of proline catabolism. *Amino Acids*. 35(4): 719-30.

Tang Y, Sahin O, Pavlovic N, LeJeune J, Carlson J, Wu Z, Dai L and Zhang Q (2017). Rising fluoroquinolone resistance in *Campylobacter* isolated from feedlot cattle in the United States. *Sci Rep*. 29;7(1): 494.

Thomas MT, Shepherd M, Poole RK, van Vliet AH, Kelly DJ and Pearson BM (2011). Two respiratory enzyme systems in *Campylobacter jejuni* NCTC 11168 contribute to growth on L-lactate. *Environ Microbiol*. 13(1): 48-61.

Tinajero-Trejo M and Shepherd M (2013). The globins of *Campylobacter jejuni*. *Adv Microb Physiol*. 63: 97-145.

Trivedi RN, Agarwal P, Kumawat M, Pesingi PK, Gupta VK, Goswami TK and Mahawar M (2015). Methionine sulfoxide reductase A (MsrA) contributes to *Salmonella typhimurium* survival against oxidative attack of neutrophils. *Immunobiology*. 220(12): 1322-7.

van der Stel AX, van Mourik A, Heijmen-van Dijk L, Parker CT, Kelly DJ, van de Lest CH, van Putten JP and Wösten MM (2015). *The Campylobacter jejuni* RacRS system regulates fumarate utilization in a low oxygen environment. *Environ Microbiol*. 17(4): 1049-64.

van der Stel AX, Boogerd FC, Huynh S, Parker CT, van Dijk L, van Putten JPM and Wösten MMSM (2017). Generation of the membrane potential and its impact on the motility, ATP production and growth in *Campylobacter jejuni*. *Mol Microbiol*. doi: 10.1111/mmi.13723.

Vegge CS, Brøndsted L, Ligowska-Marzęta M and Ingmer H (2012). Natural transformation of *Campylobacter jejuni* occurs beyond limits of growth. PLoS One. 7(9): e45467.

Vegge CS, Jansen van Rensburg MJ, Rasmussen JJ, Maiden MC, Johnsen LG, Danielsen M, MacIntyre S, Ingmer H and Kelly DJ (2016). Glucose Metabolism via the Entner-Doudoroff Pathway in *Campylobacter*: A Rare Trait that Enhances Survival and Promotes Biofilm Formation in Some Isolates. *Front Microbiol*. 7: 1877.

Velayudhan J and Kelly DJ (2002). Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. *Microbiology* 148(3): 685-94.

Velayudhan J, Jones MA, Barrow PA and Kelly DJ (2004). L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by *Campylobacter jejuni. Infect Immun*. 72(1): 260-8.

Waldenström J, Broman T, Carlsson I, Hasselquist D, Achterberg RP, Wagenaar JA and Olsen B (2002). Prevalence of *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter coli* in different ecological guilds and taxa of migrating birds. *Appl Environ Microbiol*. 68(12): 5911-7.

Weerakoon DR and Olson JW (2008). The *Campylobacter jejuni* NADH:ubiquinone oxidoreductase (complex I) utilizes flavodoxin rather than NADH. *J Bacteriol*. 190(3): 915-25.

Weerakoon DR, Borden NJ, Goodson CM, Grimes J and Olson JW (2009). The role of respiratory donor enzymes in *Campylobacter jejuni* host colonization and physiology. *Microb Pathog*. 47(1): 8-15.

Weingarten RA, Grimes JL and Olson JW (2008). Role of *Campylobacter jejuni* respiratory oxidases and reductases in host colonization. *Appl Environ Microbiol*. 74(5): 1367-75.

Weingarten RA, Taveirne ME and Olson JW (2009). The dual-functioning fumarate reductase is the sole succinate:quinone reductase in *Campylobacter jejuni* and is required for full host colonization. *J Bacteriol.* 191(16): 5293-300.

Wösten MM, van de Lest CH, van Dijk L and van Putten JP (2017). Function and Regulation of the C4-Dicarboxylate Transporters in *Campylobacter jejuni*. *Front Microbiol*. 8: 174.

Yahara K, Méric G, Taylor AJ, de Vries SP, Murray S, Pascoe B, Mageiros L, Torralbo A, Vidal A, Ridley A, Komukai S, Wimalarathna H, Cody AJ, Colles FM, McCarthy N, Harris D, Bray JE, Jolley KA, Maiden MC, Bentley SD, Parkhill J, Bayliss CD, Grant A, Maskell D, Didelot X, Kelly DJ and Sheppard SK (2017). Genome-wide association of functional traits linked with *Campylobacter jejuni* survival from farm to fork. *Environ Microbiol*. 19(1): 361-380.

Zautner AE, Johann C, Strubel A, Busse C, Tareen AM, Masanta WO, Lugert R, Schmidt-Ott R and Groß U (2014). Seroprevalence of campylobacteriosis and relevant post-infectious sequelae. *European Journal of Clinical Microbiology & Infectious Diseases*. 33(6): 1019-1027.

Chapter 2: Yahara *et al.*

Genome-wide association of functional traits linked with *Campylobacter jejuni* survival from farm to fork (2017) *Environmental Microbiology* 19(1), 361–380.

Koji Yahara, Guillaume Méric, **Aidan J. Taylor**, Stefan P. W. de Vries, Susan Murray, Ben Pascoe, Leonardos Mageiros, Alicia Torralbo, Ana Vidal, Anne Ridley, Sho Komukai, Helen Wimalarathna, Alison J. Cody, Frances M. Colles, Noel McCarthy, David Harris, James E. Bray, Keith A. Jolley, Martin C. J. Maiden, Stephen D. Bentley, Julian Parkhill, Christopher D. Bayliss, Andrew Grant, Duncan Maskell, Xavier Didelot, David J. Kelly and Samuel K. Sheppard

Preface

In collaboration with Prof Samuel Sheppard at Swansea University, we conducted a Genome-Wide Association Study (GWAS) study on the association of functional traits linked with *C. jejuni* survival from farm to fork. The GWAS study identified genetic elements significantly associated with human disease, predominately single nucleotide polymorphisms (SNPs), by sequencing and comparing 600 isolates from multiple stages of the poultry processing chain and human clinical cases. Ultimately, the significantly associated SNPs were analysed to reveal genetic variation with increased prevalence in clinical isolates. In total, 19 unique genes containing clinically associated SNP(s) were mapped onto the *C.jejuni* NCTC11168 genome. A selection of the associated genes of unknown, or partially understood, function were targeted for mutagenesis to reveal their potential role in clinical pathogenesis. This study highlights the power of combining large scale GWAS techniques with traditional microbiology to identify and characterise hitherto unknown virulence factors.

Author contributions

Isolate collection, genome sequencing and all bioinformatics leading to the mapping of clinically associated SNPs was performed primarily by Koji Yahara, Guillaume Méric and Samuel K. Sheppard. All other authors had minor contributions to isolate collection, data analysis etc. Aidan J. Taylor and David J. Kelly had no contribution.

Interpretation of the biological function of associated genes and resulting experimental design was by Aidan J. Taylor and David J. Kelly alone. All microbiology was

performed solely by Aidan J. Taylor, with the exception of the *fumC* and *nuoK* mutant strains, and associated wildtype, which were provided by Stefan P. W. de Vries *et al*. from the University of Cambridge. However all experiments with these strains were performed by Aidan J. Taylor alone.

The manuscript was written by Koji Yahara, Guillaume Méric, Aidan J. Taylor, David J. Kelly, and Samuel K. Sheppard. Figures 1-4, table 1, supplementary figures S1-3, S5-6 and supplementary tables S1, S3-5 were produced by Koji Yahara, Guillaume Méric and Samuel K. Sheppard. Figures 5-6, supplementary figure S4 and supplementary table S2 were produced by Aidan J. Taylor and David J. Kelly.

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Chapter 3: Taylor *et al*.

The Periplasmic Chaperone Network of *Campylobacter jejuni*: Evidence that SalC (Cj1289) and PpiD (Cj0694) Are Involved in Maintaining Outer Membrane Integrity (2017) *Front. Microbiol*. 8, 531.

Aidan J. Taylor, Shadi A. I. Zakai and David J. Kelly

Preface

David J. Kelly had previously demonstrated that *C. jejuni* encodes two periplasmic chaperones related to the major *E. coli* chaperone SurA and solved the crystal structure of both these enzymes (Kale *et al.* 2011). In this study we set out to complete the periplasmic chaperone network of *C. jejuni*. Bioinformatic searches identified 5 putative chaperones, all of which were targeted for mutagenesis and their phenotype relating to outer membrane integrity was assessed. Particular focus was put on Cj0694, which we show by both mutagenesis and purified enzyme assays, is the *C. jejuni* equivalent of PpiD. We also demonstrate that Cj1289 is distinct from the SurA and name it SalC, for SurA-like chaperone. This work gives a comprehensive view of outer membrane protein biogenesis in *C. jejuni* and highlights their importance in cell integrity.

Author contributions

Aidan J. Taylor and David J. Kelly conceived the study and wrote the manuscript. Mutant construction and phenotypic assays were designed and performed by Aidan J. Taylor (figures 1-2). 2D gels, protein purification and enzyme assays were performed by Shadi A. I. Zakai (figures 3-5).

Supplementary material can be found online at:

http://journal.frontiersin.org/article/10.3389/fmicb.2017.00531/full

Chapter 4: The periplasmic methionine sulfoxide reductase system of *C. jejuni*

4.1 Introduction

During invasion of the host, enteric bacteria are exposed to reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS) by the innate immune response of macrophages and neutrophils, through the activity of host NADPH oxidase, nitric oxide synthase and myeloperoxidase, respectively (Flannagan *et* al. 2015, Winterbourn and Kettle 2013). Therefore, most enteric bacteria have an arsenal of defences against oxidative species, including catalase, superoxide dismutase and peroxiredoxins to neutralise ROS, and RNS detoxifying haemoglobins, to eliminate the harmful oxidants before they cause cellular damage (see section 1.4 Stress response). Despite these defences, oxidation of bacterial proteins can still occur. Cysteine (Cys) and methionine (Met) are orders of magnitude more sensitive to oxidation than other residues due to their electron rich sulfur groups, particularly in reaction with the potent RCS hypochlorous acid (HOCl), generated *in vivo* by myeloperoxidase (Armesto *et al*. 2000). Myeloperoxidase is packaged into chlorinated neutrophil granules which are delivered to bacterial cells upon internalisation, so localised concentrations of HOCl *in vivo* are likely to be high, making HOCl-dependent oxidation of Cys and Met a physiologically relevant threat to enteric pathogens (Prokopowicz *et al*. 2012). MetSO formation is known to inactivate enzymes: for example, catalase from *Helicobacter pylori* is inactivated by HOCl, with oxidation occurring at several specific Met residues. The activity of HOCl-inactivated catalase can be recovered *in vitro* by Msr, though GroEL is also required to repair MetSO induced structural damage for full recovery of activity (Mahawar *et al*. 2011). Conversely, Met oxidation can be actively used to regulate gene expression. In *E. coli*, oxidation of three specific Met residues of the hypochlorite-responsive transcription factor, HypT, causes a conformational shift which results in the active tetrameric oligomer (Drazic *et al*. 2014). Active HypT enhances expression of Met and Cys biosynthesis genes, and represses iron uptake genes, both replenishing cellular Met and Cys levels and limiting Fenton chemistry (Gebendorfer *et al*. 2012). HypT is reduced to the inactive state by MsrAB, providing a feedback mechanism for response to HOCl (Drazic *et al*. 2013). A similar regulatory mechanism utilising oxidised Cys and turnover by glutaredoxin has been demonstrated for the oxidative stress transcriptional regulator OxyR from *E. coli* (Choi *et al*. 2001, Zheng *et al*. 1998). Oxidation of one Cys residue to sulfenic acid permits the formation of a disulfide bond with a second Cys, activating OxyR, which induces expression of oxidative stress defence genes (Storz *et al*. 1990). Thus, Met and Cys

oxidation is not simply a damaging protein modification, but can be used by bacteria to sense oxidative stress and respond accordingly.

The Cys thiol group (-SH) is a potent nucleophile, with the pK_a of free Cys around 8.6, but this value can vary significantly within proteins, dependent on the local environment. Positively charged adjacent residues can induce deprotonation, so some Cys residues within proteins exist as the even more reactive thiolate group ($-S^-$), even at physiological pH (Thurlkill *et al*. 2006). Two electron oxidants form sulfenic acid (-SOH), and single electron oxidants form thiyl radicals (-S•) which generally react with hydroxyls to form sulfenic acid. Sulfenic acid can further oxidise to irreversible sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acids, or condensate into disulfide bonds (Paulsen and Carroll 2013). Disulfide bonds are not actively introduced into cytoplasmic proteins, but do occur as part of catalytic reactions, and are reduced back by thioredoxin and glutathione pathways, which also reduce oxidised single Cys residues (Stewart *et* al. 1998). Disulfide bond formation in the periplasm is governed by the well-conserved Dsb system and this sequesters the majority of cysteines, but some periplasmic proteins do contain single Cys residues, which are susceptible to oxidation (Meehan *et al*. 2017). A periplasmic sulfenic acid reductase identified in *E. coli*, DsbG, is a thioredoxin fold enzyme with the characteristic catalytic CXXC motif which reduces sulfenic acid back to the thiol state (Depuydt *et al*. 2009). DsbG shares high sequence identity with DsbC, the disulfide bond isomerase, and, interestingly, mutation of key DsbG residues can endow the protein with disulfide bond isomerase activity (Chatelle *et al*. 2015). *C. jejuni* only encodes one DsbC/G homolog, Cj1380, which, although currently annotated as a DsbC disulfide bond isomerase, has an active site motif more closely resembling the DsbG sulfenic acid reductase from *E. coli*. Therefore, it is likely that either Cj1380 has both disulfide bond isomerase and sulfenic acid reductase activity, or there is a structurally unrelated sulfenic acid reductase yet to be identified.

Oxidation of Met residues is similar to that of Cys: the thioether group (-S-) can undergo a two electron oxidation to methionine sulfoxide (-SO-) (MetSO), or a single electron oxidation to a sulfide radical (-S•-) which further oxidises to MetSO. In the specific case of HOCl, the thioether of Met sustains a nucleophilic attack from HOCl (or $^-$OCl, depending on pH) to form a chlorosulfonium intermediate, which then condenses with a molecule of water to form the S or R isomer of MetSO (Figure 1). It is possible for MetSO to further oxidise to methionine sulfone ($-SO_2-$), a rare and irreversible modification (Vogt 1995).

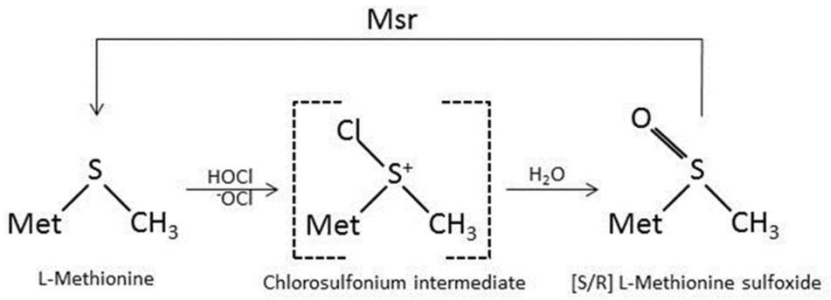

Figure 1: Oxidation of methionine to methionine sulfoxide by hypochlorous acid.

MetSO in cytoplasmic proteins is repaired by an almost universally conserved system of methionine sulfoxide reductases (Msr), normally by the stereospecific species MsrA and MsrB, for the S and R isomers, respectively, which arose as an early evolutionary response to the accumulation of O_2 in Earth's atmosphere (Delaye *et al*. 2007). Despite MsrA and MsrB being unrelated in terms of structure and sequence, their catalytic reduction of MetSO follows a similar mechanism utilising two catalytic Cys residues. In the first step, a nucleophilic Cys attacks MetSO, forming sulfenic acid on the Cys and releasing the reduced Met. The second Cys then performs a nucleophilic attack on the sulfenic acid, condensing into a disulfide bond, releasing a molecule of water (Figure 2). Finally, the disulfide bond is reduced by the thioredoxin Trx and active Msr restored (Boschi-Muller *et al*. 2005).

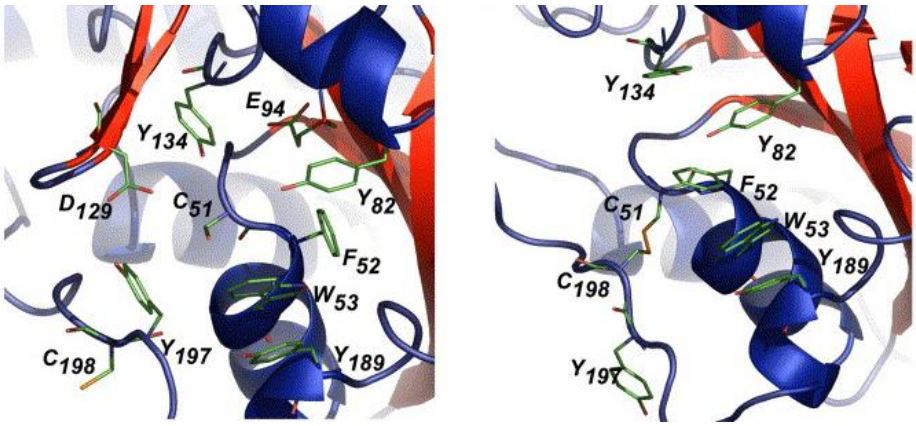

Figure 2: Representative structure of MsrA active site in the reduced (left panel) and oxidised (right panel) state (Coudevylle *et al*. 2007).

Variations of this catalytic model exist: some Msr's have three catalytic cysteines which transfer disulfide bonds for greater catalytic efficiency with Trx (Boschi-Muller *et al*. 2005). Some bacteria encode a single MsrAB fusion protein joined by a linker region which appears to improve catalytic efficiency (Han *et al*. 2016). In eukaryotes, some Msr's contain selenocysteine residues in place of Cys, which maximises catalytic efficiency (Kim and Gladyshev 2005). The stereospecificity of MsrA and MsrB has been attributed to their mirror

image structures, which contain a conserved tryptophan residue responsible for terminal methyl coordination of MetSO on opposite sides of the active site pocket (Lowther *et al*. 2002).

Interestingly, *msrAB* mutants in *E. coli* still demonstrate both S and R-MetSO reductase activity, and are able to grow on either as the sole Met source, demonstrating the existence of additional Msr activities (Spector *et al*. 2003). This led to the discovery of two further Msr proteins specific for free MetSO, the GAF domain protein fRMsr (or MsrC) and biotin sulfoxide reductase BisC (Ezraty *et al*. 2005, Lin *et al*. 2007). Although the physiological role of these proteins remains to be elucidated, the authors suggest they could function to a) reduce oxidised free Met for assimilation and use in protein synthesis, b) reduce free MetSO which is sacrificially oxidised to protect cellular proteins from oxidation, or c) to detect free MetSO as a signalling molecule for oxidative stress (Ezraty *et al*. 2005, Lin *et al*. 2007). In the perchlorate respiring bacteria *Azospira suillum* HOCl is generated as a metabolic intermediate. To combat the accumulation of this toxic intermediate, *A. suillum* expresses a periplasmic Met rich peptide, MrpX, which scavenges HOCl by sacrificial oxidation of its Met residues, which are reduced by a cognate Msr, YedY1 (Melnyk *et al*. 2015). However, bioinformatics searches reveal no obvious homologs of MsrC, BisC or MrpX in *C. jejuni*. MsrA and MsrB, however, are present and encoded by *cj0637c* and *cj1112c*, respectively (Atack and Kelly, 2008). Stereospecific MetSO reduction with the purified proteins was demonstrated, and mutagenesis demonstrated increased sensitivity to ROS and RNS sources (Atack and Kelly, 2008).

YedY is a mononuclear, molybdopterin cofactor enzyme, well conserved amongst Gram-negative bacteria, which has been of structural interest for over a decade (Loschi *et al*. 2004). The Mo binding region of YedY closely resembles the sulfite oxidase family, with the exception that isolated YedY Mo exists in the Mo^{IV}/Mo^{V} oxidation states, as opposed to the Mo^{V}/Mo^{VI} oxidation states commonly adopted by sulfite oxidases (Brokx *et al*. 2005). In addition, YedY harbours a less reduced form of pterin, designated dihydro pyranopterin dithiolate, and is the only known example of a Mo enzyme utilising this form of pterin (Figure 3) (Lee *et al*. 2016).

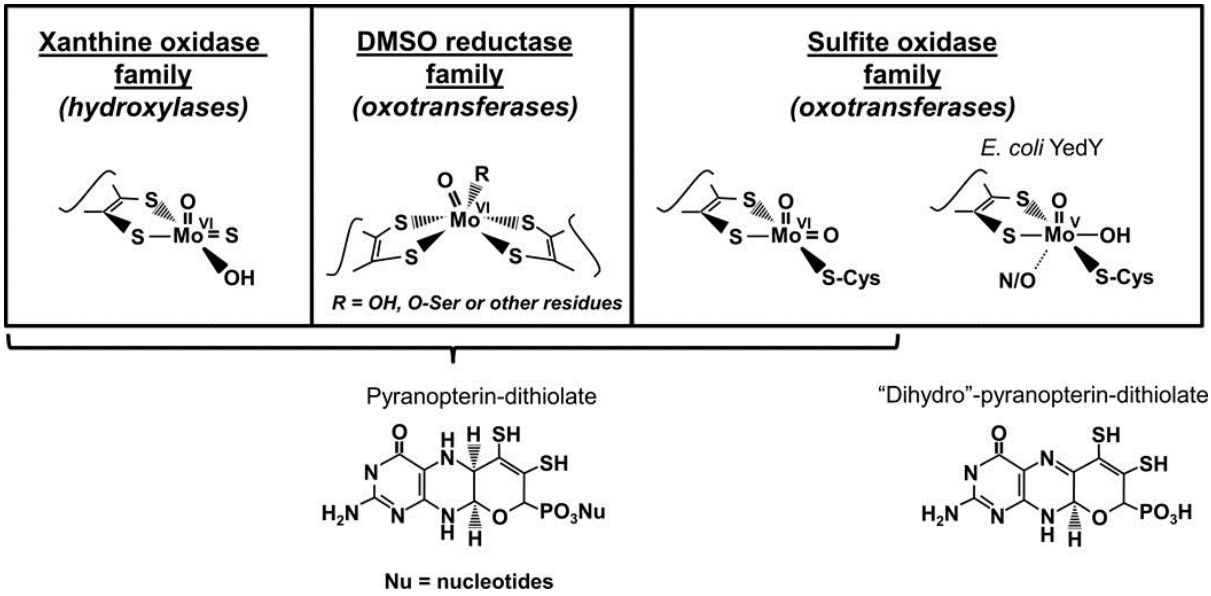

Figure 3: Active site configuration of Mo-pyranopterin-containing enzymes and YedY. The pyranopterin dithiolate ligands are abbreviated as a wavy line (Lee et al. 2016).

Adamson *et al*. (2015) utilised Fourier-transformed electrochemistry to designate the electron transfer mechanism of YedY catalysis, summarised in Figure 4. As-isolated YedY is reduced from the Mo^{V} to Mo^{IV} state by a 1 e^{-}, 1H^{+} transfer in a relatively slow reaction requiring metal centre and coordination environment changes. A second, much faster reaction occurs by a 2e^{-}, 2H^{+} transfer onto the unique dihydro-pyranopterin group, reducing to the more common tetrahydro-pyranopterin form: this transition is essential for catalytically active YedY (Adamson *et al*. 2015).

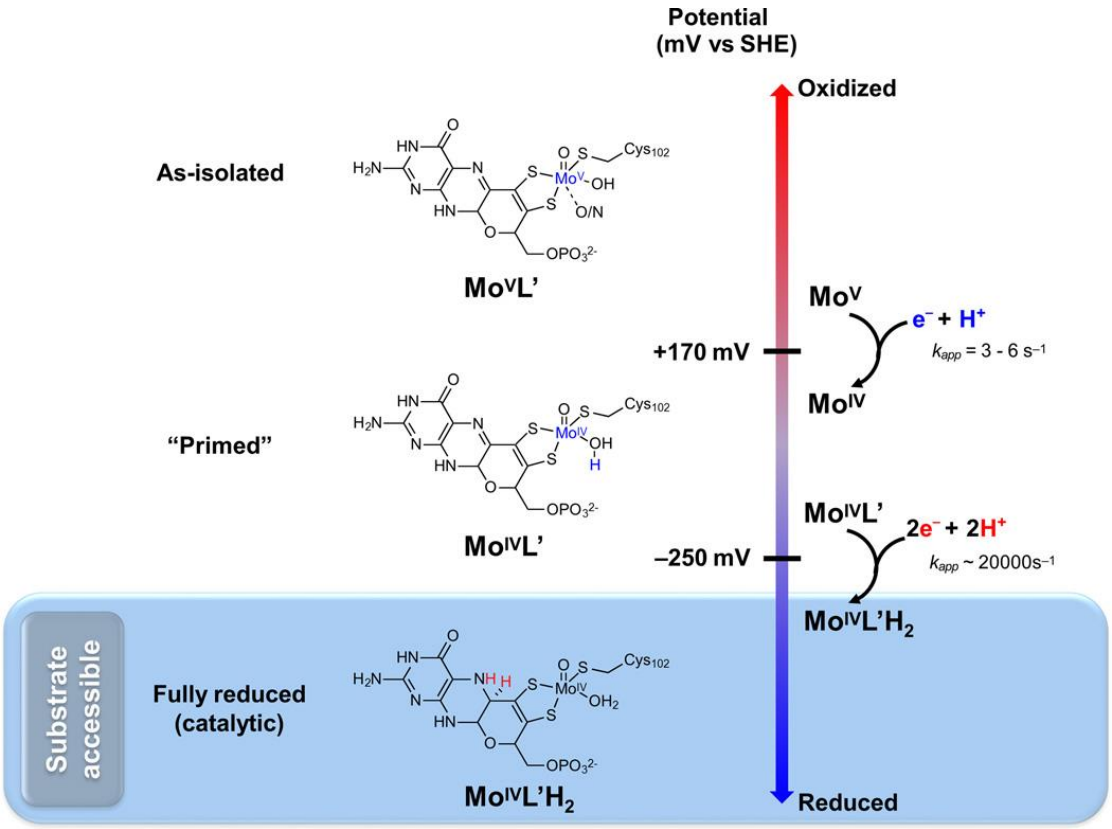

Figure 4: Summarised reduction mechanism of YedY Mo group (Lee *et al*. 2016).

Although annotated as a sulfite reductase, YedY does not show significant activity with sulfite and has historically been studied with DMSO and TMAO as substrates, though the K_m values have been poor, leaving the native substrate unclear (Sabaty *et al*. 2013). Melnyk *et al*. (2015) were the first to identify YedY as a MetSO reductase during their study on the hypochlorite quenching Met-rich protein MrpX, mentioned above, however they failed to demonstrate the general significance of their discovery. Shortly after, Gennaris *et al*. (2015) published the keystone paper describing YedY as a periplasmic Msr and cognate transmembrane electron donor protein, YedZ, which are hereafter referred to as MsrP and MsrQ, respectively.

MsrP differs fundamentally from the cytoplasmic MsrAB system, utilising molybdopterin centred catalysis, rather than a thiol-based mechanism, to non-stereospecifically reduce both the S and R-isomers of MetSO (Gennaris *et al*. 2015). MsrQ is an inner membrane, *b*-type haem protein which is the cognate electron donor for MsrP. MsrQ is believed to utilise electrons directly from the MQ pool, however recent evidence suggests in *E. coli* MsrQ contains two *b* haem groups, where the first haem receives electrons from the cytoplasmic NADPH-dependent, FMN cofactor containing flavin reductase, Fre, which transfer to the second haem group for reduction of MsrP (Figure 5) (Juillan-Binard *et al*. 2017). This mechanism resembles NOX-type systems in eukaryotes,

which have cytoplasmic NADPH reducing domains and membrane bound haem groups for reduction of extracellular species (Juillan-Binard *et al*. 2017). However, the benefit of this mechanism over utilisation of electrons directly from MQ is unclear and a lack of *in vivo* experiments on *fre* mutants brings into question the relevance of this mechanism in bacteria.

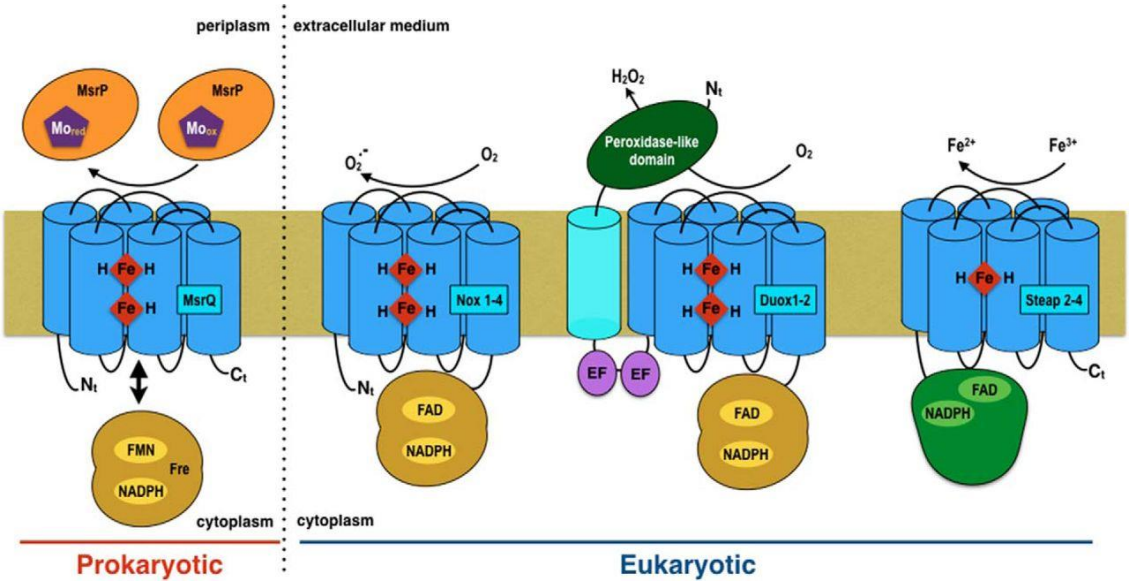

Figure 5: Comparison between the proposed bacterial MsrQ/Fre and eukaryotic NOX-type electron transfer systems. MsrQ may receive electrons from a cytoplasmic flavin reductase, Fre, as seen in eukaryotes, rather than directly from the MQ pool (Juillan-Binard *et al*. 2017).

C. jejuni encodes MsrP and MsrQ, Cj0379 and Cj0378, respectively, and limited previous work by our group, relating to Cj0379 as a TAT-exported protein, showed a *cj0379c* mutant to be strongly attenuated in chicken colonisation and demonstrated a weak nitrosative stress phenotype, leading to the conclusion of a putative role in RNS detoxification in the periplasm (Hitchcock *et al*. 2010). In this study we aimed to elucidate the role and importance of MsrPQ in *C. jejuni* by utilising a combination of mutagenesis, protein overexpression and proteomics. We show that Cj0379 does function as a periplasmic MsrP, both *in vivo* and *in vitro,* and present proteomic data which shows specific Met residues in client proteins susceptible to both oxidation and repair by MsrP, and discuss the implications for protein function.

4.2 Results

4.2.1 Overexpression, purification and enzyme activity of Cj0379

Sabaty *et al*. (2013) demonstrated that the expression and purification method used for the *E. coli* MsrP could have a significant effect on its activity with DMSO as substrate.

MsrP is a client of the twin-arginine translocase (TAT) export system, permitting insertion of its molybdopterin cofactor in the cytoplasm prior to export into the periplasm in a pre-folded state by the TAT system. Sabaty *et al*. (2013) showed that traditional C-terminal His-tagging of MsrP without TAT processing, as used in previous studies, results in very low enzyme activity with DMSO (K_m 261 ± 57 mM, V_{max} 30 ± 2 µmol.min^{-1}.mg^{-1}), while N-terminal His-tagging, utilising a 2-stage cloning method to introduce the His-tag sequence between the TAT signal sequence and mature protein sequence, maintaining TAT processing and maturation, results in much better kinetics with DMSO (K_m 71 ± 9 mM, V_{max} 49 ± 2 µmol.min^{-1}.mg^{-1}). In addition, TEV protease digestion to remove the N-terminal His-tag after purification resulted in an additional increase in activity with DMSO (K_m 61 ± 7 mM, V_{max} 56 ± 2 µmol.min^{-1}.mg^{-1}) (Sabaty *et al*. 2013). Thus processing of heterologously expressed MsrP by the TAT system, ideally in a tag-free system, is crucial for maximum activity. This work was performed before the role of MsrP as a MetSO reductase was appreciated. However, although Gennaris *et al*. (2015) demonstrated MetSO reductase activity with purified MsrP from *E. coli*, they failed to implement the above strategy of His-tag removal post purification. Their V_{max} values of 67.2 and 313.4 µmol.min^{-1}.mg^{-1} for S and R-MetSO, respectively are therefore probably an underestimate.

We sought to overexpress MsrP from *C. jejuni* using an N-terminal His-tag which could be removed by cleavage post-purification to yield maximum possible activity (Figure 6). This was achieved using the overexpression vectors pET28a and pET22b to introduce a His-tag between the TAT signal sequence (SS) and mature protein, with a thrombin digestion site for the removal of the His-tag post-purification (see Materials and Methods). The resulting plasmid was designated pETMsrP.

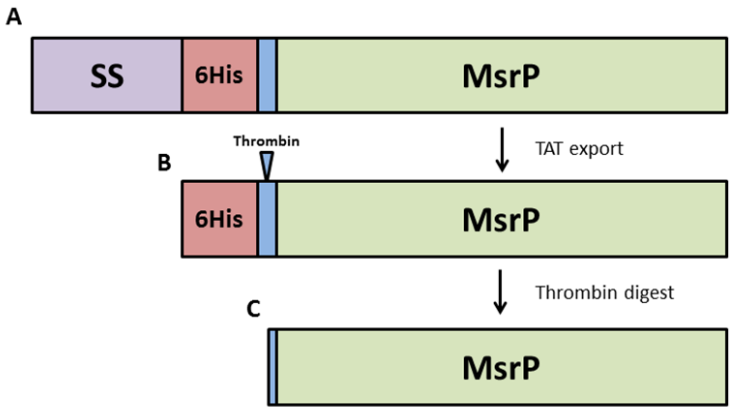

Figure 6: Strategy for overexpression of Cj0379. A) Initial cytoplasmic expression prior to TAT export. B) Periplasmic protein with signal sequence (SS) cleaved off by TAT export and maturation. C) 6His tag removal post-purification by enzymatic thrombin cleavage.

In *E. coli* there are two primary forms of molybdopterin cofactor; MoCo and molybdenum bis-MPT guanine dinucleotide (MGT). MoCo is formed from molybdenum insertion into metal-binding pterin (MPT), synthesised by the *moa* genes. MoCo can be further processed to MGT by the addition of GMP-bound MPT by the MobAB enzymes (Schwarz *et al*. 2009). *C. jejuni* has no *mob* genes and thus only uses MoCo, not MGT, in molybdenum cofactor enzyme synthesis. The *E. coli* strain chosen for heterologous expression of MsrP from pETMsrP was MG1655Δ*iscR* containing the plasmids pGP1-2 and pTPR1, for T7 polymerase expression, allowing IPTG induction, and MPT-synthesis *moa* gene overexpression, respectively. Mutation of *iscR* permits Fe-cluster biosynthesis without oxygen regulation by IscR. Expression was carried out anaerobically in custom MOPS-based glucose/nitrate fermentation media to enhance both endogenous and FNR-regulated pTPR1 cofactor synthesis.

His-tag purification using a Ni-affinity column yielded a fairly impure fraction containing 6His-MsrP and a number of *E. coli* proteins (Figure 7 A). Further purification was accomplished using a hydrophobic interaction column (HIC), yielding a very pure fraction of 6His-MsrP (Figure 7 A). 6His-MsrP was very hydrophobic and only eluted from the column at 0% salt, assisting its purification. At this stage, the protein could be further processed by thrombin digest to remove the 6His-tag, and re-purified by HIC (Figure 7 B). The enzyme activity of both the tagged and untagged version of the protein was determined by methyl viologen linked anaerobic reduction assays with racemic free L-MetSO as substrate (Figure 7 C). Due to very low protein yields (approximately 0.1-0.2 mg l^{-1}) and issues with substrate solubility, accurate K_m values were difficult to acquire, nonetheless the untagged protein was approximately 6-fold more active than the tagged protein.

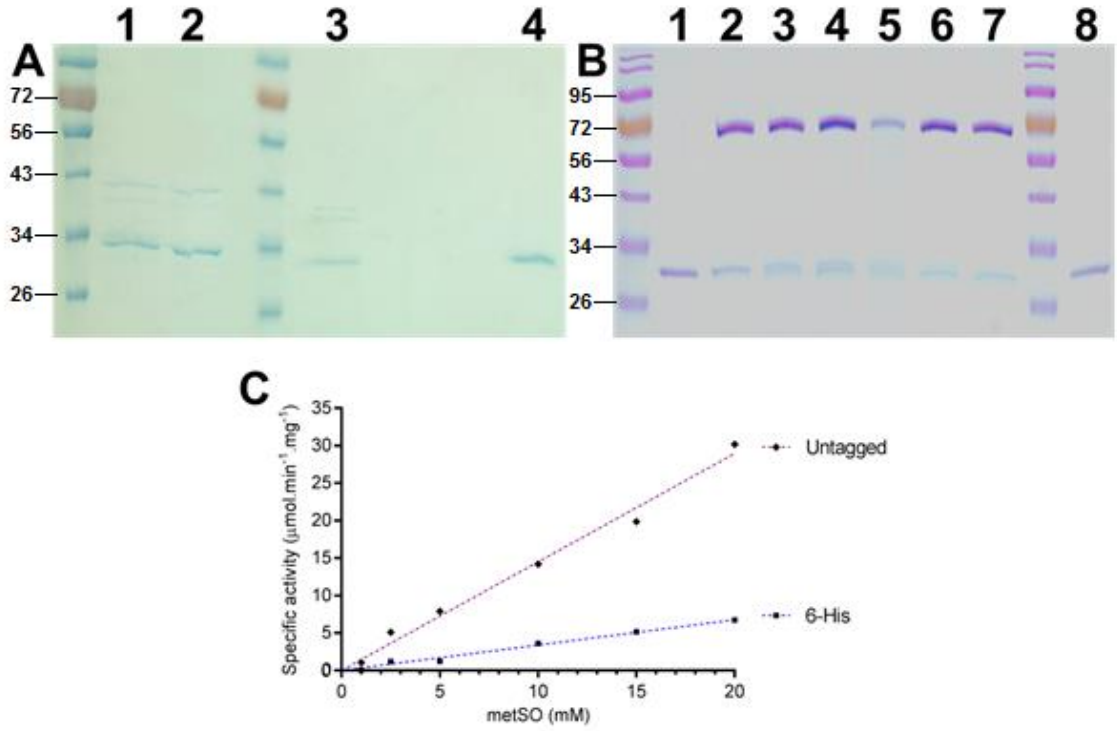

Figure 7: A) HIS and HIC purifications of MsrP. Lanes 1 and 2, 6His-MsrP containing fractions after HIS purification (imidazole elution). Lane 3, pooled HIS purification fractions. Lane 4, Purified 6His-MsrP post HIC column. **B)** Thrombin digest of 6His-MsrP. Lane 1, Purified 6His-MsrP. Lanes 2 to 7, Thrombin digest proceeding over 6 hours. Lane 8, purified untagged MsrP after HIC. Note the appearance of two bands by lane 3, which resolve down to one band by lane 7, in alignment with the predicted 2 kDa reduction in size after tag removal. **C)** Specific activity of 6His-MsrP and untagged MsrP with L-MetSO. MW shown in kDa.

Using the more active untagged protein, the V_{max} value with L-MetSO was double that of DMSO, though the K_m values were not significantly different (Figure 8). The poor K_m value with L-MetSO could be attributed to the free form not being the native substrate: it is possible MetSO within a peptide would promote stronger binding. Electrochemical experiments on MsrP with oxidised Met-containing peptides were attempted in collaboration with the authors of Adamson *et al*. (2015), however technical issues with protein binding to the electrode prevented determination of catalytic rates, though some activity was present (data not shown). Nonetheless, we obtained a V_{max} value of 161.4±30.2 µmol.min^{-1}.mg^{-1} with L-MetSO, similar to that obtained with the His-tagged protein from *E. coli* by Gennaris *et al*. (2015). Overall, despite poor expression and technical issues, we were able to show that *C. jejuni* Cj0379 is an active Msr *in vitro* with a 2-fold greater catalytic rate with L-MetSO than DMSO.

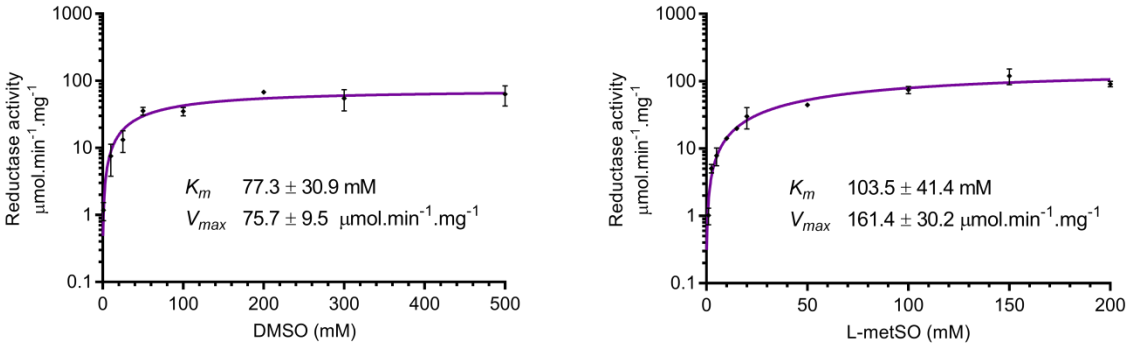

Figure 8: Specific activity of untagged MsrP with DMSO (left) and L-MetSO (right) as determined by methyl viologen linked anaerobic reduction assays.

4.2.2 Sodium hypochlorite assay development

Although commonly labelled as a ROS, HOCl is more accurately a RCS, particularly in the case of Met oxidation, as it is the chlorine atom which performs a nucleophilic attack on the sulfur of Met to form a chlorosulfonium intermediate, before condensing with H_2O to form MetSO (see introduction, Figure 1). The pH in solution strongly effects the speciation of HOCl: at pH 5.0 100% is in the form of HOCl, below pH 5.0 HOCl dissociates to molecular chlorine (Cl_2) and above pH 5.0 the hypochlorite ion (OCl^-) forms (Figure 9). At pH 7.4, solutions are a 1:1 molar ratio of HOCl and OCl^-. Commercial solutions are between pH 11.5 and 13.5, existing as the sodium salt of OCl^-, NaOCl (Wang *et al*. 2007).

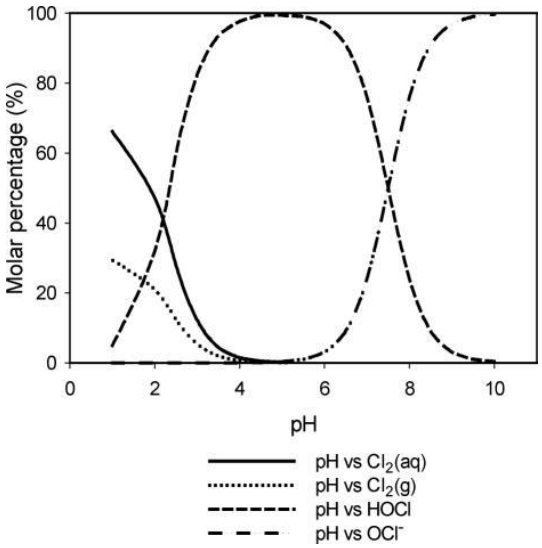

Figure 9: Chlorine speciation profile of hypochlorous acid, as a function of pH (Wang *et al*. 2007).

One issue with the use of NaOCl in bacterial studies has been the inconsistency in treatment conditions, particularly the media used. In addition to the known effect of pH, treatment in buffers with washed cells yields markedly different results to treatment in growth media, where NaOCl can either be quenched by reaction with media components (E.g. free Met and Cys) or can form secondary RCS, such as chloramines, which are toxic to cells in a mechanism disparate from HOCl (Gray *et al*. 2013). To exemplify this issue, trial experiments treating *C. jejuni* with NaOCl in standard growth media required concentrations in the mM range for a reduction in viability, while treatment in phosphate buffer required only 300 µM for complete cell death (data not shown).

To clarify the behaviour of NaOCl in buffers vs. media, and understand the stability in solution, we developed an assay using the reagent *N,N*-diethyl-1,4-phenylenediamine (DPD), commonly used in water chlorination testing kits, to accurately determine the concentration of available chlorine under assay conditions (Lee *et al*. 2007). Oxidation of DPD by RCS causes a colorimetric shift which can be assayed by measuring the absorbance at 550 nm (Figure 10 A). The relationship between absorbance at 550 nm and NaOCl concentration is linear within the practical range, allowing the concentration of free chlorine in samples to be back calculated using the determined equation (Figure 10 B). The quenching of NaOCl by growth media was demonstrated by the almost complete dissipation of available chlorine from even 2 mM NaOCl in MH broth, compared to phosphate buffer (Figure 10 C).

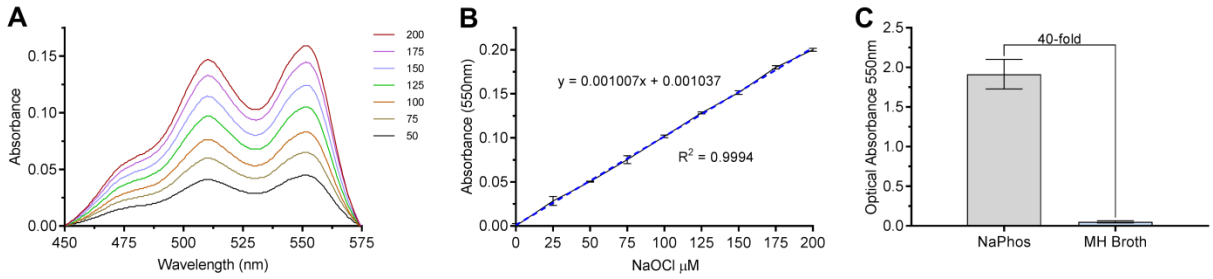

Figure 10: Development of DPD assay. **A**) DPD spectrum with increasing NaOCl concentration (µM) **B**) Linear relationship between NaOCl concentration and DPD absorbance at 550 nm **C**) Quenching of 2 mM NaOCl by MH broth compared to NaPhos buffer.

Hereafter, the treatment of cells was only performed in 20 mM phosphate buffer, pH 7.4 (NaPhos). In order to perform growth experiments with NaOCl treatment, washing steps were necessary to return cells to complex media after NaOCl treatment in buffer. Termination of treatment by washing cells is inaccurate, due to the time required for

centrifuging, and repeated washing causing variability in cell density. We therefore explored the option of NaOCl quenchers, which could be added to buffered cell suspensions to terminate treatment at a defined time (Figure 11 A). Compared to aliphatic amino acids glycine, alanine and serine, which had no quenching effect, both free Met and Cys were able to quench NaOCl at a 1:1 molar ratio. By measuring available chlorine concentration in the supernatant, we demonstrated that a 1:10 molar ratio treatment with free Met is sufficient to quench 90 – 95% NaOCl, and following with a buffer wash eliminates 100% available chlorine (Figure 11 B).

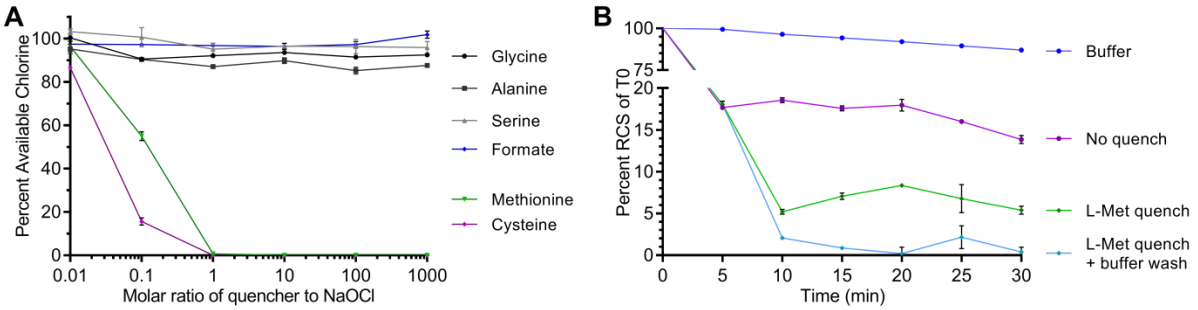

Figure 11: A) Quenching performance of select amino acids and formate. Met and Cys quench NaOCl at a 1:1 molar ratio B) Quenching performance of cell treatment methods (supernatants of cultures with an optical density of 0.1 at 600 nm). A 1:10 molar ratio of free Met causes 90-95% quenching of available chlorine, while an additional wash step completely removes all RCS.

NaOCl interaction with cells appears to occur within a <5 min time frame, reaching a maximum quench before persisting at an equilibrium state of 15-30%, perhaps due to recycling of RCS with cell surface proteins (Figure 11 B, “no quench”). We compared this behaviour to that of the canonical ROS H_2O_2, measuring H_2O_2 concentration using the well characterised FOX assay (Figure 12). Compared to the rapid dissipation of extracellular RCS, H_2O_2 degrades slowly and in linear fashion, possibly due to the enzymatic action of catalase and other enzymes. In both the case of NaOCl and H_2O_2, no significant degradation occurs in buffer alone, demonstrating these compounds are stable over the short treatment periods analysed.

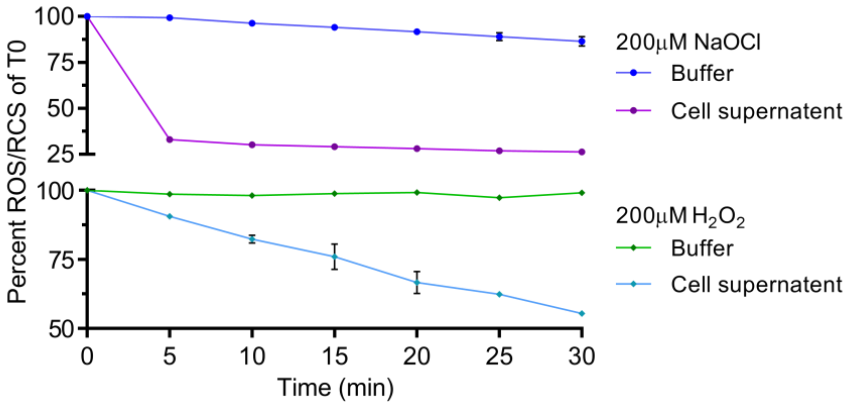

Figure 12: Comparison of the dissipation of ROS/RCS by *C. jejuni* cells (supernatants of cultures with an optical density of 0.1 at 600 nm). NaOCl is quickly dissipated to a maximum value before reaching equilibrium, while H_2O_2 is degraded in a slow, linear fashion.

Overall, the results show that NaOCl treatment in complex media is inappropriate, especially when attempting to assay Met and Cys oxidation, which is specifically targeted by HOCl, as the generation of unknown secondary RCS by reaction of NaOCl with media is likely to result in non-specific damage.

4.2.3 *C. jejuni* susceptibility to NaOCl treatment

In order to determine the appropriate treatment conditions to assay enzyme activities, we first sought to determine the sensitivity of *C. jejuni* to NaOCl (Figure 13). Wildtype cells were treated with varying concentrations of NaOCl for 10 min in NaPhos, then the reaction terminated by the addition of a 1:10 molar ratio of Met, after which cells were serially diluted and viability determined by CFU counts (Figure 13 A). A significant reduction in viability occurred between 50 and 100 μM, with 300 μM sufficient for complete killing. Given MsrP's presumed role in MetSO repair, we predicted that an *msrPQ* deletion mutant would be sensitive to NaOCl. Such a mutant was successfully constructed as described in Materials and Methods. However, under the conditions tested, the *msrPQ* mutant showed no significant change in sensitivity to acute treatment compared to wildtype (Figure 13 B).

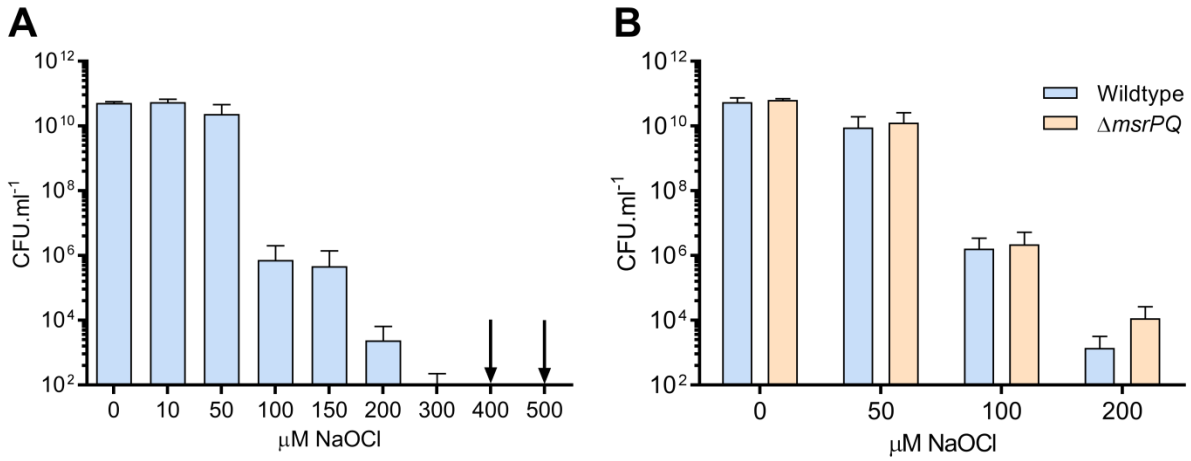

Figure 13: *C. jejuni* sensitivity assays with NaOCl A) Wildtype sensitivity range B) Wildtype vs. *msrPQ* mutant sensitivity to NaOCl is not significantly different.

4.2.4 Initial proteomics and client sensitivity assays

Prior to the realisation of the importance of NaOCl treatment in a simple buffer system, a trial proteomics run was performed on wildtype and Δ*msrPQ C. jejuni* treated with 2 mM NaOCl in MH broth. Periplasmic fractions were prepared, the proteins digested into peptides and then analysed for MetSO content by mass spectrometry. Although the results suggested Met oxidation was not strong, a few periplasmic proteins appeared to be susceptible to Met oxidation on particular residues, though an overall increased abundance above 1-fold in the *msrPQ* mutant compared to wild-type was often not demonstrable (Table 1).

Protein	Function	Met Residue	MetSO Ratio Δ*msrPQ* / WT
NapA	Periplasmic nitrate reductase	283	Mutant specific
		434	Mutant specific
		640	1.59
		417	1.454
		52	1.136
		476	1.088
		545	0.971
		769	0.955
		826	0.866
		95	0.819

Cj0358	Putative cytochrome c_{551} peroxidase	293	1.397
		262	1.342
		146	1.151
MfrA	Fumarate reductase flavoprotein subunit	366	Mutant specific
		194	Mutant specific
		270	1.827
		138	1.668
		481	1.516
		496	0.948
		36	0.862
		178	0.845
MfrB	Fumarate reductase iron-sulfur protein	190	1.459
		198	1.459
		290	1.283
		293	1.186
MfrE	Fumarate reductase subunit E	233	Mutant specific
		75	2.727
		73	2.727
		83	1.661
		71	1.161
		110	1.003
		271	1.003
FdhA	Formate dehydrogenase large subunit	813	Mutant specific
		847	0.919
Cj1516 (CueO)	Putative periplasmic oxidoreductase (multi-copper oxidase)	389	Mutant specific
		390	Mutant specific
		379	Mutant specific
		404	Mutant specific
		437	Mutant specific
		384	1.18
		193	1.05
		386	0.729

Table 1: Periplasmic proteins with apparent susceptibility to MetSO formation on certain residues.

However, this list gave us some clues as to what proteins may be clients of the MsrPQ repair system. FdhA and CueO were chosen for further study, as the activity of these enzymes can be assayed in whole cells relatively simply. Wildtype *C. jejuni* was treated with NaOCl in buffer at varying concentrations and the activity of FdhA and CueO subsequently measured (Figure 14). Both enzymes were very sensitive to NaOCl treatment, with a significant reduction in activity evident after even 10 µM.

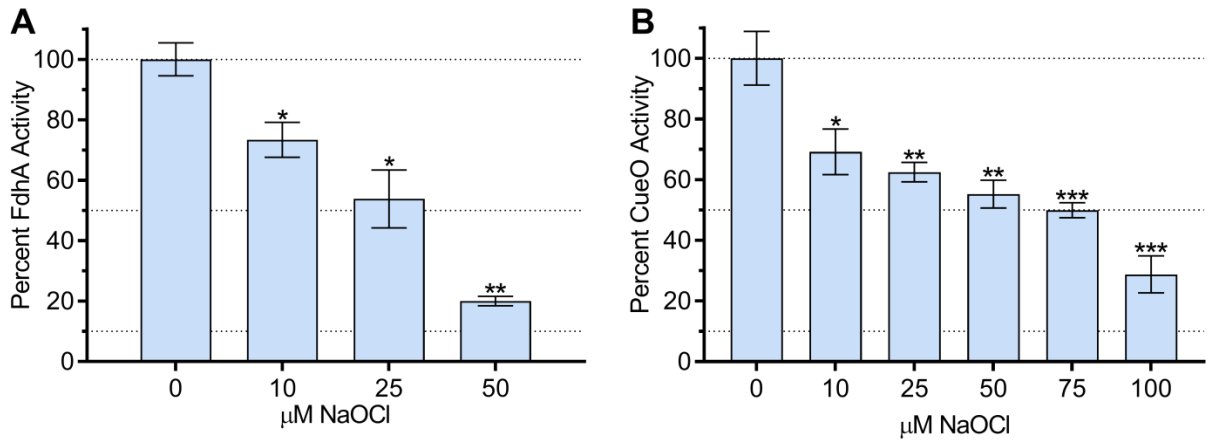

Figure 14: Enzyme activities of **A**) FdhA and **B**) CueO in whole cells treated with NaOCl, as determined by methyl viologen linked formate oxidation and DMP oxidase assay, respectively. * p<0.05, ** p<0.01, *** p<0.001.

We next attempted to demonstrate a difference in client activity between wildtype *C. jejuni* and the *msrPQ* mutant following treatment with NaOCl. As MsrP is a repair system, one would not expect the immediate reduction in client activity to be different, but only the subsequent repair to occur at different rates. To this end we attempted time course assays, measuring FdhA recovery after treatment with 50 µM NaOCl (Figure 15). Cells were treated with NaOCl in buffer, then returned to MH broth and FdhA activity periodically measured. Although activity did appear to repair, there was no difference between mutant and wildtype (Figure 15, green traces). We hypothesised the apparent repair was due to cell replication and *de novo* synthesis of new FdhA. We therefore treated cells with the DNA replication inhibiting antibiotic ciprofloxacin immediately after treatment, which would allow protein synthesis to continue (I.e. for the expression of MsrP) without cell replication (Figure 15, purple traces). Under these conditions we saw no recovery in either the mutant or wildtype.

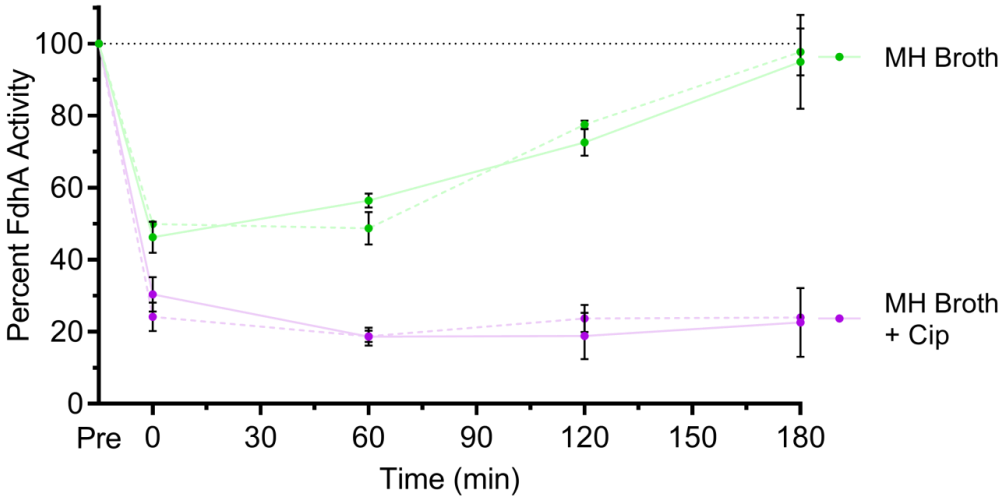

Figure 15: FdhA recovery assays after 50 µM NaOCl treatment of wildtype (solid lines) and *msrPQ* mutant (dashed lines). Cells were incubated in either standard MH broth (green traces) or MH supplemented with 200µM ciprofloxacin (Cip, purple traces).

Various permutations of this assay were performed utilising different treatment methods, antibiotic combinations and client assays, but no growth-independent recovery of client activity could be demonstrated even in wildtype, let alone any difference between wildtype and mutant. We hypothesised the problem stemmed from the acute treatment method, which is very different from the chronic RCS barrage that would be encountered *in vivo*. The acute treatment in batch culture could be preventing the cells from expressing their defence systems and repairing NaOCl mediated damage. Gennaris *et al*. (2015) showed that the *msrPQ* mutant in *E. coli* was very specifically upregulated by NaOCl, not H_2O_2. We performed some limited RT-PCR, albeit in MH broth, which demonstrated *msrP* in *C. jejuni* is induced by NaOCl in a dose dependent manner, but upregulation did not persist in response to acute treatment (Figure 16). We therefore concluded that to properly study the MsrP system, we would require a chronic treatment system in the form of continuous culture (see below).

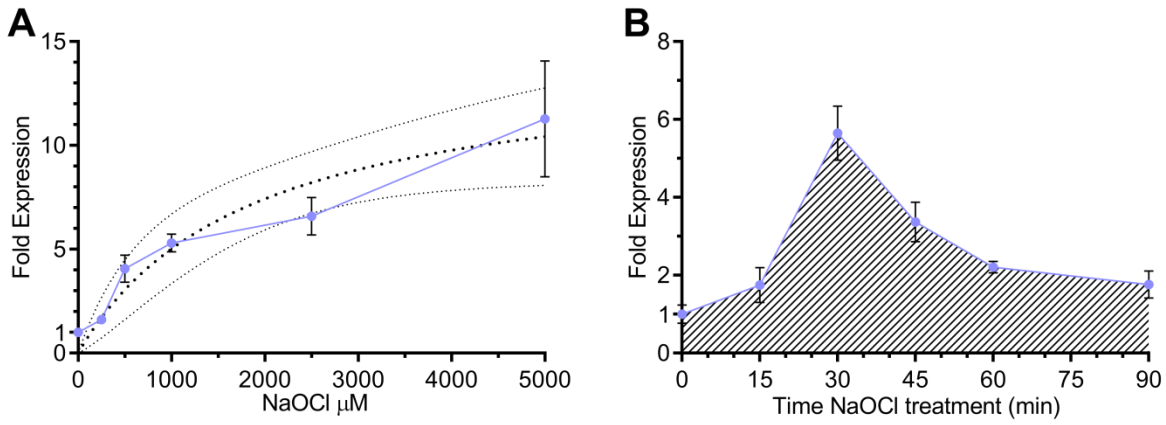

Figure 16: RT-PCR of *msrP* in response to NaOCl in *C. jejuni* wildtype. **A**) Fold increase in expression of *msrP* in response to a range of NaOCl concentrations. **B**) *msrP* expression over time following 500 μM NaOCl treatment.

4.2.5 Continuous culture and chronic treatment

Use of a chemostat to grow *C. jejuni* in continuous culture has been reported by our group previously (Guccione *et al*. 2010). The chemostat is able to finely control oxygen tension, pH and growth-limiting nutrients to create a continually dividing exponential culture of a fixed density which can be maintained indefinitely. New media feeds into the vessel as old culture exits at the same rate, creating a fixed volume exponential culture. We realised the chemostat could be used to chronically treat cells with NaOCl as it could be fed into the vessel continuously using an external peristaltic pump to maintain an effective final concentration in the culture. This would not be possible in batch culture, and has the benefit of being more reproducible as the entire population of cells are in exponential phase and so respond to stress more consistently. The chemostat uses a defined minimal media, where the carbon source L-serine is the growth limiting nutrient but which also contains low concentrations of other amino-acids. We were aware that NaOCl quenching by free Met and Cys present in the media would be an issue, but could not be avoided. Given the concentration of Met/Cys in the media and previous experiments on NaOCl quenching, we predicted a final concentration of 1 mM NaOCl would be appropriate. This was achieved by feeding in 10 mM NaOCl, stabilised in 50 mM pH 5.0 potassium phosphate buffer, at a dilution rate one tenth that of the media, to give a final effective concentration of 1 mM. Wildtype and Δ*msrPQ C. jejuni* continuous cultures were set up, first reaching a steady state without NaOCl (a steady state being defined as 5 culture volumes and/or a constant cell density is reached, approximately 24 hrs depending on feed rate), then reaching a second steady state with 1 mM NaOCl. Proteomics samples and whole cells for enzyme assays

were sampled from each steady state, yielding four sample sets, annotated as WT(-), WT(+), Δ*msrPQ*(-) and Δ*msrPQ* (+).

At the point of sampling, the activity of FdhA, NapA and CueO were measured, and the reduction in activity between (-) and (+) steady states compared between wildtype and Δ*msrPQ* (Figure 17). All three enzymes showed a strong reduction in activity in both strains, with the exception of NapA, which was largely unaffected in wildtype but strongly inhibited in the mutant. FdhA was almost completely inactivated in both strains, resulting in a non-significant difference which may be due to too strong a treatment. CueO was also strongly inhibited, but the mutant did show a significant decrease compared to wildtype.

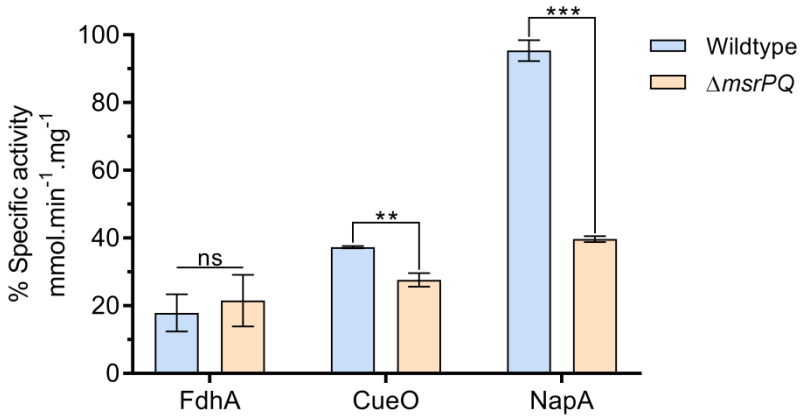

Figure 17: Specific enzyme activities of FdhA, CueO and NapA from steady state chemostat cultures. Activity is displayed as the percent activity in the treated vs. untreated samples. ** p<0.01, *** p<0.001.

Given the initial proteomics run had highlighted these three enzymes as potential targets of MetSO oxidation, and we could now, for the first time, demonstrate a reduction in their activity in the *msrPQ* mutant, we proceeded to conduct proteomics on the four steady state samples to compare i) MetSO states of periplasmic proteins in wildtype versus mutant, and ii) global protein abundance changes in the wildtype (-) vs (+) as a proxy for expression changes in response to NaOCl.

4.2.6 Proteomics

Detailed proteomics procedure can be found in Materials and Methods. Raw data is deposited in Supplementary File 1, and tables of proteins with significant changes in abundance or MetSO intensity are deposited in Supplementary File 2. The summarised results are presented in Figure 18.

The vast majority of MetSO signals detected were far more abundant in Δ*msrPQ*(+) than WT(+) (Figure 18 A), providing evidence that MsrPQ is indeed a global periplasmic MetSO repair system with many client proteins. As seen in the initial proteomics run, NapA is a clear target for NaOCl, however, with chronic treatment, MetSO ratios between mutant and wildtype are 4 – 30 fold on different residues, compared to <2 fold in the acutely treated samples (Supplementary File 2). 41% of MetSO signals were mutant specific, while only 3% were wildtype specific (Figure 18 B). Although many periplasmic proteins clearly have altered MetSO ratios in the mutant vs wild-type, the data also show that differences exist in the Met oxidation status of residues in many cytoplasmic proteins as well. This suggests deletion of *msrPQ* is somehow indirectly affecting the cytoplasmic redox environment. This will require further investigation.

Changes in protein abundance between WT(-) and WT(+) samples give a powerful insight into *C. jejuni*'s response to RCS. 36 proteins increased in abundance by >2 fold, and 50 proteins decreased in abundance by >2 fold (Figure 18 C). Of the upregulated proteins, the periplasmic NLPA family of lipoproteins, implicated in exogenous Met uptake in other bacteria, stand out as obvious targets for future study (see discussion). Several Met biosynthesis proteins were also more abundant. The downregulated proteins included many proteins involved in alternative electron transport pathways and the identity of some of these (e.g. Mfr, Cj0358) suggested the involvement of the RacRS two-component system, with the periplasmic fumarate reductase proteins MfrABE over 10-fold more reduced than any others (see discussion) (Figure 18 D).

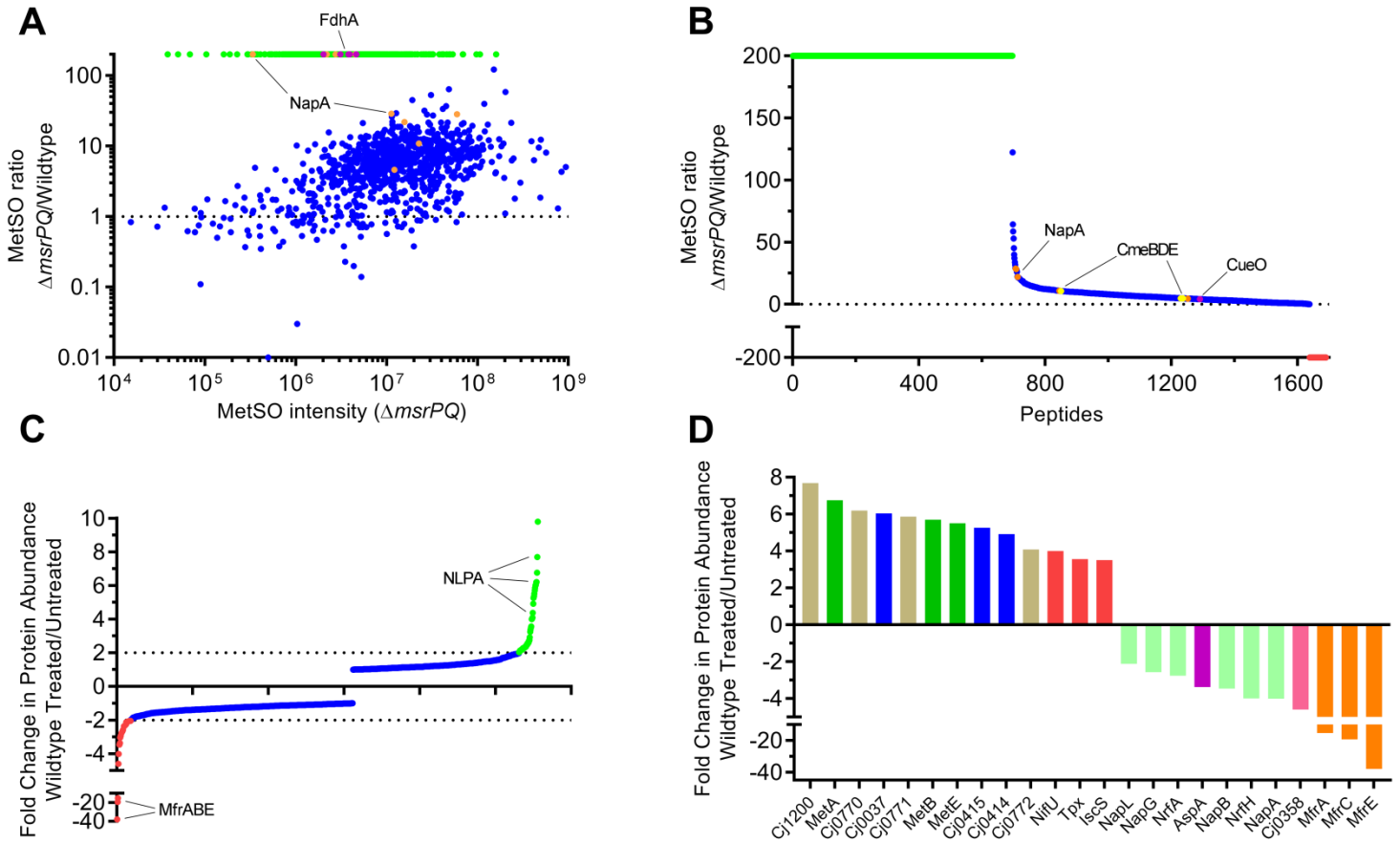

Figure 18: Summarised proteomics data. **A**) Distribution of MetSO signals as the ratio of Δ*msrPQ*/wildtype against intensity in the mutant. Example putative clients FdhA and NapA are highlighted in purple and orange, respectively. Green signals are mutant specific and assigned an arbitrary value of 200. **B**) Distribution of MetSO signals as the ratio of Δ*msrPQ*/wildtype against peptide count. Example oxidised proteins CmeBDE, CueO and NapA are highlighted in yellow, purple and orange, respectively. Green signals are mutant specific and assigned an arbitrary value of 200. Red signals are wildtype specific and assigned an arbitrary value of -200. **C**) Fold change in protein abundance in wildtype as a ratio of treated/untreated. Green and red proteins have a >2 fold increase or decrease in abundance, respectively. Example protein groups significantly changed in abundance are highlighted. **D**) Selected proteins with a >2 fold change in abundance in wildtype treated vs. untreated. Functional classification, left to right: NLPA family lipoproteins (Brown), methionine synthesis genes (Green), gluconate reduction pathway (Blue), sulfur metabolism (Red), nitrate and nitrite reductases (Light Green), aspartase (Purple), cytochrome peroxidase (Pink), fumarate reductase (Orange).

4.2.7 CueO: linking MetSO formation with functional consequences

Cj1516 (CueO) is the sole multicopper oxidase of *C. jejuni*, performing an important role in copper tolerance by oxidising toxic Cu^{+1} in the periplasm to the less toxic Cu^{+2} form (Hall *et al*. 2008). CueO activity is sensitive to NaOCl treatment (Figure 14 B), and appears to have a number of Met residues susceptible to oxidation. We were able to map the oxidation sensitive Met residues onto the known crystal structure of the *C. jejuni* CueO, as published by Silva *et al*. (2012) (Figure 19 A). Interestingly, the oxidation susceptible Met residues seemed to cluster around a single helical domain. This domain is particularly Met-rich, and in *E. coli* has been shown to bind multiple Cu^{+1} ions to "pre-load" the adjacent active site (Singh *et al*. 2011). It is clear from the binding study by Singh *et al*. (2011) that the Met residues in this domain are essential for coordinating Cu^{+1} prior to oxidation, as a ΔMet form of the protein has much lower rates of Cu^{+1} oxidase activity (Figure 19 B). We predict that the oxidation sensitive Met residues from *C. jejuni* CueO are the same residues responsible for Cu^{+1} coordination, hence their oxidation and resulting conformational change reduces Cu^{+1} binding and enzyme activity. Their particular susceptibility to oxidation is likely due to their intrinsically exposed conformation, necessary to efficiently capture free Cu^{+1} in the periplasm.

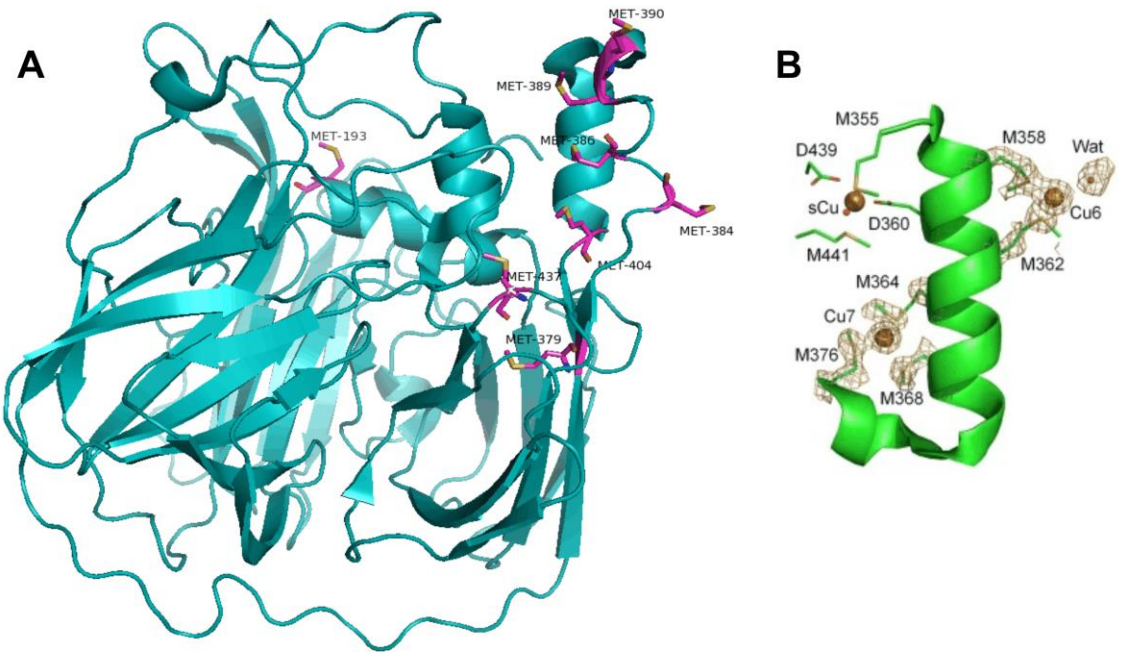

Figure 19: A) *C. jejuni* CueO structure with oxidation susceptible Met residues determined from our proteomics results highlighted in pink. Sulfur atoms are highlighted in yellow. **B**) Met-rich Cu^{+1} binding domain from *E. coli* CueO, with the spatial organisation of Cu^{+1} coordinating Met residues highlighted (Singh *et al*. 2011).

4.3 Discussion

While cytoplasmic Msr enzymes are well characterised in a range of bacteria, the function and importance of this distinct periplasmic Msr system in Gram-negative bacteria is in its infancy. We have demonstrated that the periplasmic protein Cj0379 is an active Msr *in vitro* and is the *C. jejuni* homolog of *E. coli* MsrP, with cognate electron donor protein Cj0378 (MsrQ). We show that, in agreement with a previous study, the presence of an N-terminal His-tag is detrimental to activity and may partially explain previous reports of low activity with L-MetSO (Sabaty *et al*. 2013). However, we predict that local polypeptide structure almost certainly affects the binding affinity of MetSO residues for MsrP, and therefore lower K_m values may well be demonstrated with MetSO-containing peptides as substrate. A study on the effect of neighbouring residues on MsrP binding of MetSO would be of great interest and will be the focus of future studies on the purified enzyme. Indeed, the proteomics data detailing oxidation susceptible Met residues within MsrP client proteins gathered in this study will enable the rational design of MetSO-containing peptides for use as "real" substrates.

Development of the DPD assay to readily evaluate the behaviour of NaOCl in solution allowed us to optimise our experiments to evaluate Met oxidation using the minimal concentration of NaOCl possible, limiting secondary RCS formation and cellular damage beyond protein oxidation. We believe the treatment of cells with NaOCl in complex media is a major hinderance in assaying Met and Cys oxidation of proteins and gives misleading results for the accurate study of this labile system in bacteria. Furthermore, acute treatment with NaOCl produced much lower MetSO levels and reduced differences between wildtype and *msrPQ* mutant compared to chronically treated cells from continuous culture. This may be partially due to the MsrP system failing to express well in response to acute treatment, as demonstrated by RT-PCR (Figure 16). We therefore conclude that studying cellular responses to NaOCl treatment must be conducted in continuous culture, in minimal media, where NaOCl can be added continuously to recreate its perpetual production by myeloperoxidase *in vivo*.

C. jejuni was remarkably sensitive to NaOCl in buffer, with as little as 300 µM sufficient for complete cell death (Figure 13). Surprisingly, the *msrPQ* mutant did not show any enhanced sensitivity to NaOCl *in vitro*. Proteomics data revealed the primary periplasmic targets of NaOCl to be electron transport proteins, E.g. FdhA and NapA, the inactivation of which is unlikely to affect viability under nutrient replete *in vitro* conditions. However, previous chick colonisation experiments found an *msrP* mutant unable to colonise, indicating a strong cumulative impact of periplasmic MetSO formation on *in vivo* survival (Hitchcock *et al*. 2010). FdhA and CueO were shown to be incredibly sensitive to NaOCl, with as little as

10 µM sufficient for a significant reduction in activity (Figure 14). Proteomics revealed that NapA, FdhA and CueO are all susceptible to Met oxidation on specific residues, and the ratio of this oxidation was greater in the mutant, confirming these proteins as clients for MsrP-dependent MetSO repair. Furthermore, CueO and NapA were shown to be significantly more sensitive to chronic NaOCl treatment in the *msrPQ* mutant, further supporting their role as clients of MsrP (Figure 17). Interestingly, the proteomics data showed that CueO was susceptible to oxidation of Met residues required for substrate binding, providing a functional mechanism for its inactivation by NaOCl (Figure 19). Purification of CueO to examine its oxidation susceptibility and repair by MsrP *in vitro* is of great interest and warrants further investigation.

In addition to Met oxidation states, the proteomics revealed some interesting global protein abundance changes in response to NaOCl (Figure 18 C-D). Upregulated proteins included the NlpA family lipoproteins, Cj0770-2 and Cj1200, implicated in methionine import (Zhang *et al*. 2003). *C. jejuni* strain NCTC11168 is not a methionine auxotroph (Alazzam *et al*. 2011). Our hypothesis is that the bacterium employs these NlpA homologues to scavenge Met sources from the environment in an attempt to quench the hypochlorite that it is being exposed to. In *E. coli,* Met import occurs via the high affinity complex MetNIQ, where MetN is the ATP binding protein, MetI the permease and MetQ the binding protein (Merlin *et al*. 2002). *E. coli* is predicted to encode a second, low-affinity L-Met transporter, as a *metNIQ* mutant in a Met auxotrophic background shows no major reduction in the L-Met pool, yet this secondary transport system, dubbed MetP, is yet to be identified genetically or otherwise, bringing into question its existence (Kadner and Watson, 1974). *C. jejuni* encodes MetNI homologs, encoded directly upstream of the NlpA-like genes *cj0770-2c*, by *cj0773c* and *cj0774c*, respectively. Both NlpA and MetQ of *E. coli* show homology to Cj0770, Cj0771, Cj0772 and Cj1200. One could speculate that Cj0773-4 are the permease and ATP binding subunits of an ABC type transporter, which utilise Cj0770-2 as periplasmic binding proteins. The three periplasmic proteins are unlikely to be redundant, probably specifically binding, for example, L-Met or D-Met, and a third substrate such as free MetSO. The function of Cj1200 is less obvious, being in a disparate region of the chromosome. However, recent work in *E. coli* has highlighted the role of NlpA in outer membrane vesicle formation, a possible candidate for the function of Cj1200 (Schwechheimer and Kuehn 2013, Schwechheimer *et al*. 2014). Other upregulated proteins include Met synthesis genes MetA, MetB and MetE, most likely to replenish the intracellular Met pool. The FeS cluster biosynthesis proteins NifU and IscS are also more abundant, possibly to repair oxidised Cys in FeS clusters in proteins in the cytoplasm.

The down-regulated proteins include the nitrate and nitrite reductases Nap and Nrf, respectively, and the periplasmic fumarate reductase MfrABE, the latter proteins being over 10-fold more strongly reduced than any other proteins (Figure 18 C-D). Also downregulated is the aspartase AspA and cytochrome *c* peroxidase Cj0358. It was clear from studying this list that the response mirrors that of the RacR regulon, characterised for its role in reducing fumarate respiration in the presence of nitrate in a low oxygen environment (van der Stel *et al*. 2015). RacR represses the *mfr* genes when nitrate is present, as nitrate has a much higher mid-point redox potential than fumarate (See general introduction). AspA is also repressed in the presence of nitrate by RacR as it produces fumarate from aspartate (Guccione *et al*. 2008). Cj0358 was one of the first identified proteins regulated by RacR, though its function in relation to the other metabolic targets of RacR has not been examined (Brás *et al*. 1999). We predict that the RacRS system may be sensing oxidative stress, possibly by the oxidation of cysteine residues that are present in the periplasmic domain of the membrane bound sensor RacS, which then alters the activity of RacR and represses the genes for the above proteins. The regulatory mechanism behind the nitrate and nitrite reductase suppression if unclear as no known regulators directly control these genes.

We predict the purpose of down-regulating alternative electron acceptor pathways in response to NaOCl is to decrease competition for electron flow from the MQ pool, so that a higher flux is available for MsrPQ, allowing increased substrate turnover without necessarily increasing expression levels of the system itself (Figure 20). Interestingly, the only reductase system not decreased in abundance upon HOCl treatment was that for TMAO/DMSO (TorAB). Given MsrP can reduce DMSO and TMAO non-specifically, it is likely that TorA can in turn non-specifically reduce MetSO, and may assist detoxification under stress. Up-regulation of the gluconate dehydrogenase, and possibly sulphite oxidoreductase (SorA was 2.37 fold up-regulated, see Supplementary file 2), may increase electron flow to the proton-translocating terminal oxidase Cco via the c type cytochrome Cj0037 (6.05 fold up-regulated, Figure 18 D) without consuming MQ electrons destined for MsrPQ flux.

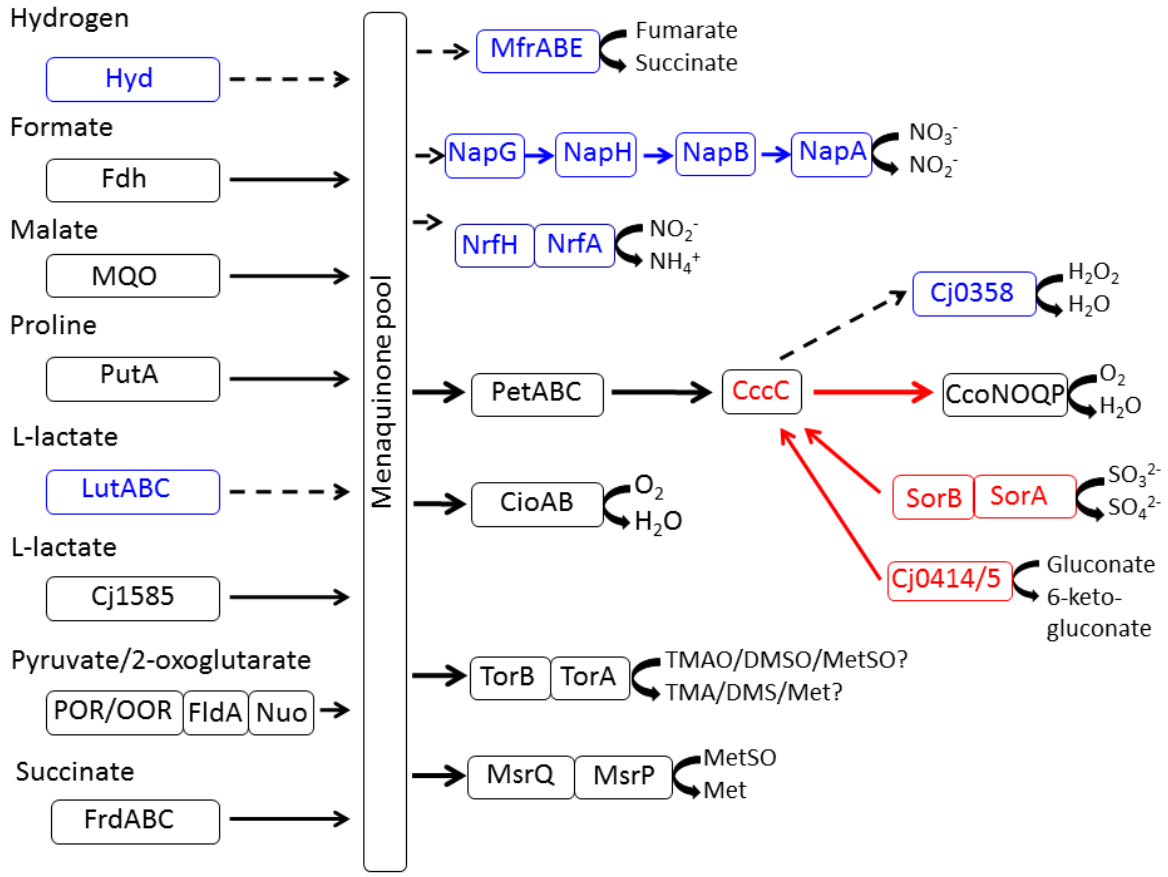

Figure 20: Putative model of expression changes in key electron transport proteins in response to NaOCl in *C. jejuni*. Proteins in blue are down-regulated in response to NaOCl, while proteins in red are up-regulated. Suppression of the alternative electron acceptor pathways for nitrate, nitrite and fumarate may increase the availability of electrons from the MQ pool and permit a higher turnover of MetSO by MsrPQ, and possibly by some non-specific activity of TorAB. Upregulation of gluconate dehydrogenase and sulphite oxidoreductase may provide additional electron flow to the terminal oxidase Cco which is required for energy conserving oxygen respiration.

Overall, the results of this study, particularly the information gleaned from the proteomic analysis of continuous cultures, has opened a myriad of avenues to explore the response of *C. jejuni* to HOCl. In particular, the regulatory pathways which appear to induce the expression of Met uptake and synthesis, while repressing periplasmic terminal reductases, are clear targets for further study. While bleach is perhaps regarded as an out-dated antibacterial agent, it is still highly effective, and, unlike antimicrobials with specific targets, is incredibly difficult for bacteria to raise resistance against. Understanding the molecular mechanism behind protein oxidation and repair, and the global response to oxidative damage, will benefit the development of preventative measures for the control of *C. jejuni*.

Indeed, in the US poultry carcasses are already treated by chlorination using chemicals similar to hypochlorite. While this practice is currently outlawed by the EU, the UK's removal from the EU in the coming years would alleviate us from these restrictions and may present an attractive import opportunity from the US.

The impact on health by the consumption of chlorinated chicken is as yet largely unproven. The US Food Safety and Inspection Service currently limits chlorine treatment to 50 ppm, a concentration which is believed to be below that required for the generation of carcinogenic compounds such as semicarbazide (Hoenicke *et al*. 2004). As such, concerns have risen that chlorinated poultry meat could induce cancer in consumers, though no data is available to date. The breakdown products of the US standardised chlorine dioxide treatment, chlorite and chlorate, are found in chlorinated poultry meat at levels considered too low to cause any realistic health risk. For example, the European Food Safety Authority concluded that an individual would have to consume 5% of their bodyweight daily to reach the recommended limit of chlorite/chlorate, equivalent to roughly 2 to 3 whole birds per day (EFSA 2015). The EU acknowledges that the consumption of chlorinated chicken is safe for the consumer, but it outlaws it as a practice due to its use in place of good hygiene and welfare practices, rather than in addition to. As such, current evidence suggests that the consumption of chlorinated poultry poses no direct health risk.

A modified treatment regime using reduced concentrations of free chlorine, possibly in a buffered process as described in this work, or in combination with other agents, could mitigate these concerns and would likely be embraced by producers if they proved to be cost effective. Ultimately, detailed analysis of the risk to human health by the consumption of chlorinated poultry is needed to win over consumer favour.

In conclusion, further research into the response of *C. jejuni* to chlorination and the impact on consumer health is desperately needed. The work described here improves our

understanding of the behaviour of hypochlorite as a chemical, and of *C. jejuni*'s response to it, perhaps providing a basis for methodology for improved poultry chlorination practices.

4.4 Materials and Methods

4.4.1 General culture conditions

Routine microaerobic growth of *C. jejuni* NCTC 11168 was conducted in MACS-VA500 Microaerobic Workstations (Don Whitley Scientific Ltd) with an atmosphere of 10% $^{v}/_{v}$ O_2, 5% $^{v}/_{v}$ CO_2 and 85% $^{v}/_{v}$ N. Batch cultures in Muller-Hinton (MH) broth supplemented with 20 mM L-serine were grown in conical flasks, sealed with a foam bung, with shaking at 140 rpm on a 19.2 mm orbit (SeaStar digital orbital shaker, Heathrow Scientific). Cultures routinely contained 10 µg ml^{-1} Amphotericin B and Vancomycin, with the selective antibiotic kanamycin at 50 µg ml^{-1} where appropriate. *E. coli* MG1655 cultures were grown in either 100 ml LB broth in 250 ml conical flasks, or 1 litre defined anaerobic media in 2 litre conical flasks, at 260 rpm shaking in constant temperature rooms at either 37^{o}C or 25^{o}C. Anaerobic cultures were subsequently transferred to sealed containers before induction of expression. Overexpression cultures contained carbenicillin and kanamycin at 50 µg ml^{-1}, and tetracycline at 5 µg ml^{-1}.

4.4.2 Mutagenesis

An isogenic *msrPQ* mutant was obtained in the *C. jejuni* 11168 background by the isothermal assembly cloning method described by Gibson *et al*. (2009). Briefly, target gene flanking regions for homologous recombination were amplified from *C. jejuni* 11168 genomic DNA, and a kanamycin resistance cassette amplified from pJMK30. The two flanking regions and kanamycin cassette were combined with linearised pGEM3ZF at a 1:1:1:1 molar ratio and incubated in an isothermal reaction at 50^{o}C for 1 hour. The primers used to amplify the fragments contain adapters such that specific recombination occurs to produce the mutant vector, pGEM*msrPQ*, which was subsequently transformed into *C. jejuni* 11168 wildtype. Mutant clones were selected for resistance to kanamycin and correct recombination confirmed by PCR screening using a combination of flanking regions primers and kanamycin resistance cassette primers (Primers are listed in Supplementary file 3).

4.4.3 MsrP overexpression

C. jejuni MsrP was expressed using the pET28a construct, allowing the introduction of a cleavable His tag between the native TAT signal sequence and mature protein. The TAT signal sequence and mature protein sequence were amplified from *C. jejuni* 11168 genomic DNA and inserted into the NcoI and NdeI sites of pET28a, respectively (Primers are listed in Supplementary file 3). In order to comply with the resistance profile of the overexpression strain, the expression cassette of pET28-MsrP was cut out at the BlpI/BglII flanking sites and ligated into similarly digested pET22a, allowing carbenicillin selection. *E. coli* MG1655Δ*iscR*

was transformed with pGP1-2 and pTPR1, conferring kanamycin and tetracycline resistance, respectively. pETMsrP was transformed into this strain using carbenicillin selection to yield the final overexpression strain. Cultures were grown in defined Glucose/Nitrate anaerobic media in sealed containers, with T7-dependent protein expression induced by the addition of 0.1 mM IPTG. Cell free extracts were prepared by French press, and purified by nickel affinity chromatography on a 5 ml HisTrap column, followed by a 5 ml hydrophobic interaction column using an Akta Prime purification system (GE Healthcare). Cleavage of the His-tag was achieved by incubation with 100 U mg^{-1} thrombin at 4^{o}C for 16 hours. 0.5 mM AEBSF was added to permanently inhibit thrombin, and the untagged protein purified by a second round of nickel affinity and hydrophobic interaction purifications.

4.4.4 Anaerobic reductase assays

The reductase activity of purified MsrP with either DMSO or L-MetSO was measured using an anaerobic methyl-viologen linked assay. A 1 ml assay mixture containing 0.1 mM methyl-viologen and varying concentrations of MsrP in 100 mM Tris-HCl buffer (50 mM NaCl, pH 7.4) was sparged in an anaerobic cuvette with oxygen-free nitrogen. The methyl-viologen was reduced by the addition of sodium dithionite until a stable reading at 585 nm was achieved. The reaction was initiated by the addition of substrate to the desired final concentration. The rate of methyl-viologen oxidation at 585nm was measured and translated into a specific rate of MsrP-dependent substrate reduction using the extinction coefficient of methyl-viologen at 585 nm of 11.8 mM^{-1} cm^{-1}.

4.4.5 Available chlorine assay

N,N-diethyl-p-phenylenediamine (DPD) was prepared as a 2x stock at 20 mM in 20 mM NaPhos buffer, pH 7.4. Aqueous samples were mixed 1:1 with 2x DPD in a 1 ml cuvette and the absorbance at 550 nm recorded. 2x DPD mixed 1:1 with buffer was used as the blank. NaOCl was used as the available chlorine standard, prepared from 10% purum solution (Sigma Aldrich).

4.4.6 FOX peroxide assay

FOX reagent was prepared as 250 µM ferrous ammonium sulfate, 100 µM sorbitol, 100 µM xylenol orange, 25 mM H_2SO_4 and 1% $^{v}/_{v}$ ethanol.100 µl aqueous sample was mixed with 900 µl FOX reagent in a 1 ml cuvette, and the absorbance at 550 nm, minus the absorbance at 800 nm, was taken after 15 min incubation. Standard curves were produced using H_2O_2 as the standard.

4.4.7 NaOCl sensitivity assay

Mid-log cells were washed twice with 20 mM NaPhos buffer, pH 7.4, and prepared to a final optical density at 600 nm of 0.8 in buffer. NaOCl, prepared in the same buffer, was mixed with cells 1:1 to yield the desired final concentration, and incubated in multi-well plates for 10 min, at which point the treatment was terminated by the addition of a 1:10 molar ratio of L-Met. Cultures were then serial diluted in 96-well plates and spotted onto CCDA agar plates. Colony counts after 48 hrs were recorded and the original CFU.ml^{-1} calculated.

4.4.8 Client activity assays

Nitrate reductase and formate dehydrogenase activities were measured using the anaerobic methyl-viologen linked assay described above, but with 50 µl concentrated whole cells rather than purified proteins. CueO activity was measured by 2,6-dimethoxyphenol oxidase (DMPO) assay. DMP is substrate for copper oxidases, forming the product 3,3',5,5'-tetramethoxydiphenylquinone, which can be monitored at 468 nm with an extinction coefficient of 14.8 mM^{-1} cm^{-1}. Assays were carried out aerobically in a 1 ml cuvette, containing 100 µl concentrated whole cells and 900 µl 100 mM NaPhos buffer, pH 5.0, with 0.1 mM $CuSO_4$ to stimulate CueO activity. The reaction was initiated by the addition of 10 mM final DMP and the oxidation rate recorded at 468 nm.

4.4.9 Chemostat culture

Continuous cultures were grown in an Infors HT Labfors 3 chemostat, controlled by Infors Iris 5 software (Infors, Switzerland). L-serine limited defined media was used as described previously (Guccione *et al*. 2010). Culture volume was maintained at 885 ml and 37^{o}C, with proportional mixing of compressed air and 10 % $^{v}/_{v}$ CO_2 (nitrogen balanced) maintained an oxygen tension of 5% O2 $^{v}/_{v}$, sufficient for 100% aerobiosis with a stirring rate of 350 rpm (Guccione *et al*. 2010). pH was monitored by an internal probe and did not significantly alter from pH 7.4, as imposed by the phosphate buffered media. The vessel was inoculated to an optical density at 600 nm of 0.1 and allowed to grow without turnover for 6 hours, allowing the culture to achieve an optical density of around 0.6. Hereafter media was fed into the vessel at a dilution rate of 0.2 hr^{-1} until a steady state was achieved, defined by 5 vessel volumes of turnover. 200 ml of culture was sampled for proteomics analysis and the culture allowed 4 hours to recover. 10 mM NaOCl in 50 mM potassium phosphate buffer, pH 5.0, was then fed in at a dilution rate of 0.02 hr^{-1} by an external peristaltic pump, resulting in a final effective concentration within the vessel of 1 mM. Once the second steady state was reached, a further 200 ml was sampled for proteomics analysis, and the vessel shut down.

4.4.10 Proteomics

Cell pellets stored at -80^{o}C were thawed and resuspended in 1.5 ml 20 mM NaPhos buffer, pH 7.4, and cell free extracts prepared by repeated sonication. Soluble protein was extracted by centrifugation and the concentration of all samples equalised to 1 mg ml^{-1} using a standard 96-well BioRad assay. 50 µg of protein per sample was denatured with 1 M urea and 100 mM ammonia bicarbonate. Peptides were trypsin digested for 2 hours at 37^{o}C. 10 µg aliquots were acidified with 1% $^{v}/_{v}$ final trifluoroacetic acid. Samples were then desalted using C18 desalting columns (Pierce) prior to loading.

Extracted peptides were analysed by nano-liquid chromatography tandem mass spectrometry (LC-MS/MS) on a LTQ Orbitrap Elite (Thermo Fisher) hybrid mass spectrometer equipped with a nanospray source, coupled with an Ultimate RSLCnano LC System (Dionex). The system was controlled by Xcalibur 2.1 (Thermo Fisher) and DCMSLink 2.08 (Dionex). Peptides were desalted on-line using a micro-Precolumn cartridge (C18 Pepmap 100, LC Packings) and then separated using a 120 min reversed phase gradient (4-32% acetonitrile/0.1% formic acid) on an EASY-Spray column, 50 cm x 50 µm ID, PepMap C18, 2 µm particles, 100 Å pore size (Thermo). The LTQ-Orbitrap Elite was operated with a cycle of one MS (in the Orbitrap) acquired at a resolution of 60,000 at m/z 400, with the top 20 most abundant multiply-charged (2+ and higher) ions in a given chromatographic window subjected to MS/MS fragmentation in the linear ion trap. An FTMS target values of 1e6 and an ion trap MSn target value of 1e4 was used and with the lock mass (445.120025) enabled. Maximum FTMS scan accumulation time of 500ms and maximum ion trap MSn scan accumulation time of 100ms were used. Dynamic exclusion was enabled with a repeat duration of 45s with an exclusion list of 500 and exclusion duration of 30s.

MS data was analysed data using MaxQuant (PMID:19029910) version 1.5.8.3. Data was searched against a *Campylobacter jejuni* NCTC 11168 UniProt sequence database using following search parameters: trypsin with a maximum of 2 missed cleavages, 7 ppm for MS mass tolerance, 0.5 Da for MS/MS mass tolerance, with Acetyl (Protein N-term) and Oxidation (Met) set as variable modifications. A protein FDR of 0.01 and a peptide FDR of 0.01 were used for identification level cut offs. Label free quantification was performed using MaxQuant calculated protein intensities (PMID:24942700).

4.4.11 Quantitative RT-PCR

C. jejuni RNA was isolated using the SV Total RNA Isolation System (Promega), following the Gram-negative bacteria protocol. RNA from this kit was further purified using the Turbo DNA-free Kit (Ambion), following the manufacturer's instructions. pRT-PCR was performed using the SensiFAST SYBR Lo-ROX Kit (Bioline). Reactions were carried out in a

MX3005P thermal cycler (Stratagene, UK) on a 96-well optical reaction plate (Applied Biosystems) using Sequence Detector System software (PE Applied Biosystems). A standard curve was produced for each primer set using genomic DNA serial dilutions. Transcript levels were calculated using the standard curve equations, with normalization to *gyrA* transcript as a control.

References

Adamson H, Simonov AN, Kierzek M, Rothery RA, Weiner JH, Bond AM and Parkin A (2015). Electrochemical evidence that pyranopterin redox chemistry controls the catalysis of YedY, a mononuclear Mo enzyme. *Proc Natl Acad Sci U S A*. 112(47): 14506-11.

Alazzam B, Bonnassie-Rouxin S, Dufour V and Ermel G (2011). MCLMAN, a new minimal medium for *Campylobacter jejuni* NCTC 11168. *Res Microbiol*. 162(2): 173-9.

Armesto XL, Canle M, Fernández MI, García MV and Santaballa JA (2000). First Steps in the Oxidation of Sulfur-Containing Amino Acids by Hypohalogenation: Very Fast Generation of Intermediate Sulfenyl Halides and Halosulfonium Cations. *Tetrahedron.* 56(8): 1103-09.

Atack JM and Kelly DJ (2008). Contribution of the stereospecific methionine sulphoxide reductases MsrA and MsrB to oxidative and nitrosative stress resistance in the food-borne pathogen *Campylobacter jejuni*. *Microbiology*. 154(8): 2219-30.

Boschi-Muller S, Olry A, Antoine M and Branlant G (2005). The enzymology and biochemistry of methionine sulfoxide reductases. *Biochim Biophys Acta*. 1703(2): 231-8.

Brokx SJ, Rothery RA, Zhang G, Ng DP and Weiner JH (2005). Characterization of an *Escherichia coli* sulfite oxidase homologue reveals the role of a conserved active site cysteine in assembly and function. *Biochemistry*. 44(30): 10339-48.

Brás AM, Chatterjee S, Wren BW, Newell DG and Ketley JM (1999). A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonization. *J Bacteriol*. 181(10): 3298-302.

Chatelle C, Kraemer S, Ren G, Chmura H, Marechal N, Boyd D, Roggemans C, Ke N, Riggs P, Bardwell J and Berkmen M (2015). Converting a Sulfenic Acid Reductase into a Disulfide Bond Isomerase. *Antioxid Redox Signal*. 23(12): 945-57.

Choi H, Kim S, Mukhopadhyay P, Cho S, Woo J, Storz G and Ryu SE (2001). Structural basis of the redox switch in the OxyR transcription factor. *Cell.* 105(1): 103-13.

Coudevylle N, Antoine M, Bouguet-Bonnet S, Mutzenhardt P, Boschi-Muller S, Branlant G and Cung MT (2007). Solution structure and backbone dynamics of the reduced form and an oxidized form of E. coli methionine sulfoxide reductase A (MsrA): structural insight of the MsrA catalytic cycle. *J Mol Biol*. 366(1): 193-206.

Delaye L, Becerra A, Orgel L and Lazcano A (2007). Molecular evolution of peptide methionine sulfoxide reductases (MsrA and MsrB): on the early development of a mechanism that protects against oxidative damage. *J Mol Evol*. 64(1): 15-32.

Depuydt M, Leonard SE, Vertommen D, Denoncin K, Morsomme P, Wahni K, Messens J, Carroll KS and Collet JF (2009). A periplasmic reducing system protects single cysteine residues from oxidation. *Science*. 326(5956): 1109-11.

Drazic A, Miura H, Peschek J, Le Y, Bach NC, Kriehuber T and Winter J (2013). Methionine oxidation activates a transcription factor in response to oxidative stress. *Proc Natl Acad Sci U S A.* 110(23): 9493-8.

Drazic A, Gebendorfer KM, Mak S, Steiner A, Krause M, Bepperling A and Winter J (2014). Tetramers are the activation-competent species of the HOCl-specific transcription factor HypT. *J Biol Chem*. 289(2): 977-86.

European Food Safety Authority (2015). Risks for public health related to the presence of chlorate in food.

Ezraty B, Bos J, Barras F and Aussel L (2005). Methionine sulfoxide reduction and assimilation in *Escherichia coli*: new role for the biotin sulfoxide reductase BisC. *J Bacteriol.* 187(1): 231-7.

Flannagan RS, Heit B and Heinrichs DE (2015). Antimicrobial Mechanisms of Macrophages and the Immune Evasion Strategies of *Staphylococcus aureus*. *Pathogens* 4(4): 826-68.

Gebendorfer KM, Drazic A, Le Y, Gundlach J, Bepperling A, Kastenmüller A, Ganzinger KA, Braun N, Franzmann TM and Winter J (2012). Identification of a hypochlorite-specific transcription factor from *Escherichia coli*. *J Biol Chem.* 287(9): 6892-903.

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA and Smith HO (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 6(5): 343-5.

Gray MJ, Wholey WY and Jakob U (2013). Bacterial responses to reactive chlorine species. *Annu Rev Microbiol*. 67: 141-60.

Guccione E, Leon-Kempis Mdel R, Pearson BM, Hitchin E, Mulholland F, van Diemen PM, Stevens MP and Kelly DJ (2008). Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol Microbiol*. 69(1): 77-93.

Guccione E, Hitchcock A, Hall SJ, Mulholland F, Shearer N, van Vliet AH and Kelly DJ (2010). Reduction of fumarate, mesaconate and crotonate by Mfr, a novel oxygen-regulated periplasmic reductase in *Campylobacter jejuni*. *Environ Microbiol*. 12(3): 576-91.

Hall SJ, Hitchcock A, Butler CS and Kelly DJ (2008). A Multicopper oxidase (Cj1516) and a CopA homologue (Cj1161) are major components of the copper homeostasis system of *Campylobacter jejuni. J Bacteriol.* 190(24): 8075-85.

Han AR, Kim MJ, Kwak GH, Son J, Hwang KY and Kim HY (2016). Essential Role of the Linker Region in the Higher Catalytic Efficiency of a Bifunctional MsrA-MsrB Fusion Protein. *Biochemistry.* 55(36): 5117-27.

Hitchcock A, Hall SJ, Myers JD, Mulholland F, Jones MA and Kelly DJ (2010). Roles of the twin-arginine translocase and associated chaperones in the biogenesis of the electron transport chains of the human pathogen *Campylobacter jejuni*. *Microbiology.* 156(10): 2994-3010.

Hoenicke K, Gatermann R, Hartig L, Mandix M and Otte S (2004). Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse? *Food Addit Contam*. 21(6): 526-37.

Juillan-Binard C, Picciocchi A, Andrieu JP, Dupuy J, Petit-Hartlein I, Caux-Thang C, Vivès C, Nivière V and Fieschi F (2017). A Two-component NADPH Oxidase (NOX)-like System in Bacteria Is Involved in the Electron Transfer Chain to the Methionine Sulfoxide Reductase MsrP. *J Biol Chem*. 292(6): 2485-2494.

Kadner RJ and Watson WJ (1974). Methionine transport in *Escherichia coli*: physiological and genetic evidence for two uptake systems. *J Bacteriol*. 119(2): 401-9.

Kim HY and Gladyshev VN (2005). Different catalytic mechanisms in mammalian selenocysteine- and cysteine-containing methionine-R-sulfoxide reductases. *PLoS Biol.* 3(12): e375.

Lee CC, Sickerman NS, Hu Y and Ribbe MW (2016). YedY: A Mononuclear Molybdenum Enzyme with a Redox-Active Ligand? *Chembiochem*. 17(6): 453-5.

Lee W, Westerhoff P, Yang X and Shang C (2007). Comparison of colorimetric and membrane introduction mass spectrometry techniques for chloramine analysis. *Water Res*. 41(14): 3097-102.

Lin Z, Johnson LC, Weissbach H, Brot N, Lively MO and Lowther WT (2007). Free methionine-(R)-sulfoxide reductase from *Escherichia coli* reveals a new GAF domain function. *Proc Natl Acad Sci U S A*. 104(23): 9597-602.

Loschi L, Brokx SJ, Hills TL, Zhang G, Bertero MG, Lovering AL, Weiner JH and Strynadka NC (2004). Structural and biochemical identification of a novel bacterial oxidoreductase. *J Biol Chem*. 279(48): 50391-400.

Lowther WT, Weissbach H, Etienne F, Brot N and Matthews BW (2002). The mirrored methionine sulfoxide reductases of *Neisseria gonorrhoeae* pilB. *Nat Struct Biol*. 9(5): 348-52.

Mahawar M, Tran V, Sharp JS and Maier RJ (2011). Synergistic roles of *Helicobacter pylori* methionine sulfoxide reductase and GroEL in repairing oxidant-damaged catalase. *J Biol Chem*. 286(21): 19159-69.

Meehan BM, Landeta C, Boyd D and Beckwith J (2017). The Disulfide Bond Formation Pathway Is Essential for Anaerobic Growth of *Escherichia coli*. *J Bacteriol*. 199(16).

Melnyk RA, Youngblut MD, Clark IC, Carlson HK, Wetmore KM, Price MN, Iavarone AT, Deutschbauer AM, Arkin AP and Coates JD (2015). Novel mechanism for scavenging of hypochlorite involving a periplasmic methionine-rich Peptide and methionine sulfoxide reductase. *MBio*. 6(3): e00233-15.

Merlin C, Gardiner G, Durand S and Masters M (2002). The *Escherichia coli* metD locus encodes an ABC transporter which includes Abc (MetN), YaeE (MetI), and YaeC (MetQ). J *Bacteriol*. 184(19): 5513-7.

Paulsen CE and Carroll KS (2013). Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. *Chem Rev*. 113(7): 4633-79.

Prokopowicz Z, Marcinkiewicz J, Katz DR and Chain BM (2012). Neutrophil myeloperoxidase: soldier and statesman. *Arch Immunol Ther Exp* 60(1): 43-54.

Sabaty M, Grosse S, Adryanczyk G, Boiry S, Biaso F, Arnoux P and Pignol D (2013). Detrimental effect of the 6 His C-terminal tag on YedY enzymatic activity and influence of the TAT signal sequence on YedY synthesis. *BMC Biochem*. 14: 28.

Schwarz G, Mendel RR and Ribbe MW (2009). Molybdenum cofactors, enzymes and pathways. *Nature*. 460(7257): 839-47.

Schwechheimer C and Kuehn MJ (2013). Synthetic effect between envelope stress and lack of outer membrane vesicle production in *Escherichia coli*. *J Bacteriol*. 195(18): 4161-73.

Schwechheimer C, Kulp A and Kuehn MJ (2014). Modulation of bacterial outer membrane vesicle production by envelope structure and content. *BMC Microbiol*. 14: 324.

Silva CS, Durão P, Fillat A, Lindley PF, Martins LO and Bento I (2012). Crystal structure of the multicopper oxidase from the pathogenic bacterium *Campylobacter jejuni* CGUG11284: characterization of a metallo-oxidase. *Metallomics*. 4(1): 37-47.

Singh SK, Roberts SA, McDevitt SF, Weichsel A, Wildner GF, Grass GB, Rensing C and Montfort WR (2011). Crystal structures of multicopper oxidase CueO bound to copper(I) and silver(I): functional role of a methionine-rich sequence. *J Biol Chem*. 286(43): 37849-57.

Spector D, Etienne F, Brot N and Weissbach H (2003). New membrane-associated and soluble peptide methionine sulfoxide reductases in *Escherichia coli*. *Biochem Biophys Res Commun*. 302(2): 284-9.

Stewart EJ, Aslund F and Beckwith J (1998). Disulfide bond formation in the *Escherichia coli* cytoplasm: an *in vivo* role reversal for the thioredoxins. *EMBO J*. 17(19): 5543-50.

Storz G, Tartaglia LA and Ames BN (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science*. 248(4952): 189-94.

Thurlkill RL, Grimsley GR, Scholtz JM and Pace CN (2006). pK values of the ionizable groups of proteins. *Protein Sci*. 15(5): 1214-8.

van der Stel AX, van Mourik A, Heijmen-van Dijk L, Parker CT, Kelly DJ, van de Lest CH, van Putten JP and Wösten MM (2015). The *Campylobacter jejuni* RacRS system regulates fumarate utilization in a low oxygen environment. *Environ Microbiol*. 17(4): 1049-64.

Vogt W (1995). Oxidation of methionyl residues in proteins: tools, targets, and reversal. *Free Radic Biol Med*. 18(1): 93-105.

Wang L, Bassiri M, Najafi R, Najafi K, Yang J, Khosrovi B, Hwong W, Barati E, Belisle B, Celeri C and Robson MC (2007). Hypochlorous acid as a potential wound care agent. *J Burns Wounds*. 6:e5.

Winterbourn CC and Kettle AJ (2013). Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid Redox Signal*. 18(6): 642-60.

Zhang Z, Feige JN, Chang AB, Anderson IJ, Brodianski VM, Vitreschak AG, Gelfand MS and Saier MH (2003). A transporter of *Escherichia coli* specific for L- and D-methionine is the prototype for a new family within the ABC superfamily. *Arch Microbiol*. 180(2): 88-100.

Zheng M, Aslund F and Storz G (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science*. 279(5357): 1718-21.

Chapter 5: Conclusion

Campylobacter is an unusual pathogen in regards to its lifestyle, physiology, metabolism and stress response pathways. Being more closely related to deep-sea vent bacteria than other canonical pathogens, the study of *Campylobacter* presents a unique challenge, as cellular mechanisms are often distinct from the well-studied analogous systems of other bacteria. Human infection by *Campylobacter* presents a large financial burden on health care systems and economies in the developed world, and can cause endemic conditions in the developing world where a lack of appropriate treatment and unsanitary conditions leads to repeated infection, especially in children, occasionally resulting in death. As such, a thorough understanding of the mechanisms that make *Campylobacter* such a successful pathogen are desperately required to control, and eventually eradicate, this troublesome bacteria.

In Chapter 2 the publication Yahara *et al*. (2017) is presented, which proved, in principle, that genome wide association studies (GWAS) can be used to identify novel virulence determinants *in silico*, which can be translated into *in vivo* mechanisms via a traditional microbiology approach. In an organism like *Campylobacter*, where a significant portion of the genome encodes proteins of unknown function, with little to no homology to other bacteria, this collaboration between state of the art genomics and traditional microbiology is a powerful means by which to dissect novel pathways. We discovered a unique nucleotide salvage pathway and selenocysteine-related ferredoxin, Cj1377, and demonstrated their function through the phenotype of the defined mutants *in vitro*. Selenocysteine synthesis is specific for formate dehydrogenase (Fdh) in *Campylobacter*, and Fdh has been of long standing interest in our lab. We are currently conducting further research into the exact function of this putative ferredoxin in selenocysteine/Fdh synthesis as this appears to be a protein completely unique to, yet well conserved in, epsilon-proteobacteria. We suspect that Cj1377 may generate the perselenide intermediate used by SelD to generate selenophosphate for downstream selenocysteine synthesis. In *E. coli* and other canonical bacteria, perselenide is generated by CsdB or SufS, for which there are no homologs present in *Campylobacter*. Therefore, we believe Cj1377 may represent an alternative family of perselenide generating proteins amongst the epsilon-proteobacteria.

In Chapter 3 the publication Taylor *et al.* (2017) is presented on the periplasmic chaperone network of *C. jejuni*. In addition to the previously characterised SurA-type chaperone PEB4, we identified four further periplasmic chaperones. Phenotype analysis of individual mutants showed outer membrane defects in keeping with a disturbed chaperone

network. Purified protein assays showed Cj0694 to be the equivalent to PpiD from *E. coli*, and Cj1289 as a unique, domain-swapped SurA-like chaperone, which we dubbed SalC (SurA-like chaperone). This paper characterises what we believe to be the complete periplasmic chaperone network of *C. jejuni*, greatly improving the understanding of how outer membrane proteins reach the outer membrane. Since cell surface proteins are of such critical importance to immune system interaction and evasion, these proteins are an obvious target for *Campylobacter* specific antibiotics. Further work in this area should concentrate on the protein changes in the outer membrane of the chaperone mutants by mass spectrometry proteomic analysis, which will define which outer membrane proteins are clients of the alternate chaperones.

Finally, I present in Chapter 4 a partially completed study on the periplasmic methionine sulfoxide reductase system of *C. jejuni*. Treatment conditions and assay development has resulted in a protocol which has allowed accurate measurement of methionine oxidation of global proteins in response to hypochlorite stress. By comparing wildtype *C. jejuni* with an isogenic *msrPQ* mutant by mass spectrometry proteomic analysis, I have discovered which periplasmic proteins of *C. jejuni* are susceptible to methionine oxidation, on what residues the oxidation occurs, and if these residues are subject to repair by MsrP. I also gained an unprecedented insight into expression changes in response to hypochlorite, which will form the basis of a further study seeking to clarify the regulatory mechanisms by which these changes occur. Based on hints from responsive systems in other bacteria, we believe *C. jejuni* encodes some unique regulators, particularly two component systems, which detect hypochlorite and induce the aforementioned cellular response. For instance, the RacRS two component system, whose primary target, *mfrABE*, is strongly suppressed in response to hypochlorite, could sense hypochlorite through the oxidation of two cysteines present in the periplasmic loop of the sensor RacS. Similarly, the CprRS two component system, of which MsrP has been shown to be within the regulon of previously, could respond to hypochlorite via oxidation of a single methionine in the periplasmic loop of the sensor CprS (Svensson *et al*. 2015). This hypothesis has precedent, as the canonical oxidative stress regulator OxyR of *E. coli*, absent in *C. jejuni*, is activated by oxidation of two cysteines to from a disulfide bridge (Choi *et al*. 2001). The hypochlorite responsive transcription factor (HypT) of *E. coli*, again absent in *C. jejuni*, is activated by oxidation of conserved methionine residues (Gebendorfer *et al*. 2012). Therefore *C. jejuni* has likely evolved equivalent systems which utilise the sensitivity of methionine and cysteine residues to detect oxidative stress.

Particularly focused on the cell envelope, this work shows that periplasmic proteins can be just as crucial to the cells integrity and survival of stress as cytoplasmic proteins,

despite generally receiving less attention in primary research. The cell envelope represents the first line of defence against the environment, creating a physical barrier to protect the cell from antimicrobial agents, host immune system and other toxic influences. In addition, the cell envelope senses the outside environment, controlling the influx of metabolites, chemotaxis, motility and a variety of signalling events to respond to indicators of stress such as metabolite availability, temperature and host factors. Much of *C. jejuni*'s metabolism occurs in the periplasm through the terminal reductases and oxidases, linked to the electron transport chain of the inner membrane. This work suggests regulation of these terminal metabolic enzymes may be an important reaction to stress and warrants further investigation. Indeed, the idea of metabolic fitness as a determinant of pathogenic ability is a rising concept which directly links basic metabolism research to pathogenicity and disease prevention (Gao *et al*. 2017). Particularly in the case of *C. jejuni*, with its apparent limited metabolic capacity, metabolic bottlenecks could be exploited to design new interventions which do not rely on generic antibiotics. The periplasmic metabolism enzymes of *C. jejuni* could present an attractive target for such interventions as they are feasibly more accessible as a drug target than cytoplasmic enzymes. Overall, this work has improved the understanding of molecular pathways in *Campylobacter* which contribute to its unique metabolism and pathogenic nature.

Choi H, Kim S, Mukhopadhyay P, Cho S, Woo J, Storz G and Ryu SE (2001). Structural basis of the redox switch in the OxyR transcription factor. *Cell*. 105(1): 103-13.

Gebendorfer KM, Drazic A, Le Y, Gundlach J, Bepperling A, Kastenmüller A, Ganzinger KA, Braun N, Franzmann TM and Winter J (2012). Identification of a hypochlorite-specific transcription factor from *Escherichia coli*. J Biol Chem. 287(9): 6892-903.

Gao B, Vorwerk H, Huber C, Lara-Tejero M, Mohr J, Goodman AL, Eisenreich W, Galán JE and Hofreuter D (2017). Metabolic and fitness determinants for in vitro growth and intestinal colonization of the bacterial pathogen *Campylobacter jejuni*. *PLoS Biol*. 15(5): e2001390.

Svensson SL, Huynh S, Parker CT and Gaynor EC (2015). The *Campylobacter jejuni* CprRS two-component regulatory system regulates aspects of the cell envelope. *Mol Microbiol.* 96(1): 189-209.