INNATE IMMUNITY EVASION THROUGH D-AMINO ACID SEQUESTRATION

D-AMINO ACID SEQUESTRATION AS A MECHANISM OF INNATE IMMUNITY EVASION IN *SALMONELLA* AND UROPATHOGENIC *ESCHERICHIA COLI* INFECTIONS

By

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ABSTRACT

The innate immune system functions to limit the spread of bacteria during an infection. This is achieved through a highly complex assault on infiltrating pathogens. One such mechanism is the production of reactive oxygen species. D-amino acid oxidase is an emerging player in the innate immunity as it is capable of producing bactericidal concentrations of reactive oxygen species. Pathogens have evolved an array of strategies to protect themselves against the innate immune system. This work focusses on two bacteria relevant to human health that have evolved two distinct D-amino acid transporters to evade reactive oxygen species produced by D-amino acid oxidase. The *Salmonella* specific gene, DalS, was found to be co-regulated with the *Salmonella* Pathogenicity Island-2, a known virulence determinant. DalS is the periplasmic binding domain of a D-alanine ATP Binding Cassette transporter capable of sequestering substrate away from D-amino acid oxidase during *Salmonella*-neutrophil infections. This work demonstrates a novel host-pathogen interaction that enhances *Salmonella* survival during an infection. The second transporter, *cycA*, is conserved across diverse taxa and transports D-alanine and D-serine. This work determined that uropathogenic *E. coli* uses CycA to sequester D-serine away from DAO during ascending urinary tract infections, and moreover provides another example of how pathogens protect themselves against DAO. Together, these findings contribute to the understanding of the intricate set of virulence strategies of two pathogens that have a significant impact on human health worldwide.

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ATP	adenosine triphosphate
AMP	Antimicrobial peptide
CBIO	6-chloro-1,2-benzisoxazol-3(2H)-one
CGD	chronic granulomatous disease
CMC	carboxymethylcellulose
DAEC	diffusely-adherent *E. coli*
DAO	D-amino acid oxidase
D-ala	D-alanine
DC	Dendritic cell
E. coli	*Escherichia coli*
EAEC	enteroaggregative *E. coli*
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
FAD	Flavin adenine dinucleotide
IBC	Intracellular bacterial community
HGT	Horizontal gene transfer
HOCl	Hypochlorous acid
H_2O_2	Hydrogen peroxide
LPS	lipopolysacchride
M cell	Microfold cell
MAMP	Microbial associated molecular pattern
MPO	Myeloperoxidase
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
NTS	Non-typhoidal *Salmonella*
PG	peptidoglycan
PMN	Polymorphonuclear
P-pili	pyelonephritis-associated pili
PRR	Pattern recognition receptor
QIR	Quiescent intracellular reservoir
RLR	Rig-like receptor
RNS	reactive nitrogen stress
ROS	Reactive oxygen species
S. bongori	*Salmonella bongori*
SCV	*Salmonella* containing Vacoule
S. enterica	*Salmonella enterica*
SPI-1	*Salmonella* Pathogenicity Island-1

SPI-2	*Salmonella* Pathogenicity Island-2
S. Typhimurium	*Salmonella* Typhimurium
T3SS	Type three secretion system
TA	teichoic acid
TLR-4	Toll-like receptor 4
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary Tract Infection
WBC	White blood cell

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1 - Impact of *Salmonella* and UPEC

The human gastrointestinal tract is colonised with a diverse flora of commensal bacteria that are critical for the health of the individual with estimates suggesting approximately an equal ratio of bacterial cells to human cells (1). Pathogens have evolved to displace commensals and occupy important niches, potentially causing severe damage to the gut, and spread throughout the host to systemic sites. These strategies can result in minor dysbiosis or gross pathology resulting in significant harm to the host. The innate immune system has evolved to prevent pathogens from gaining entry to sensitive sites within the host, however pathogens have also evolved to circumvent these antimicrobial insults. D-amino acid oxidase (DAO) forms one arm of the innate immune system and will be a major focus of this thesis. Although there are many pathogens relevant to human health this work focuses on two that directly interact with the DAO specific tissues, *Salmonella enterica* (*S. enterica*) and uropathogenic *Escherichia coli* (UPEC).

1.1 - Health Burden of *Salmonella enterica*

Salmonella are Gram-negative pathogens that can be divided into two main species, *Salmonella bongori* (*S. bongori*) and *S. enterica* (2). The *Salmonella* genus is estimated to have diverged from *Escherichia coli* (*E. coli*) approximately 120 million years ago (3). While *S. bongori* is a reptile restricted pathogen (4), *S. enterica* is capable of colonising a broad spectrum of hosts and is comprised of over 2500 different serovars, further divided into six

subspecies: *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* (3). A subset of these serovars are of particular interest with regards to human health as they can cause a range of diseases (5). *S. enterica* Typhimurium (*S.* Typhimurium) is a mammalian specific pathogen and presents as self-limiting gastroenteritis (6). There is a significant global impact of gastroenteritis due to these pathogens with over 90 million cases, over 150 000 deaths, and associated cost in the billions each year (5). *S. enterica* Typhi and Paratyphi are human adapted pathogens and present as the more severe typhoid fever. These strains are responsible for over 20 million infections and 216 000 deaths annually with most cases occurring in under developed countries in south-central and south east Asia (7, 8). Recently it has been demonstrated that Non-Typhoidal *Salmonella* (NTS), such as *S.* Typhimurium ST313, are clinically associated with invasive systemic disease rather than gastroenteritis in immune comprised individuals resulting in death of 20-25% of infected individuals (9, 10). Furthermore, the DT104 strain has been linked to several foodborne outbreaks in North America further generating interest in *Salmonella* research. This strain is of particular importance due to its resistance to ampicillin, chloramphenicol, tetracycline and several other antibiotics (11).

S. Typhimurium functions as a model organism as it results in an acute intestinal infection in humans but a typhoid like infection in mice (12). Infected mice fail to control the intestinal infection and permit bacteria to spread to secondary systemic sites within the mouse where they survive, replicate, and can continually re-seed the gut with bacteria (13). This strain is a robust model for gastrointestinal infections and will be the main *Salmonella* model used in this work.

1.2 – Health Burden of uropathogenic *Escherichia coli*

E. coli are Gram-negative, facultative anaerobes and play a significant role in human health. *E. coli* predominately exist as a commensal residing in the gastrointestinal tract, however some species have evolved to become pathogens (14), broadly characterised as diarrheagenic *E. coli* or extraintestinal pathogenic *E. coli* (ExPEC) (15). While diarrheagenic *E. coli* are typically restricted to the intestine and give rise to gastroenteritis (16), ExPEC can exist in the gut with no consequence but migrate and cause disease in other host niches such as the central nervous system, blood, and urinary tract (17).

Diarrheagenic *E. coli* can be characterised into six distinct groups of bacteria defined by the set of virulence factors they encode (pathotypes); enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and diffusely-adherent *E. coli* (DAEC), based on the pattern of virulence factors used to inflict disease (16). The ExPEC predominately implicated in urinary tract infections (UTIs) does not have a distinct pathotype but has been designated UPEC for consistency (18). Although no formal pathotype designation exists for UPEC strains it is important to note that only a subset of *E. coli* can colonise the urinary tract suggesting that some combination of virulence factors is necessary (19). Of the over 200 known strains of UPEC only 50% can be subcategorised into six pseudo pathotypes suggesting a wide range of virulence factors capable of supporting infection (19). This variance is important as it makes treatment and the tracking of infections difficult (19).

UPEC are commonly associated with human disease resulting in 75-90% of all urinary tract infections (20). The remaining 10-25% of UTIs are caused by (in order of prevalence)

Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcus faecalis, group B Streptococcus (GBS), *Proteus mirabilis, Pseudomonas aeruginosa,* and *Staphylococcus aureus.* Transmission usually occurs through contact of fecal matter with the periurethral area followed by bacterial ascension to the bladder resulting in cystitis (21). Disease prevalence is increased in females due to the close proximity of the anus and periurethral opening with incidences peaking in their early 20's and after the age of 85 and with 25% of these women experiencing a recurrent infection within 6 months (22). If gone untreated UPEC can ascend to the kidneys and establish a secondary more serious infection termed acute pyelonephritis (21). There are approximately 150-million UTI cases annually worldwide and an estimated 3-billion-dollar annual burden on the health care system in the United States alone (23, 24).

Similar to *Salmonella* Typhimurium in the gut, UPEC serves as a model for acute UTIs. Two common isolates of UPEC are typically used during a murine UTI model, CFT073 (25) and UTI89 (26). CFT073 was isolated from a human pyelonephritic infection and was used in all the UPEC modeling work described in this thesis.

2 - Pathogenesis of *S.* Typhimurium and uropathogenic *E. coli*

2.1 - *S.* Typhimurium infection and pathogenesis

Typically contracted as a zoonotic infection through contaminated food or water, *S.* Typhimurium passes through the stomach using a series of acid tolerance mechanisms and reaches the small intestine (27). Here, *Salmonella* has evolved several mechanisms allowing for the breach of the protective epithelial layer and access to the lamina propria, a region in

which *Salmonella* can target and hijack immune cells providing a replicative niche where they can evade the immune system (28). *Salmonella* can target specialised epithelial cells, termed microfold cells (M cells), found exclusively in the Peyer's Patch that are responsible for ushering bacteria to waiting immune cells (29). Using a type three secretion system (T3SS) encoded on the *Salmonella* Pathogenicity Island 1 (SPI-1) *Salmonella* can also enter non-phagocytic epithelial cells in a process termed bacterial-mediated endocytosis (30). This T3SS secretes effectors that cause cytoskeleton rearrangements and membrane ruffling allowing for *Salmonella* to invade these cells and develop within a *Salmonella*-containing vacuole (SCV) (31). The SPI-1 T3SS has also been implicated in the translocation of an effector, SopB, which forces an epithelial–mesenchymal transition of follicle associated epithelia enterocytes into M cells (32). Additionally *Salmonella* can access the lamina propria through epithelial tight junctions or through direct sampling by dendritic cells (33, 34). The sheer number of pathways *Salmonella* uses to pass the epithelial layer indicates an importance to gain access to the lamina propria.

Neutrophils and macrophages engulf *Salmonella* as they enter the lamina propria (30). Once inside, *Salmonella* exists in the specialised SCV compartment and are almost immediately subjected to antibacterial pressures (35). These include restriction of space and nutrients, decrease in pH, and exposure to antimicrobial peptides and reactive oxygen species (ROS) (36–39). *Salmonella* has evolved a second T3SS encoded on the *Salmonella* Pathogenicity Island 2 (SPI-2) which aids in vacuolar survival (40). The two-component system SsrA-SsrB acts as the primary regulatory input responsible for expression of genes within SPI-2, and through a process termed *cis*-regulatory evolution, additional genes throughout the chromosome have come under the regulatory control of SsrA-SsrB (41).

Together these virulence factors allow for *Salmonella* to survive within these immune cells. Although initial engulfment rates appear to be similar between neutrophils and macrophages, very rapidly the *Salmonella* population decreases within macrophage resulting in a higher proportion in neutrophils (42). This suggests a failure to clear *Salmonella* by neutrophils and a preferred replicative niche during infections.

Salmonella uses these immune cells to spread systemically throughout the host eventually colonising the spleen and liver (43). Within the liver *Salmonella* can descend to the gall bladder where it forms biofilms and can continually re-seed the gut through the bile duct, resulting in a persistent infection and dissemination through fecal matter (12).

2.2 - UPEC infection and pathogenesis

UPEC infections are typically initiated through the migration of ExPEC from the gastrointestinal tract to the periurethral area (21). UPEC can then colonise the urethra and using a subset of the thirty-eight identified pilus operons UPEC migrates to the bladder (44). Type 1 pili and pyelonephritis-associated (P) pili are essential for colonization, invasion, and persistence of UPEC (45). Type 1 pili are tipped with the adhesion FimH and recognise mannosylated uroplakins to initiate invasion into umbrella cells (46). Pili binding triggers a signal transduction which causes an actin rearrangement leading to internalization of UPEC (47, 48). The uroepithelium can defend from UPEC invasion by utilising a toll like receptor 4 (TLR-4) dependent expulsion mechanism which allows for the exocytosis of vacuolar UPEC (49). Through an unknown mechanism UPEC can prevent this by exiting the vacuole into the cytosol and through rapid replication form an intracellular bacterial community (IBC) (50,

51). In addition, UPEC can undergo morphological changes to evade the immune system. By blocking septation and cell division a subpopulation of UPEC in IBCs can become filamentous and upon release from umbrella cells are more resistant to engulfment by neutrophils (52). UPEC uses the toxin α-haemolysin to promote pore formation in bladder umbrella cells allowing for the acquisition of essential nutrients and further exfoliation of these host cells (53). During exfoliation the umbrella cell detaches from the epithelium and is cleared from the urinary tract allowing for the spread of UPEC to other hosts (46). The removal of these umbrella cells also exposes the transitional cells that form the lower layer of the epithelium. UPEC can colonise this exposed layer directly and form quiescent intracellular reservoirs (QIRs) which contain 4-10 bacteria and can remain viable for several months re-seeding the bladder resulting in recurrent urinary tract infections (54). A UPEC infection of the bladder is summarised in **Figure 1.1**.

It has been well documented that subpopulations of UPEC use flagella to ascend the ureters to the kidneys in large groups or waves (55). Similar to Type-1 pili, P pili play a major role in colonization of UPEC in the kidneys (56). P pili are tipped with the adhesion PapG which binds globosides containing glycolipids found in the kidneys. In addition, PapG interacts with TLR-4 to reduce the polymeric immunoglobulin receptor expression. This reduces the transport of immunoglobulin A through the lamina propria to the kidney lumen and in turn UPEC is further protected from the innate immune system (57). Neutrophils are targeted to the site of infection and transverse the submucosa and the epithelium to eliminate UPEC (58). Murine models artificially depleted of neutrophils fail to clear UPEC. If gone untreated UPEC can spread systemically to the blood and spleen eventually leading to sepsis (58).

3 - Innate Immunity

A major aspect of the life cycle of both *Salmonella* and UPEC involves subversion of the innate immune system to either remain undetected or protect against antibacterial factors employed by the host. The innate immune system is a complex system of physical barriers, such as the gut epithelium, and immune cells that functions to control the infiltration of pathogens. Unlike the adaptive immune system which provides long lasting protective immunity, the innate immune system provides an immediate defense and is found in all classes of plant and animal life (59). The innate immune system is an evolutionarily older defense system responsible for the identification of microorganisms, recruitment of immune cells, and subsequent removal of these foreign objects (60, 61). In addition, the innate immune system serves as an anatomical barrier restricting foreign objects from gaining access to sensitive tissue and further presents antigens to the adaptive immune system (62). Collectively the innate immune system establishes what is the host "self" and what is foreign "non-self".

3.1 - Leukocytes

Key players in the innate immune system are leukocytes. These cells are part of a large collection with each type having a distinct role ranging from early defense against invading pathogens to the sampling of antigens from the gut lumen and presenting these antigens to develop a strong adaptive immune system. Leukocytes typically circulate in the blood and are targeted to infection sites (63). These cells can be subcategorised into

lymphocytes and myeloid cells. Lymphocytes consist of natural killer cells, which serve to eliminate virus particles and cancer cells, and T and B cells which are the major component of the adaptive immune system (64). Myeloid cells are a more diverse class of immune cells. These cells include monocytes, basophils, eosinophils, and neutrophils (65). Monocytes are the largest white blood cell (WBC) and upon exit of the bloodstream they differentiate to macrophages and serve to remove large debris and invading pathogens (66). Basophils are mainly responsible for initiating the allergic response but have been shown to release chemical signals to attract neutrophils to infection sites (67). Eosinophils primarily remove invading parasites and are inflammatory during an allergic reaction (68). Neutrophils are the most abundant WBC and will be covered in depth below. A subcategory of myeloid cells are targeted to specific areas and remain there, one example of this is the dendritic cell (DC). DCs reside primarily within the lamina propria and function to sample antigens through the tight junctions of the adjacent epithelia cells using long dendrites presenting antigens to the adaptive immune system (69). Collectively myeloid cells help protect the host and limit the spread of invading pathogens.

3.2 - Neutrophils, phagocytosis, and bacterial clearance

Neutrophils or polymorphonuclear cells (PMN) are recognised as the major player in acute bacterial infections (70). Typically the first leukocytes recruited to the site of infection, neutrophils help dampen the initial infection (71). In the bone marrow, pluripotent hematopoietic cells are exposed to growth factors and cytokines and differentiate into myeloblasts and further mature resulting in the production of neutrophils (72). Neutrophils are short lived, usually circulating on the order of hours, but upon infection they are able to

extend their lifespan several fold (73). During homeostasis in the host, neutrophils proliferate in the bone marrow and patrol the bloodstream (74). Neutrophil reservoirs can be found in the lungs, liver, and spleen but it is unclear why they congregate in these locations (75). Once a pathogen has breached an anatomical barrier neutrophils are recruited to the site of infection. This occurs through changes on the surface of endothelium caused by the release of inflammatory mediators, such as histamine, chemokines, and cytokines, by sentinel leukocytes present in the tissue or through direct pathogen detection by pattern-recognition receptors (PRR) (70, 76, 77). This results in the neutrophil recruitment cascade which is composed of the following steps: tethering to the surface endothelia, rolling, adhesion, crawling, and transmigration at the site of infection (70). Under physiological conditions it is believed that neutrophils are targeted to the spleen and cleared from circulation (78). However during a bacterial infection a large subpopulation of neutrophils hone to the spleen and remain there, the relevance of this is unclear.

Once in the interstitial space neutrophils are exposed to a milieu filled with chemoattractants and inflammatory stimulants that establish a chemotaxis gradient (79). Using receptors, neutrophils follow this gradient to the site of infection (80, 81). Here the primary function is to eliminate the pathogen using phagocytosis, granular proteins, antimicrobial peptides, and ROS producing enzymes (71). A subset of these antimicrobials are stored in specialised compartments termed granules and include lysozyme, myeloperoxidase (MPO), elastase, and lactoferrin (82). As neutrophils become activated, their granules begin to fuse with the outer or phagosomal membrane, eventually releasing their contents into the respective environment (83). In addition to granule proteins, neutrophils also express proteins that directly interact with bacteria. These proteins, or

antimicrobial peptides (AMPs), are typically cationic allowing for the interaction with microbial surfaces potentially forming pores and destabilising the membrane (84). Of the over 800 identified AMPs, neutrophils mainly express α-defensins and cathelicidins, predominately LL-37 (71).

ROS production is perhaps the most important and well-studied antimicrobial mechanism employed by neutrophils. Patients with mutations in most neutrophil killing mechanisms fail to present any symptoms whereas patients that lack functional NADPH oxidase (chronic granulomatous disease, CGD) suffer severe consequences (85). As neutrophil activation reaches a maximum, specific granules fuse with membranes initiating the oxidative burst. This fusion permits the assembly of the NADPH oxidase complex which allows for the production of ROS. NADPH oxidase is a complex of two transmembrane proteins (gp91^{phox}and p22^{phox} which together form the large heterodimeric subunit flavocytochrome b_{558}), three cytosolic *phox* subunits (p40^{phox}, p47^{phox}, p67^{phox}), and one guanine triphosphatase (either Rap1A or Rac2) that collectively reduces molecular oxygen to superoxide (86), which in turn rapidly dismutates to hydrogen peroxide (H_2O_2) (87). Once inside the phagosome, MPO quickly converts H_2O_2 to hypochlorous acid which is believed to react with host proteins generating antimicrobial chloramines (88). During this ROS cascade the production of H_2O_2 is rate limiting (89) suggesting an auxiliary source of H_2O_2 would be beneficial. This supplementary source of H_2O_2 may be produced by D-amino acid oxidase (DAO), a host oxidase which will be discussed in more detail below.

Neutrophils can also undergo a form of programmed cell death termed NETosis (90). Neutrophil Extracellular Traps (NETs) are formed in response to a variety of pro-inflammatory stimuli, such as LPS, IL-8, and tumour necrosis factor (TNF) and serve to

snare pathogens and potentially kill them (60). These web-like structures consist mainly of chromatin, histones, and antimicrobial systems such as NADPH oxidase and MPO (91). Some evidence suggests that pathogens are able to evade these NETs by expressing endonucleases capable of degrading the chromatin mesh (92). It is suggested that formation of NETs requires the production of ROS by NADPH oxidase as neutrophils from CGD patients fail to produce NETs (93) but this phenotype is rescued when neutrophils are treated with ROS (94). NETs serve as a drastic example for the importance of eliminating pathogens during an infection.

As bacteria are cleared, neutrophils undergo apoptosis where their debris is subsequently removed by macrophages (95). This programmed cell death also functions to decrease the rate of neutrophil influx to the site of infection. As the neutrophil debris is cleared, macrophage produce anti-inflammatory signals including transforming growth factor-β (TGF-β) and IL-10 (95). Failure to clear apoptotic cells can lead to secondary necrosis and further release of pro-inflammatory signals (71). The role in neutrophil clearance by macrophage post NETosis remains uncharacterised. Limited research also suggests a small population of neutrophils can re-enter the vasculature due to a downregulation of an epithelial junction adhesion protein that typically prevents reverse migration, however the fate of these neutrophils is unclear (96).

Neutrophils function as the first responders to bacterial infection and produce an arsenal of antimicrobial factors, however many pathogens have evolved a range of mechanisms to avoid killing by these phagocytes. *Staphylococcus aureus* (*S. aureus*) expresses a polysaccharide capsule that prevents phagocytosis whereas *Helicobacter pylori* and *Yersinia pseudotuberculosis* can disrupt targeting of NADPH oxidase and cause

superoxide to accumulate extracellularly (97–99). The vast range of pathogens that have evolved to survive clearance by neutrophils further suggests the importance these immune cells have during an infection.

4 - D-amino acid oxidase; neurotransmitter modulator, antibacterial, and metabolic detoxifier

D-amino acid oxidase is a member of the flavoprotein oxidase family and was originally characterised in yeast cells in 1935 by Hans Krebs (100). It was one of the first enzymes to be described and the second flavoprotein to be discovered. DAO is a 39 kD enzyme that is active as a homodimer (101). It catalyses the dehydrogenation of D-amino acids to an imino acid and resulting in a reduced molecule of flavin adenine dinucleotide (FAD) (102). The reduced FAD molecule is further oxidised by molecular oxygen resulting in hydrogen peroxide and spontaneous reduction of the imino acid results in the production of an α-keto acid and ammonia (102). DAO is found in almost all eukaryotes and has been implicated in many roles spanning neurotransmission to regulation of the gut microbiota (103, 104). D-amino acids play a critical role in nature and will be discussed in depth below.

4.1 – DAO modulates a neurotransmitter

In mammalian brains, DAO is enriched but its role remained elusive until it was discovered that there is a significant concentration of D-amino acids in the brain, with D-serine being the most abundant (105). D-serine binds the *N*-methyl-D-Aspartate receptor and acts as a neurotransmitter in glial cells (106). DAO is responsible for the degradation of D-

serine in the brain. Interestingly abnormalities in DAO function have been linked to complex neurological disorders such as schizophrenia. Studies have demonstrated that a DAO inhibitor, chlorpromazine, is also a potent schizophrenia treatment (107–109). In addition, the small protein encoded by the *G72* gene interacts with DAO and is also linked to schizophrenia. Mutations in *G72* result in the failure of the *G72* gene product to bind DAO resulting in uncontrolled D-serine degradation and decreased levels of neuronal D-serine (110).

4.2 - DAO protects against pathogens and regulates the microbiota

In 1969, Cline and Lehrer proposed that DAO was expressed in neutrophilic leukocytes and this enzyme was able to significantly reduce growth of *E. coli* cells following the addition of D-amino acids (111). *E. coli* growth was further reduced with the addition of MPO resulting in production of hypochlorous acid (111). If neutrophilic DAO is chemically inhibited, neutrophils are less able to clear *S.* Typhimurium in tissue culture (112). DAO is found in the granule fraction of neutrophils and upon neutrophil activation it is found to be associated with the plasma and phagosome membranes (113), most likely due to granule fusion with these membranes, however this remains to be shown. Recent work has demonstrated that mice lacking functional DAO succumb to *S. aureus* infections quicker than wildtype controls (114). It has also been shown that DAO is expressed in the proximal small intestine and is secreted by goblet cells (104). Gut localised DAO was shown to clear *Vibrio cholera* infections in the proximal small intestine most likely due to the production of ROS from D-amino acids in the gut. This consumption of D-amino acids in the gut may also lead

to changes in the intestinal microbiota selecting against *Lactobacillales* and selecting for *Bacteroidales*, although the mechanism remains to be determined (104).

4.3 - DAO removes D-serine from serum

DAO is found in kidney epithelial peroxisomes located in proximal tubules (115). Its primary role is most likely to degrade D-serine found in the blood. This degradation of D-serine generates a large amount of ROS stress on the kidneys. Excess D-serine in the serum can result in kidney necrosis due to elevated ROS (116). It has been suggested that DAO may also exist outside the peroxisomes as it has been found in the urine of healthy individuals and associated with the membrane of kidney epithelial cells (117). Kidney expression of DAO may be dependent on the bacterial colonization state of the host as germ free mice were found to have minimal DAO activity compared to conventionally colonized mice (118). In addition, mice infected with *S. aureus* are found to have elevated colonization in the kidney if DAO is not functional (114). Together this suggests an antimicrobial role for DAO within the kidneys in addition to its D-serine detoxification role.

4.4 – Inhibition of DAO

In 1983 the first DAO mutant mouse strain was described in a ddY background. This strain contains a single polymorphism in the *dao* gene resulting in a glycine (GGG)-to-arginine (AGG) amino acid substitution (119). The mutation in *dao* indeed leads to complete abrogation of DAO activity, however development of non-specific effects such as motor activity began to develop after time (120). Recently the ddY mutation was backcrossed onto a C57BL/6 strain resulting in a mouse strain with a better defined genetic background (104).

DAO can also be chemically inhibited using sodium benzoate, an aromatic compound that stacks next to FAD preventing oxidation of D-amino acids (102, 121). This inhibitor however can require a dose of up to 1000 mg/kg in rats (122). Additional aromatic inhibitors have been discovered that function similar to sodium benzoate but have K_m values in the low μM range and have potential as schizophrenia treatments (122). These inhibitors include 5-chlorobenzo[d] isoxazol-3-ol (CBIO) (105, 121), compound 8 (sc- 203909; 4H-thieno[3,2-b]pyrrole-5-carboxylic acid (124, 125), 5-methylpyrazole-3-carboxylic acid (AS057278) (126), and 4-nitro-3-pyrazole carboxylic acid (NPCA) (127), as well as 3-hydroxyquinolin-2-(1H)-one (compound 2) (128) however their *in vivo* half-life in mice is under 4 hours (122).

5 – D-amino acids: Synthesis, Usage, and Transport

L-amino acids form the building blocks of proteins in nature whereas D-amino acids, their chiral opposite subunits, are responsible for more specialised tasks. In bacteria, D-amino acids are typically found extracellularly or within the periplasm, typically found in polymeric molecules such as peptidoglycan (PG) and teichoic acids (TA) (129). Mutations in D-amino acid production can lead to severe growth defects in bacteria.

5.1 – D-amino acid synthesis and usage in bacteria and eukaryotes

Bacteria typically synthesize D-amino acids through inversion of the stereochemistry about the α-carbon, a reversible reaction performed by racemase or epimerase enzymes, or stereospecific production of the α-keto acid, also reversible but requires an amino group

synthesised by a D-amino acid aminotransferase (129). In stationary culture, bacteria can generate a diverse set of D-amino acids at millimolar concentrations (130). The production of D-alanine is of great significance as D-alanine, as well as other D-amino acids, are integral in the structure of peptidoglycan. The interconversion of L to D-alanine involves the formation of a pyridoxal-phosphate α-amino acid carbanion, within the active site of the racemase, which can randomly accept a proton to either chiral face yielding either a D or L-amino acid (131). This mechanism allows for a 1:1 equilibrium of D to L-amino acids (132). Interestingly *Salmonella* contains two D-alanine racemases *dadB* and *arl* that are each sufficient for bacterial survival in media lacking D-alanine, although DadB has a V_{max} approximately 60-fold higher than Arl (133, 134). Newly synthesised D-alanine can undergo subsequent conversion back to L-alanine, become ligated to a second D-alanine residue forming a D-ala-D-ala dipeptide crucial for the formation of peptidoglycan, or be degraded to pyruvate using the D-alanine dehydrogenase DadA (132).

It is important to note that unlike bacteria, mammals cannot synthesize D-alanine, they can however generate D-serine (135). In the brain of mammals D-serine is generated through the conversion of L-serine to D-serine via a pyridoxal-phosphate α-amino acid carbanion transition state by serine racemase (SR) (105). SR is of particular interest as it is capable of producing both D and L-serine as well as degrading both to pyruvate and ammonia (136). Serum levels of D-serine exist at approximately 3% of the total serine concentration (137) but it remains unclear if this D-serine is generated in the brain and transported out or is produced by and auxiliary source such as the gut microbiota.

5.2 – Bacterial transport of D-amino acids

Like most metabolites, D-amino acids must be shuttled across the bacterial envelope through specific transporters. Although many transporters exist, my work has focussed on three, DsdX, CycA, and DalSTUV, that have been implicated in virulence of UPEC and *Salmonella*.

DsdX is a D-serine permease found in many Gram-negative bacteria and is encoded in the *dsdCXA* operon (138). DsdA is a pyridoxal phosphate-dependent D-serine deaminase and DsdC is a transcription factor responsible for the regulation of the operon (139). First hypothesised to have a role in D-cycloserine resistance, CycA is a multi-substrate transporter capable of transporting: glycine, D-serine, D-cycloserine, and D-alanine and is highly conserved across all bacteria (140–142). Work has shown that *cycA* has several regulatory inputs including the small RNA *gcvB* which has been shown to inhibit expression during elevated levels of glycine, however no work has been conducted to see the effect D-serine has on *cycA* expression (143, 144). Additionally, PhoPQ has been demonstrated to down regulate *cycA* most likely through control by *gcvB* (145). Work by the Mobley lab demonstrated an enrichment of *cycA* and *dsdX* mRNA in UPEC isolated directly from a UTI-urine sample compared to levels found during culture in LB (146). Both DsdX and CycA have a K_m of approximately 60 μM and a double deletion of both prohibits sufficient D-serine transport in UPEC, resulting in an inability to survive on D-serine as a sole carbon source (138). A Δ*dsdXA* Δ*cycA* triple mutant is attenuated for survival in an acute pyelonephritis model but not a cystitis model (147). Since D-serine is in high abundance in both the bladder and the kidneys, one might expect a need for D-serine transport in both

locations if UPEC requires D-serine as a carbon source, this however is not the case suggesting a secondary purpose for D-serine sequestration.

DalSTUV forms an Adenosine Triphosphate (ATP) Binding Cassette transporter (ABC Transporter) localised to the inner member and is exclusively found within the *Salmonella* genus (148). After the acquisition of SPI-2, DalSTUV underwent *cis*-regulatory evolution to fall under the regulation of the virulence regulator SsrB (148). Binding of D-amino acids by DalS is achieved through charged residues that stabilise the carboxy and amino groups, and a methionine residue occludes L-confirmation amino acids from binding (148). Specificity to D-alanine is most likely achieved through the occlusion of larger side chains by the shallow floor of the binding pocket. DalS shuttles D-alanine from the periplasm to the DalTUV complex situated in the inner membrane. Through hydrolysis of ATP DalSTUV transports D-alanine across the membrane into the cytoplasm (148). Deletion of *dalS* results in no detectable phenotype *in vitro*, however a *dalS* mutant is attenuated for survival in a murine infection model (148). This suggested a role in virulence rather than metabolism.

6 – Interactions between the host and the pathogen

The pathogen and the host have evolved to interact with each other during a bacterial infection. The host must protect itself from invading bacteria and the pathogen must identify its location within the host. These interactions occur through the detection of small molecules and macromolecules produced by the host and pathogen.

6.1 – Self vs. Non-self; Host sensing of bacteria

In order to maintain a state of homeostasis the host must be able to determine which cells are native and which are foreign. Failure to remove pathogens can have devastating effects and an over protective state can have serious implications on the health of the host. For the purpose of this work only detection of bacteria will be of focus. The innate immune system can recognise a large number of microbial associated molecular patterns (MAMPs) using pattern recognition receptors (PRRs) such as TLRs, Nod-Like Receptors (NLRs), and Rig-like receptors (RLRs) (149–151). These MAMPs are distinctly bacterial and include bacterial flagella, peptidoglycan (a major component of the bacterial cell wall), and lipopolysaccharide (LPS) (61). As an example TLR-4, typically expressed on sentinel cells such as macrophages and dendritic cells, recognises extracellular LPS and a conformational change recruits myeloid differentiation primary response gene 88 (MyD88) resulting in a signal cascade leading to nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and expression of chemokines and cytokines (152). This results in the recruitment of other phagocytic cells responsible for the clearance of the pathogen.

Although the innate immune system can differentiate what is a bacterium from what is the host, many MAMPs are shared between pathogenic and commensal bacteria. This raises the question of 'how does the host determine what is commensal and what is pathogenic?'. There exist two theories that may help to explain this complex problem. One possibility is a context model in which MAMPs only activate the immune system if they are stimulated in the correct location. For example, if LPS is found in intestinal crypts activation will occur but if LPS is found in the lumen this activation is absent (153). An alternate theory is the "Danger Model" in which the immune system is only activated in the presence of harm

to the host. For example if MAMPs are present but no harm is induced there is no activation of the immune system (154). Although it is unclear how the innate immune system avoids continual activation through detection of commensals, it is clear that pathogens can be detected and in many cases have evolved to evade the innate immune system.

6.2 – Location, location, location; Bacterial sensing of the environment

Identification of location during an infection is paramount for the fitness of a bacteria. As bacteria have evolved a series of virulence factors to exploit a niche within the host so too have they evolved the machinery necessary to identify where they are in the host. Similarly to the host, bacteria are able to identify small molecule signals, transmit those signals, and implement an appropriate genetic response. One example of this is the two-component regulatory system (TCS) (155). A TCS is a signal relay that senses an external signal, undergoes a conformational change and phosphorylates a receptor protein, typically a transcriptional regulator, which in turn implements a new genetic program (156). An example relevant to the current work described herein is the two-component system SsrA-SsrB in *Salmonella*. Encoded on SPI-2, SsrA-SsrB upregulates the expression of SPI-2 genes as well as genes not encoded on SPI-2 that are important for construction of a T3SS and crucial for the survival of *Salmonella* in SCV (157). In addition, it has recently been shown that SsrB potentially downregulates flagellar machinery that is no longer required during intracellular survival. The physiological cues leading to SsrA activation are beginning to be unravelled. For example, low level SsrA-SsrB activity initiates prior to transcytosis of the gut epithelium (158) priming *Salmonella* for phagocytosis and survival within the SCV. During phagocytosis bacteria are almost immediately exposed to ROS suggesting that ROS

may act as a locational cue. *Salmonella* can identify ROS using the cytosolic ROS sensing protein OxyR which is responsible for the upregulation of ROS protection genes, including the alkyl hydroperoxide reductase subunit, *ahpC* (159). Recently it has been suggested that *Salmonella* may sense ROS as an intracellular cue and further up regulate SPI-2, however the mechanism remains elusive (unpublished). Failure to correctly implement a genetic program during an infection can have devastating consequences on the survival of a bacteria.

7 - Bacterial subversion and evasion of the innate immune system

As bacteria become host adapted they evolve virulence strategies to protect themselves from various antibacterial mechanisms employed by the host in order to successfully survive, replicate, and disseminate. This co-evolution 'arms race' can establish a complex web of host-pathogen interactions. Although many host adapted pathogens exist, key examples of this adaptation are the interactions between *Salmonella* and the mammalian innate immune system and between UPEC and the urinary tract. These pathogens have been repeatedly exposed to selective pressures and as a result have evolved to effectively disseminate in their respective hosts.

For most antimicrobial mechanisms in the host, *Salmonella* has evolved a 'counter-pathway' to limit host inflicted damage. The acquisition of the genomic island SPI-1 permitted *Salmonella* to penetrate the gut epithelia. Upon acquisition of SPI-2 *Salmonella* could survive and replicate within the SCV in both epithelial cells and immune cells. The acquisitions of SPI-1 and SPI-2 permitted significant phenotypic changes that allowed access to new replicative niches. However, many individual examples exist in which *Salmonella* has

evolved to survive an insult from the innate immune system. The effector GogB targets the SCF ubiquitin ligase in order to dampen the host inflammatory response by inhibiting IκBα degradation and NFκB activation (160). SseJ promotes esterification of cholesterol and promotes vacuolar integrity (161). Exposure to antimicrobial peptides membrane perturbations activates the TCS, PhoPQ, resulting in alterations to the cell envelope that confer peptide resistance (162). As *Salmonella* is exposed to reactive nitrogen stress (RNS) within macrophages, it upregulates expression of three RNS detoxifying genes, *hmpA, ytfE,* and *hcp* (163).

Like many other antimicrobial mechanisms the neutrophil enzyme DAO exists at the host-pathogen interface. A major focus of my research has been to understand the mechanisms that *Salmonella* has evolved in order to evade the innate immune system, and as a host adapted pathogen the question remains 'Is *Salmonella* capable of evading the DAO dependent production of ROS and if so do other bacteria have similar mechanisms to survive this insult?'.

8 - Purpose and Aim of this work

The purpose of this study was to investigate how *Salmonella* and UPEC are able to protect against reactive oxygen species produced by DAO. Previous research has shown that *S.* Typhimurium requires *dalS* for survival *in vivo*. However it remained enigmatic why D-alanine import was required for virulence during an infection but dispensable *in vitro*. In contrast to the little research done on *dalS*, *cycA* has been extensively studied in several bacterial models, but the tissue specific virulence defects of a *cycA* mutant in a UPEC

infection model remained unaddressed. Since host-pathogen interactions may serve as potential targets for combating microbial infections further research in this area is warranted.

The hypothesis at the time is work was undertaken was: Bacteria can sequester D-amino acids in order to evade the host innate immune system and promote infection. The specific aims of this work were to: 1) Characterize the interaction between the D-alanine transporter, DalS, and DAO during a *Salmonella* neutrophil infection and, 2) Determine if a similar protective mechanism exists with UPEC expressed CycA and DAO during an ascending urinary tract infection.

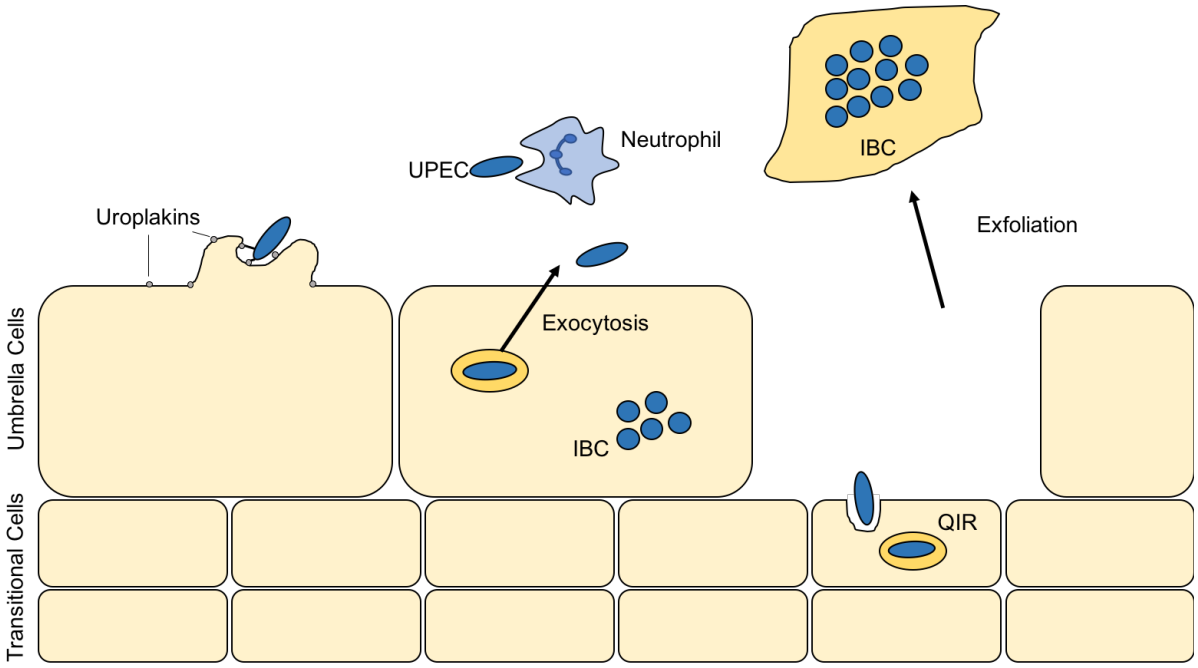
The aims of this work will be discussed in detail in the following chapters:

1) Salmonella evades D-amino acid oxidase to promote infection in neutrophils.
    - This study demonstrates that *Salmonella* uses DalS to sequester D-alanine from neutrophil DAO resulting in a reduction of ROS production and increased survival.
2) Uropathogenic *E. coli* evades D-amino acid oxidase in kidneys.
    - This study demonstrates that CycA sequesters both D-serine and D-alanine during a pyelonephritic infection to reduce exposure to kidney expressed DAO.

This work highlights the protective role played by DAO during bacterial infections and the first *in* vivo example of DAO as a neutrophil antimicrobial mechanism. Identification of two distinct transport systems that function to limit ROS exposure by DAO demonstrates a novel function for metabolic transporters in immune evasion.

Figure 1.1. UPEC infection of the bladder. UPEC utilizes several mechanisms to infect and survive within the bladder. Upon adhesion to mannosylated uroplakins, UPEC forces engulfment by bladder epithelial umbrella cells. This leads to escape of UPEC out of the vacuole or the exocytosis of UPEC into the lumen. Upon the replication of UPEC and the formation of Intracellular Bacterial Communities (IBC) umbrella cells may undergo exfoliation resulting in the liberation of the umbrella cell from the epithelial layer and subsequent excretion from the bladder. This results in the exposure of underlying transitional cells which can be targeted by UPEC. Quiescent Intracellular Reservoirs (QIR) may form resulting in a "safe haven" for UPEC to reside. Infiltrating neutrophils engulf UPEC in an attempt to control the infection. UPEC may ascend also to the kidneys resulting in a secondary site of infection.

Figure 1.1


Neutrophil
UPEC
IBC
Uroplakins
Exfoliation
Exocytosis
Umbrella Cells
IBC
QIR
Transitional Cells

CHAPTER TWO

Salmonella evades D-amino acid oxidase to promote infection in neutrophils

CO-AUTHORSHIP STATEMENT

The chapter herein has been adapted from material that has been published in *mBio*. The complete citation for the published work is as follows:

Tuinema BR, Reid-Yu SA, Coombes BK. 2014. Salmonella evades D-amino acid oxidase to promote infection in neutrophils. *mBio* **5**(6):e01886-14. doi:10.1128/mBio.01886-14.

The contributions by each author to the project are described below:

1. Strains and plasmids were created by B.R.T.

2. *In vitro* DAO assays were performed by B.R.T.

3. Neutrophil experiments were conducted by B.R.T.

4. Purity assessment of neutrophils via FACS was performed by S.A.R.

5. Western blot experiments were performed by B.R.T.

6. Competitive infections and *in vivo* imaging was conducted by B.R.T.

7. B.R.T., S.A.R., and B.K.C. contributed to the writing of the manuscript

*Note that references in the following data chapters have been compiled into one reference list at the end of the thesis in order to avoid any redundancy between sections.

Salmonella evades D-amino acid oxidase to promote infection in neutrophils

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Running title: *Salmonella* evades neutrophil DAO

ABSTRACT

Neutrophils engulf and kill bacteria using oxidative and non-oxidative mechanisms. Despite robust antimicrobial activity, neutrophils are impaired in directing *Salmonella* clearance and harbour viable intracellular bacteria during early stages of infection that can subsequently escape to more permissive cell types. The mechanisms accounting for this immune impairment are not understood. We report that *Salmonella* limits exposure to oxidative damage elicited by D-amino acid oxidase (DAO) in neutrophils by expressing an ABC importer specific for D-alanine, a DAO substrate found in peptidoglycan stem peptides. A *Salmonella dalS* mutant defective for D-alanine import was more susceptible to killing by DAO through exposure to greater oxidative stress during infection. This fitness defect was reversed by selective depletion of neutrophils, or by inhibition of DAO *in vivo* with a small molecule inhibitor. DalS-mediated subversion of neutrophil DAO is a novel host-pathogen interaction that enhances *Salmonella* survival during systemic infection.

IMPORTANCE

Neutrophils engulf *Salmonella* during early stages of infection but bacterial killing is incomplete. Very little is known about how *Salmonella* survives in neutrophils to gain access to other cell types during infection. In this study we show that D-amino acid oxidase (DAO) in neutrophils consumes D-alanine and import of this substrate protects *Salmonella* from oxidative killing by neutrophil DAO. Loss of this importer results in increased bacterial killing *in vitro*, in neutrophils, and in a mouse model of infection, all phenotypes that are lost upon inhibition of DAO. These findings add mechanistic insight into a novel host-pathogen interaction that has consequences on infection outcome.

INTRODUCTION

D-amino acid oxidase (DAO) catalyzes the flavin-dependent deamination of certain D-amino acids to yield an α-ketoacid, ammonium ion, and hydrogen peroxide (164, 165). For example, DAO regulates D-serine levels in the brain, where this amino acid co-activates glutamate-dependent N-methyl-D-aspartate receptors on post-synaptic neurons (164). Owing to its ability to generate hydrogen peroxide and its widespread conservation among vertebrates and invertebrates (166), historical work considered DAO to be a potential component of the innate host defense system. Work from the 1960's identified DAO activity in the purified granule fraction of human neutrophils (111). This activity was later localized by electron microscopy to the neutrophil phagosome following engulfment of latex beads in the presence of D-alanine (113). Studies in gnotobiotic mice showed DAO in the kidney in response to D-alanine liberated from the microbiota (118) and mice with a spontaneous mutation in DAO were shown to be more susceptible to infection with *Staphylococcus aureus* (114), further linking DAO to an understudied host defense system that is responsive to microbial input. Mammals do not synthesize D-alanine. However, in bacteria it constitutes the terminal amino acid in peptidoglycan stem peptides, making it a potential discriminator between self and non-self in the context of immunity.

In neutrophil phagosomes, hydrogen peroxide liberated from DAO-catalyzed dehydrogenation of D-alanine would be accessible to myeloperoxidase (MPO), which catalyzes the formation of hypochlorous acid (HOCl) from chloride and hydrogen peroxide. Although debated (167), this latter reaction has for some time been considered the clinically relevant and major microbicidal pathway in neutrophils, with reaction products being directly

toxic to bacteria or going on to form secondary chloramines (88, 168, 169). Experimental work indicates that hydrogen peroxide is rate limiting for MPO-catalyzed halogenation in neutrophils (89), suggesting that an auxiliary source of peroxide, in addition to dismutation of superoxide formed from the more widely studied NADPH oxidase, would be beneficial towards neutrophil killing activity.

Previously, we identified an ATP-binding cassette transporter in *Salmonella enterica* that imports D-alanine (148). This transporter underwent regulatory evolution for expression in the intracellular environment following host cell invasion (41), providing a clue that its function was related to intracellular survival. Structural work confirmed the specificity and chiral selectivity for D-alanine, showing that DalS, the periplasmic binding component, restricts the beta-carbon of alanine to a D-configuration due to steric hindrance of the L-isomer (148). Interestingly, DalS was dispensable for *S.* Typhimurium growth *in vitro,* and for peptidoglycan composition. However, D-alanine import was required for competitive fitness during infection of mice, and mice infected with DalS-deficient *Salmonella* had a significantly extended survival time compared to mice infected with wild type *Salmonella* (148). Our previous results suggested a possible interaction between DalS and the host immune system, however its function remained enigmatic. Using a murine model of systemic infection with the pathogen *Salmonella enterica* serovar Typhimurium, which is widely used as a model for the host-restricted *S.* Typhi, we show that DalS helps protect *Salmonella* from DAO-dependent killing in neutrophils. *Salmonella* mutants with a deletion of *dalS* were exposed to greater DAO-dependent oxidative stress during host infection, leading to a loss of competitive fitness and increased killing by neutrophils. However, this defect could be repaired upon inhibition of DAO activity or by host neutropenia. Thus, DalS-mediated

subversion of neutrophil DAO is an important host-pathogen interaction that enhances bacterial survival during early stages of infection. These data help explain, in part, the incomplete killing of *Salmonella* by neutrophils, allowing secondary dissemination to more permissive cell types (163).

RESULTS

DalS protects *S.* Typhimurium from the antimicrobial activity of DAO

The flavin-dependent deamination of D-alanine by DAO yields the α-ketoacid, ammonium ion, and hydrogen peroxide (Figure 2.1A). To study the susceptibility of *S.* Typhimurium to the activity of DAO, we purified recombinant DAO and confirmed its hydrogen peroxide generating activity in the presence of 5 mM D-alanine or 50 mM D-serine (Figure 2.1B and see Figure 2.S1 in the supplemental material). Increasing the concentration of D-serine was necessary as this substrate produces only 10% of the Vmax activity compared to D-alanine (111). DalS-deficient bacteria were more sensitive to killing by purified DAO in the presence of D-alanine (Figure 2.1C). This killing was blocked by the addition of thiourea, a potent hydroxyl radical scavenger that mitigates the toxic effects of hydrogen peroxide on bacterial cells by reducing the formation of hydroxyl radicals from hydrogen peroxide (170, 171). As expected, the addition of myeloperoxidase to the *in vitro* reaction increased the magnitude of killing of both wild type and *dalS* mutants, yet the enhanced susceptibility of Δ*dalS* bacteria persisted (Figure 2.1C). These data established that hydrogen peroxide was the toxic DAO reaction product in these *in vitro* reactions and that *Salmonella* mutants lacking DalS are more susceptible to DAO-dependent killing.

To determine whether DalS-deficient bacteria allowed a higher concentration of enzymatic hydrogen peroxide product to form by DAO, we constructed an *S.* Typhimurium reporter strain, previously validated to report hydrogen peroxide stress, by fusing the OxyR-

dependent *ahpC* promoter (172, 173) to *luxCDABE* from *Photorhabdus luminescens*. We confirmed the activity of this strain in response to hydrogen peroxide as low as 2 μM and showed dose-dependent luminescence in response to peroxide up to 100 μM (Figure 2.1D), well below the steady-state peroxide concentration in neutrophil phagosomes estimated to be ~ 5 μM by kinetic models (88). Importantly, we confirmed that this reporter responded equally in wild type and *dalS* mutants exposed to non-enzymatically generated hydrogen peroxide at relevant concentrations (Figure 2.1E). However, upon exposure to hydrogen peroxide generated enzymatically by DAO in the presence of D-alanine, reporter activity was significantly greater in *dalS* mutants compared to wild type (Figure 2.1F). This was consistent with an impaired ability of the *dalS* mutant to reduce an initial 5 mM extracellular concentration of D-alanine to the same extent as wild type (Figure 2.1G), in agreement with our previous data (148). As a control, both wild type and Δ*dalS* bacteria were killed to equivalent levels following exposure to non-enzymatic sources of hydrogen peroxide or hypochlorous acid (HOCl) (Figure 2.1H) indicating that increased killing of the *dalS* mutant was DAO-dependent and not due to an inherent sensitivity to hydrogen peroxide or HOCl. Complementing Δ*dalS* with the *dalS* gene under the control of its native promoter restored bacterial survival to wild type levels in the presence of DAO and D-alanine (Figure 2.1I). Together these data indicated that DalS-deficient bacteria allowed a higher concentration of DAO-dependent hydrogen peroxide product to form, resulting in increased killing.

Salmonella lacking DalS are more susceptible to DAO killing in neutrophils

Neutrophils express DAO (111, 113) and are an early host cell target for *Salmonella* (20, 21). To test whether *dalS* mutant *S.* Typhimurium were more susceptible to the microbicidal activity of neutrophils, we infected purified neutrophils with wild type or *dalS* mutants and monitored bacterial survival. After 2 h, 40% of wild type *Salmonella* had survived whereas only ~25% of the *dalS* mutants remained viable (Figure 2.2A). To confirm that this killing phenotype was dependent on DAO, we used the chemical inhibitor 6-chloro-1,2-benzisoxazol-3(2H)-one (CBIO) that blocks the dehydrogenation reaction catalyzed by DAO (123) (Figure 2.2B). We also confirmed that CBIO had no effect on *S.* Typhimurium growth and did not bind to the *S.* Typhimurium DalS protein as measured by fluorescence thermal shift of purified DalS (see Figure 2.S2 in the supplemental material) (148). In the presence of CBIO, the ability of neutrophils to kill *Salmonella* was impaired and there was no longer a survival defect of the *dalS* mutant (Figure 2.2A). To confirm that this killing phenotype was linked to DAO-dependent hydrogen peroxide stress, we infected neutrophils with wild type and *dalS* mutants carrying the OxyR-dependent *ahpC* reporter in the presence or absence of CBIO. Consistent with the killing phenotypes observed, peroxide reporter activity in the *dalS* mutant was double that seen in wild type *Salmonella* during neutrophil infections (Figure 2.2C). However, when DAO activity was inhibited with CBIO, the reporter activity from wild type and *dalS* mutants was reduced by ~35% and ~65%, respectively, and were no longer different (Figure 2.2C). Together these data established that DalS enhances *S.* Typhimurium survival in neutrophils in a manner that depends on functional DAO.

S. Typhimurium lacking DalS are sensitized to neutrophil DAO *in vivo*

Neutrophils are the primary source of DAO among polymorphonuclear leukocytes (111, 113) and their numbers increase rapidly in the spleen in response to *S.* Typhimurium infection (173, 174). Given this, we reasoned that the levels of splenic DAO would increase following *S.* Typhimurium infection due to neutrophil influx. We collected spleens from infected and uninfected mice and probed them for DAO using a DAO antibody. Uninfected mice had no detectable DAO in the spleen or cecum using this method (Figure 2.3A). In contrast, splenic DAO increased dramatically upon *S.* Typhimurium infection (Figure 2.3A). Hepatic DAO has been detected in some studies but not others (175). We detected DAO in liver isolated from uninfected mice, but this did not increase dramatically upon infection when normalized to a control host protein (Figure 2.3A). To verify neutrophils were the source of splenic DAO we rendered mice neutropenic by antibody-based neutrophil depletion and verified this by flow cytometry (see Figure 2.S3 in the supplemental material). In infected neutropenic mice, splenic DAO became undetectable, and liver DAO was reduced to ~25% of that seen in non-neutropenic controls (Figure 2.3B). The residual DAO signal in neutropenic mouse liver is likely from hepatocytes, which are a source of DAO activity in a variety of animals (176).

Given that DalS protected *S.* Typhimurium from neutrophil DAO, we hypothesized that depleting neutrophils would restore virulence to the *dalS* mutant *in vivo*. Indeed, in neutropenic mice, *dalS* mutant *S.* Typhimurium competed equally with wild type bacteria

whereas they remained significantly attenuated in control-treated animals containing neutrophils (Figure 2.3C). To verify that this restoration of virulence in the mutant was linked to the attendant loss of DAO following neutrophil depletion, we alternatively inhibited DAO activity in infected mice by *in vivo* administration of CBIO (122). These data showed that the effect of neutropenia on the *dalS* mutant was phenocopied by DAO inhibition (Figure 2.3D). The restoration of virulence to *dalS* mutants following DAO inhibition *in vivo* was specific to DalS and not a generalized immune dysfunction because a *Salmonella* mutant (Δ*SPI2*) containing a lesion in the type III secretion system involved in intracellular replication (177) remained attenuated in mice in the presence of CBIO (Figure 2.3D). To confirm the importance of DalS for *Salmonella* during infection, and to further connect this virulence factor to host DAO, single infections were performed with wild type or the *dalS* mutant in mice in which DAO was inhibited or not. Mice infected with wild type *S.* Typhimurium carried a significantly higher splenic bacterial burden than mice infected with a *dalS* mutant (Figure 2.3E), confirming that DalS contributes to bacterial fitness in the host. Treatment of mice with the DAO inhibitor, CBIO, significantly increased the bacterial load associated with Δ*dalS* infection (Figure 2.3E). Together, these experiments clearly linked the phenotype of the *dalS* mutant to the expression and activity of neutrophil DAO.

DAO contributes to bacterial peroxide stress independent of NOX2

The NADPH oxidase system in neutrophils is a source of superoxide that can spawn an array of reactive oxygen species. Prominent among these is hydrogen peroxide that is consumed by myeloperoxidase to generate hypochlorous acid (HOCl) within the neutrophil

phagosome (178). However, our experiments indicated that neutrophils killed Δ*dalS S.* Typhimurium more effectively than wild type bacteria even in the presence of functional NADPH oxidase, suggesting that DAO is a relevant source of innate immune activity. If this were the case, then the fitness defect in the Δ*dalS* mutant would persist in a host lacking NADPH oxidase. To quantify the contribution of DAO to host protection independent of NADPH oxidase, we infected *CYBB*/gp91/NOX2 mice lacking the flavocytochrome b-245 heavy chain, an essential component of NADPH oxidase. In the liver, *dalS* mutant bacteria remained attenuated to similar levels as in wild type mice. Interestingly, in the spleen where neutrophils accumulate after infection (163, 174), the defect of *dalS* mutants was significantly amplified in *CYBB* $^{-/-}$ mice compared to their defect in wild type mice (Figure 2.4A). The fitness defect of the *dalS* mutant was repaired upon treatment of *CYBB* $^{-/-}$ mice with CBIO (Figure 2.4B), implicating DAO as a relevant source of innate immune protection during *S.* Typhimurium infections. These data indicated that DalS increases early *Salmonella* survival, particularly in the spleen, even in the absence of NADPH oxidase.

To confirm that this source of bactericidal peroxide was DAO, we used the OxyR-dependent *ahpC-lux* transcriptional reporter constructed earlier and performed whole-animal *in vivo* imaging of infected mice to quantify hydrogen peroxide stress in *S.* Typhimurium following DAO inhibition. *S.* Typhimurium produced OxyR-dependent luminescence in both wild type mice and in *CYBB* $^{-/-}$ mice lacking NADPH oxidase, which was reduced in both cases when mice were treated with the DAO inhibitor CBIO (Figure 2.4C). Importantly, at these early time points after infection, the bacterial load among the groups was found to be

similar at necropsy (data not shown). OxyR-dependent luminescence in wild type mice was greater than that from *CYBB*$^{-/-}$ mice, a result that was expected because NADPH oxidase activity is broadly conserved among immune cells that interact with *Salmonella* (174, 179), whereas DAO is restricted to neutrophils (111, 113). Luminescence normalized to bacterial load in the spleen was quantified in both wild type and *CYBB*$^{-/-}$ mice by *ex vivo* imaging of freshly excised organs (Figure 2.4D). This analysis showed that inhibition of DAO activity in both wild type and *CYBB*$^{-/-}$ mice significantly reduced OxyR-dependent luminescence from splenic *S.* Typhimurium by ~60% confirming that DAO was a relevant source of hydrogen peroxide during infection in the presence or absence of NOX2.

Our previous results showed that *S.* Typhimurium lacking DalS are exposed to greater DAO-dependent oxidative stress during infection. We quantified this in mice lacking NADPH oxidase by infecting *CYBB*$^{-/-}$ mice with DalS-deficient *Salmonella* carrying the OxyR-dependent luminescence reporter. OxyR-dependent luminescence in mice infected with the Δ*dalS* mutant was significantly greater than from mice infected with wild type *Salmonella.* Treatment of mice with CBIO to inhibit DAO activity significantly reduced this luminescence signal (Figure 2.4E). Together, these experiments revealed that splenic *S.* Typhimurium is exposed to DAO-dependent peroxide stress in mice and that DalS reduces the magnitude of this stress to promote infection.

DISCUSSION

The majority of work on *Salmonella* infection of immune cells has focused on macrophages. However, neutrophils are the major cell type infected by *Salmonella* during the first two days of infection. In the gut, 70% of luminal *S.* Typhimurium are inside neutrophils at day 1 (180) and in the spleen, a neutrophil-enriched population harboured 100% of the viable intracellular *S.* Typhimurium population in the first 24 h (42), and 70% of this bacterial population on day 2 with a concomitant increase in the number of infected macrophages (174). Despite robust antimicrobial activity of neutrophils, which indeed kills many *Salmonella* during infection (163, 181), neutrophils are unable to direct full *S.* Typhimurium clearance (182). This incomplete killing is thought to allow *Salmonella* to spread to a more permissive macrophage population, which have been shown to generate sub-lethal oxidative bursts (163). The mechanisms to account for this immune subversion of neutrophils are incomplete but likely involve resistance to the microbicidal effector functions of these cells (71). Interestingly, despite the importance of this host-pathogen interaction, very little is known mechanistically about how *Salmonella* survives in neutrophils. Our data are consistent with neutrophil DAO functioning to exert bactericidal activity on *S.* Typhimurium at early stages of infection. These data provide insight into a host-pathogen interaction in systemic neutrophils that has bearing on the outcome of *Salmonella* infection. Of importance, it is likely that this immune subversion mechanism is also conserved in *S.* Typhi, as the DalS transport system is 98% identical in this human pathogen.

Hydrogen peroxide production is rate limiting for MPO-catalyzed halogenation and killing of bacteria in neutrophils (89). These data suggest that an auxiliary source of peroxide, in addition to dismutation of superoxide formed from the more widely studied NADPH oxidase, would be beneficial towards neutrophil killing activity. In mice lacking NADPH oxidase, we showed that *Salmonella* indeed senses an auxiliary source of peroxide stress that can be inhibited by CBIO, a specific small molecule inhibitor of DAO. This DAO-catalyzed source of oxidative stress was biological relevant because it elicited a larger response in DalS mutants and was able to better control growth of *dalS* mutant *Salmonella* that lack the D-alanine import system. In our *in vitro* experiments with purified DAO, exogenous D-alanine was added to elicit *Salmonella* killing by DAO, but bacterial derived D-alanine was sufficient for killing by DAO in neutrophils. Neutrophils are a rich source of membrane-perturbing antimicrobial peptides, which might sensitize *Salmonella* to the effects of neutrophil DAO due to liberation of D-alanine during membrane damage. This is supported by the fact that neutrophil antimicrobial peptides act synergistically with peptidoglycan recognition proteins to kill bacteria (183). In addition, bacterial killing by purified DAO is likely mediated by the terminal hydrogen peroxide product that forms, requiring higher concentrations to reach toxic levels. However, DAO-derived hydrogen peroxide in neutrophils would be converted by MPO to more toxic secondary products. Indeed, steady state levels of hydrogen peroxide in the low micromolar range appears sufficient to support killing in neutrophil phagosomes (88).

Salmonella has been reported to actively disrupt the trafficking of NADPH oxidase to the *Salmonella* containing vacuole (184), although the molecular basis for this observation has not been uncovered. The subversion by *Salmonella* of this additional oxidative stress might provide a more complete inhibition of oxidative killing in neutrophils, allowing *Salmonella* time to access more permissive splenic macrophages at later stages of infection (163). As predicted by our model, the presence of DalS confers a selective advantage to *Salmonella* in wild type mice, and even more so in NADPH oxidase-deficient mice, but is dispensable when DAO is inhibited or if neutrophils are depleted. These data highlight a novel interaction between host and microbe that adds to the growing complexity of bacterial pathogenesis. In this context, evasion of neutrophil DAO is a virulence mechanism operating in conjunction with other mechanisms of immune subversion that each confer different, but important fitness gains on the infecting pathogen.

Patients with chronic granulomatous disease (CGD) that lack NADPH oxidase activity are highly susceptible to a variety of bacterial infections. However, these patients are rarely infected with catalase-negative organisms (185), suggesting that a source of hydrogen peroxide stress independent of NADPH oxidase may be selective for certain infections in CGD patients (167). A proposed explanation for this was that the bacteria themselves generate enough hydrogen peroxide to mediate MPO-catalyzed halogenation. However this idea has been challenged because catalase negative bacteria are equally virulent in a CGD model system and produce approximately two orders of magnitude less peroxide than CGD phagocytes with non-protective levels of residual NADPH oxidase activity (186). Instead,

others have argued that the ability of CGD neutrophils to kill even catalase-positive organisms suggests an incomplete loss of hydrogen peroxide and/or an alternate system of intracellular killing (187). Our results are consistent with both of these suggestions. These findings provide rationale for the targeted augmentation of DAO activity in certain immune deficiencies. For example, polyethylene glycol-conjugated DAO can restore bactericidal activity to congenitally defective CGD neutrophils *in vitro* (188). In this disease, even modest residual oxidative activity offers significant protection from severe illness and greater likelihood of long-term survival (189). Thus, augmenting oxidative killing mechanisms in neutrophils may have clinical benefit in some cases.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care using protocols approved by the Animal Review Ethics Board at McMaster University.

Bacteria, cloning and reagents

All bacterial strains are isogenic derivatives of *Salmonella enterica* serovar Typhimurium strain SL1344 and are listed in Supplementary Table 1. The mRNA encoding porcine DAO was amplified by PCR and cloned with a 6-histidine tag as described (114) using primers BRT139 (GGA ATT CCA TAT GCG TGT GGT GGT GAT TGG) and BRT140 (GGA AGA TCT TCA GTG GTG GTG GTG GTG GTG GAG GTG GGA TGG TGG CAT T). DNA was digested with BglII and NdeI and pET3a was digested with BamHI and NdeI and then ligated. All cloning steps were confirmed by sequencing and plasmids transformed into *E. coli* BL21 (DE3). The OxyR-dependent *ahpC-lux* transcriptional fusion was generated as described previously with slight modifications (173). Three-hundred bp upstream of the *S.* Typhimurium *ahpC* gene was amplified using primers BRT169 (CGG GAT CCG TAA TGT AGA GCG CAA CAC TT) and BRT171 (CGT ACG TAT ACT TCC TCC GTG TTT TCG TT) and cloned as a SnaBI/BamHI fragment in pGEN-*luxCDABE*.

Protein purification

Purification of DalS-6HIS was performed as described previously (148). Briefly *E. coli* BL21 (DE3) carrying pDalS-6HIS was grown in LB at 37 deg. C. and then induced with 0.5 mM IPTG at 22 deg. C. for an additional 3 h. Cells were lysed by sonication and soluble supernatant was loaded onto a Ni-NTA bead column (Qiagen) and eluted with 80 mM imidazole. Recombinant DAO was purified by growing *E. coli* BL21 (DE3) carrying pDAO-6HIS in LB at 37 deg. C. to an $A_{600\ nm} = 0.55$ and then induced with 0.1 mM IPTG and grown at 37 deg. C. for an additional 20 h. Cultures were centrifuged at 4000 *g* for 13 min at 12 deg. C., resuspended in lysis buffer (20 mM Tris, pH 7.5, 0.5 M NaCl, protease inhibitors), and lysed via sonication (Misonix Sonicator, Ultrasonic Processor S-400, at 40% amplitude with 6 pulses of 30 s in 1 min intervals). Lysates were pelleted at 10000 *g* for 30 min at 4 deg. C. Supernatant was loaded onto a Ni-NTA bead column and washed with an imidazole gradient (10, 20, 40 mM) in TBS (40 mM Tris, pH 7.5, 0.5 M NaCl). DAO-6HIS was eluted in TBS containing 80-320 mM imidazole and purity was determined using SDS-PAGE. Purified DAO was dialysed in TBS, concentrated using 3K Amicon Ultra Centrifugal filters (Millipore, UFC800324) and stored at -80 deg. C.

Kinetic assays *(i) DAO production of hydrogen peroxide.* Enzymatic activity of DAO was measured by colorimetric assay using peroxidase-coupled oxidation of *o*-dianisidine as previously described (1). Briefly DAO (5 µg/mL), 5 mM amino acid (D-alanine, D-serine, D-valine, L-alanine, Sigma), horseradish peroxidase (6.6 µg/mL, Sigma), and 6-Chloro-1,2-benzisoxazol-3(2H)-one (CBIO) (0, 0.15, 1.5, 15 µM, Sigma) were incubated at room

temperature in PBS containing *o*-dianisidine (75 µg/mL) and A_{460nm} was measured. *(ii) Estimation of D-alanine concentration*. *S.* Typhimurium was grown to mid-log phase in LPM medium, pH 5.8) (190) at 37 deg. C. Bacteria were washed twice in phosphate buffered saline (PBS) and 1 x 10^7 *S.* Typhimurium were incubated with 5 mM D-alanine for 3 h at 37 deg. C. in LPM. Cultures were pelleted at 10,000 *g* for 2 min, supernatant was filter sterilised, and incubated with DAO (5 µg/mL), horseradish peroxidase (6.6 µg/mL, Sigma) at room temperature in PBS containing 75 µg/mL *o*-dianisidine and A_{460nm} was measured after 1 h. D-alanine concentration was determined by comparison to a standard curve. *(iii) Fluorescent Thermal Shift (FTS) assay*. DalS binding was determined by FTS assay as described previously (13). Briefly, the assay was performed with a 480 Lightcycler (Roche) (498 nm excitation, 610 nm emission). Each reaction contained DalS-6HIS (10 mM), SyPro Orange (5 µM, Invitrogen) and indicated amino acid or CBIO in 100 mM HEPES, 150 mM NaCl, pH 7.5 in a 96-well plate (Roche). A ΔT_m of 2°C upon addition of ligand was considered positive binding.

Bactericidal activity assays with purified DAO

S. Typhimurium was grown to mid-log phase in LPM at 37 deg. C. and washed twice in PBS. 1 x 10^7 *S.* Typhimurium were incubated with 5 µg/mL DAO and 5 mM D-alanine in the presence or absence of 150 mM thiourea, 0.1% hydrogen peroxide, 0.01% HOCl, or 2.5 U/mL MPO in LPM at 37 deg. C. with shaking for 2 h. Cultures were serial diluted in PBS and plated on LB to determine killing activity. Colony forming units were determined and normalised to 0 h growth.

Isolation of mouse peritoneal neutrophils and bactericidal activity

Female 6-10 week old C57BL/6 mice (Charles River) were injected with 1 mL 2% Biogel (Biorad) in PBS. Neutrophils were harvested via peritoneal lavage 12-16 h later with 6 mL RPMI (10% FBS, 1 x HEPES, 1 x sodium pyruvate, 1 x β-mercaptoethanol, 1 x essential amino acids). Cells were passed through a 40 μm cell strainer to remove Biogel and purity was determined by Giemsa staining (Sigma). Approximately 2 x 10^7 neutrophils were obtained per mouse. Neutrophils were exposed to 3 μM CBIO in PBS or PBS alone at 37 deg. C., 5% CO_2 in RPMI for 60 min. Overnight cultures of wild type and Δ*dalS S.* Typhimurium with or without *ahpC-luxCDABE* were diluted in RPMI medium and added to neutrophils at a multiplicity of infection of 1:100 in tissue culture wells. Infected cells were incubated at 37 deg. C., 5% CO_2 in RPMI for 30 min to allow bacterial uptake. Infected cells were washed five times in PBS and incubated for an additional 90 min in RPMI or 30 min for *ahpC-luxCDABE* assays. Luminescence was measured in bacteria containing the *ahpC-lux* reporter. Bacteria were washed five times with PBS and lysed using in 250 μL of lysis buffer (1% Triton X-100, 0.1% SDS). Lysates were serial diluted and plated for cfu determination. Bacterial killing was determined as the ratio of cfu at 2 h/0.5h and normalised to survival of wild type bacteria.

Animal experiments

For *in vivo* experiments, C57BL/6 (Charles River) or B6.129S6-*CYBB*^{tm1Din}/J (Jackson Laboratory) mice were infected via the peritoneum with 2 x 10^5 *Salmonella* for competitive

experiments as described previously (191). At 24 h (single infections) or 48 – 60 h (competitive infections) post-infection mice were sacrificed by cervical dislocation and liver and spleen were harvested. Organs were homogenised in PBS with a Mixer Mill (10 min, 30 Hz) (Retsch), serial diluted in PBS, and plated on LB containing streptomycin. Colonies were replica plated onto LB agar containing either chloramphenicol or streptomycin to determine the ratio of wild type to mutant colonies. To induce neutropenia, mice were injected i.p. with 0.15 mg αLy6G clone 1A8 antibody (BioXcell) daily for two consecutive days prior to infection. To confirm neutropenia by flow cytometry, blood was mixed with ACD anticoagulant, and placed on ice. Cells were harvested by centrifugation at 5000 *g* for 10 min at 4°C. Red blood cells were eliminated with ACK lysis buffer. Cells were first Fc blocked with anti-mouse CD16/32 (1:100, eBiosciences), and then stained with anti-mouse CD3e-PE-CF594 (1:200, BD Biosciences), anti-mouse CD11b-PE (1:200, eBiosciences), and anti-mouse Gr-1 APC-eFluor 780 (1:200, eBiosciences) antibodies. All samples were run using an LSRII flow cytometer (BD Biosciences), and analyzed using FlowJo software (Tree Star, Inc.). For inhibition of DAO *in vivo*, mice were injected i.p. with 25 mg/kg CBIO in 0.5% carboxymethylcellulose (CMC) (Sigma) or 0.5% CMC only every 6 h during the course of infection.

Immunoblotting

Neutropenic or immune replete C57BL/6 mice were infected with 2 x 10^5 *S.* Typhimurium in 0.1 M HEPES pH 8.0, 0.9% NaCl. Spleen, liver, and cecum were isolated after 48 h and homogenised as described above in 50 μM Tris, 150 μM NaCl, protease inhibitor cocktail

(Roche), pH 7.5. Samples were centrifuged at 12,000 *g* for 20 min at 4 deg. C. Supernatants were diluted in an equal volume of SDS-PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 0.002% (w/v) bromophenol blue, and 100 mM DTT) and boiled for 5 min. 5 µL of each sample, including 5 µg/mL rDAO-HIS as a control, was separated on 12% polyacrylamide gels and blotted with goat anti-DAO (1:2000, Sigma) and sheep anti-goat IgG-HRP (1:5000, Abcam). Blots were stripped and re-probed using mouse anti-GAPDH (1:1000, Novus Biologicals) and goat anti-mouse IgG-HRP (1:10 000, Cedarlane). Conjugated HRP was detected using chemiluminescence (Western Lightning Plus, PerkinElmer).

Bioluminescent reporter assay

S. Typhimurium containing the P*ahpC-luxCDABE* reporter was grown to mid-log phase in 96-well plates. Bacteria were treated with hydrogen peroxide (0, 2, 4, 6, 8, 10, 100 µM) and $A_{600\ nm}$ and luminescence was measured every 10 min. Luminescence was normalized to the $A_{600\ nm}$.

***In vivo* bioluminescent imaging**

C57BL/6 or B6.129S6-*CYBB*^{tm1Din}/J mice were dosed with 25 mg/kg CBIO in 0.5% CMC or 0.5% CMC only and infected i.p. with 1 x 10^7 *S.* Typhimurium containing the *ahpC-luxCDABE* reporter 1 h post treatment. Mice were anesthetised (2% isofluorane carried in 2% oxygen) 2 h post-infection and imaged for 5 sec in a Spectrum *in vivo* Imaging System (IVIS) (Caliper Life Sciences). Spleens were isolated and imaged *ex vivo*. Tissues were

homogenised as described above, serial diluted, and plated on LB agar to obtain total cfu per organ. Total flux was normalised to tissue cfu.

Statistical analysis

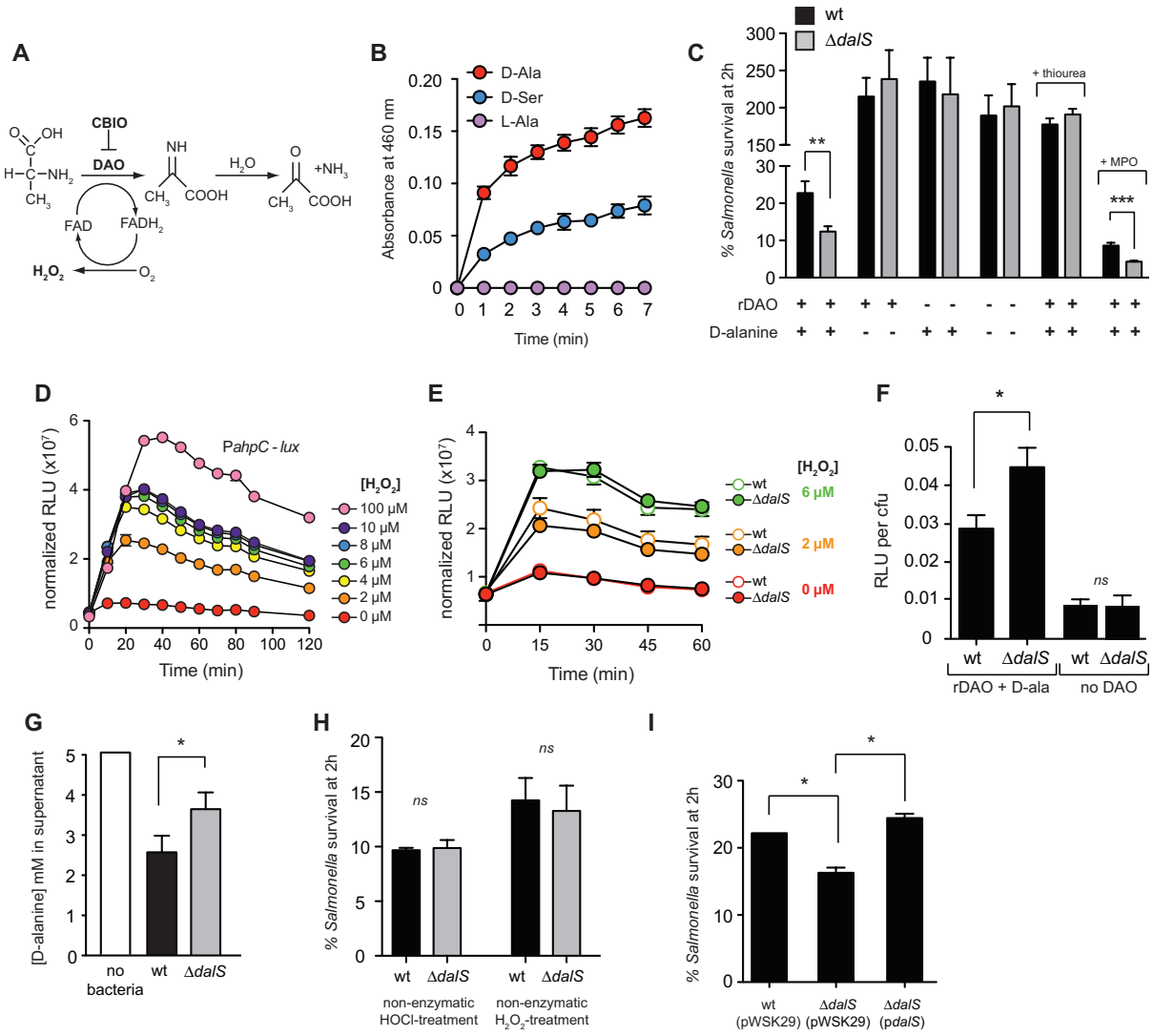
Treatment groups were compared using a non-parametric Mann-Whitney test. All analyses were performed using Graph Prism 4.0 (GraphPad Software Inc. San Diego, CA). A *P* value of .05 or less was considered significant.

ACKNOWLEDGMENTS

We are grateful to members of the Coombes lab for helpful discussion on this work, and Gerry Wright, Eric Brown, and Tim Gilberger for comments on the manuscript. This work was supported by the Canadian Institutes of Health Research (MOP 82704), the Canada Foundation for Innovation, and the Canada Research Chairs program (to B.K.C.). S.A.R. and B.R.T. are recipients of Ontario Graduate Scholarships. B.K.C. is the Canada Research Chair in Infectious Disease Pathogenesis.

Figure 2.1. DalS protects *S.* Typhimurium from the antimicrobial activity of DAO. **A**) DAO catalyzes the flavin-dependent deamination of D-alanine to yield the α-ketoacid, NH_4^+, and hydrogen peroxide. **B**) Recombinant DAO produces hydrogen peroxide upon addition of D-alanine and D-serine as measured by peroxidase-coupled oxidation of *o*-dianisidine. Data are the means with standard error for three experiments. **C**) *S.* Typhimurium *dalS* mutants are more susceptible to the reaction products of DAO. Bacteria were incubated with purified DAO in the presence (+) or absence (-) of exogenous D-alanine. Survival data are the means with standard error from three independent experiments. **D**) The OxyR-dependent P*ahpC-lux* reporter strain is sensitive to hydrogen peroxide. Data are mean relative light units (RLU) normalized to culture optical density. **E**) Wild type and *dalS* mutant *Salmonella* sense non-enzymatic hydrogen peroxide equally. Data are the means with standard error for three experiments. RLU data is normalized to the culture optical density. Reporter activity between wild type and *dalS* mutants for each of the treatment groups are not significantly different. **F**) A *dalS* mutant is exposed to greater hydrogen peroxide stress compared to wild type following exposure to DAO and D-alanine. Data is from strains carrying the P*ahpC-lux* reporter and are the means with standard error from three separate experiments. **G)** *S.* Typhimurium lacking DalS are defective for D-alanine import. The ability of *Salmonella* to remove D-alanine (5 mM) from the culture media was estimated by DAO-catalyzed peroxidase-coupled oxidation of *o*-dianisidine. **H**) Δ*dalS* mutants have wild type sensitivity to exogenous hydrogen peroxide (0.1%) and HOCl (0.01%) generated non-enzymatically. **I**) Sensitivity of *dalS* mutants to DAO-dependent killing is normalized to wild type levels upon complementation with a *dalS*-encoding plasmid (p*dalS*). All experiments were performed independently three times. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Mann-Whitney).

Figure 2.1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CBIO
DAO
FAD
FADH2
H2O2
O2
H2O
+NH3
B
D-Ala
D-Ser
L-Ala
Absorbance at 460 nm
Time (min)
C
wt
ΔdalS
% Salmonella survival at 2h
+ thiourea
+ MPO
rDAO
D-alanine
D
PahpC - lux
normalized RLU (x10⁷)
[H2O2]
100 µM
10 µM
8 µM
6 µM
4 µM
2 µM
0 µM
Time (min)
E
normalized RLU (x10⁷)
[H2O2]
6 µM
2 µM
0 µM
Time (min)
F
RLU per cfu
ns
rDAO + D-ala
no DAO
G
[D-alanine] mM in supernatant
no bacteria
H
% Salmonella survival at 2h
non-enzymatic HOCl-treatment
non-enzymatic H2O2-treatment
I
% Salmonella survival at 2h
(pWSK29)
(pdalS)

Figure 2.2. DalS-deficient *Salmonella* are killed more efficiently in neutrophils in a DAO-dependent manner. A) Δ*dalS S.* Typhimurium are sensitized to neutrophil killing. Bacterial killing by purified neutrophils was measured after 2 h. Survival was measured as the ratio of viable bacteria at 2 h compared to the starting internalised bacteria. The sensitivity of Δ*dalS* mutants to neutrophil killing was inhibited by DAO inhibition with CBIO. Data are from three experiments. **B**) CBIO inhibits DAO enzyme activity *in vitro* ([CBIO]: ●0 μM, ▼ 0.15 μM, ■ 1.5 μM, ▲ 15 μM). Data are the means with standard error from three experiments. **C**) *Salmonella* lacking DalS are exposed to greater hydrogen peroxide stress in neutrophils. Hydrogen peroxide stress levels are restored when DAO is inhibited with CBIO. Data is from strains carrying the P*ahpC-lux* reporter 30 min following infection of purified neutrophils and are the means with standard error from three separate experiments. * $P<0.05$ (Mann-Whitney)

Figure 2.2

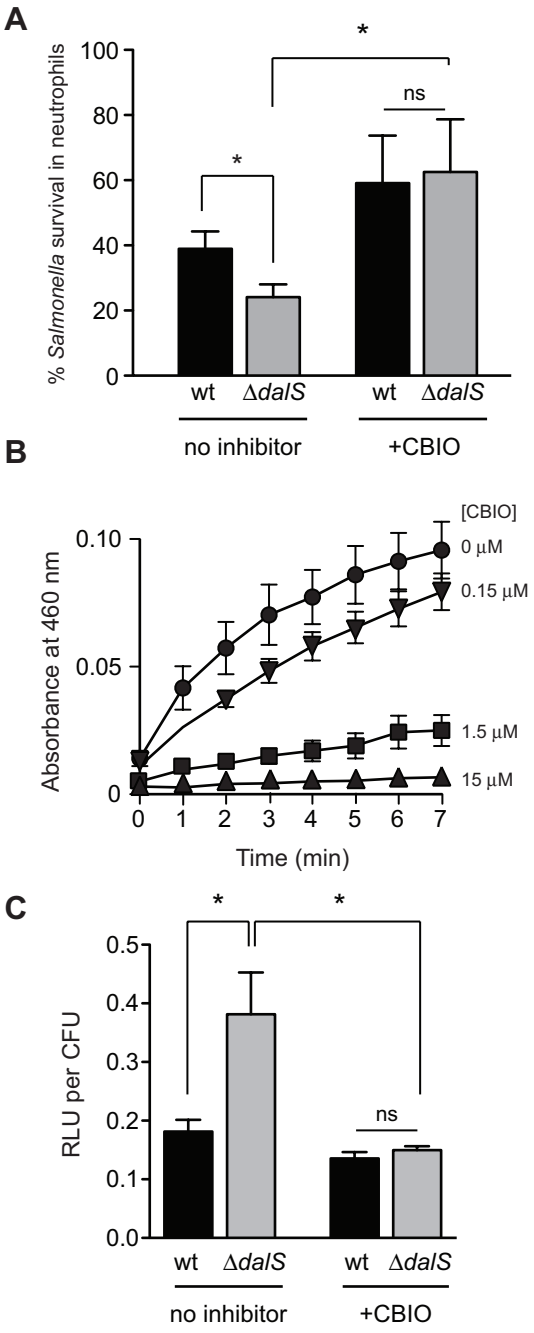

A
% Salmonella survival in neutrophils
100
80
60
40
20
0
*
*
ns
wt
ΔdalS
wt
ΔdalS
no inhibitor
+CBIO
B
Absorbance at 460 nm
0.10
0.05
0
0 1 2 3 4 5 6 7
Time (min)
[CBIO]
0 μM
0.15 μM
1.5 μM
15 μM
C
RLU per CFU
0.5
0.4
0.3
0.2
0.1
0.0
*
*
ns
wt
ΔdalS
wt
ΔdalS
no inhibitor
+CBIO

Figure 2.3. *S.* Typhimurium lacking DalS are more susceptible to neutrophil DAO killing *in vivo.* **A)** Western blot of DAO in liver, spleen, and cecum from uninfected or *S.* Typhimurium –infected mice. The relative density of the DAO signal adjusted to GAPDH signal is shown below each panel. **B)** Neutropenic mice fail to accumulate splenic DAO following *S.* Typhimurium infection. Neutropenic mice were infected with *S.* Typhimurium and organs were probed for DAO (2x spleen vol., represents twice the amount of sample loaded). **C)** Neutropenia normalizes the fitness defect of Δ*dalS S.* Typhimurium. Control mice and neutropenic mice were infected with an equal mixture of wild type and Δ*dalS S.* Typhimurium for 2 days. Shown is the competitive index data from two experiments. **D**) Inhibition of DAO *in vivo* restores virulence to Δ*dalS* mutants. Mice were infected with an equal mixture of wild type and Δ*dalS S.* Typhimurium (first set) or an equal mixture of wild type and Δ*SPI-2 S.* Typhimurium (second set) and treated with either carboxymethylcellulose as a control or the DAO inhibitor CBIO. Shown is the competitive index data from two experiments. **E)** CBIO restores *dalS* mutant defect in singly infected mice. Mice were infected with either wild type or *dalS* deficient *S.* Typhimurium and treated with CBIO or CMC control for 24 hr. Shown is the cfu normalized to spleen mass. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (Mann-Whitney).

Figure 2.3

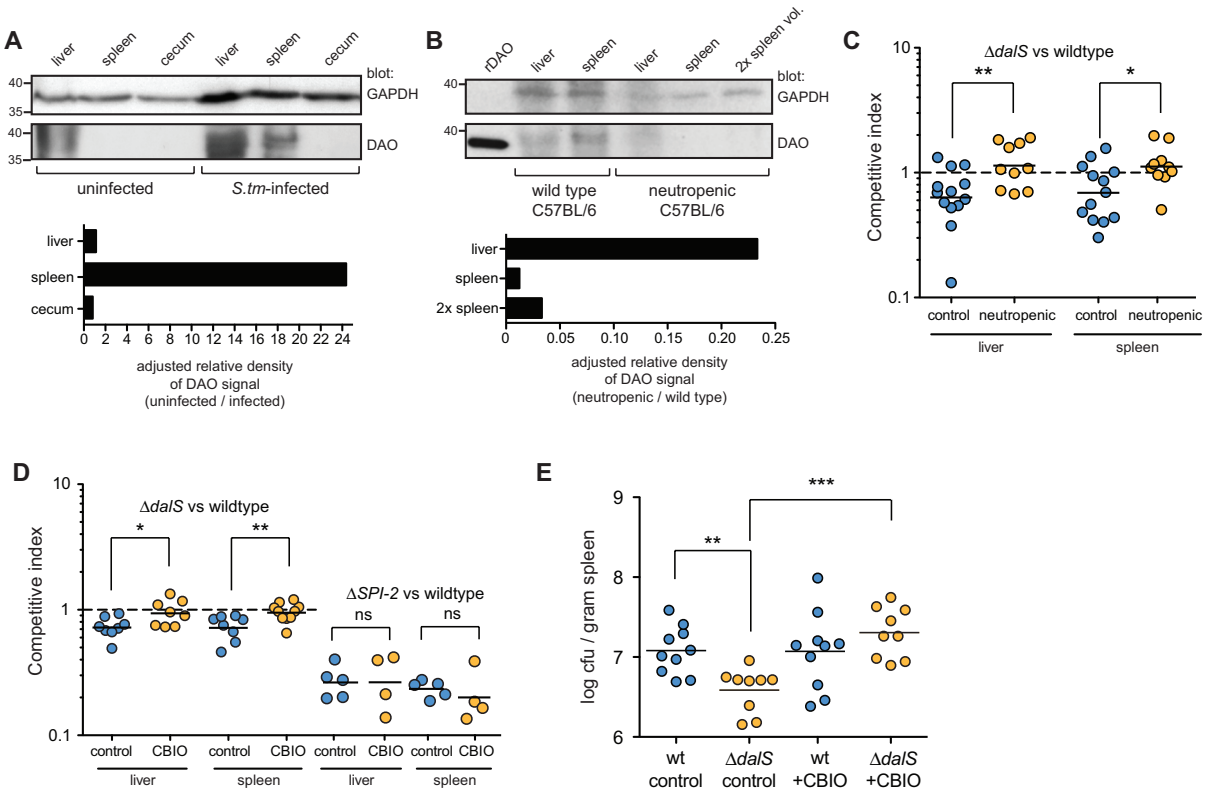

A
liver
spleen
cecum
liver
spleen
cecum
blot:
GAPDH
DAO
uninfected
S.tm-infected
liver
spleen
cecum
adjusted relative density
of DAO signal
(uninfected / infected)
B
rDAO
liver
spleen
liver
spleen
2x spleen vol.
blot:
GAPDH
DAO
wild type
C57BL/6
neutropenic
C57BL/6
liver
spleen
2x spleen
adjusted relative density
of DAO signal
(neutropenic / wild type)
C
ΔdalS vs wildtype
Competitive index
control
neutropenic
control
neutropenic
liver
spleen
D
ΔdalS vs wildtype
ΔSPI-2 vs wildtype
ns
ns
Competitive index
control
CBIO
liver
spleen
E
log cfu / gram spleen
wt
control
ΔdalS
control
wt
+CBIO
ΔdalS
+CBIO

Figure 2.4. DAO contributes to bacterial peroxide stress independent of NOX2. A) DalS mutants remain attenuated in mice lacking functional NOX2/NADPH oxidase. Wild type mice (B6) or *CYBB* $^{-/-}$ mice were infected with an equal mixture of wild type and Δ*dalS S.* Typhimurium and the competitive index was calculated after 2 days. **B)** DalS mutant defect is restored with CBIO treatment in NADPH oxidase deficient mice. *CYBB-/-* mice were infected with and equal mixture of wild type and Δ*dalS S.* Typhimurium and were treated with control or CBIO. Competitive index was calculated after 2 days. **C)** DAO elicits peroxide stress in mice. Wild type and *CYBB* $^{-/-}$ mice with functional (control) or inhibited DAO (CBIO) were infected with the peroxide-reporter strain and imaged after 2 h. Images represent 5 sec integrations and are representative of three separate experiments. **D)** *Ex vivo* imaging of excised spleens from mice in (*B).* Images (inset) were integrated over 5 sec and plotted as total photon flux was normalized to viable colony counts. **E)** DalS mutants are exposed to greater peroxide stress than wild type *in vivo. CYBB*$^{-/-}$ mice were infected with wild type *S.* Typhimurium or Δ*dalS* mutants containing the OxyR-dependent luminescent reporter, and then treated with the DAO inhibitor CBIO, or carboxymethylcellulose (control). Shown are representative whole body images from infected mice with normalized luminescence flux plotted above each image. Plotted data represent three mice per group. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ (Mann-Whitney).

Figure 2.4

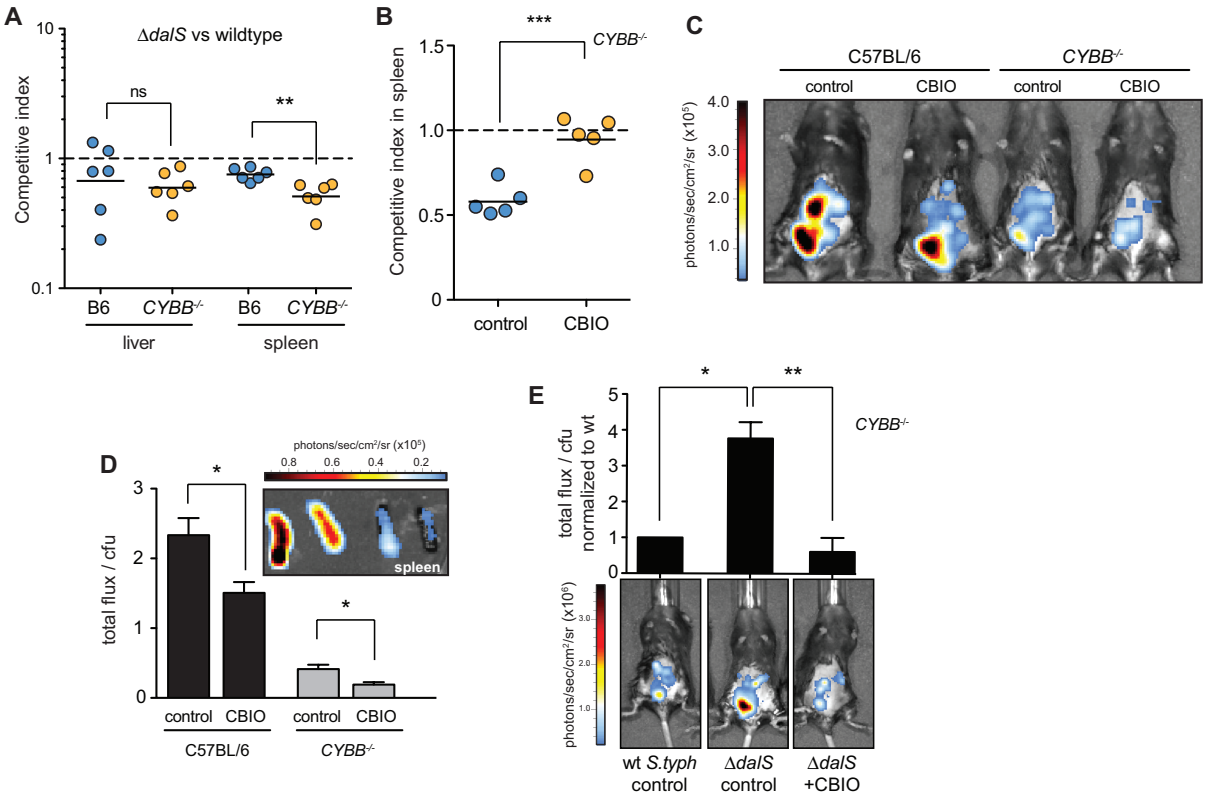

A
ΔdalS vs wildtype
Competitive index
ns
**
B6
CYBB-/-
liver
spleen
B
***
CYBB-/-
Competitive index in spleen
control
CBIO
C
C57BL/6
CYBB-/-
control
CBIO
photons/sec/cm²/sr (x10⁵)
D
photons/sec/cm²/sr (x10⁵)
spleen
total flux / cfu
C57BL/6
CYBB-/-
E
total flux / cfu normalized to wt
CYBB-/-
wt S.typh control
ΔdalS control
ΔdalS +CBIO

Figure 2.S1. Recombinant DAO produces hydrogen peroxide upon addition of D-alanine. DAO dependent oxidation of 0 – 20 mM D-alanine as measured by peroxidase-coupled oxidation of *o*-dianisidine. Data are the means with standard error for three experiments.

Figure 2.S1

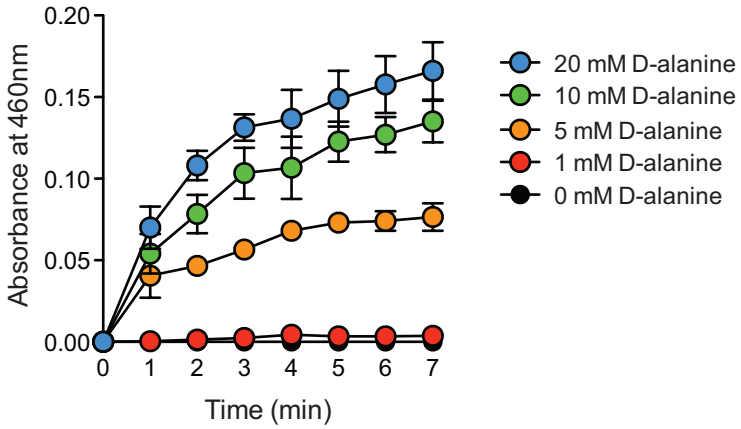

0.20
0.15
0.10
0.05
0.00
Absorbance at 460nm
0
1
2
3
4
5
6
7
Time (min)
20 mM D-alanine
10 mM D-alanine
5 mM D-alanine
1 mM D-alanine
0 mM D-alanine

Figure 2.S2. The DAO inhibitor CBIO does not bind to DalS or impair bacterial growth. (A). Fluorescence thermal shift experiments were conducted with purified DalS without ligand, and in the presence of D-alanine, D-valine, or CBIO as described in Methods. Data are the means with standard errors from three separate experiments. **(B).** Wild type *S.* Typhimurium and *dalS* mutants were grown in the presence or absence of CBIO in the culture medium. Optical density was measured over time. Data are the means with standard errors from three separate experiments.

Figure 2.S2

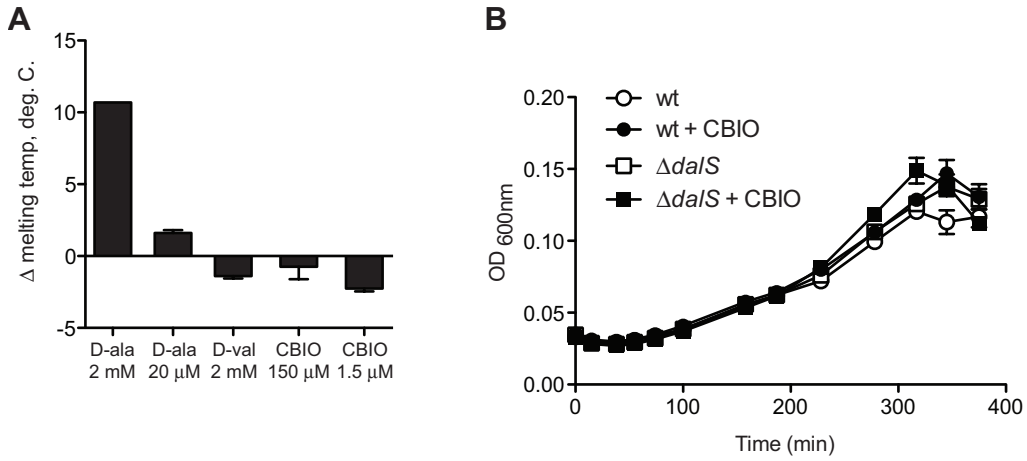
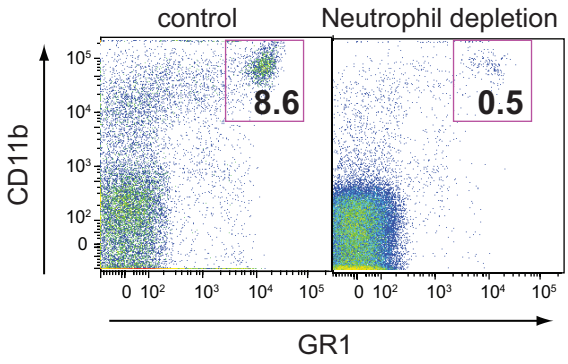

A
Δ melting temp, deg. C.
15
10
5
0
-5
D-ala 2 mM
D-ala 20 μM
D-val 2 mM
CBIO 150 μM
CBIO 1.5 μM
B
OD 600nm
0.20
0.15
0.10
0.05
0.00
wt
wt + CBIO
ΔdalS
ΔdalS + CBIO
0
100
200
300
400
Time (min)

Figure 2.S3. Flow cytometry verifying neutrophil depletion in neutropenic animals.

Neutrophil gate was defined as CD3-CD11b+Gr-1hi.

Figure 2.S3


control
Neutrophil depletion
8.6
0.5
CD11b
GR1
0
10²
10³
10⁴
10⁵

CHAPTER THREE

Uropathogenic *E. coli* evades D-amino acid oxidase in kidneys

CO-AUTHORSHIP STATEMENT

At the time of this thesis publication this manuscript was intended to be submitted in March 2017.

The following individuals are intended to be co-authors on this manuscript: Brian R. Tuinema, Bushra Ilyas, and Brian K. Coombes

The contributions by each author to the project are described below:

1. Strains and plasmids were created by B.R.T.

2. *In vitro* DAO assays were performed by B.R.T.

3. Neutrophil experiments were conducted by B.R.T.

4. Competitive infections, kidney isolation, and *in vivo* imaging was conducted by B.R.T.

5. RNA isolation and qRT-PCR was performed by B.I.

6. B.R.T., B.I., and B.K.C. contributed to the writing of the manuscript

*Note that references in the following data chapters have been compiled into one reference list at the end of the thesis in order to avoid any redundancy between sections.

Uropathogenic *E. coli* evades D-amino acid oxidase in kidneys

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ABSTRACT

Uropathogenic *E. coli* (UPEC) is associated with over 80% of all urinary tract infections (UTI). In order to colonize the bladder and further ascend to the kidneys UPEC requires an arsenal of virulence factors to evade the innate immune system. D-amino acid oxidase (DAO) forms one arm of the innate immune system and produces reactive oxygen species (ROS) through the oxidation of D-serine in the kidneys. Here we show that UPEC uses two D-serine transporters to sequester D-amino acids away from DAO thereby limiting exposure to ROS in the kidneys. A UPEC Δ*cycA*Δ*dalS* mutant defective for D-serine import was more susceptible to killing by DAO through exposure to greater oxidative stress during infection. This fitness defect was partially restored by depletion of neutrophils, or by inhibition of DAO *in vivo* with a small molecule inhibitor. Clinical isolates from patients experiencing recurrent UTIs had elevated levels of *cycA* expression when isolated from infected *ex vivo* kidneys relative to isolates from acute infections. This work demonstrates the second example of DAO subversion through transport of D-amino acids, suggesting a possible conserved virulence strategy among many human relevant pathogens.

INTRODUCTION

D-amino acid oxidase (DAO) is an emerging player in host control of microbes at mucosal surfaces. DAO catalyzes the oxidative deamination of D-amino acids to their respective α-keto acid and ammonia, resulting in hydrogen peroxide as a bi-product. Recently DAO has been implicated in bacterial control during acute infections of *Staphylococcus aureus* (114), *Salmonella* Typhimurium (112), and *Vibrio cholera* (104). DAO is expressed in the brain, neutrophils, gut epithelial, and proximal tubule epithelial cells in the kidney (104, 113, 115, 192). The role of DAO in the brain is most well-characterized where it degrades the neurotransmitter D-serine. DAO has also been proposed to remove circulating D-serine within the kidneys however the exact purpose of this is unclear. Expressed mainly in kidney tubule peroxisomes, DAO found in urine suggests that it may also be excreted from these tubule cells (117).

Previously we identified an ABC transporter, DalSTUV, that sequesters D-alanine, thereby limiting this substrate from neutrophil DAO. In doing so this prevents the production of reactive oxygen species (112, 148). Due to the range of hosts and tissues that express DAO we predicted that other bacteria might employ a similar virulence strategy. Since DalSTUV is exclusively found in *Salmonella* we searched for other D-amino acid transporters capable of protecting bacteria from DAO. CycA is an D-amino acid permease and unlike DalS it is conserved across diverse taxa. Mutants in *cycA* are impaired in D-alanine and D-serine transport but were unaffected in import of the L-enantiomers (193). *E. coli* lacking *cycA* are attenuated in mice suggesting a potential role for virulence.

Uropathogenic *Escherichia coli* (UPEC) is the causative agent in over 80% of urinary tract infections (20). Upon infection UPEC uses pili to adhere to bladder epithelial cells (56). If gone untreated UPEC can ascend the ureters and colonise the proximal tubules in the kidney, establishing a secondary infection called acute pyelonephritis (57). UPEC can further breach this epithelial layer where they can be phagocytosed by infiltrating neutrophils (194). To survive within the host UPEC uses an arsenal of virulence and fitness factors (195). Among these, two D-serine transporters, DsdX and CycA have been shown to be important fitness factors in an ascending UTI model (138, 196). Previous work has demonstrated that these transporters are required for survival within mice, indicating a need for D-serine import (138). UPEC lacking *cycA, dsdX*, and a D-serine deaminase, *dsdA*, are significantly attenuated in the kidneys but not the lower urinary tract (147), a phenotype that is consistent with the expression patterns of DAO in the urinary tract. Using a murine renal infection model we demonstrate that CycA and DsdX protect UPEC from DAO. Mutants lacking both *cycA* and *dsdX* are exposed to elevated ROS *in vivo* which results in a survival defect compared to wild type. This phenotype is partially restored by chemically inhibiting DAO or by rendering mice neutropenic suggesting that both kidney and neutrophil expressed DAO play a role in UPEC clearance. Clinical isolates of UPEC from patients with recurrent UTI expressed higher levels of *cycA* and *dsdX* mRNA compared to those isolated from patients experiencing an acute infection when grown in sterile urine or isolated from infected kidneys compared to isolates grown in LB. Together this work demonstrates that *cycA* and *dsdX* serve to protect UPEC from reactive oxygen stress during pyelonephritis.

RESULTS

CycA and DsdX protect UPEC CFT073 from DAO

To study the antimicrobial activity of DAO on UPEC CFT073 we isolated recombinant DAO and tested its ROS producing ability in the presence of D-serine (5 mM, 10 mM, 25 mM, and 50 mM) and compared it to D-alanine (5 mM). An increased concentration of D-serine (50 mM) was required to match the ROS output produced by 5 mM as the V_{max} of D-serine was approximately 10% of D-alanine (Figure 3.1A). We next tested whether DAO was capable of killing UPEC using D-serine as a substrate. Testing three concentrations of D-serine we found that both 50 mM and 100 mM D-serine was sufficient to kill UPEC by approximately 50% and 75% respectively however 10 mM failed to limit UPEC survival (Figure 3.1B). Interestingly we found that in both the 50 mM and 100 mM groups a *cycA dsdX* double mutant was significantly sensitized compared to wildtype (Figure 3.1B). Since CycA is capable of transporting D-alanine we also tested UPEC survival in the presence D-alanine as a DAO substrate. We found that both a *cycA* and a *cycA dsdX* double mutant were more susceptible to DAO killing compared to wildtype (Figure 3.1C). To confirm that this susceptibility was due to an inability to remove substrate from the reaction we incubated UPEC with 50 mM D-serine (Figure 3.1D) or 5 mM D-alanine (Figure 3.1E) and measured the remaining D-amino acid concentration in the supernatant (112). Mutations in either *cycA* or *dsdX* did not result in D-serine transport defects, however mutants lacking *cycA* were able to take up D-alanine from the supernatant than wildtype. Mutations in both *cycA* and *dsdX* resulted in a decrease in D-serine and D-alanine transport.

Together this data indicates that UPEC CFT073 requires both *cycA* and *dsdX* to protect against DAO *in vitro*.

D-amino acid transporters protect UPEC from DAO-dependent killing

The *in vitro* killing data suggested that the increased DAO-dependent killing of Δ*cycA* and Δ*dsdX* mutants was due their inability to sequester DAO substrate from the enzyme, which would lead to an excess production of ROS. To test this, we generated UPEC strains that report on ROS stress through OxyR-dependent expression of luciferase. To ensure that the CFT073 transporter mutants were able to sense ROS generated non-enzymatically, we added exogenous hydrogen peroxide to cultures and measured luminescence, indicating that both wild type cells and the transporter mutants sensed external hydrogen peroxide equally. (Figure 3.2A). Next we tested whether the increased DAO-dependent killing was linked to increased ROS exposure. When exposed to DAO and exogenous D-alanine, the Δ*cycA* and Δ*cycA* Δ*dsdX* double mutant had ~ 6.5 and 9.4-fold increase in luminescence compared to wildtype UPEC. When D-serine was used as the DAO substrate, luminescence was significantly increased in both the Δ*dsdX* and Δ*cycA* Δ*dsdX* double mutant (Figure 3.2B). UTI are accompanied by neutrophil influx into the infected site (194) and could be a major source of DAO for antibacterial responses. To test whether DAO conferred a neutrophil effector function toward UPEC, bacteria were exposed to primary isolated peritoneal neutrophils in the presence or absence of the DAO inhibitor, CBIO. Neutrophil killing was measure 90 min post infection. A *cycA* and *cycA-dsdX* double mutant were sensitized to neutrophil killing compared to wildtype UPEC (Figure 3.2C). Exposure to ROS in both a *cycA* mutant and a *cycA dsdX* double mutant was significantly higher in the

presence of neutrophils, this phenotype was restored in the presence of CBIO (Figure 3.2D). These data suggest that CycA and DsdX protect UPEC from ROS produced by DAO *in vitro* and neutrophils.

CycA and DsdX protect against DAO produced hydrogen peroxide during urinary tract infections

To characterise the contribution CycA and DsdX have on UPEC viability *in vivo* we competed each of the D-amino acid transporter mutants against wildtype UPEC in a murine UTI model. In uncontrived CBA/J mice a *cycA* deletion mutant was attenuated for fitness however the single *dsdX* mutant was not. Interestingly the *cycA* fitness defect was exacerbated upon deletion of both *cycA* and *dsdX* (Figure 3.3A). This phenotype could be partially reverted by rendering mice neutropenic with an α-Ly6G antibody or by inhibiting DAO *in vivo* with CBIO (Figure 3.3A). To link this phenotype with DAO-generated ROS, we infected mice with UPEC strains harbouring the P*ahpC-lux* reporter and found that *cycA* and *dsdX* mutants were exposed to higher ROS levels *in vivo* (Figure 3.3B), which could be normalized upon DAO inhibition (Figure 3.3D). ROS exposure was quantified *ex vivo* in infected kidneys treated with CMC or CBIO (Figure 3.3C and 3.3E respectively). Kidneys infected with transporter mutants showed elevated ROS exposure, however in the prescence of DAO this ROS exposure decreased. Together these data suggest that *cycA* and *dsdX* play a role in protection against DAO produced ROS in the kidney.

UPEC isolated from recurrent UTI express higher levels *cycA* and *dsdX* compared to acute infections

Twenty five percent of women suffering from an acute UTI can have a recurrent episode of infection within 6 months of the initial infection. Up to 68% of recurring UTIs are caused by *E. coli* that are genetically identical to the original infecting strain (197). We tested whether *cycA* and *dsdX* were upregulated in *E. coli* isolated from patients suffering from recurrent UTIs compared to strains from acute infections. Clinical isolates were collected from 5 patients presenting with acute UTIs and 5 with recurrent infections (two incidences within one year). These strains were grown in LB and freshly collected sterile urine with RNA levels being measured thereafter. Strains isolated from recurrent UTIs displayed elevated expression of both *cycA* and *dsdX* in urine compared to LB (Figure 3.4A). We next measured gene expression of *cycA* and *dsdX* in an ascending UTI model of infection. The expression of these genes isolated from *ex vivo* kidneys relative to LB was increased in recurrent isolates (Figure 3.4B).

DISCUSSION

An increasing number of studies have demonstrated that DAO acts as a source of antimicrobial ROS that serves to protect mucosal surfaces from bacterial pathogens (104, 112, 114). While previous work has been limited to the gut and neutrophils, there is no data on whether this innate pathway protects the urinary tract. DAO is expressed in the kidneys, suggesting that its role in host protection may be more broadly distributed. DAO was originally suggested to remove excess D-serine from the body (176), however D-serine is non-toxic to mammals and only becomes damaging upon oxidation by DAO (121). This supports an alternative role for D-serine oxidation by kidney DAO as an innate immune factor. As pyelonephritis worsens UPEC can breach the kidney epithelium and become exposed to neutrophils resulting in potential exposure to DAO in the kidney and neutrophils.

The transporters DsdX and CycA are essential for the uptake of D-serine and CycA is additionally able to import D-alanine (138). Mutants lacking either transporter are able to survive on a sole nitrogen and carbon source of D-serine however a double mutant fails to grow (138). Our competitive infection data showed that a *dsdX* mutant was as fit as wildtype in an ascending infection model of UTI, whereas a *cycA* mutant was attenuated for survival. This may suggest that CycA performs a non-redundant role to DsdX, perhaps in the sequestration of D-alanine in a similar manner to the D-alanine transporter, DalS, in *Salmonella*. Compared to control mice, a *cycA* mutant survived better in mice that were neutropenic or in which DAO was inhibited, suggesting that both neutrophil and perhaps kidney-derived DAO are involved in host protection in this model. Only UPEC strains with disruptions in the *cycA* gene were sensitized to neutrophil killing *in vitro,* further suggesting

a role in neutrophil survival possibly due to its ability to transport D-alanine similar to that of DalS. Interestingly, a double transporter mutant is further attenuated for survival during a murine infection, potentially due to a complete inability to transport D-serine. During a *Salmonella* infection model chemically inhibiting DAO with CBIO restored a *dalS* mutant survival defect completely, however in our ascending UTI model we failed to completely restore the transporter mutant defect. This survival phenotype is most likely a result of increased exposure to DAO produced ROS as shown by UPEC strains capable of sensing small changes in ROS stress, a phenotype that was altered by CBIO treatment.

Previously *dsdX* and *cycA* genes were shown to be expressed under different physiological conditions. Expression of *cycA* is controlled by the nitrogen-scavenging system (Nac) under low nitrogen (198, 199) and the *gcvB* small RNA that is responsive to glycine concentrations (143), whereas *dsdX* expression is controlled by DsdC and requires D-serine for transcriptional activity (200). Previous work (201) and this paper have demonstrated that both *dsdX* and *cycA* are up regulated when UPEC is exposed to urine or isolated from UTI patients. In a murine UTI model UPEC can reach the kidneys as early as 2 hours post infection (202). This activation in urine may act as a priming mechanism to rapidly increase expression of D-serine importers prior to colonization of the kidneys. UPEC isolates from patients with recurrent UTIs expressed higher levels of *cycA* and *dsdX* compared to isolates from single acute. Recurrent UTIs have been demonstrated, in many cases, to stem from quiescent intracellular reservoirs of UPEC found within the renal epithelium, suggesting that such strains may have additional mechanisms to persist in the face of host defenses. The upregulation of D-amino acid transporters that protect the bacteria from host-mediated killing may be one mechanism underlying this ability to persist.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care using protocols approved by the Animal Review Ethics Board at McMaster University. All experiments using human samples were conducted according to the guidelines set by the Hamilton Integrated Research Ethics Board.

Bacteria, cloning, and deletion mutants

Bacterial strains are isogenic derivatives of uropathogenic *Escherichia coli* stain CFT073 or human clinical isolates that were obtained from Harry Mobley (University of Michigan). All strains are listed in Table 3.1. The OxyR-dependent *ahpC-lux* transcriptional reporter was generated as described previously with slight modifications (112). Three-hundred bp upstream of the UPEC CFT073 *ahpC* gene was amplified using primers BRT192 and BRT193 and cloned as a SnaBI/BamHI fragment in pGEN-*luxCDABE*. Deletion mutants were generated using the Lambda Red recombinase system (203). Chloramphenicol resistance cassettes were amplified from pKD3 with either BRT184 and BRT185 to create the *cycA* deletion construct, or BRT186 and BRT187 to create the *dsdX* deletion construct. DNA fragments were electroporated into UPEC CFT073 containing the pKD46 plasmid. Colonies were screened by colony PCR. Resistance cassettes were removed using the FLR recombinase encoded on pCP20 for *in vitro* assays but were untouched for *in vivo* competitive infections.

Protein purification

Purification of DAO-6HIS was performed as described previously (112). Recombinant DAO was purified by growing *E. coli* BL21 (DE3) carrying pDAO-6HIS in LB at 37 deg. C. and inducing in mid-log phase with 0.1 mM IPTG for 20 h at 37 deg. C. Cultures were centrifuged at 4000 *g* for 13 min at 12 deg. C., resuspended in lysis buffer (20 mM Tris, pH 7.5, 0.5 M NaCl, protease inhibitors), and lysed via sonication. Lysates were centrifuged at 10000 *g* for 30 min at 4 deg. C. Supernatant was loaded onto a Ni-NTA bead column and washed with an imidazole gradient (10, 20, 40 mM) in TBS (40 mM Tris, pH 7.5, 0.5 M NaCl). DAO-6HIS was eluted in TBS containing 80-320 mM imidazole and purity was determined using SDS-PAGE. Purified DAO was buffer exchanged and concentrated in TBS using 3K Amicon Ultra Centrifugal filters (Millipore, UFC800324) and stored at -80 deg. C.

Enzyme assays

(i) DAO production of hydrogen peroxide. Enzymatic activity of DAO was measured by a colorimetric assay using peroxidase-coupled oxidation of *o*-dianisidine as previously described (112). Briefly DAO (5 µg/mL), D-alanine (5 mM) or D-serine (5, 10, 25, or 50 mM), and horseradish peroxidase (6.6 µg/mL, Sigma) were incubated at room temperature in PBS containing *o*-dianisidine (75 µg/mL). After 60 min, A_{460nm} was measured. *(ii) Estimation of D-serine concentration*. CFT073 was grown to mid-log phase in LB medium at 37 deg. C. Bacteria were washed twice in (PBS) and 1 x 10^7 UPEC were incubated with 50 mM D-serine for 3 h at 37 deg. C. in PBS. Cultures were pelleted at 10,000 *g* for 2 min, supernatant was filter sterilised, and incubated with DAO (5 µg/mL), horseradish peroxidase

(6.6 µg/mL, Sigma) at room temperature in PBS containing 75 µg/mL *o*-dianisidine and A_{460nm} was measured after 1 h.

Bactericidal activity assays with purified DAO

UPEC was grown to mid-log phase in LB at 37 deg. C. and washed twice in PBS. 1 x 10^7 UPEC were incubated with 5 µg/mL DAO and D-alanine (5 mM) or D-serine (5, 10, 25, or 50 mM) in PBS at 37 deg. C. with shaking for 2 h. Cultures were serial diluted in PBS and plated on LB to measure the remaining viable cfu. Colony forming units were determined and normalised to cultures grown in buffer.

Isolation of mouse peritoneal neutrophils and bactericidal activity

Female 6-10 week old CBA/J mice (Jackson Laboratory) were injected via the peritoneum with 1 mL 2% Biogel (Biorad) in PBS. Neutrophils were harvested via peritoneal lavage 12-16 h later with 6 mL RPMI (10% FBS, 1 x HEPES, 1 x sodium pyruvate, 1 x β-mercaptoethanol, 1 x essential amino acids). Cells were passed through a 40 µm cell strainer to remove Biogel and purity was determined by Giemsa staining (Sigma). Neutrophils were treated with 3 µM CBIO in 0.5% CMC to inactivate DAO, or with CMC alone at 37 deg. C., 5% CO_2 in RPMI for 60 min. Overnight cultures of CTF073 strains with *ahpC-luxCDABE* were diluted in RPMI medium and added to neutrophils at a multiplicity of infection of 100:1 in 96-well tissue culture plates. Infected cells were incubated at 37 deg. C., 5% CO_2 in RPMI for 30 min to allow bacterial uptake. Infected cells were washed five times in PBS and incubated for an additional 30 min for *ahpC-luxCDABE* assays. Luminescence was measured

in bacteria containing the *ahpC-lux* reporter. Neutrophils were washed five times with PBS and lysed using in 250 µL of lysis buffer (1% Triton X-100, 0.1% SDS). Lysates were serial diluted and plated for cfu determination. Luminescence was normalised to cfu.

Animal experiments

For *in vivo* experiments, anesthetised CBA/J mice (Jackson Laboratory) were infected via the urethra with 1 x 10^8 UPEC described previously (204). At 96 hrs post-infection mice were sacrificed by cervical dislocation and kidneys were harvested. Organs were homogenised in PBS with a Mixer Mill (15 min, 30 Hz) (Retsch), serial diluted in PBS, and plated on LB. Colonies were replica plated onto LB agar containing either chloramphenicol or LB alone to determine the ratio of wild type to mutant colonies. To induce neutropenia, mice were injected i.p. with 0.15 mg αLy6G clone 1A8 antibody (BioXcell) for two consecutive days prior to infection. For inhibition of DAO *in vivo*, mice were injected i.p. with 25 mg/kg CBIO in 0.5% carboxymethylcellulose (CMC) (Sigma) or 0.5% CMC every 8 h during the course of infection.

Bioluminescent reporter assay

UPEC CFT073 wild type and transporter mutants containing the P*ahpC-luxCDABE* reporter were grown to mid-log phase in 96-well plates. Bacteria were treated with hydrogen peroxide (1, 10, 100 µM) and $A_{600\ nm}$ and luminescence was measured every 10 min for 60 min. Luminescence was normalized to the $A_{600\ nm}$.

In vivo bioluminescent imaging

CBA/J mice were dosed with 25 mg/kg CBIO in 0.5% CMC or 0.5% CMC control and infected via the urethra with 1 x 10^8 UPEC containing the *ahpC-luxCDABE* reporter 1 h post treatment. Mice were anesthetised (2% isofluorane carried in 2% oxygen) 4 h after infection and imaged for 5 min in a Spectrum *in vivo* Imaging System (IVIS) (Caliper Life Sciences). Kidneys were isolated, diced, and imaged *ex vivo*. Tissues were homogenised as described above, serial diluted, and plated on LB agar to obtain total cfu per organ. Total flux was normalised to tissue cfu.

RNA isolation

Clinical UPEC isolates were grown in 10 mL of LB or freshly collected urine for 3 hrs at 37°C with shaking. Cell pellets were lysed in 2 ml TRIzol reagent (Life Technologies) and incubated at room temperature for five minutes, or frozen at -20 deg. C. for later processing. Phase separation was done by phenol-chloroform extraction, following the manufacturer's instructions. Two-hundred μL of chloroform (Biobasic) was vigorously mixed with 1 mL of TRIzol, followed by centrifugation at 12000 *g* for 15 minutes at 4 deg. C. The aqueous phase was collected and RNA was isolated by isopropanol precipitation using 500 μL of 100% isopropanol (Biobasic) per 1 mL of TRIzol reagent. After a 10 minute incubation at room temperature, RNA was pelleted by centrifugation at 4 deg. C. for 15 minutes at 12000 *g*. RNA was then washed with 75% ethanol and centrifuged once more under the same conditions. The resulting pellet was allowed to air dry for 5-10 minutes to remove any remaining ethanol, and resuspended in 100 μL of RNase free water (Ambion). RNA was treated with DNase I (Ambion) for one hour at 37 deg. C., followed by DNase inactivation.

The RNA was then mixed with isopropanol and incubated at -20 deg. C. for thirty minutes or overnight. RNA was pelleted by centrifugation at 4 deg. C. for 15 minutes at 12000 *g*, washed once with 75% ethanol, re pelleted, and resuspended in water.

cDNA and qRT-PCR

Prior to cDNA synthesis, RNA from all isolates was adjusted to identical concentrations. Six-hundred ng total RNA from each sample was used for reverse transcription using the Quanta Biosciences qScript cDNA supermix. Controls reactions omitted reverse transcriptase. First strand cDNA was diluted 1:10 prior to quantitative PCR, carried out in 96-well format using a Light Cycler 480 (Roche) with SYBR Green (PerfeCTa SYBR green supermix, Quanta Biosciences). Primers BRT 194 and BRT 195 were used to amplify *cycA*, BRT 196 and BRT 197 for *dsdX* and BRT 198 and BRT 199 were used for *rsmC*. Cycle threshold (Ct) values were used to calculate RNA concentrations based on a genomic standard curve for each primer pair. RNA concentrations for *cycA* and *dsdX* were normalized to the housekeeping gene *rsmC*, and normalized to levels in LB. Each experiment used two technical replicates, and was repeated three times.

Statistical analysis

Treatment groups were compared using a non-parametric Mann-Whitney test. All analyses were performed using Graph Prism 4.0 (GraphPad Software Inc. San Diego, CA). A *P* value of .05 or less was considered significant.

ACKNOWLEDGEMENTS

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Figure 3.1. CycA and DsdX protect Uropathogenic *E. coli* CFT073 by sequestering D-amino acids. **A)** Recombinant DAO produces hydrogen peroxide upon addition of D-alanine and D-serine as measured by peroxidase-coupled oxidation of *o*-dianisidine. CFT073 *cycAdsdX* double mutant is more susceptible to the reaction products of DAO. Bacteria were incubated with purified DAO in the presence of D-serine (10, 50, 100 mM) **(B)** or D-alanine (5 mM) **(C)**. CFT073 lacking both *cycA* and *dsdX* are defective for D-serine and D-alanine import. The ability of CFT073 to remove D-serine (50 mM) **(D)** and D-alanine (5 mM) **(E)** from PBS was estimated by DAO-catalyzed peroxidase-coupled oxidation of *o*-dianisidine. All experiments were performed independently three times. * $P<0.05$, ** $P<0.01$ (T-test)

Figure 3.1

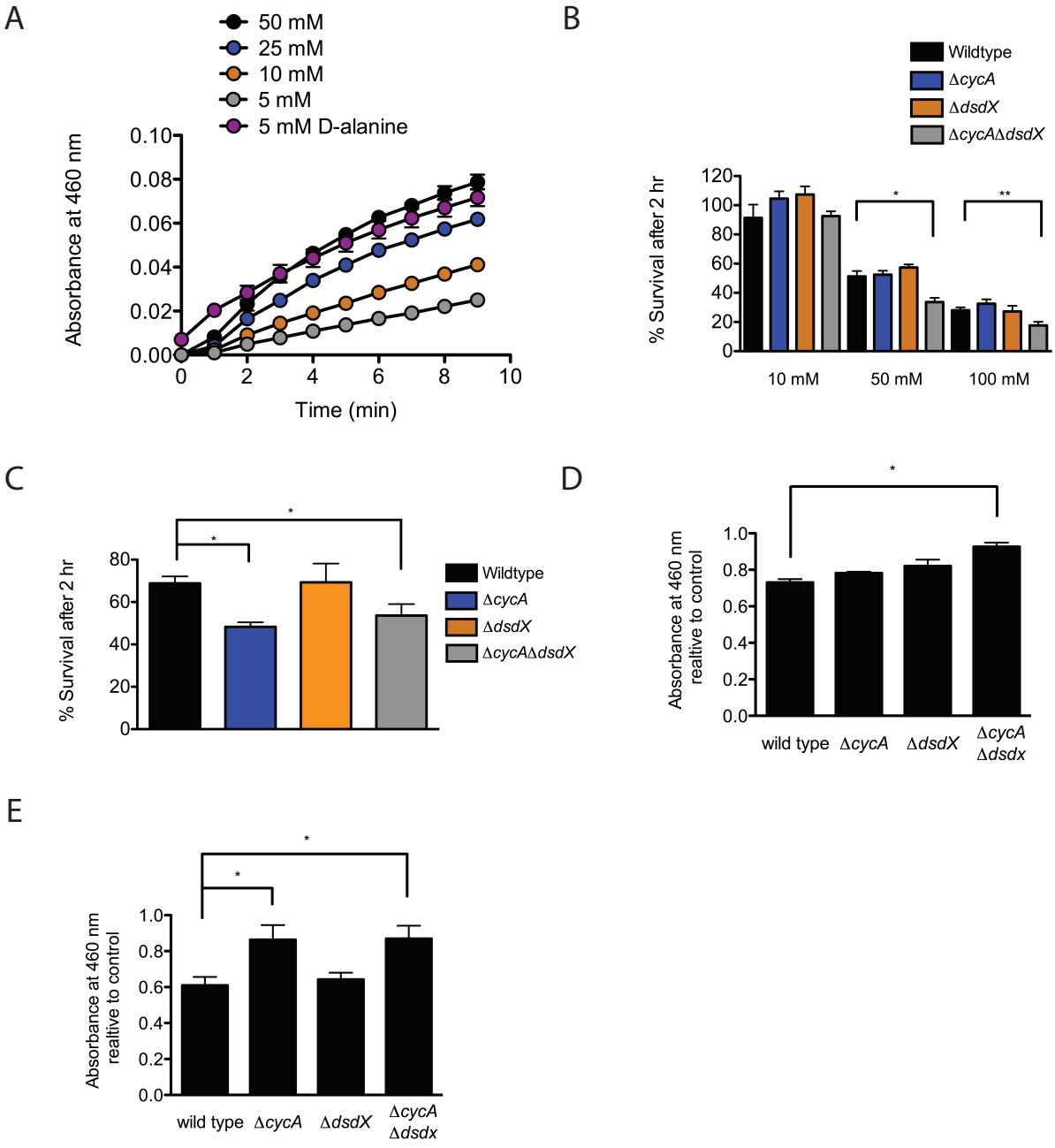
A
50 mM
25 mM
10 mM
5 mM
5 mM D-alanine
Absorbance at 460 nm
Time (min)
B
Wildtype
ΔcycA
ΔdsdX
ΔcycAΔdsdX
% Survival after 2 hr
10 mM
50 mM
100 mM
C
% Survival after 2 hr
Wildtype
ΔcycA
ΔdsdX
ΔcycAΔdsdX
D
Absorbance at 460 nm realtive to control
wild type
ΔcycA
ΔdsdX
ΔcycA Δdsdx
E
Absorbance at 460 nm realtive to control
wild type
ΔcycA
ΔdsdX
ΔcycA Δdsdx

Figure 3.2. CycA and DsdX limit exposure of CFT073 to DAO produced oxidative stress. A) The OxyR-dependent P*ahpC-lux* reporter strain is sensitive to hydrogen peroxide. Wild type, *cycA, dsdX,* and a *cycAdsdX* double mutant sense non-enzymatic hydrogen peroxide equally. RLU data is normalized to the culture optical density. Reporter activity between wild type, *cycA, dsdX,* and a *cycA-dsdX* double mutants for each of the treatment groups are not significantly different. **B)** A *cycA* mutant is exposed to greater hydrogen peroxide stress compared to wild type following exposure to DAO and D-alanine. Both *cycA* and *dsdX* provide protection from oxidative stress when CFT073 is exposed to DAO and D-serine. Data is from strains carrying the P*ahpC-lux* reporter and are the means with standard error from three separate experiments. **C)** A *cycA* and *cycA-dsdX* double mutant are sensitized to neutrophil killing. Neutrophil killing was measured 90 min post infection in CBIO or control treated neutrophils. **D)** CFT073 lacking *cycA* are exposed to greater hydrogen peroxide stress in neutrophils. Hydrogen peroxide stress levels are restored when DAO is inhibited with CBIO. Data is from strains carrying the P*ahpC-lux* reporter 30 min following infection of purified neutrophils and are the means with standard error from three separate experiments. * $P<0.05$, ** $P<0.01$, ns-not significant (T-test)

Figure 3.2

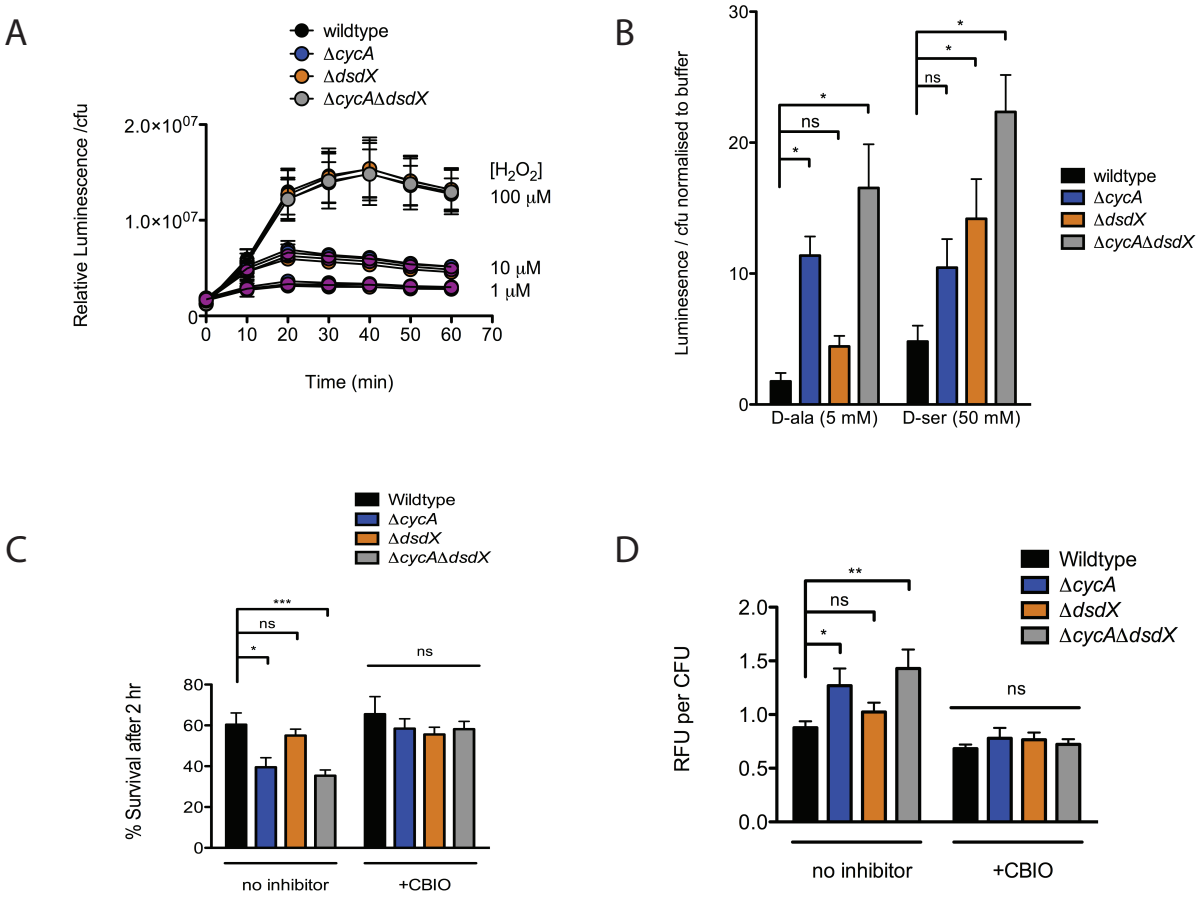
A
wildtype
ΔcycA
ΔdsdX
ΔcycAΔdsdX
Relative Luminescence /cfu
2.0×10^07
1.0×10^07
0
[H2O2]
100 μM
10 μM
1 μM
0 10 20 30 40 50 60 70
Time (min)
B
Luminescence / cfu normalised to buffer
30
20
10
0
*
ns
*
*
*
ns
D-ala (5 mM)
D-ser (50 mM)
wildtype
ΔcycA
ΔdsdX
ΔcycAΔdsdX
C
Wildtype
ΔcycA
ΔdsdX
ΔcycAΔdsdX
% Survival after 2 hr
80
60
40
20
0
***
ns
*
ns
no inhibitor
+CBIO
D
Wildtype
ΔcycA
ΔdsdX
ΔcycAΔdsdX
RFU per CFU
2.0
1.5
1.0
0.5
0.0
**
ns
*
ns
no inhibitor
+CBIO

Figure 3.3. CFT073 requires *cycA* and *dsdX* for protection against DAO *in vivo*. A) Inhibition of DAO and neutropenia normalizes the fitness defect of *cycA* CFT073 and *cycA-dsdX* CFT073. Control (CMC), DAO inhibited (CBIO) and neutropenic (Ly6G) mice were infected with an equal mixture of wild type and mutant CFT073 for 4 days. Shown is the competitive index data from three experiments. CFT073 transporter mutants are exposed to greater peroxide stress than wild type *in vivo*. CBA mice were infected with either wild type, *cycA, dsdX,* or *cycA-dsdX* mutants containing the OxyR-dependent luminescent reporter, and then treated with carboxymethylcellulose (control, **B**) or DAO inhibitor CBIO **(D)**. Images represent 5 min integrations of infected mice from three independent experiments. **(C, E)** *Ex vivo* imaging of excised kidneys from mice in **(B and D**) were quantified and normalised cfu and wildtype expression.

Figure 3.3

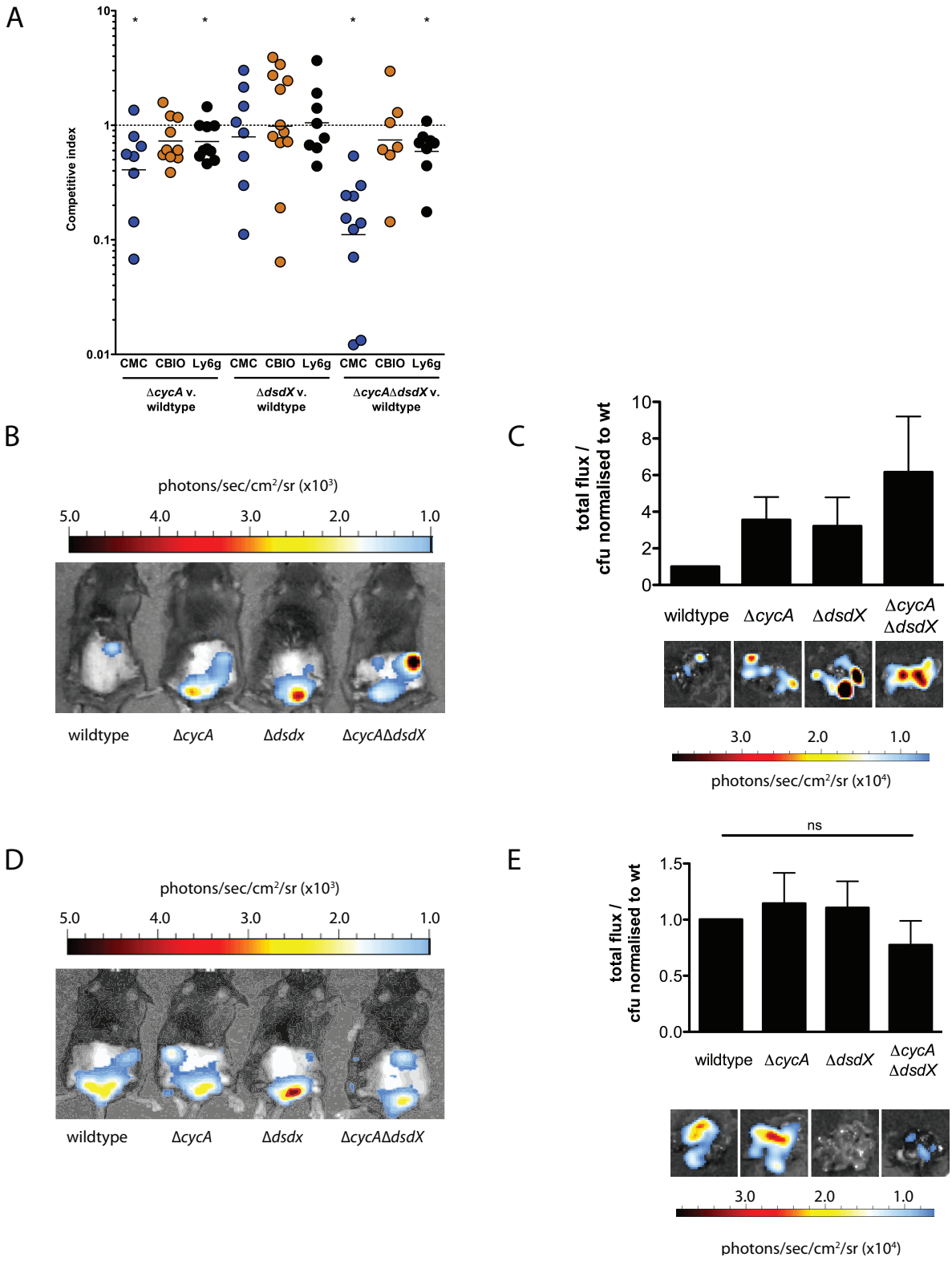
A
Competitive index
10
1
0.1
0.01
*
*
*
*
CMC CBIO Ly6g CMC CBIO Ly6g CMC CBIO Ly6g
ΔcycA v. wildtype
ΔdsdX v. wildtype
ΔcycAΔdsdX v. wildtype
B
photons/sec/cm²/sr (x10³)
5.0 4.0 3.0 2.0 1.0
wildtype ΔcycA Δdsdx ΔcycAΔdsdX
C
total flux / cfu normalised to wt
10
8
6
4
2
0
wildtype ΔcycA ΔdsdX ΔcycA ΔdsdX
3.0 2.0 1.0
photons/sec/cm²/sr (x10⁴)
D
photons/sec/cm²/sr (x10³)
5.0 4.0 3.0 2.0 1.0
wildtype ΔcycA Δdsdx ΔcycAΔdsdX
E
ns
total flux / cfu normalised to wt
1.5
1.0
0.5
0.0
wildtype ΔcycA ΔdsdX ΔcycA ΔdsdX
3.0 2.0 1.0
photons/sec/cm²/sr (x10⁴)

Figure 3.4. *cycA* is upregulated in urine and during a kidney infection. A) *cycA* and *dsdX* expression were measured in 10 UPEC clinical isolates taken from either acute or recurrent infections and grown in fresh urine and normalised to samples from LB. **B)** Mice were infected with 10 UPEC clinical samples and *cycA* and *dsdX* gene expression was measured in *ex vivo* kidneys.

Figure 3.4

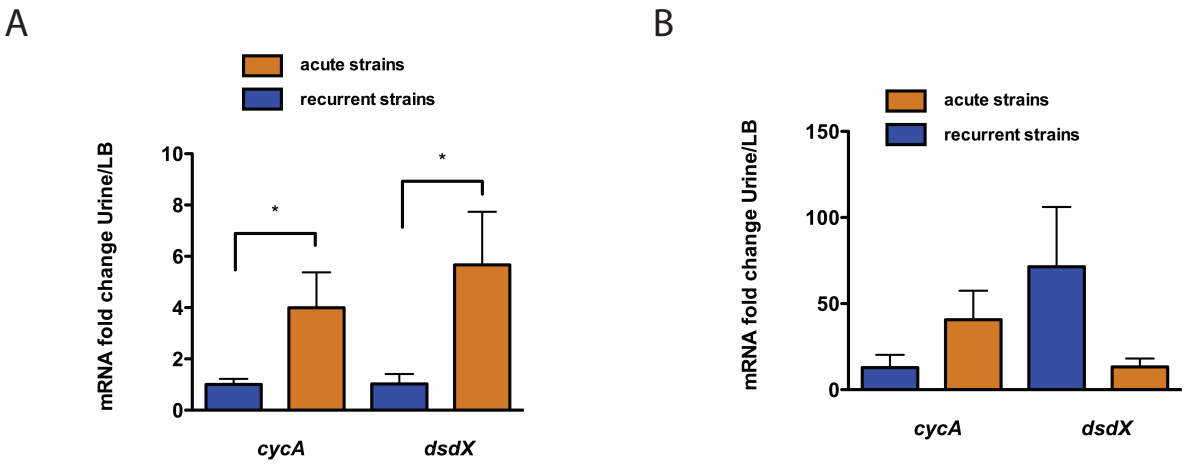
A
acute strains
recurrent strains
mRNA fold change Urine/LB
10
8
6
4
2
0
*
*
cycA
dsdX
B
acute strains
recurrent strains
mRNA fold change Urine/LB
150
100
50
0
cycA
dsdX

Table 3.1. Strains used in this study.

Strain	Reference or source
Uropathogenic *E. coli* CFT073 (wild type)	Our collection
CFT073 Δ*cycA*	This study
CFT073 Δ*dsdX*	This study
CFT073 Δ*cycA*Δ*dsdX*	This study
CFT073 Δ*cycA*::Cm	This study
CFT073 Δ*dsdX*::Cm	This study
CFT073 Δ*cycA*Δ*dsdX*::Cm	This study
E. coli BL21 (DE3) pET3a::DAO-6HIS	(Tuinema et al., 2016)
CFT073 wild type P*ahpC*-pGEN-*luxCDABE*	This study
CFT073 Δ*cycA* P*ahpC*-pGEN-*luxCDABE*	This study
CFT073 Δ*dsdX* P*ahpC*-pGEN-*luxCDABE*	This study
CFT073 Δ*cycA* Δ*dsdX* P*ahpC*-pGEN-*luxCDABE*	This study
HM 01	Mobley Lab
HM 03	Mobley Lab
HM 06	Mobley Lab
HM 46	Mobley Lab
HM 68	Mobley Lab
HM 26	Mobley Lab
HM 27	Mobley Lab
HM 35	Mobley Lab
HM 57	Mobley Lab
HM 61	Mobley Lab

Table 3.2. List of oligonucleotides used in this study.

Oligonucleotide	Sequence
BRT 184	atggtagatcaggtaaaagtcgttgccgatgatcaggctccggctgaagtgtaggctggagctgcttcg
BRT 185	ttatttccgcagttcagcagcccgtttcttaccaataaacagccagcccatatgaatatcctcctta
BRT 186	atgcactctcaaatctgggttgtgagcacgctgcttatcagcatcgtggtgtaggctggagctgcttcg
BRT 187	atagtatttaaatgtttcattgagcgtagcgccgcaatattgcttcaccatatgaatatcctcctta
BRT 192	cgggatccagcttagatcaggtgattgcc
BRT 193	cgtacgtaccgtttttgaatgcctggttt
BRT 194	atcactgccacgcgcgatt
BRT 195	gctttcggtgccacacctt
BRT 196	atatgttgccaataagctggg
BRT 197	caatcgttttaaccaacatcag
BRT 198	gcacggatatttttgtcgttg
BRT 199	gccacagccgacatccag

CHAPTER FOUR

DISCUSSION

DISCUSSION

Summary of Main Findings

Human pathogens have a significant impact on health outcomes worldwide. Characterising the underlying mechanisms of how these pathogens evade innate immunity will assist in the development of novel strategies to control infections. The importance of this research is further compounded as researching these pathogens provides insights into the underlying mechanisms of the innate immune system and human health. The work in this thesis focussed around two pathogenic bacteria species *Salmonella enterica* and uropathogenic *E. coli.* and characterised two distinct amino acid transport systems involved in virulence. Taken together this work describes a novel host-pathogen interaction common between *Salmonella*, UPEC, and potentially other pathogens.

Salmonella is commonly contracted through contaminated food and water and is of great concern in both developing and developed countries (6). This pathogen has undergone several important horizontal gene transfer events resulting in the acquisition of multiple pathogenicity islands, the most important to this work is that of SPI-2 (3). The characterization of the SPI-2 regulator, SsrB, using functional genomic techniques has led to the identification of several genes that have undergone regulatory evolution to come under the control of SsrB. One such gene, *dalS,* provides a fitness advantage during infection by transporting D-alanine and yet a protective mechanism remained enigmatic. As such we tested the putative role of DalS during *Salmonella* infection. Deletion of *dalS* was found to sensitize *Salmonella* to *in vitro* killing mediated by the DAO dependent production of ROS (Figure 2.1), as well as survival within neutrophils (Figure 2.2). Using a murine model of systemic salmonellosis, we demonstrated that a *dalS* mutant is less fit than wildtype *in vivo*

(Figure 2.3) due to elevated levels of DAO produced ROS (Figure 2.4). This work demonstrates the first example of an amino acid transporter sequestering substrate to evade the innate immune system. In addition, it outlines an *in vivo* antimicrobial role for DAO during bacterial infections.

Over 80% of all urinary tract infections are caused by uropathogenic *E. coli* (58). It has previously been demonstrated that a triple deletion of the D-serine transporters, *cycA* and *dsdX*, and the D-serine deaminase, *dsdA*, results in a fitness defect during murine pyelonephritis (147). However the underlying mechanism accounting for this fitness defect was unclear. DAO is highly expressed in kidneys and rapidly degrades excess D-serine prior to excretion, resulting in the production of ROS (115). We tested whether UPEC CycA and DsdX sequester D-serine to prevent DAO killing. Exposing transporter mutants to recombinant DAO and exogenous D-serine resulted in a statistically significant increase in ROS dependent killing of all strains and a sensitization of the transporter mutant (Figure 3.1). Using an ascending urinary tract infection model we demonstrated that UPEC requires these transporters to survive ROS produced by DAO (Figure 3.3). This work outlines a function for two previously identified D-serine transporters crucial for the survival of UPEC and highlights a possible conserved mechanism of innate immune system subversion by bacterial pathogens.

Despite the progress this work makes to better understand bacterial virulence strategies and the innate immune system, the following questions still remain: Do other transport systems exist that protect bacteria from DAO or other antimicrobial mechanisms? Do DalS or CycA provide a secondary role during virulence? When is DAO functional during an infection? What is the biological source of DAO substrate during an infection?

What is the fate of DAO produced hydrogen peroxide? Does DAO have a secondary role within neutrophils? These questions will be the focus of the following sections.

Future Directions for Bacterial Evasion of DAO

The identification of *dalS* and *cycA* demonstrated a new mechanism by which *Salmonella* and UPEC evade the immune system, however it is unclear if *dalS* and *cycA* have alternate roles during infection; to what extent this virulence strategy exists in other infection systems, and whether vancomycin resistance inadvertently protects against DAO.

We identified D-alanine as the biological substrate of DalS however it appears glycine (148) and D-serine (unpublished) may also be transported by DalS. Using a radiolabelled uptake assay it was demonstrated that DalS does not appear to directly import glycine however this has not been tested with D-serine. D-serine is of particular interest with regards to DalS as it is a biological substrate for DAO. Although it is unlikely that D-serine transport protects against DAO during an intracellular neutrophil infection we cannot rule out an alternative role for D-serine transport in other stages of a *Salmonella* infection. Of interest is the sensitization of *Salmonella* to macrophage killing upon deletion of *dalS* (148). DAO is not expressed, to our knowledge, in macrophages indicating a possible secondary role of DalS in this cell type. The transport of D-serine or another undetermined substrate may play a role in this sensitization.

During infection of mice, a *cycA dalS* double mutant had a more severe decrease in fitness than either individual mutant. This suggests that the roles of *cycA* and *dalS* are not completely redundant as one would expect a phenotype would only be present in the double

deletion and not either of the single mutants. Although we demonstrated a role for both *dalS* and *cycA* in protection against DAO there may exist differences between these two transporters. Work discussed in the Appendix has suggested that *cycA* and *dalS* are regulated differentially during a murine infection. This may suggest that these two transporters may provide protection against DAO at different time points or perhaps under different metabolic conditions as DalS is part of an active ABC transporter and CycA is a transmembrane passive permease. Alternatively, the redundancy of these genes may suggest the importance of protecting against DAO produced hydrogen peroxide.

Recently it was demonstrated that *Vibrio cholera* is sensitive to DAO killing *in vitro* and in the proximal small intestine of mice (104). This sensitization appears to be more drastic than that observed with *S.* Typhimurium, *E. coli*, and *Listeria monocytogenes.* Although *cycA* homologues are found in most bacteria a homologue is absent in *Vibrio cholera*. The lack of *cycA* may explain an increased sensitivity to DAO killing. Testing the ability of *Vibrio* to sequester D-amino acids away from DAO may provide insights into why some bacteria are sensitized to DAO. Interestingly, Osborne *et al.* demonstrated that a *dalS* mutant is attenuated in the cecum of mice after an oral competitive infection with *Salmonella*. The mechanism for this remains unclear but is potentially due to DAO secreted by goblet cells and located in the proximal intestine providing a selective barrier during a *Salmonella* infection. A second possibility is that a *dalS* mutant escapes the intestine unaffected but is sensitized during neutrophil engulfment changing the population distribution. This new population of *Salmonella* can subsequently re-seed the gut through the bile duct resulting in a what appears to be a cecum localised phenotypic defect. Alternatively this fitness defect may be a result of exposure to infiltrating neutrophils during an intestinal

infection. During *Salmonella* induced gastroenteritis, intestinal epithelial cells can release proinflammatory cytokines. Neutrophils are attracted by these signals, transverse the epithelium, and can target pathogens in the intestinal lumen (182). Understanding where this occurs may illuminate how DAO plays a role in acute gastroenteritis of *Salmonella* and *Vibrio*.

Multi-drug resistance is becoming an increasing problem in health care due to limited treatment options available to combat these pathogens. Vancomycin targets the cell wall of Gram positive bacteria by binding the D-alanyl-D-alanine moiety of N-acetylmuramic acid and N-acetylglucosamine peptides which in turn prevents the polymerization of these peptides. Vancomycin resistance results in a substitution of the terminal D-alanine residue in the stem peptide of peptidoglycan to a D-lactate residue. Considering this I hypothesize that vancomycin resistance inadvertently results in protection against DAO. This removal of D-alanine effectively reduces the concentration of D-alanine by half in the cell wall of these bacteria therefore limiting the overall substrate for DAO. This resistance only occurs when in the presence of vancomycin suggesting that during an untreated infection this would not be beneficial, however this adaptation may provide increased virulence in the presence of vancomycin. Although we have demonstrated that Vancomycin-resistant *Enterococci* does have less D-alanine available for DAO oxidation after exposure to vancomycin (unpublished) it is still unclear whether this plays a role in virulence. More work is required to test whether this phenotype extends to an *in vivo* model.

Future Directions for the Characterization of DAO

DAO is responsible for several key functions in mammals. This work has demonstrated DAO as an important arm of the innate immune system, however several questions remain. Perhaps the most paramount of which is the source of DAO substrate during bacterial infections. It is clear that the source of D-alanine must be bacterial in origin but whether it is produced by the microbiota and circulates through serum to sites of infections or it is directly produced by the pathogen remains to be elucidated. If DAO plays a role in bacterial clearance in the intestinal lumen D-alanine produced by the microbiota may be a viable substrate. Circulating serum concentrations of D-alanine represent approximately 5% of all alanine in healthy individuals (205). Although D-alanine is present in the blood it may be occluded from the lamina propria and other infection sites limiting the concentration of microbiota derived D-alanine. I hypothesize that DAO works synergistically with membrane perturbing antimicrobial peptides that liberate D-alanine from *Salmonella*. We have demonstrated that sub-inhibitory concentrations of LL-37 are capable of potentiating DAO killing especially in the presence of a *dalS* mutant (Figure A.2). To further test this, a more rigorous approach is required. Using a series of AMPs and DAO at different concentrations to calculate a fractional inhibitory concentration it is possible to determine if these enzymes work in concert. In addition, by inhibiting select AMP's *in vivo* while simultaneously inhibiting DAO it may be possible to determine if this synergy occurs in neutrophils.

During engulfment, pathogens are exposed to reactive oxygen species generated by NADPH oxidase. In neutrophils, the ROS produced by NADPH oxidase is converted to

hypochlorous (HOCl) acid by myeloperoxidase which is believed to be the prominent antimicrobial compound. The kinetics of NADPH oxidase are well characterised (86) however it is unclear when ROS is produced by DAO and to exactly what extent it is required during neutrophil killing. It has been shown that the limiting step in this pathway is the production of hydrogen peroxide (89) and therefore an auxiliary source of ROS could be beneficial for the elimination of pathogens. Chapter two demonstrated that DAO may be the source of auxiliary ROS production (Figure 2.4). Work from the early 2000's demonstrated that *Salmonella* requires the SPI-2 T3SS to prevent NADPH oxidase assembly resulting in an incomplete respiratory burst and increased *Salmonella* survival (184). However there exists controversy surrounding this data as Aussel *et al.* demonstrated that *Salmonella* lacking a SPI-2 T3SS were exposed to equivalent NADPH oxidase produced ROS and were equally attenuated compared to wildtype in both wildtype and *CYBB*$^{-/-}$ mice. Although this data has been questioned it still outlines a possible need for a second source of ROS during an infection. Alternatively, the role of DAO may be to generate ROS prior to NADPH oxidase assembly. Upon engulfment of a bacterium NADPH oxidase complex requires time to assemble whereas DAO does not require this assembly step. This would allow for DAO to generate ROS prior to NADPH oxidase. This initial burst may be crucial for the clearance of pathogens, however this remains unclear. To understand the role of DAO during neutrophil engulfment it is key to understand the kinetics of DAO during an infection. By monitoring a single live cell infection, it may be possible to understand how both DAO and NADPH oxidase function during infections.

During this work we have assumed the biological function of DAO is to generate antimicrobial levels of ROS either directly or through an MPO intermediate. In 1981 using *S.*

aureus and human phagocytes, Segal demonstrated that within neutrophils there existed a slight increase in phagosomal alkalinity followed by a delay in phagosomal acidification in neutrophils compared to macrophages (206). The authors argued that this temporary increase in pH allows for the priming of neutral proteases and was therefore not required in macrophages. It was proposed that this was due to NADPH oxidase and was independent of the particle being phagocytosed. This was later contested as Jankowski in 2002 demonstrated that this was not the case and no alkalinity occurred when these phagocytes were exposed to synthetic beads (207). I hypothesise that both results are correct however the nature of the particle is important and DAO is the source of alkalinity. During the oxidative deamination by DAO the basic compound ammonia is produced potentially increasing the pH or delaying acidification. If DAO is the source of alkalinity than the nature and D-alanine content of the particle would be crucial and explain why this only occurs in neutrophils. Similar to bacteria neutral proteases are important for the clearance of fungi (208) however unlike bacteria, fungi do not produce D-alanine. To date no work has been conducted to determine if this acidification delay occurs when fungi are phagocytosis. Understanding this problem may answer the question as to why neutrophil phagosome acidification is delayed compared to macrophage.

Anti-virulence as a mechanism for pathogen control

Antibiotic resistance is becoming an increasingly dire problem. The rate that antibiotic resistance is occurring shadows the rate at which new antibiotic classes are discovered (209). A major problem with antibiotic resistance is the ease at which resistance

can be transferred between species of bacteria. This resistance stems from antibiotic selective pressure imposed on bacteria thorough human intervention (210). One possible solution to limit this resistance is to target virulence machinery of the pathogen limiting the damage inflicted on the host. In doing so the innate immune system can more readily clear these pathogens. Although resistance towards these anti-virulence compounds can occur it is proposed that it occurs at a slower rate than traditional antibiotics (211, 212). In addition, the risk of transfer of resistance decreases due to specie specific targets and contextual exposure. If a bacterium acquires a resistant version of a virulence factor this may provide no benefit if that bacterium is unable to employ that specific virulence strategy (213). Also, the activation of particular virulence strategies are extremely environmentally dependent, and therefore the acquisition of a resistant virulence factor *ex vivo* may be too costly and not become stable in the population as readily as an antibiotic resistant gene (212). As such targeting virulence genes may serve as a better method for pathogen clearance. DalS, CycA, and DsdX may provide suitable targets for anti-virulence targets. These transporters have been demonstrated to be important for virulence within a host however their necessity *ex vivo* is limited. One caveat of this strategy may be that deletions of these transporters individually have only minor phenotypes compared to other virulence factors, however a *cycA dsdX* double mutant has a more dramatic phenotype and creating a compound that targets both may be more amenable to an anti-virulence strategy.

Concluding Remarks

The work presented in this thesis has sought to address a novel virulence strategy employed by several human pathogens. This was achieved by elucidating the specific function of two D-amino acid transporters, DalS in *Salmonella* and CycA in uropathogenic *E. coli.* As the number of infections caused by Gram-negative pathogens increases, as well as the increase of antibiotic resistance within these pathogens, the need to develop novel strategies to combat these pathogens also increases. In addition, by studying how these pathogens survive within the host we can achieve a better understanding of how the innate immune system limits infections. The development of novel treatment stems from a deep understanding of how these bacteria survive within the host and how the host controls infections. This work represents one strand of the complex web of host-pathogen interactions relevant to human health.

APPENDIX

CycA and DalS Protect against DAO in *Salmonella* Infections

CO-AUTHORSHIP STATEMENT

The following individuals are have contributed to this work: Brian R. Tuinema, Vivian Lau, Scott Carlaw, and Brian K. Coombes

The contributions by each author to the project are described below:

1. Strains and plasmids were created by B.R.T., S.C.

2. *In vitro* DAO assays were performed by B.R.T., V.L.

3. Neutrophil experiments were conducted by B.R.T., V.L.

4. Competitive infections and *in vivo* imaging was conducted by B.R.T.

5. Fluorescent microscopy was conducted by B.R.T.

6. B.R.T. and B.K.C. contributed to manuscript preparation

*Note that references in the following data chapters have been compiled into one reference list at the end of the thesis in order to avoid any redundancy between sections.

INTRODUCTION

During a bacterial infection, neutrophils are a major immune cell type that protect the host against invading pathogens during early stages of an infection. Using a series of antimicrobial pathways, these phagocytic cells engulf and kill pathogens to limit systemic spreading throughout the host. Recent work has demonstrated that the production of ROS through the oxidation of D-alanine by DAO is one such pathway (112). In neutrophils, DAO has been shown to be localised to the membrane and phagosomes (113). However, this analysis was conducted using hydrogen peroxide production as a proxy for DAO location (113). Since hydrogen peroxide is also a downstream product of NADPH oxidase, it is unclear what the true source of hydrogen peroxide is. Other studies have also found DAO in the granule fraction of neutrophil isolates (111) which potentially fuse with the phagosome during phagosome maturation.

Eukaryotes are unable to produce D-alanine *de novo* therefore all D-alanine present is either produced by bacteria or ingested. D-alanine makes up part of the stem peptide in peptidoglycan and is required for the construction of the bacterial cell wall (132). *Salmonella* generates D-alanine through the epimerization of L-alanine and these two isomers exist in a 1:1 equilibrium throughout the bacterial cytoplasm (132). During interactions with *Salmonella,* neutrophil DAO must use D-alanine produced by the host microbiota or D-alanine generated by *Salmonella* itself. Little is known about the concentration of free D-alanine generated by the microbiota at the bacteria-neutrophil interface, however it is known that some neutrophil antimicrobial peptides perturb the bacterial envelope causing cytosolic metabolites to leak from the cell (84).

Salmonella is able to prevent complete clearance by neutrophils during an infection, in part, through its ability to sequester D-alanine using the DalSTUV ABC transporter (148). In an effort to uncover similar DAO protection systems we identified a second D-alanine transporter in *Salmonella*, CycA. As previously discussed in Chapter 3, *cycA* is conserved across diverse taxa including *Salmonella*, and is responsible for the uptake of glycine, D-serine, and D-alanine (142, 193) making it an alternative candidate for protection against DAO. Similar to the *E. coli* homologue, *Salmonella cycA* is regulated, in part, by the small RNA *gcvB* (144) however it is unclear whether other regulatory inputs exist.

Understanding how and when DAO is functional in neutrophils will provide insights into how *Salmonella* evades these immune cells. As previously demonstrated *Salmonella* sequesters D-amino acids to prevent DAO produced ROS, however it remains unclear what the source of D-alanine is during an infection and if DalS is the only transporter capable of protecting against DAO. Here we show that DAO is localised to the neutrophil plasma membrane and upon infection this localization shifts to LAMP-1-positive vacuoles. LL-37 may liberate D-alanine from *Salmonella* which in turn is available as a substrate for DAO. CycA functions to sequester D-alanine from DAO and a deletion of both *cycA* and *dalS* results in decreased fitness. DalS appears to function during very early infection time points where the expression of *cycA* is delayed. This data sheds light onto how *Salmonella* is protected against DAO.

RESULTS

DAO localizes to LAMP-1 positive vacuoles after *Salmonella* infection

Robinson, Briggs, and Karnovsky demonstrated that hydrogen peroxide was localised to neutrophil membrane and upon infection with *E. coli* this signal redistributed to the vacuoles (113). ROS production was only observed in the presence of D-alanine suggesting a role by DAO. Hydrogen peroxide is also produced through the conversion of superoxide produced by NADPH oxidase. To improve the specificity of DAO localization we tested whether antibodies targeted directly against DAO demonstrated a similar localization pattern as previously observed. In uninfected neutrophils, DAO was co-localized with the membrane marker CD43 (Figure A.1A). Upon infection with *Salmonella,* a redistribution of DAO to the vacuole and co-localization with LAMP-1occured (Figure A.1A). Using the Pearson coefficient, we quantified this relocation of DAO in neutrophils (Figure A.1B).

A *dalS* mutant is sensitized to LL-37 assisted DAO killing

To test whether LL-37 is able to liberate D-alanine from *Salmonella,* we exposed cultures to either purified DAO, LL-37, or both in cultures lacking exogenous D-alanine. Upon treatment with 5 μg/mL of DAO *Salmonella* was able to survive as well as a buffer treated control whereas *Salmonella* exposed to 10 μg/mL of LL-37 were slightly attenuated for survival (Figure A.2). Upon combination of both DAO and LL-37 a *dalS* mutant was sensitized to killing. These data suggested that combining LL-37 and DAO may potentiate killing.

CycA and DalS protect *Salmonella* against DAO produced ROS

To determine if CycA could protect *Salmonella* against DAO *in vitro* we exposed bacteria to DAO and exogenous D-alanine. We found that *Salmonella* lacking *cycA*, *dalS,* or both showed a decrease in survival. This phenotype was restored by removal of DAO produced ROS from culture through treatment with a ROS scavenger, thiourea (Figure A.3A). These transporter mutants were less able to survive compared to wild type in neutrophils (Figure A.3B), a phenotype that was restored upon exposure to the DAO chemical inhibitor CBIO (Figure A.3C). We next tested the ability of a *cycA* mutant to compete against wildtype during a murine infection. Similar to a *dalS* mutant, *cycA* mutants were less fit than wildtype (Figure A.3C). Interestingly a transporter double mutant was further attenuated compared to a *cycA* single mutant (Figure A.3D). This fitness defect was restored through treatment with the chemical inhibitor CBIO (Figure A.3E). To test whether the fitness defect of these transporter mutants was due exposure to elevated ROS levels we infected *CYBB*$^{-/-}$ mice with either wildtype or a *cycA* mutant harbouring a ROS reporter capable of sensing minute changes in ROS (112). Mutants lacking *cycA* displayed elevated ROS exposure compared to wildtype and treatment with CBIO decreased this exposure significantly (Figure A.3F). Together this data suggests that *cycA* and *dalS* function to sequester D-alanine during infection limiting the available substrate for DAO.

cycA* and *dalS* are differentially expressed *in vivo

Previous work has demonstrated that *dalS* is regulated, in part, by the SPI-2 master regulator SsrA-SsrB (41, 148). Chromatin immunoprecipitation work has demonstrated a possible interaction between SsrB and the *cycA* promoter, similar to *dalS*, however SsrB

appears to not play a role in the expression of *cycA* (41). We next aimed to measure the expression *in vivo* of both genes using a transcriptional luciferase fusion. Interestingly the *dalS* reporter appeared to activate immediately after infection whereas the expression of *cycA* was delayed by approximately 40 min (Figure A.4A-B).

DISCUSSION

There remain several unanswered questions in this project that will serve to clarify the interaction between *Salmonella* and DAO. Although preliminary results suggest that DAO undergoes a re-localization in neutrophils upon infection with *Salmonella*, further work is required to clarify this localization. Furthermore, we did not investigate the distribution of DAO associated with granules within naïve and infected neutrophils. This may provide insights into the timing of DAO mediated killing during an infection. Using fluorescently labelled DAO, it could be possible to track DAO during a *Salmonella* neutrophil infection in real time and better understand the localization pattern of DAO. DAO utilises bacterial produced D-alanine however how this D-alanine is liberated from bacteria remains elusive. Further work is required to address the synergy between DAO and AMPs. Neutrophils express several AMPs as well as other antibacterial factors that may liberate D-alanine from bacteria as a consequence of their antimicrobial function. Our research demonstrates that *Salmonella* requires CycA to protect against DAO even in the presence of DalS. We demonstrated that *cycA* and *dalS* expression differ suggesting a temporal requirement for both transporters, however this requires further experimentation. A more rigorous approach is required to elucidate the expression of these two transporters. In addition, traditionally redundant systems typically fail to present a phenotype when one system is removed. Phenotypes only exist if both systems are not present. When either transporter is deleted we still saw individual phenotypes that become more apparent upon double deletions. One alternate possibility is that *cycA* has a secondary function that is not captured in our experiments.

Understanding the temporal localisation of DAO within neutrophils and the biological source of D-alanine will provide insights into how *Salmonella* is protected against DAO. Since *cycA* is conserved across diverse taxa it may serve a non-DAO related purpose in bacteria that do not encounter DAO. The intracellular lifestyle of *Salmonella* may have led to the regulatory evolution of *cycA* in order to protect against ROS damage caused by neutrophilic DAO.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted according to guidelines set by the Canadian Council on Animal Care using protocols approved by the Animal Review Ethics Board at McMaster University. All experiments using human samples were conducted according to the guidelines set by the Hamilton Integrated Research Ethics Board.

Bacteria, cloning, and deletion mutants

Bacterial strains are isogenic derivatives of *Salmonella* Typhimurium SL1344. Deletion mutants were generated using the Lambda Red recombinase method (203). Chloramphenicol resistance cassettes were amplified with BRT 131 (atggtagatcaggtaaaagtcgcagccgacgaa-caggctccggctgaagtgtaggctggagctgcttcg) and BRT 132 (ttaacgcattcccgccatccgtttcttacctatca-acagccagcccagcatatgaatatcctcctta). DNA was electroporated into *S.* Typhimurium containing the pKD46 plasmid. Correct mutants were screened and resistance cassettes were removed using the FLR recombinase encoded on pCP20.

Isolation of human peripheral blood neutrophils and fluorescence microscopy

Human blood was layered onto Lympholyte-poly (Cederlane CL5071) and centrifuged at 20 deg. C for 30 min at 500 *g*. The neutrophil layer was removed and osmolarity was stabilised with RPMI (10% FBS, 1 x HEPES, 1 x sodium pyruvate, 1 x β-mercaptoethanol, 1 x essential amino acids). Neutrophils were washed and resuspended in RPMI. Neutrophils were seeded onto glass coverslips and infected with *Salmonella* at an MOI of 10:1 for 2

hours. Coverslips were washed with PBS and fixed with 4% paraformaldehyde. Neutrophils were permeabilized with 0.2% Triton for 5 min, washed 3 times with PBS, and blocked with 5% normal goat serum (NGS). Slides were stained with primary antibodies against DAO (Abcam) 1:100, LAMP-1 (DSHB)1:200, CD43 (Abcam) 1:100 in 1% NGS. Slides were washed 5 times with 0.1% bovine serum albumin and 0.1% Tween-20 in PBS and stained with secondary antibodies conjugated with Alexa fluorophores (Abcam)1:500. Slides were washed 5 times followed by mounting with Prolong Diamond with DAPI (Thermo Fisher Scientific). Pearson coefficient was determined by the ImageJ plugin JACoP.

Protein purification

Purification of DAO-6HIS was performed as described previously (112). Recombinant DAO was purified from *E. coli* BL21 (DE3) containing the plasmid pDAO-6HIS. Cells were grown in LB at 37 deg. C. to mid-log phase then induced with 0.1 mM IPTG for 20 h at 37 deg. C. Cells were centrifuged at 4000 *g* for 13 min at 12 deg. C., resuspended in lysis buffer (20 mM Tris, pH 7.5, 0.5 M NaCl, protease inhibitors), and lysed via sonication. Lysates were pelleted at 10000 *g* for 30 min at 4 deg. C. Supernatant was loaded onto a Ni-NTA bead column and washed with an imidazole gradient (10, 20, 40 mM) in TBS (40 mM Tris, pH 7.5, 0.5 M NaCl). DAO-6HIS was eluted in TBS containing 80-320 mM imidazole and purity was determined using SDS-PAGE. Purified DAO was buffer exchanged and concetrated in TBS using 3K Amicon Ultra Centrifugal filters (Millipore, UFC800324) and stored at -80 deg. C.

Bactericidal activity assays with purified DAO

Salmonella was grown to mid-log phase in LB at 37 deg. C. and washed twice in PBS. 1 x 10^7 *Salmonella* were incubated with 5 µg/mL DAO and 5 mM D-alanine or DAO alone, LL-37 alone, or both in PBS at 37 deg. C. with shaking for 2 h. Cultures were serial diluted in PBS and plated on LB to determine killing activity. Colony forming units were determined and normalised to a buffer only control.

Isolation of mouse peritoneal neutrophils and bactericidal activity

Female 6-10 week old C57BL/6 mice (Jackson Laboratory) were injected intraperitoneally with 1 mL 2% Biogel (Biorad) in PBS. Neutrophils were harvested via peritoneal lavage 12-16 h later with 6 mL RPMI. Cells were passed through a 40 µm cell strainer to remove Biogel and purity was determined by Giemsa staining (Sigma). Neutrophils were exposed to 3 µM CBIO in CMC or CMC alone at 37 deg. C., 5% CO_2 in RPMI for 60 min. Overnight cultures of SL1344 strains were diluted in RPMI medium and added to neutrophils at a multiplicity of infection of 100:1 in tissue culture wells. Infected cells were incubated at 37 deg. C., 5% CO_2 in RPMI for 30 min to allow bacterial uptake. Neutrophils were further incubated for 90 mins. Cultures were washed five times with PBS and lysed using in 250 µL of lysis buffer (1% Triton X-100, 0.1% SDS). Lysates were serial diluted and plated for cfu determination.

Animal experiments

For *in vivo* experiments, C57BL/6 mice (Jackson Laboratory) were infected intraperitoneally with 1 x 10^6 *Salmonella* described previously (112). At 48 hrs post-infection mice were sacrificed by cervical dislocation and liver and spleens were harvested. Organs were homogenised in PBS with a Mixer Mill (15 min, 30 Hz) (Retsch), serial diluted in PBS, and plated on LB. Colonies were replica plated onto LB agar containing either chloramphenicol or LB alone to determine the ratio of wild type to mutant colonies. For inhibition of DAO *in vivo*, mice were injected i.p. with 25 mg/kg CBIO in 0.5% carboxymethylcellulose (CMC) (Sigma) or 0.5% CMC only every 8 h during the course of infection.

***In vivo* bioluminescent imaging**

C57BL/6 mice were dosed with 25 mg/kg CBIO in 0.5% CMC or 0.5% CMC only and infected intraperitoneally with 1 x 10^8 *Salmonella* containing the *ahpC-luxCDABE* reporter 1 h post treatment. Mice were anesthetised (2% isofluorane carried in 2% oxygen) 4 h post-infection and imaged for 5 min in a Spectrum *in vivo* Imaging System (IVIS) (Perkin Elmer). Total flux was normalised to input cfu.

Statistical analysis

Treatment groups were compared using a non-parametric Mann-Whitney test. All analyses were performed using Graph Prism 4.0 (GraphPad Software Inc. San Diego, CA). A *P* value of .05 or less was considered significant.

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Figure A.1. DAO co-localizes with LAMP-1 in infected neutrophils. A) Human peripheral neutrophils were stained with antibodies against DAO (green), CD43 (red), and or LAMP-1 (red) and imaged after exposure to *Salmonella* (infected) or no treatment (uninfected). Co-localization was quantified using the Pearson's coefficient **(B)**. ** P<0.01, ***P<0.001 DAPI (blue).

Figure A.1

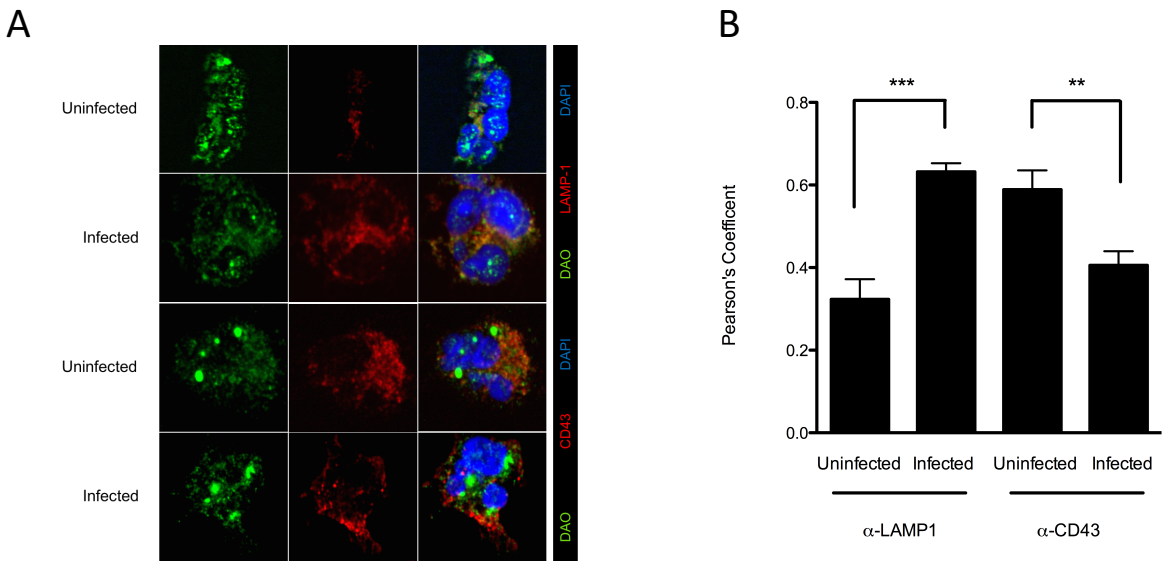

A
Uninfected
Infected
Uninfected
Infected
DAPI
LAMP-1
DAO
DAPI
CD43
DAO
B
Pearson's Coefficent
0.8
0.6
0.4
0.2
0.0
***
**
Uninfected
Infected
Uninfected
Infected
α-LAMP1
α-CD43

Figure A.2. DAO mediated killing of a *dalS* mutant increases with treatment of LL-37. Wild type and a *dalS* mutant were exposed to DAO, LL-37, or both in the absence of exogenous D-alanine. A *dalS* mutant was more susceptible to killing by both LL-37 and DAO compared to either DAO or LL-37 alone and compared to wildtype. * $P<0.05$, *** $P<0.001$

Figure A.2

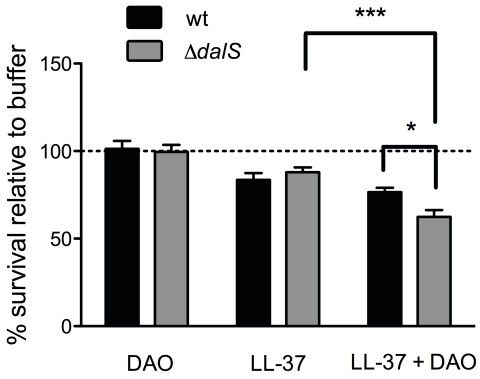

wt
ΔdalS
***
*
% survival relative to buffer
150
100
50
0
DAO
LL-37
LL-37 + DAO

Figure A.3. CycA and DalS protect *Salmonella* from DAO produced ROS. Transporter mutants were exposed to exogenous D-alanine and DAO and bacterial killing was monitored in the presence or absence of thiourea **(A)**. **B)** Deletion mutants of *cycA*, *dalS,* and a double mutant are sensitized to neutrophil killing. Bacterial survival was measured 2 h post infection and killing was normalised to the initial uptake of *Salmonella* after 30 min. Sensitivity was restored through chemical inhibition of DAO by CBIO **(C).** A *cycA* mutant has decreased fitness compared to wildtype *in vivo*. This fitness is further decreased upon deletion of *dalS* in combination with *cycA* **(D)**. Inhibition of DAO with CBIO restores this *in vivo* fitness defect **(E)**. A *cycA* mutant is exposed to elevated levels of ROS produced by DAO *in vivo*. *CYBB*$^{-/-}$ mice were infected with wildtype or *cycA* mutants containing the P*ahpC-lux* reporter. Deletion of *cycA* results in elevated ROS stress but is restored in the absence of DAO. * $P<0.05$ ** $P<0.01$ ***$P<0.001$

Figure A.3

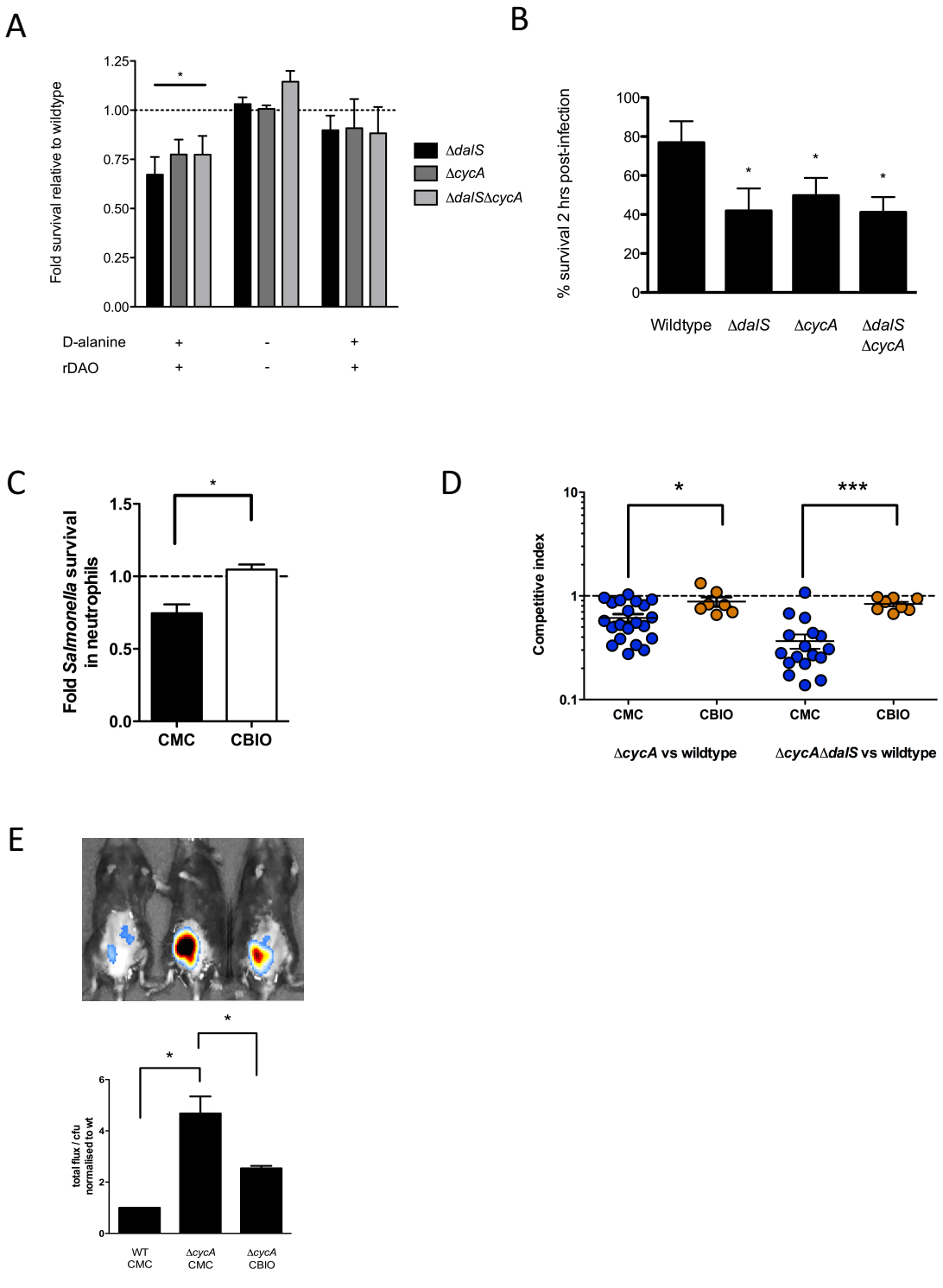
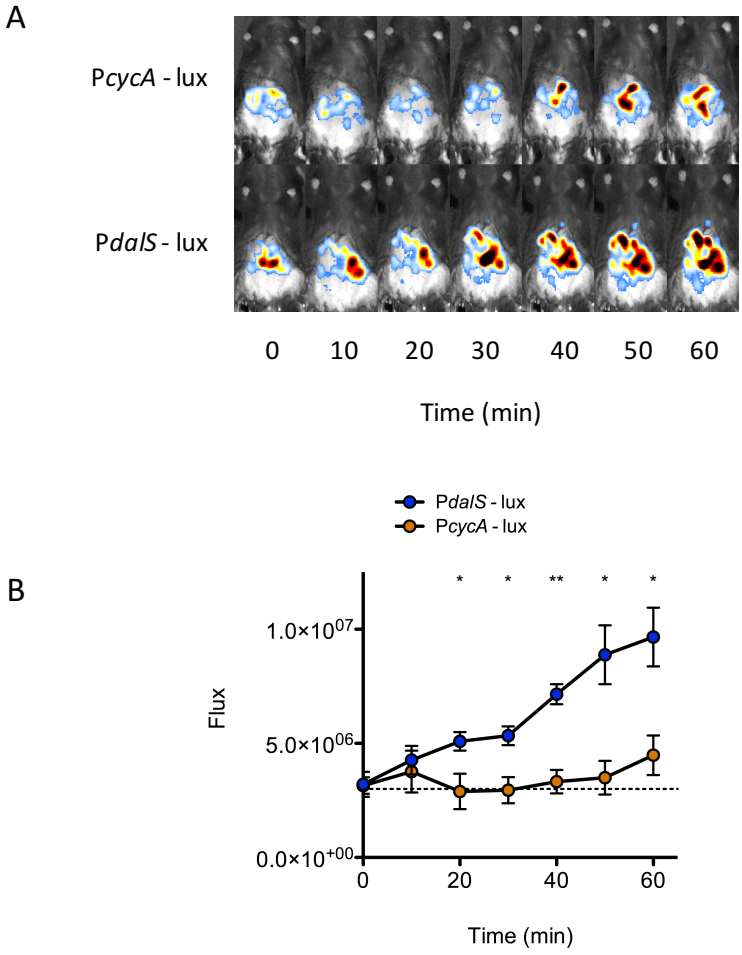

A
Fold survival relative to wildtype
1.25
1.00
0.75
0.50
0.25
0.00
*
ΔdalS
ΔcycA
ΔdalSΔcycA
D-alanine + - +
rDAO + - +
B
% survival 2 hrs post-infection
100
80
60
40
20
0
*
*
*
Wildtype
ΔdalS
ΔcycA
ΔdalS ΔcycA
C
Fold Salmonella survival in neutrophils
1.5
1.0
0.5
0.0
*
CMC
CBIO
D
Competitive index
10
1
0.1
*
***
CMC
CBIO
CMC
CBIO
ΔcycA vs wildtype
ΔcycAΔdalS vs wildtype
E
*
*
total flux / cfu normalised to wt
6
4
2
0
WT CMC
ΔcycA CMC
ΔcycA CBIO

Figure A.4. *cycA* and *dalS* are differentially regulated *in vivo.* C57BL/6 mice were infected with *Salmonella* containing either P*dalS-lux* or P*cycA-lux*. Expression of P*dalS* occurs immediately upon infected where as P*cycA* is delayed by 40 mins **(A).** Total flux was measured every 10 mins **(B).** * $P<0.05$, ** $P<0.01$

Figure A.4


A
PcycA - lux
PdalS - lux
0
10
20
30
40
50
60
Time (min)
B
PdalS - lux
PcycA - lux
*
*
**
*
*
1.0×10^07
5.0×10^06
0.0×10^+00
Flux
0
20
40
60
Time (min)

REFERENCES

1. **Sender R**, **Fuchs S**, **Milo R**. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol **14**:e1002533.

2. **Akiba M**, **Kusumoto M**, **Iwata T**. 2011. Rapid identification of *Salmonella enterica* serovars, Typhimurium, Choleraesuis, Infantis, Hadar, Enteritidis, Dublin and Gallinarum, by multiplex PCR. J Microbiol Methods **85**:9–15.

3. **Ochman H**, **Groisman EA**. 1994. The origin and evolution of species differences in *Escherichia coli* and *Salmonella* typhimurium. EXS **69**:479–93.

4. **Fookes M**, **Schroeder GN**, **Langridge GC**, **Blondel CJ**, **Mammina C**, **Connor TR**, **Seth-Smith H**, **Vernikos GS**, **Robinson KS**, **Sanders M**, **Petty NK**, **Kingsley RA**, **Bäumler AJ**, **Nuccio S-P**, **Contreras I**, **Santiviago CA**, **Maskell D**, **Barrow P**, **Humphrey T**, **Nastasi A**, **Roberts M**, **Frankel G**, **Parkhill J**, **Dougan G**, **Thomson NR**. 2011. *Salmonella bongori* provides insights into the evolution of the *Salmonellae*. PLoS Pathog **7**:e1002191.

5. **Majowicz SE**, **Musto J**, **Scallan E**, **Angulo FJ**, **Kirk M**, **O'Brien SJ**, **Jones TF**, **Fazil A**, **Hoekstra RM**. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clin Infect Dis **50**:882–9.

6. **Haraga A**, **Ohlson MB**, **Miller SI**. 2008. Salmonellae interplay with host cells. Nat Rev Microbiol **6**:53–66.

7. **Crump JA**, **Luby SP**, **Mintz ED**. 2004. The global burden of typhoid fever. Bull World Health Organ **82**:346–53.

8. **Crump JA**, **Mintz ED**. 2010. Global trends in typhoid and paratyphoid Fever. Clin Infect Dis **50**:241–6.

9. **Feasey NA**, **Dougan G**, **Kingsley RA**, **Heyderman RS**, **Gordon MA**. 2012. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet **379**:2489–2499.

10. **Ramachandran G**, **Perkins DJ**, **Schmidlein PJ**, **Tulapurkar ME**, **Tennant SM**. 2015. Invasive *Salmonella* Typhimurium ST313 with naturally attenuated flagellin elicits reduced inflammation and replicates within macrophages. PLoS Negl Trop Dis **9**:e3394.

11. **Hall RM**. 2010. *Salmonella* genomic islands and antibiotic resistance in *Salmonella enterica*. Future Microbiol **5**:1525–38.

12. **Monack DM**, **Bouley DM**, **Falkow S**. 2004. *Salmonella* typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ Mice and can be reactivated by IFNγ neutralization. J Exp Med **199**.

13. **Menendez A**, **Arena ET**, **Guttman JA**, **Thorson L**, **Vallance BA**, **Vogl W**, **Finlay BB**. 2009. *Salmonella* infection of gallbladder epithelial cells drives local inflammation and injury in a model of acute typhoid fever. J Infect Dis **200**:1703–13.

14. **Kaper JB**, **Nataro JP**, **Mobley HLT**. 2004. Pathogenic *Escherichia coli.* Nat Rev Microbiol **2**:123–140.

15. **Croxen MA**, **Finlay BB**. 2009. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol **8**:26.

16. **Nataro JP**, **Kaper JB**. 1998. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev **11**:142–201.

17. **Johnson JR**, **Russo TA**. 2002. Extraintestinal pathogenic *Escherichia coli* : "The other bad *E. coli* ." J Lab Clin Med **139**:155–162.

18. **Foxman B**, **Zhang L**, **Palin K**, **Tallman P**, **Marrs CF**. 1995. Bacterial virulence characteristics of *Escherichia coli* isolates from first-time urinary tract infection. J Infect Dis **171**:1514–21.

19. **Marrs CF**, **Zhang L**, **Foxman B**. 2005. *Escherichia coli* mediated urinary tract infections: Are there distinct uropathogenic *E. coli* (UPEC) pathotypes? FEMS Microbiol Lett **252**.

20. **Flores-Mireles AL**, **Walker JN**, **Caparon M**, **Hultgren SJ**. 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol **13**:269–284.

21. **Hooton TM**. 2012. Clinical practice. Uncomplicated urinary tract infection. N Engl J Med **366**:1028–37.

22. **Foxman B**. 1990. Recurring urinary tract infection: incidence and risk factors. Am J Public Health **80**:331–3.

23. **Stamm WE**, **Norrby SR**. 2001. Urinary tract infections: disease panorama and challenges. J Infect Dis **183**:S1–S4.

24. **Litwin MS**, **Saigal CS**, **Yano EM**, **Avila C**, **Geschwind SA**, **Hanley JM**, **Joyce GF**, **Madison R**, **Pace J**, **Polich SM**, **Wang M**. 2005. Urologic diseases in America Project: analytical methods and principal findings. J Urol **173**:933–7.

25. **Welch RA**, **Burland V**, **Plunkett G**, **Redford P**, **Roesch P**, **Rasko D**, **Buckles EL**, **Liou S-R**, **Boutin A**, **Hackett J**, **Stroud D**, **Mayhew GF**, **Rose DJ**, **Zhou S**, **Schwartz DC**, **Perna NT**, **Mobley HLT**, **Donnenberg MS**, **Blattner FR**. 2002. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. Proc Natl Acad Sci U S A **99**:17020–4.

26. **Chen SL**, **Hung C-S**, **Xu J**, **Reigstad CS**, **Magrini V**, **Sabo A**, **Blasiar D**, **Bieri T**, **Meyer RR**, **Ozersky P**, **Armstrong JR**, **Fulton RS**, **Latreille JP**, **Spieth J**, **Hooton TM**, **Mardis ER**, **Hultgren SJ**, **Gordon JI**. 2006. Identification of genes subject to positive selection in uropathogenic strains of *Escherichia coli*: a comparative genomics approach. Proc Natl Acad Sci U S A **103**:5977–82.

27. **Coburn B**, **Grassl GA**, **Finlay BB**. 2007. *Salmonella*, the host and disease: a brief review. Immunol Cell Biol **85**:112–118.

28. **Santos RL**, **Bäumler AJ**. 2004. Cell tropism of *Salmonella enterica*. Int J Med Microbiol **294**:225–233.

29. **Hase K**, **Kawano K**, **Nochi T**, **Pontes GS**, **Fukuda S**, **Ebisawa M**, **Kadokura K**, **Tobe T**, **Fujimura Y**, **Kawano S**, **Yabashi A**, **Waguri S**, **Nakato G**, **Kimura S**, **Murakami T**, **Iimura M**, **Hamura K**, **Fukuoka S-I**, **Lowe AW**, **Itoh K**, **Kiyono H**, **Ohno H**. 2009. Uptake through glycoprotein 2 of FimH+ bacteria by M cells initiates mucosal immune response. Nature **462**:226–230.

30. **Frost AJ**, **Bland AP**, **Wallis TS**. 1997. The early dynamic response of the calf ileal epithelium to *Salmonella* typhimurium. Vet Pathol **34**:369–86.

31. **Hänisch J**, **Ehinger J**, **Ladwein M**, **Rohde M**, **Derivery E**, **Bosse T**, **Steffen A**, **Bumann D**, **Misselwitz B**, **Hardt W-D**, **Gautreau A**, **Stradal TEB**, **Rottner K**. 2010. Molecular dissection of *Salmonella*-induced membrane ruffling versus invasion. Cell Microbiol **12**:84–98.

32. **Tahoun A**, **Mahajan S**, **Paxton E**, **Malterer G**, **Donaldson DS**, **Wang D**, **Tan A**, **Gillespie TL**, **O'Shea M**, **Roe AJ**, **Shaw DJ**, **Gally DL**, **Lengeling A**, **Mabbott NA**, **Haas J**, **Mahajan A**. 2012. *Salmonella* transforms follicle-associated epithelial cells into M cells to promote intestinal invasion. Cell Host Microbe.

33. **Chieppa M**, **Rescigno M**, **Huang AYC**, **Germain RN**. 2006. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med **203**:2841–2852.

34. **Niess JH**. 2005. CX3CR1-Mediated Dendritic Cell Access to the Intestinal Lumen and Bacterial Clearance. Science **307**:254–258.

35. **Oh Y-K**, **Alpuche-Aranda C**, **Berthiaume E**, **Jinks T**, **Miller SI**, **Swanson JA**. 1996. Rapid and complete fusion of macrophage lysosomes with phagosomes containing *Salmonella* typhimurium. Infect Immun **64**:3877–3883.

36. **Fields PI**, **Groisman EA**, **Heffron F**. 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243**:1059–62.

37. **Miller SI**, **Mekalanos JJ**. 1990. Constitutive expression of the phoP regulon attenuates *Salmonella* virulence and survival within macrophages. J Bacteriol **172**:2485–90.

38. **Shiloh MU**, **MacMicking JD**, **Nicholson S**, **Brause JE**, **Potter S**, **Marino M**, **Fang F**, **Dinauer M**, **Nathan C**. 1999. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity **10**:29–38.

39. **Vazquez-Torres A**, **Jones-Carson J**, **Mastroeni P**, **Ischiropoulos H**, **Fang FC**. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. J Exp Med **192**:227–36.

40. **Shea JE**, **Hensel M**, **Gleeson C**, **Holden DW**. 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella* typhimurium. Proc Natl Acad Sci U S A **93**:2593–7.

41. **Tomljenovic-Berube AM**, **Mulder DT**, **Whiteside MD**, **Brinkman FSL**, **Coombes BK**. 2010. Identification of the Regulatory Logic Controlling *Salmonella* Pathoadaptation by the SsrA-SsrB Two-Component System. PLoS Genet **6**:e1000875.

42. **Dunlap NE**, **Benjamin WH**, **Berry AK**, **Eldridge JH**, **Briles DE**. 1992. A "safe-site" for *Salmonella* typhimurium is within splenic polymorphonuclear cells. Microb Pathog **13**:181–190.

43. **Worley MJ**, **Ching KHL**, **Heffron F**. 2000. *Salmonella* SsrB activates a global regulon of horizontally acquired genes. Mol Microbiol **36**:749–761.

44. **Wurpel DJ**, **Beatson SA**, **Totsika M**, **Petty NK**, **Schembri MA**. 2013. Chaperone-usher fimbriae of *Escherichia coli*. PLoS One **8**:e52835.

45. **Hadjifrangiskou M**, **Gu AP**, **Pinkner JS**, **Kostakioti M**, **Zhang EW**, **Greene SE**, **Hultgren SJ**. 2012. Transposon mutagenesis identifies uropathogenic *Escherichia coli* biofilm factors. J Bacteriol **194**:6195–205.

46. **Hannan TJ**, **Totsika M**, **Mansfield KJ**, **Moore KH**, **Schembri MA**, **Hultgren SJ**. 2012. Host-pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic *Escherichia coli* bladder infection. FEMS Microbiol Rev **36**:616–48.

47. **Eto DS**, **Jones TA**, **Sundsbak JL**, **Mulvey MA**. 2007. Integrin-Mediated host cell invasion by Type 1–piliated uropathogenic *Escherichia coli*. PLoS Pathog **3**:100.

48. **Martinez JJ**, **Hultgren SJ**. 2002. Requirement of Rho-family GTPases in the invasion of Type 1-piliated uropathogenic *Escherichia coli*. Cell Microbiol **4**:19–28.

49. **Song J**, **Bishop BL**, **Li G**, **Grady R**, **Stapleton A**, **Abraham SN**. 2009. TLR4-mediated expulsion of bacteria from infected bladder epithelial cells. Proc Natl Acad Sci **106**:14966–14971.

50. **Anderson GG**. 2003. Intracellular bacterial biofilm-like pods in urinary tract infections. Science **301**:105–107.

51. **Hannan TJ**, **Mysorekar IU**, **Hung CS**, **Isaacson-Schmid ML**, **Hultgren SJ**. 2010. Early severe inflammatory responses to uropathogenic *E. coli* predispose to chronic and recurrent urinary tract infection. PLoS Pathog **6**.

52. **Justice SS**, **Hunstad DA**, **Seed PC**, **Hultgren SJ**. 2006. Filamentation by *Escherichia coli* subverts innate defenses during urinary tract infection. Proc Natl Acad Sci **103**:19884–19889.

53. **O'Hanley P**, **Lalonde G**, **Ji G**. 1991. Alpha-hemolysin contributes to the pathogenicity of piliated digalactoside-binding *Escherichia coli* in the kidney: efficacy of an alpha-hemolysin vaccine in preventing renal injury in the BALB/c mouse model of pyelonephritis. Infect Immun **59**:1153–61.

54. **Blango MG**, **Ott EM**, **Erman A**, **Veranic P**, **Mulvey MA**. 2014. Forced resurgence and targeting of intracellular uropathogenic *Escherichia coli* reservoirs. PLoS One **9**:e93327.

55. **Walters MS**, **Lane MC**, **Vigil PD**, **Smith SN**, **Walk ST**, **Mobley HLT**. 2012. Kinetics of uropathogenic *Escherichia coli* metapopulation movement during urinary tract infection. MBio **3**.

56. **Wright KJ**, **Hultgren SJ**. 2006. Sticky fibers and uropathogenesis: bacterial adhesins in the urinary tract. Future Microbiol **1**:75–87.

57. **Rice JC**. 2005. Pyelonephritic *Escherichia coli* expressing P fimbriae decrease immune response of the mouse kidney. J Am Soc Nephrol **16**:3583–3591.

58. **Sivick KE**, **Mobley HLT**. 2010. Waging war against uropathogenic *Escherichia coli*: winning back the urinary tract. Infect Immun **78**:568–85.

59. **Iwasaki A**, **Medzhitov R**. 2015. Control of adaptive immunity by the innate immune system. Nat Immunol **16**:343–353.

60. **Cooper MD**, **Alder MN**. 2006. The evolution of adaptive immune systems. Cell **124**:815–822.

61. **Kumar H**, **Kawai T**, **Akira S**. 2011. Pathogen recognition by the innate immune system. Int Rev Immunol **30**:16–34.

62. **Hooper L V.**, **Littman DR**, **Macpherson AJ**. 2012. Interactions between the microbiota and the immune system. Science **336**.

63. **Springer TA**. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell **76**:301–314.

64. **Gasteiger G**, **Rudensky AY**. 2014. Interactions between innate and adaptive lymphocytes. Nat Rev Immunol **14**:631–639.

65. **Kawamoto H**, **Minato N**. 2004. Myeloid cells. Int J Biochem Cell Biol **36**:1374–1379.

66. **Ginhoux F**, **Jung S**. 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat Rev Immunol **14**:392–404.

67. **Voehringer D**. 2013. Protective and pathological roles of mast cells and basophils. Nat Rev Immunol **13**:362–375.

68. **Rosenberg HF**, **Dyer KD**, **Foster PS**. 2012. Eosinophils: changing perspectives in health and disease. Nat Rev Immunol **13**:9–22.

69. **Merad M**, **Sathe P**, **Helft J**, **Miller J**, **Mortha A**. 2013. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol **31**:563–604.

70. **Phillipson M**, **Kubes P**. 2011. The neutrophil in vascular inflammation. Nat Med **17**:1381–1390.

71. **Amulic B**, **Cazalet C**, **Hayes GL**, **Metzler KD**, **Zychlinsky A**. 2012. Neutrophil function: from mechanisms to disease. Annu Rev Immunol **30**:459–489.

72. **Galli SJ**, **Borregaard N**, **Wynn TA**. 2011. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. Nat Immunol **12**:1035–1044.

73. **Colotta F**, **Re F**, **Polentarutti N**, **Sozzani S**, **Mantovani A**. 1992. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood **80**:2012–20.

74. **Summers C**, **Rankin SM**, **Condliffe AM**, **Singh N**, **Peters AM**, **Chilvers ER**. 2010. Neutrophil kinetics in health and disease. Trends Immunol **31**:318–24.

75. **Peters AM**. 1998. Just how big is the pulmonary granulocyte pool? Clin Sci **94**:7–19.

76. **Ley K**, **Laudanna C**, **Cybulsky MI**, **Nourshargh S**. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol **7**:678–689.

77. **Sadik CD**, **Kim ND**, **Luster AD**. 2011. Neutrophils cascading their way to inflammation. Trends Immunol **32**:452–460.

78. **Hong C**, **Kidani Y**, **A-Gonzalez N**, **Phung T**, **Ito A**, **Rong X**, **Ericson K**, **Mikkola H**, **Beaven SW**, **Miller LS**, **Shao W-H**, **Cohen PL**, **Castrillo A**, **Tontonoz P**, **Bensinger SJ**. 2012. Coordinate regulation of neutrophil homeostasis by liver X receptors in mice. J Clin Invest **122**:337–47.

79. **Zarbock A**, **Ley K**. 2008. Mechanisms and consequences of neutrophil interaction with the endothelium. Am J Pathol **172**:1–7.

80. **Sabroe I**, **Dower SK**, **Whyte MKB**. 2005. The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. Clin Infect Dis **41**:421–426.

81. **Parker LC**. 2005. The expression and roles of Toll-like receptors in the biology of the human neutrophil. J Leukoc Biol **77**:886–892.

82. **Faurschou M**, **Borregaard N**. 2003. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect **5**:1317–27.

83. **Borregaard N**, **Cowland JB**. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood **89**:3503–21.

84. **Brogden KA**. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol **3**:238–250.

85. **Segal BH**, **Leto TL**, **Gallin JI**, **Malech HL**, **Holland SM**. 2000. Genetic, biochemical, and clinical features of chronic granulomatous disease. Medicine (Baltimore) **79**:170–200.

86. **Panday A**, **Sahoo MK**, **Osorio D**, **Batra S**. 2015. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. Cell Mol Immunol **12**:5–23.

87. **Bogdan C**, **Röllinghoff M**, **Diefenbach A**. 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol **12**:64–76.

88. **Winterbourn CC**, **Hampton MB**, **Livesey JH**, **Kettle AJ**. 2006. Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: implications for microbial killing. J Biol Chem **281**:39860–39869.

89. **Rosen H**, **Crowley JR**, **Heinecke JW**. 2002. Human neutrophils use the myeloperoxidase-hydrogen peroxide-chloride system to chlorinate but not nitrate bacterial proteins during phagocytosis. J Biol Chem **277**:30463–8.

90. **Brinkmann V**. 2004. Neutrophil extracellular traps kill bacteria. Science **303**:1532–1535.

91. **Urban CF**, **Ermert D**, **Schmid M**, **Abu-Abed U**, **Goosmann C**, **Nacken W**, **Brinkmann V**, **Jungblut PR**, **Zychlinsky A**. 2009. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. PLoS Pathog **5**:e1000639.

92. **Buchanan JT**, **Simpson AJ**, **Aziz RK**, **Liu GY**, **Kristian SA**, **Kotb M**, **Feramisco J**, **Nizet V**. 2006. DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. Curr Biol **16**:396–400.

93. **Metzler KD**, **Fuchs TA**, **Nauseef WM**, **Reumaux D**, **Roesler J**, **Schulze I**, **Wahn V**, **Papayannopoulos V**, **Zychlinsky A**. 2011. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood **117**.

94. **Yost CC**, **Cody MJ**, **Harris ES**, **Thornton NL**, **McInturff AM**, **Martinez ML**, **Chandler NB**, **Rodesch CK**, **Albertine KH**, **Petti CA**, **Weyrich AS**, **Zimmerman GA**. 2009. Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. Blood **113**.

95. **Kennedy AD**, **DeLeo FR**. 2009. Neutrophil apoptosis and the resolution of infection. Immunol Res **43**:25–61.

96. **Woodfin A**, **Voisin M-B**, **Beyrau M**, **Colom B**, **Caille D**, **Diapouli F-M**, **Nash GB**, **Chavakis T**, **Albelda SM**, **Rainger GE**, **Meda P**, **Imhof BA**, **Nourshargh S**. 2011. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils *in vivo*. Nat Immunol **12**:761–769.

97. **Luong TT**, **Lee CY**. 2002. Overproduction of type 8 capsular polysaccharide augments *Staphylococcus aureus* virulence. Infect Immun **70**:3389–95.

98. **Allen L-AH**, **Beecher BR**, **Lynch JT**, **Rohner O V**, **Wittine LM**. 2005. *Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. J Immunol **174**:3658–67.

99. **Green ER**, **Clark S**, **Crimmins GT**, **Mack M**, **Kumamoto CA**, **Mecsas J**. 2016. Fis is essential for *Yersinia pseudotuberculosis* virulence and protects against reactive oxygen species produced by phagocytic cells during Infection. PLoS Pathog **12**.

100. **Krebs HA**. 1935. Metabolism of amino-acids: Deamination of amino-acids. Biochem J **29**:1620–44.

101. **Molla G**, **Sacchi S**, **Bernasconi M**, **Pilone MS**, **Fukui K**, **Pollegioni L**. 2006. Characterization of human D-amino acid oxidase. FEBS Lett **580**:2358–2364.

102. **Umhau S**, **Pollegioni L**, **Molla G**, **Diederichs K**, **Welte W**, **Pilone MS**, **Ghisla S**. 2000. The x-ray structure of D-amino acid oxidase at very high resolution identifies the chemical mechanism of flavin-dependent substrate dehydrogenation. Proc Natl Acad Sci **97**:12463–12468.

103. **Snyder SH**, **Kim PM**. 2000. D-amino acids as putative neurotransmitters: focus on D-serine. Neurochem Res **25**:553–560.

104. **Sasabe J**, **Miyoshi Y**, **Rakoff-Nahoum S**, **Zhang T**, **Mita M**, **Davis BM**, **Hamase K**, **Waldor MK**. 2016. Interplay between microbial d-amino acids and host d-amino acid oxidase modifies murine mucosal defence and gut microbiota. Nat Microbiol **1**:16125.

105. **Wolosker H**, **Dumin E**, **Balan L**, **Foltyn VN**. 2008. D-Amino acids in the brain: d-serine in neurotransmission and neurodegeneration. FEBS J **275**:3514–3526.

106. **Hashimoto A**, **Nishikawa T**, **Oka T**, **Takahashi K**. 1993. Endogenous D-serine in rat brain: N-methyl-D-aspartate receptor-related distribution and aging. J Neurochem **60**:783–6.

107. **Iwasa S**, **Tabara H**, **Song Z**, **Nakabayashi M**, **Yokoyama Y**, **Fukushima T**. 2011. Inhibition of D-amino acid oxidase activity by antipsychotic drugs evaluated by a fluorometric assay using D-kynurenine as substrate. Yakugaku Zasshi **131**:1111–1116.

108. **Yagi K**, **Ozawa T**, **Nagastu T**. 1960. Mechanism of inhibition of D-amino acid oxidase. IV. Inhibitory action of chlorpromazine. Biochim Biophys Acta **43**:310–7.

109. **Sawa A**. 2002. Schizophrenia: diverse approaches to a complex disease. Science **296**:692–695.

110. **Chumakov I**, **Blumenfeld M**, **Guerassimenko O**, **Cavarec L**, **Palicio M**, **Abderrahim H**, **Bougueleret L**, **Barry C**, **Tanaka H**, **La Rosa P**, **Puech A**, **Tahri N**, **Cohen-Akenine A**, **Delabrosse S**, **Lissarrague S**, **Picard F-P**, **Maurice K**, **Essioux L**, **Millasseau P**, **Grel P**, **Debailleul V**, **Simon A-M**, **Caterina D**, **Dufaure I**, **Malekzadeh K**, **Belova M**, **Luan J-J**, **Bouillot M**, **Sambucy J-L**, **Primas G**, **Saumier M**, **Boubkiri N**, **Martin-Saumier S**, **Nasroune M**, **Peixoto H**, **Delaye A**, **Pinchot V**, **Bastucci M**, **Guillou S**, **Chevillon M**, **Sainz-Fuertes R**, **Meguenni S**, **Aurich-Costa J**, **Cherif D**, **Gimalac A**, **Van Duijn C**, **Gauvreau D**, **Ouelette G**, **Fortier I**, **Realson J**, **Sherbatich T**, **Riazanskaia N**, **Rogaev E**, **Raeymaekers P**, **Aerssens J**, **Konings F**, **Luyten W**, **Macciardi F**, **Sham PC**, **Straub RE**, **Weinberger DR**, **Cohen N**, **Cohen D**. 2002. Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. Proc Natl Acad Sci **99**:13675–13680.

111. **Cline MJ**, **Lehrer RI**. 1969. D-amino acid oxidase in leukocytes: A possible D-amino-acid-linked antimicrobial system. Microbiology **62**:756–63.

112. **Tuinema BR**, **Reid-Yu SA**, **Coombes BK**. 2014. *Salmonella* evades D-amino acid oxidase to promote infection in neutrophils. MBio **5**.

113. **Robinson JM**, **Briggs RT**, **Karnovsky MJ**. 1978. Localization of D-amino acid oxidase on the surface of human polymorphonuclear leukocytes. J Cell Biol **77**:59–71.

114. **Nakamura H**, **Fang J**, **Maeda H**. 2012. Protective role of D-amino acid oxidase against *Staphylococcus aureus* infection. Infect Immun **80**:1546–53.

115. **Usuda N**, **Yokota S**, **Hashimoto T**, **Nagata T**. 1986. Immunocytochemical localization of D-amino acid oxidase in the central clear matrix of rat kidney peroxisomes. J Histochem Cytochem **34**:1709–18.

116. **Maekawa M**, **Okamura T**, **Kasai N**, **Yuuichi Hori**, **Summer KH**, **Konno R**. 2005. d-Amino-acid Oxidase Is Involved in d-Serine-Induced Nephrotoxicity. Chem Res Toxicol **18**:1678–1682.

117. **Kawasaka K**, **Tatsumi N**. 1998. D-amino acid oxidase activity in urine obtained from patients with renal disorders. Clin Nephrol **49**:214–20.

118. **Lyle LR**, **Jutila JW**. 1968. D-amino acid oxidase induction in the kidneys of germ-free mice. J Bacteriol **96**:606–608.

119. **Konno R**, **Yasumura Y**. 1983. Mouse mutant deficient in D-amino acid oxidase activity. Genetics **103**.

120. **Zhao W**, **Konno R**, **Zhou X-J**, **Yin M**, **Wang Y-X**. 2008. Inhibition of D-amino-acid oxidase activity induces pain relief in mice. Cell Mol Neurobiol **28**:581–591.

121. **Williams RE**, **Lock EA**. 2005. Sodium benzoate attenuates d-serine induced nephrotoxicity in the rat. Toxicology **207**:35–48.

122. **Gong N**, **Gao Z-Y**, **Wang Y-C**, **Li X-Y**, **Huang J-L**, **Hashimoto K**, **Wang Y-X**. 2010. A series of D-amino acid oxidase inhibitors specifically prevents and reverses formalin-induced tonic pain in rats. J Pharmacol Exp Ther **336**.

123. **Ferraris D**, **Duvall B**, **Ko Y-S**, **Thomas AG**, **Rojas C**, **Majer P**, **Hashimoto K**, **Tsukamoto T**. 2008. Synthesis and biological evaluation of D-amino acid oxidase inhibitors. J Med Chem **51**:3357–3359.

124. **Sparey T**, **Abeywickrema P**, **Almond S**, **Brandon N**, **Byrne N**, **Campbell A**, **Hutson PH**, **Jacobson M**, **Jones B**, **Munshi S**, **Pascarella D**, **Pike A**, **Prasad GS**, **Sachs N**, **Sakatis M**, **Sardana V**, **Venkatraman S**, **Young MB**. 2008. The discovery of fused pyrrole carboxylic acids as novel, potent d-amino acid oxidase (DAO) inhibitorsBioorganic & Medicinal Chemistry Letters.

125. **Adage T**, **Trillat A-C**, **Quattropani A**, **Perrin D**, **Cavarec L**, **Shaw J**, **Guerassimenko O**, **Giachetti C**, **Gréco B**, **Chumakov I**, **Halazy S**, **Roach A**, **Zaratin P**. 2008. *In vitro* and *in vivo* pharmacological profile of AS057278, a selective D-amino acid oxidase inhibitor with potential anti-psychotic properties. Eur Neuropsychopharmacol **18**:200–214.

126. **Adage T**, **Trillat A**, **Quattropani A**, **Perrin D**. 2008. *In vitro* and *in vivo* pharmacological profile of AS057278, a selective D-amino acid oxidase inhibitor with potential anti-psychotic properties. European.

127. **Fang Q**, **Hopkins S**, **Heffernan M**, **Chytil M**. 2011. Pyrrole and pyrazole DAAO inhibitors. US Pat 7,893,098.

128. **Duplantier AJ**, **Becker SL**, **Bohanon MJ**, **Borzilleri KA**, **Chrunyk BA**, **Downs JT**, **Hu L-Y**, **El-Kattan A**, **James LC**, **Liu S**, **Lu J**, **Maklad N**, **Mansour MN**, **Mente S**, **Piotrowski MA**, **Sakya SM**, **Sheehan S**, **Steyn SJ**, **Strick CA**, **Williams VA**, **Zhang L**. 2009. Discovery, SAR, and pharmacokinetics of a novel 3-hydroxyquinolin-2(1 H)-one series of potent D-amino acid oxidase (DAAO) inhibitors. J Med Chem **52**:3576–3585.

129. **Radkov AD**, **Moe LA**. 2014. Bacterial synthesis of d-amino acids. Appl Microbiol Biotechnol **98**:5363–5374.

130. **Lam H**, **Oh D-C**, **Cava F**, **Takacs CN**, **Clardy J**, **de Pedro MA**, **Waldor MK**. 2009. D-amino acids govern stationary phase cell wall remodeling in bacteria. Science **325**:1552–1555.

131. **Faraci WS**, **Walsh CT**. 1988. Racemization of alanine by the alanine racemases from *Salmonella* typhimurium and *Bacillus stearothermophilus*: energetic reaction profiles. Biochemistry **27**:3267–76.

132. **Walsh CT**. 1989. Enzymes in the D-alanine branch of bacterial cell wall peptitoglycan assembly. J Biol Chem **264**:2393–2396.

133. **Wasserman SA**, **Daub E**, **Grisafi P**, **Botstein D**, **Walsh CT**. 1984. Catabolic alanine racemase from *Salmonella* typhimurium: DNA sequence, enzyme purification, and characterization. Biochemistry **23**:5182–7.

134. **Galakatos NG**, **Daub E**, **Botstein D**, **Walsh CT**. 1986. Biosynthetic alr alanine racemase from *Salmonella* typhimurium: DNA and protein sequence determination. Biochemistry **25**:3255–60.

135. **Baumgart F**, **Rodríguez-Crespo I**. 2008. D-amino acids in the brain: the biochemistry of brain serine racemase. FEBS J **275**:3538–45.

136. **Foltyn VN**. 2004. Serine racemase modulates intracellular D-serine levels through an elimination activity. J Biol Chem **280**:1754–1763.

137. **Silbernagl S**, **Völker K**, **Dantzler WH**. 1999. D-Serine is reabsorbed in rat renal pars recta. Am J Physiol - Ren Physiol **276**.

138. **Anfora AT**, **Welch RA**. 2006. DsdX is the second D-serine transporter in uropathogenic *Escherichia coli* clinical isolate CFT073. J Bacteriol **188**.

139. **Roesch PL**, **Redford P**, **Batchelet S**, **Moritz RL**, **Pellett S**, **Haugen BJ**, **Blattner FR**, **Welch RA**. 2003. Uropathogenic *Escherichia coli* use D-serine deaminase to modulate infection of the murine urinary tract. Mol Microbiol **49**:55–67.

140. **Kaback HR**, **Kostellow AB**. 1968. Glycine uptake in *Escherichia coli*. I. Glycine uptake by whole cells of *Escherichia coli* W+ and a D-serine-resistant. J Biol Chem **243**:1384–9.

141. **Cosloy SD**. 1973. D-serine transport system in *Escherichia coli* K-12. J Bacteriol **114**:679–84.

142. **Robbins JC**, **Oxender DL**. 1973. Transport systems for alanine, serine, and glycine in *Escherichia coli* K-12. J Bacteriol **116**:12–8.

143. **Pulvermacher SC**, **Stauffer LT**, **Stauffer G V**. 2009. Role of the sRNA GcvB in regulation of *cycA* in *Escherichia coli*. Microbiology **155**:106–14.

144. **Sharma CM**, **Papenfort K**, **Pernitzsch SR**, **Mollenkopf H-J**, **Hinton JCD**, **Vogel J**. 2011. Pervasive post-transcriptional control of genes involved in amino acid metabolism by the Hfq-dependent GcvB small RNA. Mol Microbiol **81**:1144–1165.

145. **Coornaert A**, **Chiaruttini C**, **Springer M**, **Guillier M**. 2013. Post-transcriptional control of the *Escherichia coli* PhoQ-PhoP two-component system by multiple sRNAs involves a novel pairing region of GcvB. PLoS Genet **9**.

146. **Subashchandrabose S**, **Hazen TH**, **Brumbaugh AR**, **Himpsl SD**, **Smith SN**, **Ernst RD**, **Rasko DA**, **Mobley HLT**. 2014. Host-specific induction of *Escherichia coli* fitness genes during human urinary tract infection. Proc Natl Acad Sci **111**:18327–18332.

147. **Anfora AT**, **Haugen BJ**, **Roesch P**, **Redford P**, **Welch RA**. 2007. Roles of serine accumulation and catabolism in the colonization of the murine urinary tract by *Escherichia coli* CFT073. Infect Immun **75**:5298–5304.

148. **Osborne SE**, **Tuinema BR**, **Mok MCY**, **Lau PS**, **Bui NK**, **Tomljenovic-Berube AM**, **Vollmer W**, **Zhang K**, **Junop M**, **Coombes BK**. 2012. Characterization of DalS, an ATP-binding cassette transporter for D-Alanine, and its role in pathogenesis in *Salmonella enterica*. J Biol Chem **287**:15242–15250.

149. **Kawai T**, **Akira S**. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol **11**:373–384.

150. **Takeuchi O**, **Akira S**. 2010. Pattern recognition receptors and inflammation. Cell **140**:805–820.

151. **Medzhitov R**. 2007. Recognition of microorganisms and activation of the immune response. Nature **449**:819–826.

152. **Akira S**, **Takeda K**. 2004. Toll-like receptor signalling. Nat Rev Immunol **4**:499–511.
153. **Coers J**. 2013. Self and non-self discrimination of intracellular membranes by the innate immune system. PLoS Pathog **9**.

154. **Matzinger P**. 2002. The danger model: A renewed sense of self. Science **296**:301–305.

155. **Stock AM**, **Robinson VL**, **Goudreau PN**. 2000. Two-component signal transduction. Reactions **69**:183–215.

156. **Stock JB**, **Ninfa AJ**, **Stock AM**. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol Rev **53**:450–90.

157. **Ochman H**, **Soncini FC**, **Solomon F**, **Groisman EA**. 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proc Natl Acad Sci U S A **93**:7800–4.

158. **Osborne SE and**, **Coombes BK**. 2011. Transcriptional priming of *Salmonella* Pathogenicity Island-2 precedes cellular invasion. PLoS One **6**:e21648.

159. **Papp-Szabò E**, **Firtel M**, **Josephy PD**. 1994. Comparison of the sensitivities of *Salmonella* typhimurium oxyR and katG mutants to killing by human neutrophils. Infect Immun **62**:2662–8.

160. **Pilar AVC**, **Reid-Yu SA**, **Cooper CA**, **Mulder DT**, **Coombes BK**. 2012. GogB is an anti-inflammatory effector that limits tissue damage during *Salmonella* infection through interaction with human FBXO22 and Skp1. PLoS Pathog **8**:e1002773.

161. **Nawabi P**, **Catron DM**, **Haldar K**. 2008. Esterification of cholesterol by a type III secretion effector during intracellular *Salmonella* infection. Mol Microbiol **68**:173–185.

162. **Richards SM**, **Strandberg KL**, **Conroy M**, **Gunn JS**. 2012. Cationic antimicrobial peptides serve as activation signals for the *Salmonella* Typhimurium PhoPQ and PmrAB regulons in vitro and in vivo. Front Cell Infect Microbiol **2**:102.

163. **Burton NA**, **Schürmann N**, **Casse O**, **Steeb AK**, **Claudi B**, **Zankl J**, **Schmidt A**, **Bumann D**. 2014. Disparate impact of oxidative host defenses determines the fate of *Salmonella* during systemic infection in mice. Cell Host Microbe **15**:72–83.

164. **Sacchi S**. 2013. D-Serine metabolism: new insights into the modulation of D-amino acid oxidase activity. Biochem Soc Trans **41**:1551–1556.

165. **Umhau S**, **Pollegioni L**, **Molla G**, **Diederichs K**, **Welte W**, **Pilone MS**, **Ghisla S**. 2000. The x-ray structure of D-amino acid oxidase at very high resolution identifies the chemical mechanism of flavin-dependent substrate dehydrogenation. Proc Natl Acad Sci U S A **97**:12463–8.

166. **Pollegioni L**, **Piubelli L**, **Sacchi S**, **Pilone MS**, **Molla G**. 2007. Physiological functions of D-amino acid oxidases: from yeast to humans. Cell Mol Life Sci **64**:1373–1394.

167. **Segal AW**. 2005. How neutrophils kill microbes. Annu Rev Immunol **23**:197–223.

168. **Lehrer RI**, **Hanifin J**, **Cline MJ**. 1969. Defective bactericidal activity in myeloperoxidase-deficient human neutrophils. Nature **223**:78–79.

169. **Grisham MB**, **Jefferson MM**, **Melton DF**, **Thomas EL**. 1984. Chlorination of endogenous amines by isolated neutrophils **259**:10404–10413.

170. **Imlay JA**, **Chin SM**, **Linn S**. 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. Science **240**:640–2.

171. **Kohanski MA**, **Dwyer DJ**, **Hayete B**, **Lawrence CA**, **Collins JJ**. 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell **130**:797–810.

172. **Christman MF**, **Morgan RW**, **Jacobson FS**, **Ames BN**. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella* typhimurium. Cell **41**:753–62.

173. **Aussel L**, **Zhao W**, **Hébrard M**, **Guilhon AA**, **Viala JPM**, **Henri S**, **Chasson L**, **Gorvel JP**, **Barras F**, **Méresse S**. 2011. *Salmonella* detoxifying enzymes are sufficient to cope with the host oxidative burst. Mol Microbiol.

174. **Geddes K**, **Cruz F**, **Heffron F**. 2007. Analysis of cells targeted by *Salmonella* Type III Secretion *in vivo*. PLoS Pathog **3**:e196.

175. **Konno R**, **Sasaki M**, **Asakura S**, **Fukui K**, **Enami J**, **Niwa A**. 1997. D-amino-acid oxidase is not present in the mouse liver. Biochim Biophys Acta **1335**:173–81.

176. **D'aniello A**, **D'onofrio G**, **Pischetola M**, **D'aniello G**, **Vetere A**, **Petrucellin L**, **Fishefl GH**. 1993. Biological role of D-amino acid oxidase and D-aspartate oxidase. J Biol Chem **268**:26941–26949.

177. **Knodler LA**, **Vallance BA**, **Hensel M**, **Jäckel D**, **Finlay BB**, **Steele-Mortimer O**. 2003. *Salmonella* type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes. Mol Microbiol **49**:685–704.

178. **Winterbourn CC**, **Kettle AJ**. 2013. Redox reactions and microbial killing in the neutrophil phagosome. Antioxid Redox Signal **18**:642–660.

179. **Nauseef WM**. 2008. Nox enzymes in immune cells. Semin Immunopathol **30**:195–208.

180. **Loetscher Y**, **Wieser A**, **Lengefeld J**, **Kaiser P**, **Schubert S**, **Heikenwalder M**, **Hardt W-D**, **Stecher B**. 2012. *Salmonella* transiently reside in luminal neutrophils in the inflamed gut. PLoS One **7**:e34812.

181. **Mayadas TN**, **Cullere X**, **Lowell CA**. 2014. The multifaceted functions of neutrophils. Annu Rev Pathol Mech Dis **9**:181–218.

182. **Cheminay C**, **Chakravortty D**, **Hensel M**. 2004. Role of neutrophils in murine salmonellosis. Infect Immun **72**:468–77.

183. **Royet J**, **Dziarski R**. 2007. Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences. Nat Rev Microbiol **5**:264–277.

184. **Vazquez-Torres A**, **Xu Y**, **Jones-Carson J**, **Holden DW**, **Lucia SM**, **Dinauer MC**, **Mastroeni P**, **Fang FC**. 2000. *Salmonella* pathogenicity island 2–dependent evasion of the phagocyte NADPH oxidase. Science **287**:1655–1658.

185. **Winkelstein JA**, **Marino MC**, **Johnston RB**, **Boyle J**, **Curnutte J**, **Gallin JI**, **Malech HL**, **Holland SM**, **Ochs H**, **Quie P**, **Buckley RH**, **Foster CB**, **Chanock SJ**, **Dickler H**. 2000. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore) **79**:155–69.

186. **Messina CGM**, **Reeves EP**, **Roes J**, **Segal AW**. 2002. Catalase negative *Staphylococcus aureus* retain virulence in mouse model of chronic granulomatous disease. FEBS Lett **518**:107–10.

187. **Pitt J**, **Bernheimer HP**. 1974. Role of peroxide in phagocytic killing of pneumococci. Infect Immun **9**:48–52.

188. **Nakamura H**, **Fang J**, **Mizukami T**, **Nunoi H**, **Maeda H**. 2012. PEGylated d -amino acid oxidase restores bactericidal activity of neutrophils in chronic granulomatous disease via hypochlorite. Exp Biol Med **237**:703–708.

189. **Kuhns DB**, **Alvord WG**, **Heller T**, **Feld JJ**, **Pike KM**, **Marciano BE**, **Uzel G**, **DeRavin SS**, **Priel DAL**, **Soule BP**, **Zarember KA**, **Malech HL**, **Holland SM**, **Gallin JI**. 2010. Residual NADPH oxidase and survival in chronic granulomatous disease. N Engl J Med **363**:2600–2610.

190. **Coombes BK**, **Brown NF**, **Valdez Y**, **Brumell JH**, **Finlay BB**. 2004. Expression and secretion of *Salmonella* Pathogenicity Island-2 virulence genes in response to acidification exhibit differential requirements of a functional Type III secretion apparatus and SsaL. J Biol Chem **279**:49804–49815.

191. **Coombes BK**, **Wickham ME**, **Lowden MJ**, **Brown NF**, **Finlay BB**. 2005. Negative regulation of *Salmonella* pathogenicity island 2 is required for contextual control of virulence during typhoid. Proc Natl Acad Sci U S A **102**:17460–5.

192. **Gaunt GL**, **de Duve C**. 1976. Subcellular distribution of D-amino acid oxidase and catalase in rat brain. J Neurochem **26**:749–59.

193. **Wargel RJ**, **Shadur CA**, **Neuhaus FC**. 1970. Mechanism of D-cycloserine action: transport systems for D-alanine, D-cycloserine, L-alanine, and glycine. J Bacteriol **103**:778–88.

194. **Chassin C**, **Vimont S**, **Cluzeaud F**, **Bens M**, **Goujon J-M**, **Fernandez B**, **Hertig A**, **Rondeau E**, **Arlet G**, **Hornef MW**, **Vandewalle A**. 2008. TLR4 facilitates translocation of bacteria across renal collecting duct cells. J Am Soc Nephrol **19**:2364–74.

195. **Subashchandrabose S**, **Mobley HLT**. 2015. Virulence and fitness determinants of uropathogenic *Escherichia coli*. Microbiol Spectr **3**.

196. **Wiles TJ**, **Kulesus RR**, **Mulvey MA**. 2008. Origins and virulence mechanisms of uropathogenic *Escherichia coli.* Exp Mol Pathol **85**:11–9.

197. **Mysorekar IU**, **Hultgren SJ**. 2006. Mechanisms of uropathogenic *Escherichia coli* persistence and eradication from the urinary tract. Proc Natl Acad Sci U S A **103**:14170–5.

198. **Hua Q**, **Yang C**, **Oshima T**, **Mori H**, **Shimizu K**. 2004. Analysis of gene expression in *Escherichia coli* in response to changes of growth-limiting nutrient in chemostat cultures. Appl Environ Microbiol **70**:2354–66.

199. **Zimmer DP**, **Soupene E**, **Lee HL**, **Wendisch VF**, **Khodursky AB**, **Peter BJ**, **Bender RA**, **Kustu S**. 2000. Nitrogen regulatory protein C-controlled genes of *Escherichia coli*: scavenging as a defense against nitrogen limitation. Proc Natl Acad Sci U S A **97**:14674–9.

200. **Nørregaard-Madsen M**, **McFall E**, **Valentin-Hansen P**. 1995. Organization and transcriptional regulation of the Escherichia coli K-12 D-serine tolerance locus. J Bacteriol **177**:6456–61.

201. **Subashchandrabose S**, **Hazen TH**, **Brumbaugh AR**, **Himpsl SD**, **Smith SN**, **Ernst RD**, **Rasko DA**, **Mobley HLT**. 2014. Host-specific induction of *Escherichia coli* fitness genes during human urinary tract infection. Proc Natl Acad Sci U S A **111**:18327–32.

202. **Lane MC**, **Alteri CJ**, **Smith SN**, **Mobley HLT**, **Greenberg P**. 2007. Expression of flagella is coincident with uropathogenic *Escherichia coli* ascension to the upper urinary tract. Proc Natl Acad Sci **104**:16669–16674.

203. **Datsenko KA**, **Wanner BL**. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97**:6640–5.

204. **Hung C-S**, **Dodson KW**, **Hultgren SJ**. 2009. A murine model of urinary tract infection. Nat Protoc **4**:1230–1243.

205. **Nagata Y**, **Masui R**, **Akino T**. 1992. The presence of free D-serine, D-alanine and D-proline in human plasma Research Articles. Experientia **48**.

206. **Segal AW**, **Geisow M**, **Garcia R**, **Harper A**, **Miller R**. 1981. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. Nature **290**:406–9.

207. **Jankowski A**, **Scott CC**, **Grinstein S**. 2002. Determinants of the phagosomal pH in neutrophils. J Biol Chem **277**:6059–6066.

208. **Hogan D**, **Wheeler RT**. 2014. The complex roles of NADPH oxidases in fungal infection. Cell Microbiol **16**:1156–1167.

209. **Blair JMA**, **Webber MA**, **Baylay AJ**, **Ogbolu DO**, **Piddock LJ V.** 2014. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol **13**:42–51.

210. **Laxminarayan R**. 2014. Antibiotic effectiveness: Balancing conservation against innovation. Science **345**.

211. **Maeda T**, **García-Contreras R**, **Pu M**, **Sheng L**, **Garcia LR**, **Tomás M**, **Wood TK**. 2012. Quorum quenching quandary: resistance to antivirulence compounds. ISME J **6**:493–501.

212. **Cegelski L**, **Marshall GR**, **Eldridge GR**, **Hultgren SJ**. 2008. The biology and future prospects of antivirulence therapies. Nat Rev Microbiol **6**:17–27.

213. **Allen RC**, **Popat R**, **Diggle SP**, **Brown SP**. 2014. Targeting virulence: can we make evolution-proof drugs? Nat Rev Microbiol **12**:300–308.