Quantitative PCR Analysis of the *Bartonella henselae carD* Gene during Bacterial Stress

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Abstract

The *Bartonella* genus is comprised of arthropod-borne, intracellular bacterial pathogens that colonise the mammalian bloodstream. A large number of mammalian species are hosts for one or more *Bartonella* species, as either reservoir or incidental hosts. *Bartonella* species are only able to invade and replicate in host red blood cells in the reservoir host, and to be taken up by an associated haematophagous arthropod vector to complete transmission and the bacterial life cycle. Humans are the reservoir hosts for *B. quintana* and *B. bacilliformis*, and are incidental hosts for more than 16 additional zoonotic *Bartonella* species, including *B. henselae*, which is normally carried by cats. *B. henselae* infection, usually acquired through cat scratches or bites, can result in several clinical manifestations, with varying degrees of severity; the most common of these is cat scratch disease, where symptoms commonly range from enlarged lymph nodes to severe fever. Although usually a mild illness, *B. henselae* infection can occasionally lead to severe symptoms, affecting neurological and other major organ systems.

During their life cycle the *Bartonellae* must adapt to various toxic host environments; such adaptation is mediated by several bacterial stress pathways, which modify bacterial transcription. However, many gaps remain in the understanding of *B. henselae* stress response pathways. The object of this study, the *carD* gene, was identified as a possible component of the *Bartonella* stress response. The *carD* gene has been shown to be critical for stress defence in other bacterial species, including *Mycobacterium tuberculosis, Thermus thermophilus,* and *Myxococcus xanthus.*

Our study aimed to investigate whether *carD* played as significant a role during the *B. henselae* response to stresses as it does in other bacterial genera. We first attempted to perform growth comparisons between a *B. henselae carD* mutant strain and a wild type strain during exposure to stress conditions; however, our mutagenic *carD* plasmid interfered with bacterial growth of *Escherichia coli* cultures, which hindered transformation and generation of a *B. henselae carD* mutant. As an alternative, we investigated the expression of *B. henselae carD* under stress conditions, comparing *carD* expression during stress against a non-stressed *B. henselae* control, using quantitative PCR. We found no significant difference of expression of the *carD* gene between the control and any of our conditions, although a trend of increased *carD* expression was found in several stress conditions. We believe that these findings merit further study into the role of *carD* in the *B. henselae* stress response.

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Abbreviations used in this thesis

Δ*carD*	*carD* null mutant strain
cDNA	Complementary DNA
CSD	Cat scratch disease
ddH$_2$O	Distilled and Deionised H$_2$O
EtOH	Ethanol
gDNA	Genomic DNA
Gent	Gentamicin
IFA	Indirect fluorescent-antibody assay
IL-10	Interleukin- 10
LB	Luria Broth
M199	Media 199
M199S	Media 199 supplemented with fetal bovine calf serum, L-glutamate, and Sodium pyruvate
mRNA	Messenger RNA
OD$_x$	Optical density at a wavelength of x
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SOC	Super Optimal Broth with added glucose
TRCF	Transcription repair coupling factor
T4SS	Type 4 secretion system
WT	Wild Type

Chapter 1. Introduction

In recent years, research has shown that 60.3% of all emerging infectious diseases are due to zoonotic infections, of which 54.3% are bacterial in origin[1,2]. One group that makes up a large portion of zoonotic bacteria is the genus of *Bartonella*. *Bartonella* are fastidious, Gram-negative alphaproteobacteria, capable of intracellular invasion of host tissues. Each *Bartonella* species has co-evolved to persist in specific host/vector parasitic relationships. Such relationships always involve a warm-blooded mammalian reservoir host and a haematophagous (blood sucking) arthropod vector, responsible for transmission of the pathogen[3]. A wide range of mammalian species act as *Bartonella* reservoir hosts, including canids, felines, ruminants, ungulates, rodents, marsupials, and chiroptera[4,5]. Whereas prior to 1993 the *Bartonella* genus consisted of one species[6], there are now over 40 identified species and likely many more that remain undiscovered.

The *Bartonella* life cycle begins when the infected haematophagous arthropod vector takes a blood meal from its mammalian host, during which faeces, containing *Bartonella*, are deposited onto the skin. The bacteria enter the mammalian host body through the skin, infecting several intracellular niches. From these niches, *Bartonella* is released into the circulatory system, where the bacteria invade red blood cells. Infected host erythrocytes are taken up into the gut of the arthropod vector during a blood meal, restarting the cycle. Though each *Bartonella* species has evolved to invade only the specific erythrocytes of a mammalian reservoir host, *Bartonella* species may also cause non-erythrocytic infections in other mammalian species, which are known as incidental hosts. In the life cycle of pathogenic organisms, a reservoir host is a species whose infection will contribute to the life cycle of the pathogen, e.g. *B. quintana* in humans. In

contrast, an incidental host is a species whose infection cannot be passed on to another species. Incidental host infection will not contribute to the life cycle of the pathogen, e.g. *B. henselae* in humans and dogs.

Unlike many other bacterial diseases, *Bartonella* infections cannot be easily diagnosed using *in vitro* culturing. The bacteria have a slow growth rate and require specific growth conditions, including haemoglobin enriched media and capnophilic (high carbon dioxide) and microaerophilic (low oxygen) conditions. *Bartonella* also frequently have a low bacterial load in infected individuals, requiring extra enrichment steps to enable detection. Samples of patient tissue, serum, or whole blood may be incubated in supplemented insect media (such as Schneider's Drosophila Medium supplemented with serum[7], or Bartonella Alpha Proteobacteria Growth Medium, BAPGM[8]) to enrich bacteria before samples can be analysed to diagnose *Bartonella* infections[9].

In healthy reservoir hosts, *Bartonella* infection will usually result in persistent bacteraemia, with bacteria present in the blood and tissues of a host over a period of months or years[10-12]. In many cases, a reservoir host infection will remain sub-clinical, although *Bartonella* infections can predispose otherwise healthy hosts to secondary infections and other health issues[13,14]. In young, old and infirm reservoir hosts, and in incidental hosts, *Bartonella* exposure can lead to long-lasting relapsing infection, known as bartonellosis, resulting in a broad spectrum of medical complications[15]. Treatment of *Bartonella* infection has improved to the point where the correct combination of antibiotics generally resolves the infection - with the right treatment *Bartonella* infections are rarely fatal. Treatment can vary slightly depending on the species of *Bartonella*, immune status of the host, whether the infection is chronic or acute, or if

there are additional complications caused by the infection such as endocarditis or bacillary angiomatosis[16,17]. Gentamicin, doxycycline, and rifampin are common choices to treat *Bartonella* infections, and standard chemotherapy protocol recommendations have been published by clinicians[16].

1.1. Human-Specific *Bartonella* Species

Currently two *Bartonella* species have been found to use humans as reservoir hosts, *B. bacilliformis* and *B. quintana*. Infection with either of these two *Bartonella* species can result in serious health problems, although risk of infection is low due to the limited prevalence of the relevant arthropod vectors. *B. bacilliformis* is restricted to the Andes region of South America, the geographic range of its associated sandfly vector, while *B. quintana* is prevalent in lower socioeconomic groups, who suffer elevated exposure to the human body louse vector.

1.1.1. *Bartonella bacilliformis*

B. bacilliformis, described in 1909, is spread by *Lutzomyia* sand flies endemic to the Andes region[18]. *B. bacilliformis* was the only member of the *Bartonella* genus until 1993 when, following the discovery of related species, the *Bartonella* and *Rochalimaea* genera were merged[6]. *B. bacilliformis* differs from other species of *Bartonella* in virulence and lethality, as invasion of host erythrocytes by *B. bacilliformis* results in the potentially lethal haemolysis of the red blood cells. Untreated infection leads to Verruga peruana (pathological angiogenesis resulting in tumour-like growths on the skin) and Oroya fever (haemolytic anaemia)[16,19].

1.1.2. *Bartonella quintana*

B. quintana is seen worldwide, and is transmitted by *Pediculus humanus*, the human body louse. *B. quintana* is believed to have diverged at some point from *B. henselae*, although an accurate timescale for such an event is unknown. One hypothesis is that divergence occurred as the human body louse split from the human head louse, ~72,000 years ago[20], around the time when humans began wearing clothing. However, it has been suggested that the level of divergence between *B. quintana* and *B. henselae* indicates an earlier split is more likely[21]. The earliest documented evidence of *B. quintana* infection in humans is found in 4000 year old remains[22]. In modern times, the infection rate of *B. quintana* is very low due to the relative rarity of the human body louse ectoparasite; advances in public health, personal hygiene, and more readily available hot water have reduced the prevalence of the parasite, and in turn *B. quintana*.

B. quintana is responsible for trench fever, a disease characterised by a five day fever cycle, with symptoms that include, but are not limited to, severe headaches, sore muscles, and bone and eye pain[16,19]. The largest outbreaks of trench fever were among soldiers serving on the French front in World War I[23,24], and on the eastern front in World War II[25,26]. Trench fever is not usually lethal, but had one of the highest morbidities of any disease over the duration of World War I; of all British troops sent home from the trenches due to sickness in 1917, 15.0% of cases were due to trench fever[26]. Both the human body louse and a blood-borne bacterium were proposed as the agents responsible in 1916[27,28], and later confirmed in 1918[29,30]. Since World War II, prominent *B. quintana* outbreaks have been seen in several African nations[31,32], as well as isolated cases in homeless patients in France, Russia, and the United States[33-36]. Urban homelessness, together with alcoholism and intravenous drug addictions, often

lead to body louse infestations, which enables the transmission of *B. quintana*[37,38]. Coupled with the fact that these populations are also especially vulnerable to contracting HIV/AIDS and related immune deficiency diseases, co-infection of the virus and *B. quintana* re-emerged in the 1980s and 1990s as a new public health problem[12,39]. This was characterised by a rise in trench fever, along with bacillary angiomatosis and endocarditis, also due to *B. quintana*[12,40].

1.2. Zoonotic *Bartonella* Species

Zoonotic *Bartonella* infections in humans are far more prevalent than the reservoir host infections of *B. bacilliformis* and *B. quintana*. Currently 16 *Bartonella* species, along with two candidate species, are known to act as disease causative agents in humans (see **Table 1.1**), for which a variety of mammals act as hosts, sometimes of multiple species[9,10]. Incidental host infections differ from reservoir host infections; *Bartonella* in incidental hosts colonise similar cellular and tissue sites as in reservoir hosts (excluding erythrocytic cells), but appear to have more evident secondary illnesses associated with infections. The most apparent difference between reservoir and incidental hosts is that in most cases *Bartonella* in incidental hosts cannot invade erythrocytes, preventing spread through arthropod vectors[41]; research has shown that *B. henselae* invasion of human erythrocytes is possible due to the similarity between *B. quintana* and *B. henselae*, but occurs at too low a frequency to enable transmission[42].

1.2.1. Groups at Risk of *Bartonella* Infection

Exposure to animal-associated *Bartonella* species generally occurs through contact with animals (e.g., scratches or bites). Exposure to arthropod vectors may play a role, although this remains a heavily debated topic within the field of *Bartonella* research. Transmission of zoonotic *Bartonella* is a known occupational hazard for those who interact with animals on a regular basis – veterinarians, animal handlers, farmers, and pet owners are at heightened risk.

Also at a possible heightened risk are those who receive blood transfusions, in the United States in 2011 around 40,000 units of whole blood or red blood cells were collected, with 38,000 units transfused[43]. The American Food and Drug Administration mandates that red blood cells can be refrigerated for a maximum of 42 days, yet *B. henselae* remains viable in stored red blood cell units for 35 days[44,45]. *B. henselae* is transmissible through needle pricks in humans[46,47], and is transmissible through intravenous blood transfusions in both mice and cat animal models[48-50]. This raises the question as to whether *B. henselae,* or other *Bartonella* species, may be transmitted between human hosts through blood transfusions. Prevalence studies of *Bartonella* in blood donors are limited, with the majority focused on *B. henselae* (see **Table 1.2**) the seroprevalence (number of patients whose serum tested positive for *Bartonella* antibodies) of *B. henselae* in blood donors varies between countries, ranging from 0.6% to 34.4%, but the average worldwide is about 10.0%, making *Bartonella* transmission via blood transfusions a notable risk for recipients.

Table 1.1.   –   Human Pathogenic *Bartonella* Species All *Bartonella* species listed here (with the exception of those in bold) are zoonotic human pathogens. An example of reservoir and vector host species is noted for each *Bartonella* species.

Bartonella spp.	Reservoir spp.	Vector spp.	Incidental spp.
B. alsatica[51]	Rabbits- *O. cuniculus*	Rabbit Flea- *S. cuniculi*?	Human
B. bacilliformis[52]	**Humans- *H.sapiens***	**Sandfly- *L. verrucarum***	**None**
B. clarridgeiae[53]	Cats- *F. catus*	Cat Flea- *C. felis*	Human, Dog
B. doshiae[54]	Voles and Rats- *M. agentiis*	Rodent Flea- *X. cheopis*?	Human
B. elizabethae[55]	Rats- *R. norvegicus*	Oriental Rat Flea- *X cheopis*	Human, Dog
B. grahamii[56]	Mice- *C. glareolus*	Rodent Flea- *X. cheopis*?	Human
B. henselae[56]	Cats- *F. catus*	Cat Flea- *C. felis*	Human, Dog, Horse, Porpoise
B. koehlerae[56]	Cats- *F. catus*	Cat Flea- *C. felis*	Human, Dog
B. melophagi[57]	Sheep- *O. aries*	Sheep Ked- *M. ovinus*	Human
B. quintana[58]	**Humans- *H. sapiens***	**Human Body Louse- *P. humanis***	**Cat, Dog**
B. rattimassiliensis[59]	Rats- *R. rattus*?	Rat Flea- *X. cheopis*?	Human
B. rochalimae[55]	Canids- *C. familiaris*	Dog Fleas- *P. irritans*	Human, Dog, Cat
B. schoenbuchensis[54]	Deer- *C. capreolus*	Deer Ked- *L. cervi*	Human
B. tamiae[60]	Rats- *R. rattus*?	Chigger Mites and Ticks- Trombiculidae	Humans
B. tribocorum[54]	Rats- *R. rattus*	Rat Flea- *X. cheopis*?	Human
B. vinsonii arupensis[56]	Mice- *P leucopu*	Unknown- ?	Human
B. vinsonii berkhoffii[56]	Canids- *C. latrans*	Unknown- ?	Human, Cat
B. washoensis[61]	Squirrel- *S. beecheyii*	Rodent Flea- *O. montana*	Human, Dog
B. ancashi[62] (Canditatus)	Unknown- ?	Sandfly- *L. verrucarum*?	Human
B. mayotimonensis[63] (Canditatus)	Bats?- ?	Bat Flies and Fleas- ?	Human

1.3. *Bartonella henselae*

The most prevalent zoonotic *Bartonella* species is *B. henselae*, the causative agent of cat scratch disease (CSD), as well as several other illnesses. Due to genomic similarities, it has been suggested to be the progenitor species of *B. quintana*[21]. The reservoir host of *B. henselae* is the house cat, *Felis catus,* and *B. henselae* is transmitted between cats by the faeces or flea 'dirt' of the cat flea, *Ctenocephalides felis.* The majority of human *B. henselae* infections are due to cat bites and scratches[64-66], and is rarely transmitted to humans through flea faeces[67,68]. Though most cases of zoonotic *B. henselae* infection are in humans, *B. henselae* has also been known to infect dogs, horses, and porpoises as secondary hosts[69,70].

1.3.1. History of *B. henselae* and Cat Scratch Disease

Previously undocumented in modern medicine, CSD was identified independently in patients in 1931 by Robert Debré in France, and in 1932 by Lee Foshay in the United States; the pair met in 1947[71], leading to the first published clinical description of CSD in French by Debré in 1950[72]. The first report of CSD written in English was by Greer et al, who presented findings to medical professionals at the American Clinical and Climatological Association in late 1950 and published in 1951[71,73]. Numerous studies have been published since 1950, describing case studies, epidemiology[74-76], related diseases[77], and transmission to animals[71]. However, CSD aetiology remained unknown until a bacterial presence was noted in patients whose immune systems were suppressed by HIV/AIDS in the 1980s[78]. A Gram negative, rod-shaped bacterium, later identified as *B. henselae*, was identified in histological sections of a Warthin-Starry stained lymph node from a CSD patient in 1983[79]. The bacterium was first linked to the aetiology of bacillary angiomatosis[80], bacillary peliosis hepatis[81], and fever and

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bacteraemia in 1990[82], before being shown as the causative agent of CSD in 1992[83,84]. The bacterium was isolated and characterised as *Rochalimaea henselae* in 1992[85,86], then renamed *Bartonella henselae* and merged into the *Bartonella* genus alongside *B. quintana* and the related zoonotic pathogens, *B. elizabethae* and *B. vinsonii*, in 1993[87].

1.3.2.   *Bartonella henselae, Felis catus, and Ctenocephalides felis*

The housecat is a common pet, with an ownership average (defined as the percentage of households with at least one cat) of 25.0% for Europe[88], 35.0% for North America[89,90], and 49.0% in New Zealand[91]. *B. henselae* prevalence is highest in cat populations that have a high rate of flea infestations; seroprevalence is greatest in young and stray cats[92,93], and in regions with a warm climate and moderate precipitation[94]. Cats infected with *B. henselae* often suffer subclinical, persistent bartonellosis[10], but infection can alternatively result in clinical manifestations or predispose the affected cat to a number of ailments[13,14].

Ctenocephalides felis (*C. felis*) is the primary ectoparasite of both cats and dogs worldwide[95-97]; under the right conditions, over 90.0% of cats can be infected by *C. felis* in a population of feral cats[98,99]. Areas with a warm climate and moderate precipitation favour *C. felis*, with fleas growing faster and larger[100]. Adult *C. felis* act as the haematophagous vector of *B. henselae* infection in cat-to-cat transmissions. Fleas living on bacteremic cats were shown to harbour bacteria in 1994[101], and to transmit *B. henselae* in 1996[102,103]. As in the majority of arthropod vectors, fleas take up *B. henselae* during a blood meal from an infected cat. Erythrocytes are lysed in the arthropod midgut, where *Bartonella* survives exposure to high levels of haemin, persists and replicates. Voided gut contents and dirt may be culture positive for *B. henselae*,

with bacteria harvested from flea faeces remaining viable for three days[102,104,105]. Both voided gut contents and dirt are crucial for the flea life cycle, acting as nutrients for larvae[106], and providing a possible method of flea-to-flea transmission of *Bartonella*[107]. Currently, *B. henselae* are known to maintain themselves for 12 days in the *C. felis* midgut, the entire lifespan of fleas fed from artificial blood-membrane system[108]. No studies investigating *B. henselae* persistence in fleas fed on animals have been performed, where *C. felis* can live and reproduce up to a hundred days[109].

1.3.3.   Cat Scratch Disease

In 1997, 24,000 people were diagnosed with CSD in the United States; 80.0% of these were children between two and 14 years old[76,110,111]. Cases of CSD increase in autumn and winter, potentially due to the increased time cats spend indoors and in contact with humans[112]. Previously, CSD in humans was believed to be a self-limiting disease, although recent studies show that this may not always be the case[11,113-115]. In a study of 24 patients with severe CSD related arthropathy (joint pain), 80.0% of patients fully recovered within six weeks, while the remainder had chronic disease, lasting between 16 and 53 months[116].

Around 85.0% of CSD patients suffer from 'typical' CSD symptoms[117], which involve a raised bump or lesion on the site of infection, followed by lymphadenopathy (swelling of the lymph node nearest to the site) and minor symptoms also typical of other *Bartonella* infections[118]. Bartonellosis symptoms vary between patients depending on health and age. Common symptoms are often non-specific, including chronic fatigue, malaise, headaches, acute fever, mygalia (muscle pain), arthralgia (joint pain), paresthesia (loss of sensation), photophobia (sensitivity to light), and memory

loss. In the remaining 15.0% of 'atypical' CSD infections, and especially in immunocompromised patients, a range of neurological and major organ symptoms may appear, including bacillary angiomatosis (formation of tumour-like lesions), pericarditis and endocarditis (inflammation of the pericardium and endocardium heart tissue), pleural and pericardial effusion (build-up of fluid around the lungs and heart), bacillary splenitis (lesion of the spleen), peliosis hepatis (blood filled spaces in the liver), meningitis (inflammation of membranes surrounding the brain and spinal cord), myelitis (inflammation of the spinal cord), neuroretinitis (inflammation of the retina or optic nerve), cerebral arteritis and artery occlusion (inflammation of arteries in the brain and blockage of arteries), seizures and convulsions, and hemiplegia (paralysis)[119-131]. *Bartonella* species have also been named as a probable causative agent of cerebral bacillary angiomatosis[132], and of dementia in HIV-positive patients[133,134].

1.3.4. Prevalence of *Bartonella henselae*

Studies have been undertaken in the last 25 years to determine the prevalence of *Bartonella* species in humans and animals worldwide. In particular, the prevalence of *B. henselae* has been assessed using indirect fluorescent-antibody (IFA) assays. Other techniques, such as PCR, have become more common as bacterial enrichment techniques have improved. IFA assays detect *Bartonella* genus- or species-specific IgG or IgM antibodies in the patient's bloodstream, to determine if a patient has been exposed to the bacteria, giving a snapshot of the prevalence of *Bartonella* in a sample population. IFA results will only be positive if the correct antibodies are present, although the level of each antibody appears to fluctuate over the course of the disease; levels of IgM decrease within three months after initial infection, while IgG levels remain detectable for several years following infection[135,136]. Combined with PCR and

culturing techniques, IFA serology is accurate at diagnosing *Bartonella* infections; however, previous patient exposure to *Bartonella* can give false positives if serology is the sole diagnosis technique used.

Seroprevalence varies considerably within country (depending on climate, urban versus rural location, population of humans and cats etc). Serological studies are a good starting point for determining the prevalence of *Bartonella*. Interestingly, the majority of *B. henselae* seroprevalence studies currently are performed in healthy adults and blood donors, rather than in children, who are the main sufferers of CSD. **Table 1.2** demonstrates that seroprevalence of *B. henselae* follows several trends, with some exceptions. High seroprevalence is seen in countries with warm, wet climates and in those that have a high cat population (domesticated or stray). Climate appears to be a large factor in seroprevalence, most likely due to favourable conditions for *C. felis*. The exceptions to these trends are in Germany and Canada, where despite lower temperatures (and Germany's low cat ownership) both nations have higher seroprevalence than similar nations. Unexpectedly high seroprevalence levels can likely be explained by how animals are regarded in a community, and the laws and regulations of a nation – a society that has a positive attitude towards stray cats will have higher interactions with the animals (e.g. feeding and socialisation), which can result in increased rate of infection. Conversely, negligence in caring for pet cats, in controlling the cat population, or in dealing with abandoned pets may all increase the rate of infection[89,137,138]. New Zealand has a reported seroprevalence of 5.0% and a household cat ownership of 49.0%[89]; of cats tested in Auckland, 17.0% were culture positive for *B. henselae*, and of *C. felis* from three North Island Veterinaries, 11.0% were PCR positive for *B. henselae*[139,140].

Table 1.2.   – Serological *B. henselae* Prevalence Studies All serology studies were performed using IFA serology. Bd= Blood donors, Vs= Veterinarian students, Ha= Healthy adults, Hc= Healthy children. Cat ownership per country is given as an estimate of the percentage of households that own at least one cat. N/A signifies the country currently has no reliable cat population statistics available.

Prevalence		Country	Sample Size	Group Studied	Year	Cat Ownership (Household)
≤ 5.0%	0.6%	Denmark	159	Bd	2004[141]	18.0%[88]
	1.2%	Sweden	498	Bd	2005[142]	17.0%[88]
	1.5%	UK	200	Bd	1999[143]	19.0%[88]
	4.5%	Japan	155	Vs	2001[144]	N/A
	5.0%	New Zealand	140	Bd	2007[145]	49.0%[91]
	5.0%	USA	199	Bd	1996[36]	35.0%[90]
≤10.0%	5.5%	Thailand	163	Bd	2000[146]	N/A
	6.0%	Turkey	800	Bd	2009[147]	9.0%[88]
	8.7%	Spain	218	Ha	2008[148]	19.0%[88]
≤ 15.0%	11.0%	Jordan	482	Hc	2004[149]	N/A
	13.0%	Germany	116	Hc	2001[150]	13.0%[88]
	13.7%	Brazil	437	Bd	2005[151]	N/A
	14.8%	China	351	Ha	2010[152]	4.1%[153]
≤ 30.0%	21.6%	Greece	491	Bd	2008[155]	13.0%[88]
	24.7%	Spain	146	Bd	2005[154]	19.9%[88]
	30.0%	Germany	270	Ha	1998[155]	13.0%[88]
≤ 40.0%	34.4%	Brazil	125	Bd	2010[156]	N/A
	36.8%	Canada	142	Ha	2000[157]	37.0%[89]
≥ 50.0%	50.0%	Croatia	100	Bd/Hc	2009[158]	N/A
	61.6%	Italy	508	Hc	2004[159]	19.0%[88]

1.4.   Pathology and Host Cell Interactions of *Bartonella*

1.4.1.   *Bartonella* Life Cycle

The life cycle of *Bartonella* in reservoir hosts can be characterised by two major phases, the vector phase and the host phase. Though the vector and host vary for each *Bartonella* species, the life cycle, as shown below in **Figure 1.1** is largely similar for all members of the genus, with the exception of *B. bacilliformis*.
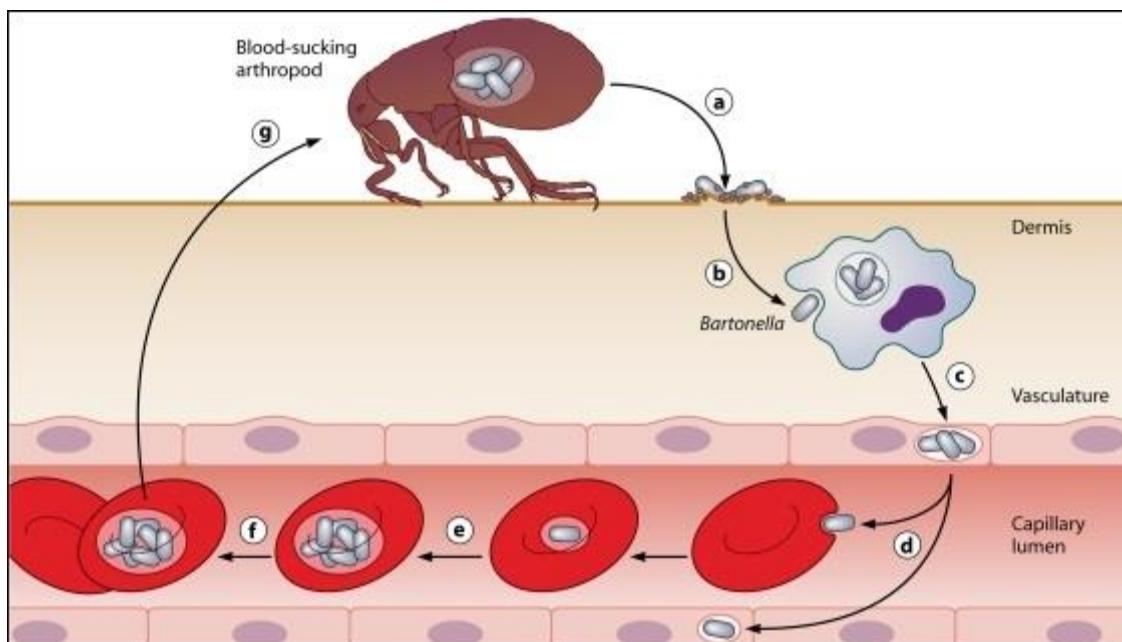
Figure 1.1. – *Bartonella* Reservoir Host Life Cycle a) Bacteria are deposited on the skin of the mammalian host. b) *Bartonella* enter the host and are likely taken up by migratory cells. c) Transport to the vascular endothelium occurs, where the bacteria are thought to persist intracellularly. d) From endothelial and other infected cells, the bacteria are seeded into the bloodstream, where they invade erythrocytes and possibly other cell types. e and f) After limited replication inside the red blood cell, *Bartonella* persist in the intra-erythrocytic niche. g) Red blood cells are taken up into the haematophagous arthropod vector to restart the cycle. Figure has been reproduced from: Clinical Microbiology Review, 25(1), Harms and Dehio, Intruders below the radar: molecular pathogenesis of Bartonella spp, pages 42-78, 2012, with permission from the American Society for Microbiology[19].

In the first phase, erythrocytes containing *Bartonella* are taken up by a haematophagous arthropod vector during a blood meal from an infected mammalian host. Lysis of erythrocytes releases bacteria into the arthropod gut, where *Bartonella* replicates for a

sustained period of time. The bacteria remain viable in both regurgitated gut contents and faeces of the vector. Transmission to a new mammalian host occurs when the infected arthropod takes a blood meal; the arthropod gut expands during feeding, expelling bacteria-laden faeces onto the skin of the new host. *Bartonella* enters the new host via abrasions in the skin, often assisted by the host scratching the affected area.

After infiltrating the host, *Bartonella* enters the second phase, moving through the body, using the blood and lymph systems as vehicles to reach deeper host tissues, possibly via dendritic cells[160,161]. As the bacteria move through the circulatory system, *Bartonella* spp. bind and invade immunologically privileged sites, known as the primary or replicative niches[41]. A hotly debated topic, many cellular sites have been proposed to be the primary niche, with vascular, endothelial, microglia, and hematopoietic progenitor cells all suggested as candidates[19,162,163]. Within these cellular niches, *Bartonella* replicate and influence cellular function and expression. *Bartonella* can invade multiple cell types *in vitro*, including endothelial, epithelial, hematopoietic progenitor, dendritic, microglial, and monocytic cells[163-166]. As of yet, the *in vivo* relevance has not been fully elucidated for all these cell types.

From the primary niche, *Bartonella* are proposed to be periodically reseeded into the circulatory system in recurring synchronous waves of infection[41,167], yet this release has not been noted in all species. In the circulatory system, *Bartonella* bind to and invade erythrocytes; *Bartonella* has been shown to replicate within the erythrocytes of reservoir hosts, although the number of bacteria per erythrocyte is species specific and limited – an average of eight *B. tribocorum* bacteria per erythrocyte in rats, five *B. quintana* per erythrocyte in humans, and just one *B. henselae* bacterium per erythrocyte in cats[19,41]. The proportion of erythrocytes infected in the blood of the reservoir host is also limited,

with around 1.0% of all erythrocytes infected in *B. quintana* and *B. tribocorum*, and up to 5.0% in *B. henselae*. These figures differ greatly in *B. bacilliformis*, which exhibits traits similar to malaria, in that the percentage of infected erythrocytes can reach up to 100.0%, and replication within the erythrocyte will continue until haemolysis[19]. Though haemolysis in common in *B. bacilliformis,* no other *Bartonella* species has been shown to severely disrupt reservoir host erythrocyte function; erythrocytes remain infected and function for their lifespan or until taken up by a haematophagous vector during a blood meal, restarting the cycle[41].

1.4.2. Pathology and Mechanism of Infection of *B. henselae*

Although infection with *B. henselae* and other *Bartonella* species leads to clinical symptoms in some reservoir and in most incidental hosts, infection in a healthy reservoir host will often be at sub-clinical levels. During infection of a reservoir host, *Bartonella* can remain undetected for prolonged periods of time, this is due to both its ability to evade and subvert the immune system of a host, and its ability to hide in immunologically protected sites.

One means of immune evasion is through the unique characteristics of *Bartonella* lipopolysaccharide. Normally bacterial lipopolysaccharide interacts with host pattern recognition receptors to elicit a large immune response, but *Bartonella* lipopolysaccharide differs in structure, leading to a much reduced binding rate to host Toll-like receptor 4, relative to other Gram negative bacterial pathogens[168,169]. There is evidence that *Bartonella* also undergoes phase and antigenic variation as a means of avoiding the adaptive immune response[170]. *Bartonella* also actively modulates the immune system, through mechanisms not fully understood, stimulating secretion of high levels of the cytokine IL-10, a key regulator of immunity. IL-10 suppresses the functions

of various immune cells, interfering with innate immunity and the establishment of an adaptive immune response. IL-10$^{-/-}$ knockout mice infected with *B. birtlesii* did not become bacteraemic[171], demonstrating the crucial role of IL-10 expression in *Bartonella* infection. *Bartonella* trimeric auto-transporter adhesins and the VirB/D4 type 4 secretion system (T4SS) may also affect the immune system via host cell interactions[19,168]. The most widely studied trimeric auto-transporter adhesin, the *B. henselae* Bartonella Adhesin A, BadA, governs cell binding and also promotes angiogenesis through up-regulation of hypoxia inducible factor 1[18,170,172], and the immune system through NF-κB[173], suggesting that other trimeric auto-transporter adhesins could fulfil a similar function.

In the circulatory system, interactions between *B. henselae* and endothelial cells are mediated by BadA. BadA facilitates cellular invasion by first promoting auto-aggregation of *B. henselae*, and by adhering to endothelial cells and the extracellular matrix[19,170,174]. Once *B. henselae* adheres to a host endothelial cell, the VirB/D4 T4SS is used to 'inject' *Bartonella* effector proteins (Beps) into the target cell, bypassing the bacterial inner and outer membranes and the host cell membrane, and additionally preventing endothelial cell apoptosis[175]. Though not all of their functions have been discovered, the Beps generally target and modify host cell signalling; some Beps have anti-apoptotic and pro-angiogenic functions[176], while others mediate and assist in the uptake of *Bartonella* into the cell[177]. There are two ways for an endothelial cell to take up *B. henselae*, either by endocytosis of a single bacterium into a *Bartonella*-containing vacuole or by uptake of an bacterial aggregate mass into an invasome, via the Beps (e.g. BepG prevents endocytosis, resulting in accumulation of the aggregate mass)[177]. *Bartonella*-containing vacuoles have been found to lack typical endocytic markers such

as Lamp1, preventing fusion with lysosomes[178]. *B. henselae* has been proposed to replicate within the primary niche and be released back into the circulatory system.

Within the circulatory system, *Bartonella* species-specific binding to reservoir host erythrocytes is mediated by the Trw T4SS[17,41,175,176]; when deleted in *B. henselae*, bacteria are found to be present in the bloodstream, but cannot invade reservoir host erythrocytes[175].

1.5. *Bartonella* Stress Response

To survive and thrive in a given environment, bacteria must overcome stresses; a 'stress' is a broad label for anything that puts pressure on the bacteria – high or low temperatures, pH, nutrient deprivation, competition with other organisms, and antibiotic assault are common stresses. In pathogenic bacteria, the ability to adapt to diverse environments and their respective stresses is paramount to survival; in the *Bartonella* life cycle these environments occur in the arthropod midgut and the mammalian host circulatory system or intracellular niches. Stresses in the arthropod midgut include a high concentration of haemin (an iron chelating product of red blood cells similar to haem), a low temperature (28°C), and possible nutrient starvation (between invertebrate blood meals). Stresses in the circulatory system for *Bartonella* include antibiotics (genotoxic or toxic by other mechanisms), oxidative stresses (from reactive oxygen species generated by neutrophils and macrophages), and other host defences, such as antimicrobial peptides or nutrient deprivation (nutritional immunity). Stresses in host intracellular niches include oxidative stress (from reactive oxygen species), and other cellular defences such as the low pH of lysosomes.

When bacteria experience exposure to an environmental stress, a global change in bacterial transcription takes place to adapt to that environment. Alterations to cellular processes and metabolism simultaneously slow growth and unnecessary functions while promoting survival and nutrient uptake. This process is driven by various pathways, known collectively as bacterial stress response pathways[179-181]. Though a specific stress may activate a specific stress response pathway, i.e., a high temperature will activate the heat shock response, bacterial stress response pathways have extensive crosstalk and work in conjunction to respond to stresses. Stress adaptation is controlled by sigma factors (σ factors) that modulate bacterial expression to promote bacterial survival and downregulate non-essential systems. The components of stress pathways are essential in regular cellular functions such as growth, division, transcription, and virulence[182-184]. The best studied *Bartonella* stress response pathways are the general stress response and the stringent response pathways.

1.5.1. *Bartonella* General Stress Response

The general stress response protects Alphaproteobacteria (e.g. *B. henselae*), Gammaproteobacteria (e.g. *E. coli*), and Firmicutes (e.g. *Bacillus subtilis*) against non-specific stresses, such as oxidative stress, heat and pH, and provides cross resistance to multiple stresses (carbon-starved gammaproteobacteria cells gain resistance to H_2O_2, low pH, and high temperatures)[185]. The general stress response is present in several bacterial lineages, although the mechanisms vary between phylum and class of bacteria.

In *B. henselae*, the general stress response is regulated through the extracytoplasmic function (ECF) sigma factor (σ^{EcfG}) partner switching mechanism (see **Figure 1.2** below). When stress is low, σ^{EcfG} is inactivated by binding to an anti-sigma

factor, NepR. When a histidine kinase sensor detects an environmental stress, the sensor undergoes an autophosphorylation reaction, subsequently transferring a phosphoryl group from to PhyR. Phosphorylated PhyR interacts with NepR, releasing the σ^{EcfG} sigma factor and enabling it to interact with RNA polymerase enzyme to promote stress-related transcription.
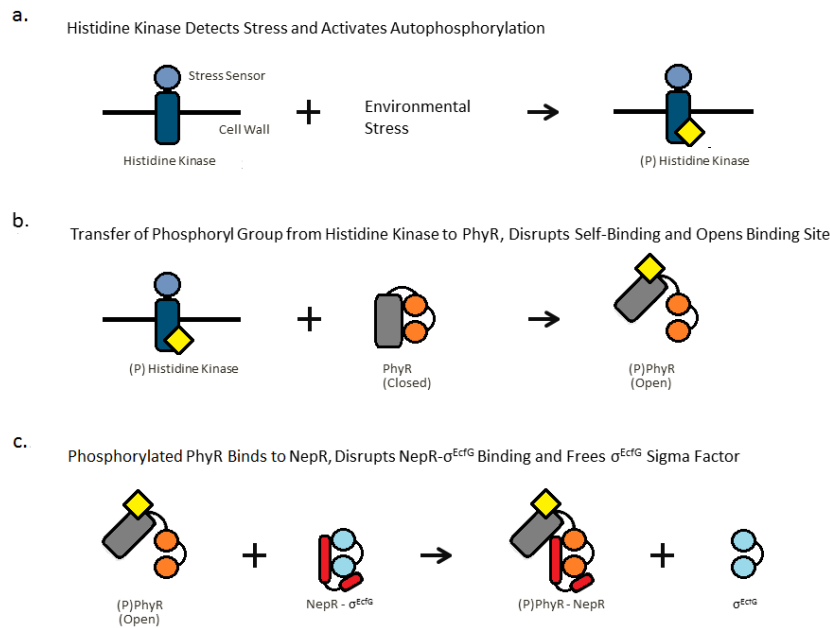
Figure 1.2. – *Bartonella* general stress response partner switching mechanism. PhyR = anti-anti-sigma factor, NepR = anti-sigma factor, σ^{EcfG} = EcfG Sigma factor[186]. a) An environmental stress external to the bacterial cell activates the histidine kinase sensor embedded in the cell wall, leading to autophosphorylation of the histidine kinase. b) The phosphoryl group of histidine kinase is transferred to the inactive PhyR, opening the binding site. c) The open binding site of the phosphorylated PhyR group disrupts binding of NepR- σ^{EcfG}, freeing σ^{EcfG} to interact with RNA polymerase to activate a transcriptional response.

1.5.2. *Bartonella* Stringent Response

The stringent response is a process which is ubiquitous in bacteria, but also occurs on several plant species, and in some higher organisms[187]. Originally thought to solely be a response to amino acid nutrient deprivation, the stringent response has been found to

be critical for the cellular response to various stresses in *E. coli* and other bacteria. Major cellular processes that are affected by the stringent response include cellular growth, adaptation to new conditions, survival, persistence, cellular division, and virulence[188-193]. The stringent response plays an important role in defence against several classes of antibiotics, including fluoroquinolones[190,194]. Inhibiting the stringent response lowers bacterial resistance to hydrogen peroxide, a common reactive oxygen species (ROS) generated by the respiratory burst of macrophages and neutrophils[195-197].

The stringent response is characterised by the presence of (p)ppGpp alarmones, the collective name of the GDP and GTP analogues guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp)[198,199]. (p)ppGpp is important in regulating normal cellular division, growth, virulence, and DNA replication, among other pathways[182,184]. In the stringent response, (p)ppGpp levels regulate transcription and metabolism. This can be done through direct binding to RNA polymerase[200], by interacting with σ factors (indirectly via assisting in alternate σ factor binding to RNA polymerase or by directly via binding to multiple σ factors)[201-203], or by promoting GTP and amino acid uptake and production[204,205]. Accumulation of cellular (p)ppGpp promotes survival, while low concentration of (p)ppGpp promotes cellular growth[206]. Levels of (p)ppGpp are regulated by RelA/SpoT homologues and small alarmone synthetase or hydrolase proteins; all bacteria have a combination of (p)ppGpp regulatory genes. *E. coli* encodes the two originally identified *relA* and *spoT* genes, from which *relA*/*spoT* homologues are named. RelA synthesises (p)ppGpp from GTP, GDP or ATP, while SpoT can either synthesise or hydrolyse (p)ppGpp into the base components of GDP and pyrophosphate (P_i) or GTP and PP_i.

In *Bartonella*, the stringent response is controlled by DksA and SpoT, which regulate the two component system BatR/BatS[207], and the alternate sigma factor RpoH1. Interestingly, in *B. henselae* both VirB/D4 and Trw T4SSs are regulated by the bacterial stringent response (see **Figure 1.3**[207,208]. The stringent response is hypothesised to detect small environmental changes in stress (e.g. nutrients and pH) to modulate bacterial virulence in early and late *Bartonella* infection – in nutrient starvation the stringent response triggers up-regulation of VirB/D4 T4SS mediated invasion of the primary niche, while Trw T4SS is down-regulated. However, in the nutrient rich bloodstream, down-regulation of the stringent components SpoT and DksA and the up-regulation of BatR activates the Trw T4SS to mediate invasion of mammalian erythrocytes.

Additionally DksA in *Bartonella*, *E. coli*, and other Gram-negative bacteria (commonly in alpha- and gamma-proteobacteria) assists in stabilising the (p)ppGpp-RNA polymerase complex by binding to the secondary channel of RNA polymerase[209,210]. DksA in *E. coli* plays a role in stimulating the stringent response, by potentiating (p)ppGpp activity via binding to RNA polymerase[211]. In *Mycobacteria*, the role of DksA in the stringent response appears to be carried out by a CarD homologue protein[212]. Despite a lack of CarD sequence homology to DksA, *M. smegmatis* CarD could functionally replace DksA in *E. coli*, although the reverse was not true. While both bind to RNA polymerase, DksA binds to the secondary channel of the complex, while CarD binds to the β-subunit. Interestingly, *Bartonella* species have both CarD and DksA proteins.
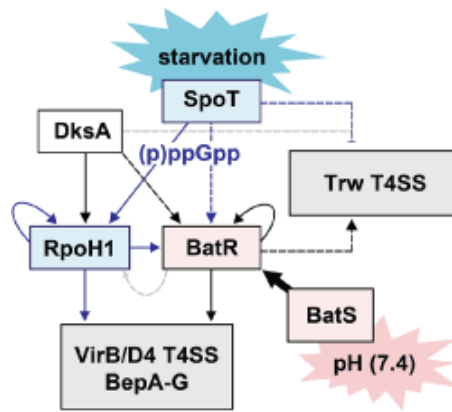
Figure 1.3. – Hypothetical *B. henselae* stringent response control over the VirB/D4 and Trw type 4 secretion systems (T4SS) Unbroken arrows denote positive effect, dashed lines possible indirect effects (i.e. through other proteins such as KorA/KorB). Figure has been reproduced from: Molecular Microbiology, 90/4, Québatte, et al, Dual input control: activation of the *Bartonella henselae* VirB/D4 type IV secretion system by the stringent sigma factor RpoH1 and the BatR/BatS two-component system, pages 756-75, 2013, with permission from John Wiley and Sons[207].

1.6. CarD

The CarD protein was first described in *Myxococcus xanthus*, where the protein acts as a global transcriptional regulator, through binding with RNA polymerase and DNA. The protein regulates several cellular functions, such as carotenoid synthesis and formation of fruiting bodies during starvation[213,214]. However, the CarD protein reported in the literature for *Mycobacterium* and *Thermus* genera, along with the *Bartonella* CarD protein that is the focus of this study, are actually homologues of the *M. xanthus* CdnL (CarD N-terminal Like protein), rather than *M. xanthus* CarD. Though both *M. xanthus* CarD and CdnL act as transcriptional regulators, and have a high degree of structural protein homology (a conserved Tudor-like structure at the N-terminal domain), they have divergent functions; while CarD deletion inhibits *M. xanthus* growth, a CdnL deletion is lethal[215-217].

In *Mycobacterium*, a genus that includes *M. tuberculosis*, a human pathogen that is the causative agent of tuberculosis, the CarD (*M. xanthus* CdnL) homologue is essential[212]. CarD from *M. tuberculosis* directly binds to RNA polymerase and DNA to regulate

rRNA transcription during both regular bacterial function and in response to environmental stresses[212,218]. During transcription, the RNA polymerase holoenzyme binds to a promoter site, unwinding the DNA to form the open promoter complex[216], a process in which CarD assists in several ways. The CarD N-terminal domain binds to the β-subunit of RNA polymerase to stabilise the RNA polymerase-promoter complex[219]. In addition, the CarD C-terminal domain binds to a site upstream of the promoter site, revealed by the open promoter complex, to prevent collapse of the transcription bubble[220,221].   Through these interactions with RNA polymerase and DNA, CarD regulates expression of a wide array of genes and as such can be labelled a global regulator[212,222]. Interestingly, although *M. xanthus* CdnL cannot bind to DNA, it is still involved in stabilisation of the open promoter complex, through its interaction with RNA polymerase[215].

Among other bacterial processes, *Mycobacterium* CarD is involved in the bacterial response to stress, through the stringent response pathway. Transient deletion of *carD* in *M. smegmatis* lead to increased sensitivity to ciprofloxacin, hydrogen peroxide and starvation[212,223]. In *M. smegmatis* cultures exposed to starvation and H_2O_2 conditions, an accumulation of (p)ppGpp and a down-regulation of rRNA was observed. However, when expression of c*arD* was artificially lowered, in cultures exposed to starvation and H_2O_2 conditions, a reduced accumulation of (p)ppGpp was observed, while rRNA levels observed were the same as for unstressed cells[212].

As mentioned previously, CarD in *Mycobacterium* appears to carry out the same function as *E. coli* DksA in the bacterial stringent response[211,212]. This is especially interesting, considering that *Bartonella* species have both CarD and DksA proteins[207], although as of yet, the functionality of CarD has not been described. In contrast to

M. tuberculosis, little is known about the structure or function of CarD in *Bartonella* species, although bioinformatic analysis of the gene indicates that it likely binds RNA polymerase similar to other bacterial species (for protein sequence comparison alignments see **Appendix Figure 8.1**). Similarly to *E. coli* DksA, neither *B. henselae* CarD nor DksA appear to be essential genes for growth, in contrast with several other bacterial CarD homologues[215,216,224]. Deletion has a negative effect on bacterial pathways[176,207], as Δ*carD B. birtlesii* lacked the ability to establish bacteraemia in a mouse, and Δ*dksA B. henselae* mutants had reduced *in vitro* invasome formation during endothelial cell invasion, through disruption of the VirB/D4 T4SS system[207]. This differs from *M. xanthus*, where both *carD* and *dksA* were shown to be essential genes[225].

1.7.   Research Aims

The overall aim of this thesis was to elucidate the role of the CarD homologue in the *B. henselae* response to common environmental stresses. To this end we undertook two strategies to investigate the activity of *B. henselae carD*. Our first aim was to create a Δ*carD* mutant strain of *B. henselae*. This involved inducing homologous recombination of our wild-type *B. henselae* and mutagenic plasmids containing sequence of *B. henselae carD* gene; the result of this would be to knock out function of the *carD* gene. Wild-type and Δ*carD* mutant strains of *B. henselae* would be subjected to environmental stresses, then growth measured to determine the effect of *carD* deletion on *B. henselae* growth. Despite multiple attempts to generate a Δ*carD* strain, a mutant was not obtained. The second aim was to carry out transcriptional analysis of *B. henselae carD* expression during exposure to environmental stresses. Before this aim could be achieved, a series of stress response assays were carried out on *B. henselae* colonies to determine the

appropriate stress conditions to be used in our quantitative PCR experiments. The overall goal of this thesis was to determine if the *B. henselae carD* gene behaved in a similar manner to *carD* genes in other well-studied bacterial models.

Chapter 2. Methods

2.1. Oligonucleotide Primers

All primers were synthesised and lyophilised for shipment by Integrated DNA Technologies (IDT; Singapore). Primers were resuspended at a concentration of 100 μM in autoclaved 0.22 μm filter-sterilized ddH$_2$O, and stored at -20°C. Working stocks were diluted to 10 μM in autoclaved filter-sterilized ddH$_2$O, and stored at -20°C. Primers are listed by reaction pair in **Table 2.1**.

Primers prJS01 forward and prJS02 reverse were designed with engineered BamHI restriction enzyme sites at the 5' end (**Table 2.1**, underlined), and prJS02 reverse 2 was designed with an engineered SalI restriction site (underlined). Primers prJS01 reverse and prJS02 forward were designed with complementary sequences to enable internal priming and generation of a PCR fusion product (**Table 2.1**, bold).

Table 2.1. – **Oligonucleotide Primers** Underlined primer sequences denote restriction sites. Bold sequences denote overlapping nucleotides. * prJS02 reverse 2 primer is a duplicate of the original prJS02 reverse primer, albeit with a BamHI restriction site instead of a SalI site.

Primer Name	Primer Sequence	Primer Target/Function
prJS01		
prJS01_Fwd	5' - GCG<u>GGATCC</u>CCTGCAGAAGCCATTAAACC - 3'	*B. henselae carD* 3'
prJS01_Rev	5' - **TGGGAGAGATGTCGCAAAATGA**ATGACGAAAT - 3'	Flanking Region
prJS02		
prJS02_Fwd	5' - **TCATTTTGCGACATCTCTCCCA**AAAGCCAAA - 3'	*B. henselae carD* 5'
prJS02_Rev	5' - GCG<u>GTCGAC</u>CGTTCAGTGTTGCCTGCT - 3'	Flanking Region
prJS02_Rev2*	5' - GC<u>GGATCC</u>CGTTCAGTGTTGCCTGCTAA - 3'	
prJS03		
prJS03_Fwd	5' - TGCGCAAATATCTGCTGTC - 3'	*B. henselae carD* primer for
prJS03_Rev	5' - TTCGTTAACTGCGCTTTT - 3'	qPCR
prJS04		
prJS04_Fwd	5' - TCCGTGGGTGCTTGATACA - 3'	*B. henselae rpsL*
prJS04_Rev	5' - GCTTCGCGCCATACTTGA - 3'	primer for qPCR
M13		
M13_Fwd	5' - GTAAAACGACGGCCAG - 3'	Universal Sequencing Primers
M13_Rev	5' - CAGGAAACAGCTATGAC - 3'	

2.2.   Bacterial Strains

All bacterial strains used in this study were suspended in glycerol freezer stocks and stored at -80°C. Strains are listed by species in **Table 2.2**.

The *B. henselae* Houston-1 strain was streaked onto Columbia blood agar (CBA) by the ESR Culture Collection, and streaked and grown on CBA or chocolate agar plates. Confluent *B. henselae* plates were harvested and resuspended in tryptic soy broth containing 20% glycerol and stored at -80°C. S17-1 *E. coli* cells were transported in gel plugs by BioMedal Life Sciences (Spain), and used to inoculate fresh LB media. S17-1 cells were harvested and resuspended in *E. coli* glycerol stocks (LB with 20% glycerol) and stored at -80.0°C.

Table 2.2.   – Strains of Bacteria

Strain	Characteristics	Source
B. henselae		
Houston-1[85]	Wild type, Cefazolin, Nalidixic acid, and sucrose resistant, Kanamycin and Gentamicin sensitive	New Zealand Reference Culture Collection (ESR)
E. coli		
DH10B	High competency (electrocompetent) cloning strain	Life Technologies
DH10B pEX18gm plasmid	DH10B containing pEX18gm plasmid, Gentamicin resistant	This study
DH10B pJM05 plasmid	DH10B containing pJM05 plasmid, Kanamycin resistant	pJM05 from Dr Koehler, UCSF
DH10B pJS01 plasmid	DH10B containing pJS01 plasmid, Gentamicin resistant, sucrose sensitive	This study
DH10B pJS02 plasmid	DH10B containing pJS02 plasmid, Kanamycin resistant, sucrose sensitive	This study
S17-1	*E. coli* strain for biparental conjugations, λpir lysogen	BioMedal Life Science (Spain)
S17-1 pJS01 plasmid	*E. coli* S17-1 containing pJS01 plasmid	This study
S17-1 pJS02 plasmid	*E. coli* S17-1 containing pJS02 plasmid	This study

2.3.   Plasmids

All plasmids used in this study were suspended either in autoclaved 0.22 μm filter-sterilized ddH$_2$O or elution buffer (10 mM Tris-HCl), and stored at -20°C. Plasmids are listed in **Table 2.3**. The plasmid supplied by Dr Jane Koehler (UCSF) was lyophilised for transportation, then resuspended in 0.22 μm filter-sterilized ddH$_2$O and stored at -20°C.

Table 2.3.   – Bacterial Plasmids

Plasmid	Characteristics	Source	Size (bp)
pEX18gm	Gentamicin resistant, *oriT* (origin of transfer), *sacB* (sucrose sensitive), derived from pEX18ap	Hoang et al[226], kindly provided by Dr David Ackerley (VUW)	~5,830
pJS01	Mutagenic plasmid with *carD* flanking sequence, cloned into pEX18gm	This study	~6730
pJM05	Kanamycin resistant, *oriT*, *sacB*, derived from pRS14[227]	MacKichan et al[228], kindly provided by Dr Jane Koehler (UCSF)	~8,600
pJS02	Mutagenic plasmid with *carD* flanking sequence, cloned into pJM05	This study	~9,910

2.4.   Growth Media

2.4.1.   Liquid Media

Lysogeny Broth, LB (1 L) –
Premixed powder (20 g; Duchefa Biochemie) was mixed in distilled water (1 L), and then autoclaved for 15 minutes at 120°C. Media was stored for up to two months at room temperature.

Schneider's Drosophila Medium containing 10% Fetal Calf Serum (10 mL) –

Schneider's Drosophila Medium (9 mL; Gibco™) was supplemented with Fetal Calf Serum (FCS, 1 mL) inside a biosafety cabinet, and then used immediately.

M199S medium (10 mL) –

M199 medium (8 mL; Gibco™) was supplemented with GlutaMAX (100 µL of 200 mM; Gibco™), Sodium Pyruvate (100 µL of 100 mM; Gibco™), and FCS (2 mL) inside a biosafety cabinet, sterilised by 0.22 µm filter and then used immediately.

SOC Media (1 L) –

Tryptone (20 g), yeast extract (5 g), NaCl (2 mL of 5M), KCl (2.5 mL of 5 M) were mixed in distilled water (1 L), and then autoclaved for 15 minutes at 120°C. Media was cooled to 50°C, and then supplemented with sterile, autoclaved $MgCl_2$ (10 mL of 1 M) and 0.22 µm filter-sterilized glucose (20 mL of 1 M). Media was stored up to two months at 4°C.

E. coli Freezer stock (50 mL) –

LB (25 mL) and glycerol (25 mL of 80%) were combined and then autoclaved for 15 minutes at 120°C. Media was cooled and stored up to a month.

B. henselae Freezer stock (50 mL) –

Tryptic Soy Broth (40 mL) and glycerol (10 mL of 80%) were combined and autoclaved for 15 minutes at 120°C. Media was cooled and used immediately.

2.4.2. Solid Media

LB agar plates –

Premixed LB agar powder (32 g; Life Technologies) was mixed in distilled water (1 L), and then autoclaved for 15 minutes at 120°C. Supplements were added when molten

media was cooled to 50°C. Media was pipetted (20 mL) into standard 90 mm petri dishes in a sterile biosafety cabinet. LB plates were cooled at room temperature until the agar solidified, at which point they were stored at for two months at 4°C or one month if supplemented with antibiotics.

Chocolate agar plates[229] –

GC agar solution (36 g of GC agar powder, in 500 mL distilled water; Thermo Fisher Scientific) and haemoglobin solution (10 g of 2% freeze-dried bovine haemoglobin powder, in 500 mL distilled water; BD BBL) were prepared separately by gentle mixing. Solutions were gently heated (~60 to 70°C) on a magnetic stirring plate, and were then autoclaved for 15 minutes at 120°C. Solutions were cooled to 50°C, and then combined along with IsoVitaleX™ media enrichment (10 mL; Becton Dickinson) and any other supplements in a Class II bio-safety cabinet. Media was carefully pipetted (27 mL) into 90 mm petri dishes, cooled at room temperature and then stored for one month at 4°C.

2.4.3. Media Supplements

All media supplements or antibiotics were made up to the noted stock concentration in the appropriate solvent and stored at -20°C, with the exceptions of haemin (4°C), hydrogen peroxide (room temperature), and sucrose (used immediately). Media supplements are listed in **Table 2.4**.

Ciprofloxacin was suspended in dilute HCl (25 mg/mL in 0.1 M) then diluted to desired stock concentration and stored at -20°C. Haemin was prepared as described by Roden et al[230], and stored for one week at 4°C. Sucrose was resuspended in 0.22 μL filter-sterilized ddH$_2$O, and used immediately.

Table 2.4. – **Media Supplements and Concentrations** *E.c* denotes *E. coli*, while *B. h* denotes *B. henselae*

Supplement	Media	Stock Concentration	Media Concentration
Ciprofloxacin	Schneider's Medium	10 mg/mL	10, 25 μg/mL
Haemin	Schneider's Medium	1 mM	0.1, 0.5 mM
Hydrogen Peroxide (H_2O_2)	Schneider's Medium	36% w/v	0.1, 1 mM
Kanamycin	LB medium, LB agar plates, Chocolate agar	50 mg/mL (*E. c*) 30 mg/mL (*B. h*)	50 μg/mL (*E. c*) 30 μg/mL (*B. h*)
Gentamicin	LB medium, LB agar plates, Chocolate agar	10 mg/mL	10 μg/mL
Cefazolin	Chocolate agar	50 mg/mL	20 μg/mL
Nalidixic acid	Chocolate agar	10 mg/mL	2 μg/mL
Sucrose	Chocolate agar	50% Sucrose	10% Sucrose

2.5.   Culture Conditions of Bacteria

B. henselae on Chocolate Agar Plates –

For confluent plates, -80°C glycerol stocks of *B. henselae* were streaked onto plain chocolate agar plates in a biosafety cabinet. Plates were incubated for seven to 10 days in a humidified 5% CO_2, 37°C stationary incubator. *B. henselae* were harvested and re-streaked onto fresh chocolate agar plates, and then incubated for five to seven days to generate confluent *B. henselae* plates. Confluent plates were passaged several times before being replaced with fresh -80°C *B. henselae* glycerol stocks.

E. coli on LB Plates and in LB Broth –

E. coli was streaked onto plain LB agar plates from -80°C glycerol stocks in a biosafety cabinet, and then incubated overnight in a 37°C stationary incubator. Fresh LB broth (10 mL) in a 50 mL Falcon tube was inoculated with a single colony, and then grown overnight in a 37°C rotary incubator. Overnight culture was recovered in the morning; fresh LB media (9 mL) was inoculated with overnight culture (1 mL), and then incubated for ~three hours at 37°C in a rotary incubator until the OD_{600} reached 1.0. The

OD$_{600}$ (optical density of liquid at a wavelength of 600 nm) was measured in 10x10x45 mm clear plastic cuvettes using a Nicolet Evolution 100 UV-Vis Spectrophotometer (Thermo Scientific).

Preparation of *E. coli* Electrocompetent Cells

An *E. coli* liquid culture (10 mL) was prepared from a -80°C Glycerol stock as per above then incubated overnight. The culture was diluted 1:10 and then incubated for several hours in a 37°C rotary incubator, during which the growth rate was monitored until the OD$_{600}$ reached 0.35 to 0.4; the culture was removed from the incubator and cooled for 15 to 20 minutes on ice. Chilled cultures were transferred to pre-chilled Falcon tubes and centrifuged for 10 minutes at 3,000 x *g*, at 4°C. The supernatant was poured off and ice-cold 25% glycerol (25 to 50 mL) added. The Falcon tubes were gently rotated and inverted to remove latent salts from the walls of the tube, taking care not to disturb the pellet. The suspensions were washed via centrifugation a further three times in ice-cold 25% glycerol (50 mL) for 10 minutes at 3,000 x *g*, at 4°C. After each spin, the supernatant was poured off, fresh glycerol was added, and the tubes rotated and inverted as before. After the final centrifugation step, the supernatant was poured off and the pellet resuspended in glycerol (40%) as a freezer stock (5 mL). Aliquots of electrocompetent *E. coli* cultures (100 μL) were divided into cryovials and either used immediately or stored at -80°C.

Preparation of *B. henselae* Electrocompetent Cells

Preparation of electrocompetent *B. henselae* cells was carried out essentially as described in Riess et al, with several modifications[231]. Two confluent *B. henselae* chocolate agar plates were harvested and then resuspended in ice-cold M199S medium (10 mL) in a 50 mL Falcon tube and cooled alongside autoclaved 10% glycerol for

10 to 15 minutes on ice. Cultures were then centrifuged for six minutes at 3,000 x g, at 4°C. The supernatant was removed and pellets were resuspended in ice-cold 10% glycerol (5 mL). Cultures were washed via centrifugation a further three times in ice-cold 10% glycerol (5 mL). After each spin, the supernatant was poured off, and the pellets were resuspended in fresh 10% glycerol. After the final spin, supernatant was poured off and the pellet resuspended in 10% glycerol. Aliquots of electrocompetent *B. henselae* cultures (100 μL) were used immediately.

2.6. Molecular Biology Protocols

2.6.1. Isolation of Genomic DNA

Bacterial genomic DNA was purified using the CTAB extraction method[232]. Briefly, *B. henselae* was scraped from plates and suspended in TE buffer (567 μl; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in a 1.5 mL microcentrifuge tube. Proteinase K (3 μl of 20 mg/mL) and SDS (30 μL of 10%) were added, and the suspension was incubated for one hour in a 37°C stationary incubator. Next, NaCl (100 μl of 5 M) was added to the suspension and mixed thoroughly, before CTAB-NaCl (80 μl; 10% CTAB [hexadecyltrimethyl ammonium bromide] in 0.7 M NaCl) was added. The suspension was incubated for 10 minutes at 65°C on a heating block. An equivalent volume (~0.7 to 0.8 ml) of chloroform:isoamyl alcohol (24:1) was added, vortexed, then centrifuged for five minutes at 10,000 x *g*. After centrifugation, the upper, aqueous phase was removed with a narrow pipette and transferred to a fresh 1.5 mL microcentrifuge tube. An equivalent volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed, and centrifuged for five minutes at 10,000 x *g*. Isopropanol (0.6 volume) was added, then the tube was inverted gently until a visible DNA precipitate formed. The

DNA was pelleted by centrifugation and then washed with ethanol (500 μL of 70% EtOH). The tube was gently inverted to mix the solution, and then centrifuged for five minutes at 10,000 x g. The supernatant was removed and the pellet was air dried. The pellet was then resuspended in TE buffer (100 μl) for freezer storage at -20°C.

2.6.2. Polymerase Chain Reaction (PCR)

All standard PCR reactions in this study were performed using either Advantage® 2 Polymerase (Clontech) or with Phusion® High-Fidelity PCR Polymerase Mastermix (New England Biolabs) in 0.2 mL PCR tubes. Components and quantities of each PCR are listed in **Table 2.5** and PCR conditions in **Table 2.6**.

Table 2.5. – Standard PCR Components and Quantities

PCR Components	Quantity
Advantage Reaction Mixture	
Forward Primer (10 μM)	1 μL (200 nM)
Reverse Primer (10 μM)	1 μL (200 nM)
dNTPs (10mM of each dNTP)	1 μL (200 μM)
Advantage Buffer (10x)	5 μL (1x)
Advantage Polymerase (50x)	1 μL (1x)
DNA Template	x μL (100 to 250 ng)
ddH$_2$O	up to 50 μL
	50 μL Total
Phusion Reaction Mixture	
Forward Primer (10 μM)	0.5 μL (200 nM)
Reverse Primer (10 μM)	0.5 μL (200 nM)
Phusion Master Mix (2x)	12.5 μL (1x)
DNA Template	x μL (100 to 250 ng)
ddH$_2$O	up to 25 μL
	25 μL Total

Table 2.6. – Standard PCR Conditions *Extension temperature was set according to the optimum primer T$_m$, 56.5°C for *carD* qPCR primers and 62.8°C for *rpsL* qPCR primers, although if required both are functional at 58°C.

PCR Conditions	Temperature	Hold Time	Cycle Number
Advantage PCR Conditions			
Pre-heat	95°C	1 Minute	
Denaturation	95°C	30 Seconds	
Annealing	60°C	1 Minute	30 cycles
Extension	68°C	1 Minute	
Post Cycle Extension	68°C	3 Minutes	
Hold	4°C	Infinite hold	
Phusion PCR Conditions			
Pre-heat	98°C	1 Minute	
Denaturation	98°C	10 Seconds	
Annealing	56.5 or 62.8°C*	20 Seconds	30 cycles
Extension	72°C	20 Seconds	
Post Cycle Extension	72°C	5 Minutes	
Hold	4°C	Infinite hold	

2.6.3. Agarose Gel Electrophoresis

For visualising DNA samples, DNA (5 µL with 1 µL loading buffer 6x) was run alongside a DNA reference ladder, Hyperladder™ I (5 µL; Bioline), on agarose electrophoresis gels; agarose gels were 1% w/v of agarose in 1x TAE buffer (100 mL of 40 mM Tris-acetate, 1mM EDTA). Electrophoresis gels were run for 30 to 60 minutes at 100 to 120 V in 1x TAE running buffer. Agarose gels were post-stained in Gelred™ nucleic acid gel stain (10 µL of 10,000x solution; Biotium) for one to two hours, and then visualised and photographed with a GelDoc (Cell Biosciences) under ultraviolet light.

2.6.4. Isolation of Plasmids

E. coli containing the desired plasmid was cultured overnight on selective LB media (50 µg/mL Kanamycin for pJM05 or 10 µg/mL Gentamicin for pEX18gm or their derivatives) from either *E. coli* -80°C glycerol stocks or from recovered *E. coli*

electroporation mixtures. Plasmids were isolated from overnight cultures using a GenepHLow™ kit according to the plasmid isolation protocol, eluting in 30 µL of either 0.22 µm filter-sterilized ddH$_2$O, elution buffer (1 mL of 10 mM Tris-HCl), or TE buffer (1 mL of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Elutions were quantified (1 µL per sample) on a NanoDrop ND-1000™ (Thermo Fisher) machine as per manufacturer's to determine concentration. Eluted plasmids (10 to 25 µL) to be used for electroporation were purified by drop dialysis on MF-Millipore™ Nitrocellulose Membrane Filters (Merck Millipore), floating on top of ddH$_2$O for one hour. Plasmids were then carefully moved by pipette into fresh 1.5 mL microcentrifuge tubes and stored at -20°C.

2.6.5. Purification and Quantification of DNA products

To clean and purify plasmids and PCR products, GenepHLow™ Gel Extraction and PCR Cleanup Kit (Geneaid) or DNA Clean and Concentrator™ kit (Zymo) were used as per the manufacturer's instructions. Elutions were quantified by NanoDrop as described above and stored at -20°C.

2.6.6. Size-Specific Purification of DNA products by Gel Electrophoresis

DNA purification of size-specific PCR products was performed using gel electrophoresis and column purification. PCR products (10 to 20 µL) were run on an agarose gel alongside Hyperladder™ I as described. Agarose gels were viewed under UV light to identify desired products, which were excised from the gel using a razorblade. Gel plugs were then heated and purified with the GenepHLow™ kit, according to the kit instructions. Elutions were quantified by NanoDrop as per above and stored at -20°C.

2.6.7.   Restriction Digest and Ligation Reactions

Restriction digests of DNA products were performed with BamHI and SalI in a double restriction digest or BamHI restriction enzymes (all Thermo Scientific). Purified and quantified DNA products were thawed, added to reaction mixture in 20 μL reactions (PCR components are listed in **Table 2.7**) and then incubated for one hour at 37°C for optimal enzyme conditions. Digested products were column purified using DNA Clean and Concentrator™ kit (Zymo) to concentrate and remove salts and enzymes, and then stored at -20°C. Before column purification of digested pJM05, the plasmid was treated with Calf Intestinal Alkaline Phosphatase (1 μL) for five minutes at 37°C in a stationary incubator, preventing self-ligation of plasmid BamHI ends.

All DNA ligation reactions in this study were performed using T4 DNA Ligase (New England Biolabs). Digested and purified plasmid and *carD* PCR Fusion insert DNA products were combined into a ligation reaction (reaction protocol is listed in **Table 2.7**) and incubated for one hour at room temperature. Ligated plasmid was purified by drop dialysis using Membrane Filters (Merck Millipore) for one hour to remove salts and then transformed into cloning *E. coli* strains.

Table 2.7. – **Restriction Digest and Ligation Reactions Components and Quantities** DNA templates were mixed together at a molar ratio of about 1:6 (where *x*1 = ng of vector and *y*1 = ng of Insert), using the Ligation Template Ratio equation below

Reaction Components	Quantity
BamHI Restriction Digest	
BamHI Enzyme	0.5 µL (5 units)
NE Buffer 3.1 (10x)	2 µL (1x)
DNA Template	x µL (≤ 500 ng)
ddH₂O	up to 20 µL
	20 µL Total
BamHI/SalI Restriction Digest	
BamHI Enzyme	0.5 µL (5 units)
SalI Enzyme	0.5 µL (5 units)
NE Buffer 3.1 (10x)	2 µL (1x)
DNA Template	X µL (≤ 500ng)
ddH₂O	up to 20 µL
	20 µL Total
T4 DNA Ligation Reaction	
T4 DNA Ligase Enzyme	0.5 µL (10 units)
Ligation Buffer (10x)	1 µL (1x)
Mixed Digested DNA Templates*	x µL (≤ 200 ng)
ddH₂O	Up to 10 µL
	10 µL Total

.

$$\text{Vector: Template Ligation Ratio} = \frac{x1}{\text{bp}} : \frac{y1}{\text{bp}}$$

2.6.8. Electroporation into *E. coli* Cells

Plasmids containing the *carD* flanking region insert were introduced into DH10B *E. coli* cells (Life Technologies) by electroporation. Electrocompetent *E. coli* (40 µL) were thawed on ice, and then pipetted into pre-chilled 1.5 mL microcentrifuge tubes on ice. Purified plasmid (2 µL, 100 to 300 ng DNA) was added to each sample with gentle pipetting, and chilled for 30 to 60 seconds on ice. Pre-chilled 0.2 mm electroporation cuvettes were completely dried and the electroporation mix was pipetted into the cuvettes, gently tapping to remove air bubbles and settle the mixture. A pulse was

delivered (25 μF capacitance, 2.5 kV/cm, and 200 Ω resistance; Biorad Gene Pulser Xcell), pre-warmed 37°C SOC (1 mL) was immediately added to the cuvettes cells to recover the cells, and then the mixture was transferred to a fresh microcentrifuge tube. The electroporated cells were incubated for one hour at 37°C with shaking to recover cultures. Recovered electroporation mixes were then spread on selective LB agar plates (kanamycin or gentamicin) at varied concentrations and incubated overnight at 37°C in a stationary incubator.

2.6.9. DNA Sequencing and DNA Sequence Analysis

For sequencing of DNA products, samples were prepared in 20 μL aliquots at ~100 ng of total DNA. Samples were shipped to Macrogen (Korea) for sequencing, using Universal M13 primers. Returned sequencing outputs were analysed using Geneious bioinformatics software program 8.1.7[233], multiple sequence alignment tool.

For analysis and comparison of bacterial DNA and protein amino acid sequences, alignments were performed using Clustal Omega, a multiple sequence alignment program[234].

2.7. Construction of the pJS01 Plasmid

To generate the pJS01 plasmid, sequences flanking the *carD* gene were amplified by PCR. We amplified 0.5 to 0.6 kb of sequence on either side of the *carD* gene, providing enough sequence for homologous recombination to take place. Purified *B. henselae* gDNA (1 to 5 μL, <250 ng) was used as a template for two concurrent PCR reactions (for diagram see **Appendix Figure 8.2**) using Advantage® Polymerase (PCR components are listed in **Table 2.5** and PCR conditions in **Table 2.6**). The first PCR

used the prJS01 primer pair to yield the 5' flank product, while the second PCR used the prJS02 forward and reverse primers to yield the 3' flank product (primers are listed in **Table 2.1**). Both PCR products were predicted to be 0.65 kb. The PCR control reaction with no DNA template was run concurrently.

The resulting PCR products were combined in a 1:1 ratio and used as a template for another PCR reaction, using primers prJS01 forward and prJS02 reverse. Combining both PCR products as a single template enabled internal priming, and then "fusion" of PCR products to occur. The resulting product was predicted to be about 1.3 kb in size. The PCR fusion product was confirmed by gel electrophoresis, then was subjected to a double digest using BamHI and SalI. The plasmid pEX18gm was simultaneously digested, also with BamHI and SalI, and the digestion products combined and ligated to form the pJS01 plasmid. The ligations were electroporated into DH10B cells, which were selected on LB plates with gentamicin. The resulting plasmid was verified by restriction digest and sequencing (Macrogen), and subsequently used for conjugation of *B. henselae*. Confirmed pJS01 plasmids were electroporated into S17-1 *E. coli* donor strain.

2.8. Construction of the pJS02 Plasmid

When conjugations with plasmid pJM01 did not result in merodiploid colonies, we explored constructing a mutagenic plasmid with pJM05, which has been used previously to mutagenise *Bartonella*. Because pJM05 lacks a SalI site in the cloning site, the *carD* flanking region was only changed to adjust the restriction sites for cloning. The *carD* flanking region PCR fusion product (described above) was used as a template for a round of PCR using primers prJS01 forward and prJS02 reverse 2; this introduced

BamHI sites at each end of the PCR fusion product. Because the PCR product was cloned into a single restriction site, the vector was treated with calf intestine alkaline phosphatase to prevent the ends re-joining, following restriction digest. The product was ligated into the BamHI site of pJM05, and the ligation product electroporated into *E. coli*. Bacteria were selected on kanamycin LB plates, and resulting plasmid was verified by restriction digest. Confirmed pJS02 plasmids were transformed into S17-1 as above.

2.9. Transformation of *Bartonella henselae*

2.9.1. *B. henselae* Bi-Parental Conjugation

Transfer of either the pJS01 or pJS02 plasmid into *B. henselae* was carried out by bi-parental conjugation of S17-1 and *B. henselae*, essentially as described by Roden *et al*[230]. S17-1 *E. coli* containing the plasmid was cultured overnight in fresh selective LB media (10 mL, kanamycin or gentamicin) in a 50 mL Falcon tube at 37°C with shaking (250 rpm). The following morning, overnight cultures were diluted 1:10 in fresh LB media (to a volume of 5 mL), then returned to the 37°C incubator for about three hours. The OD_{600} was continuously monitored until it reached an OD_{600} of 1.0 (~$1x10^9$ cells/mL). The S17-1 culture (1 mL) was washed three times in M199S media by centrifugation for one minute at 10,000 x *g*. After each centrifugation step, the supernatant was carefully removed with a narrow pipette and pellets were resuspended in fresh M199S (1 mL). After the final centrifugation and re-suspension step, the resuspended pellet was diluted with 1:50 in fresh M199S media (10 μL into 500 μL). The diluted S17-1 culture containing the plasmid was then pipetted directly (all 510 μL) onto a confluent plate of *B. henselae*. Bacteria were mixed on the plate surface, air-dried

for five minutes, and then were incubated facing up for five hours in a 5% CO_2, 37°C stationary incubator, to allow conjugation to occur. Exconjugants were scraped from the plate, resuspended in fresh M199S (500 µL), and various dilutions were immediately plated (150 mL) onto selective chocolate agar plates. The selective plates contained 10 µg/mL gentamicin or 30 µg/mL kanamycin (to select for transconjugants), and 20 µg/mL cefazolin, and 2 µg/mL nalidixic acid, to suppress the growth of *E. coli*. Selective plates were incubated for 10 to 14 days in a humidified CO_2, 37°C stationary incubator.

2.9.2. *B. henselae* Electroporation

Transformation of mutagenic plasmids into *B. henselae* was carried out as described in the literature[235,236], with several adjustments. Electrocompetent *B. henselae* cells (40 µL) were thawed on ice, and then pipetted into pre-chilled 1.5 mL microcentrifuge tubes placed alongside 1 mm electroporation cuvettes on ice. Purified plasmid (2 µL; 200 to 800 ng of total DNA) was added to each sample with gentle pipetting, and chilled for 30 to 60 seconds on ice. The electroporation mix was pipetted into the cuvettes, gently tapping to remove air bubbles and to settle the mixture. Cuvettes were dried on the outside and inserted into the electroporation machine; a pulse was delivered (25 µF capacitance, 2.5 kV/cm, and 200 Ω resistance). Pre-warmed 37°C Schneider's Drosophila Medium containing FCS (1 mL) was added immediately to the cuvettes to recover the cells. The electroporation mixtures were transferred to fresh microcentrifuge tubes and were incubated overnight in a 5% CO_2, 35°C stationary incubator. Recovered electroporation mixes were spread (100 µL) onto selective chocolate agar plates at various concentrations. Selective plates contained 10 µg/mL gentamicin or 30 µg/mL

kanamycin. Selective plates were incubated for 10 to 14 days in a 5% CO_2, 35°C incubator.

2.10. Growth and Stress Response Assays

2.10.1. Acclimatised *B. henselae* Bacterial Suspension Preparation

Three to four confluent *B. henselae* chocolate agar plates were harvested and resuspended in Schneider's Drosophila Medium containing 10% FCS (1 mL) in 1.5 mL microcentrifuge tubes (one tube per plate). Resuspended *B. henselae* cultures were washed three times in Schneider's Medium via centrifugation for five minutes at 3,000 x *g*. After each centrifugation step, the supernatant was carefully removed with a narrow pipette and pellets were resuspended in fresh Schneider's Medium containing FCS (1 mL). After the final centrifugation and re-suspension step, the washed suspensions were combined in a 50 mL Falcon tube then diluted into pre-warmed 37°C Schneider's Medium containing FCS to an OD_{600} equal to 1.0. The diluted *B. henselae* suspension was then incubated for two hours in a humidified 5% CO_2, 37°C stationary incubator to acclimate the bacteria to the liquid culture condition. The acclimatised *B. henselae* suspension was further diluted either 1:10 for growth assays or 3:10 for stress assays, and divided into replicates in 50 mL Falcon tubes using pre-warmed 37°C Schneider's Medium containing FCS. The result was *B. henselae* bacterial suspensions in Schneider's Medium containing FCS at an OD_{600} of 0.1 or 0.3. Each growth assay replicate was carried out in a volume of 30 mL, while each stress assay replicate was carried out in a volume of 10 mL.

2.10.2. Baseline Growth Control

Three acclimatised *B. henselae* bacterial suspensions in Schneider's Drosophila Medium containing FCS (at an OD_{600} of 0.1, of a volume of 30 mL) were prepared as above, and incubated for 14 days in a humidified CO_2, 37°C incubator; the OD_{600} was taken for 14 days to generate a growth curve using GraphPad Prism Version 6.0 graphing software.

2.10.3. Low Temperature Growth Assay

Three acclimatised *B. henselae* bacterial suspensions in Schneider's Drosophila Medium containing FCS (at an OD_{600} of 0.1, of a volume of 30 mL) were prepared as above, with the following adjustments. Each suspension was diluted with pre-warmed 28°C Schneider's Medium containing FCS, and suspensions were incubated in a CO_2 GENbox (BioMérieux) inside a humidified 28°C stationary incubator; the OD_{600} was taken once daily for 14 days to generate a growth curve as above.

2.10.4. Haemin Stress Assay

Three acclimatised *B. henselae* bacterial suspensions in Schneider's Drosophila Medium containing FCS (at an OD_{600} equal to 0.3, of a volume of 10 mL) were prepared as above and treated with haemin. The first suspension was a non-supplemented control, with the remaining two *B. henselae* suspensions supplemented with haemin (Sigma) at 0.1 mM and 0.5 mM respectively. Suspensions were incubated for 24 hours in a humidified 5% CO_2, 37°C stationary incubator, during which samples were taken regularly (twice from each suspension). Each sample was serially diluted in Schneider's Medium containing FCS; dilutions were streaked (100 µL per dilution) onto plain chocolate agar plates, and then incubated for 14 days in a CO_2, 37°C incubator. Colonies on each plate were counted by hand or using colony counting software (OpenCFU[237])

to determine rate of survival in each haemin condition, which was graphed using GraphPad Prism Version 6.0.

2.10.5. Ciprofloxacin Assay

Acclimatised *B. henselae* bacterial suspensions in Schneider's Drosophila Medium containing FCS (at an OD_{600} equal to 0.3, of a volume of 10 mL) were prepared as above and treated with ciprofloxacin (Sigma). Alongside an untreated control, bacterial suspensions were supplemented with ciprofloxacin at 10 µg/mL or 25 µg/mL. Suspensions were incubated for 10 hours in a 5% CO_2, 37°C incubator, during which samples were taken regularly; each sample was prepared by serial dilution as before, plated onto plain chocolate agar plates, and then incubated for 14 days in a CO_2, 37°C incubator. Colonies were counted and the rate of survival determined as for haemin above.

2.10.6. Hydrogen Peroxide (H_2O_2) Assay

Acclimatised *B. henselae* bacterial suspensions in Schneider's Drosophila Medium containing FCS (at an OD_{600} equal to 0.3, of a volume of 10 mL) were prepared as above and treated with H_2O_2. Alongside the untreated control, the remaining bacterial suspensions were supplemented with H_2O_2 at 0.1 mM or 1 mM and then incubated for one and a half hours in a 5% CO_2, 37°C incubator. As for previous assays, samples were taken regularly throughout the incubation, serially diluted onto chocolate agar and incubated for 14 days in a CO_2, 37°C incubator. Resulting colonies were counted and the rate of survival was determined as for haemin-treated colonies as above.

2.10.7. Starvation Assay

An undivided, acclimatised *B. henselae* suspension in Schneider's Drosophila Medium containing FCS (at an OD_{600} equal to 1) was washed a further three times in 1x PBS via centrifugation to remove all nutrients. *B. henselae* suspended in pre-warmed 37°C 1x PBS was divided into three aliquots and diluted to reach an OD_{600} of 0.3, with one suspension diluted in Schneider's Medium containing FCS to act as a control, and two suspensions diluted in 1x PBS; suspensions were incubated for 10 hours in a 5% CO_2, 37°C incubator. Samples were regularly taken from each suspension, serially diluted and then plated onto chocolate agar plates for incubation, 14 days at CO_2, 37°C. Colonies were counted and the rate of survival was determined as for haemin assays above.

2.11. qPCR Analysis of *carD* Stress Induced Expression

2.11.1. Selection of *B. henselae* Stress Conditions

The results of the *B. henselae* stress condition assays were analysed, and for each stress condition, the concentration of stressor and length of stress exposure were chosen to correspond with *B. henselae* survival of 80 to 90%. The only exception was for the 37°C and low temperature growth assays, where the bacteria continued to replicate. All the stress conditions are listed in **Table** 2.8 below.

Table 2.8. – *B. henselae* Stressors and Stressor Conditions

Stress Condition	Supplement Concentration	Time of Stress Exposure
Baseline Growth Control (37°C)	N/A	Two Hours
Low Temperature (28°C)	N/A	Two Hours
Haemin	0.1 mM	One Hour
Ciprofloxacin	10 ng/µL	One Hour
Hydrogen Peroxide (H_2O_2)	0.1 mM	10 Minutes
Starvation	PBS	30 Minutes

2.11.2. Collection of *B. henselae* Cultures under Treatment Conditions

Acclimatised *B. henselae* in Schneider's Drosophila Medium containing FCS (at an OD_{600} of 1.0) were prepared from six to seven confluent *B. henselae* plates. The suspension was diluted by 3:10 and divided into six volumes in 50 mL Falcon tubes using pre-warmed Schneider's Medium containing FCS (five at 37°C and one at 28°C, each at a volume of 10 to 15 mL). The result was six suspensions of *B. henselae* in Schneider's Medium containing FCS at an OD_{600} equal to 0.3. Each *B. henselae* culture was supplemented with their respective stressor as indicated in **Table 2.8** and incubated at 5% CO_2, 37°C for the indicated time (or for low temperature, at 28°C in a CO_2 GENbox). In the case of the starvation condition, the culture was washed in 1x PBS via centrifugation as per above and then incubated at 5% CO_2, 37°C.

After the cultures were exposed to the stressor for the designated time, a volume of each culture containing approximately $1x10^8$ bacteria was combined with a RNA stop solution (100 µL of 5% phenol in absolute ethanol) in 1.5 µL microcentrifuge tubes to prevent additional transcription of RNA. Cultures were mixed by inversion and immediately centrifuged at maximum speed for two to three minutes to pellet bacteria. The supernatant was removed by pipetting and the pellets were immediately frozen at -80°C freezer overnight.

2.11.3.   *B. henselae* RNA Extraction using TRIzol and RNeasy

All RNA work was performed using RNase-free reagents in a biosafety cabinet. Pelleted *B. henselae* colonies from each stress condition were removed from the -80°C freezer and briefly thawed at room temperature. Each pellet was resuspended in lysozyme (50 µL of 0.4 mg/mL lysozyme in TE; Sigma) with gentle pipetting and incubated for five minutes at room temperature to weaken the cell wall. TRIzol reagent (0.95 mL; Life Technologies) was added to each microcentrifuge tube, vortexed for 30 to 60 seconds, and then incubated for five minutes at room temperature. Chloroform (200 µL) was added to each microcentrifuge tube; tubes were sealed and shaken by hand for 15 seconds, and then incubated for two to three minutes at room temperature. Phases were separated by centrifugation for 15 minutes at 12,000 x g. Taking care not to disturb the middle or lower phases, the colourless upper phase containing RNA was pipetted into fresh 50 mL falcon tubes, with one Falcon tube per stress condition; up to four bacterial pellets for each sample were combined.

To prepare the RNA suspensions for RNeasy® (Qiagen) columns, absolute ethanol (of a volume equivalent to 0.5 of the volume of RNA) was slowly added to each 50 mL Falcon tube via a pipette whilst vortexing at the lowest setting to mix. The RNA-ethanol suspension was loaded onto RNeasy® columns in sequential 700 µL aliquots, and centrifuged at maximum speed for 30 seconds after each aliquot addition, discarding the flow-through after each spin. Once all the RNA was loaded onto the columns, Buffer RW1 (0.35 mL) was added to the column and centrifuged at maximum speed for 30 seconds, discarding the flow-through. An on-column DNA digestion using DNase I (Qiagen) was then performed. For each column, 10 µl of DNase I stock solution was combined with 70 µl Buffer RDD, and the total (80 µl) was added to each column

and incubated for 30 minutes at room temperature. Buffer RW1 (0.35 mL) was added to each column, followed by centrifugation for 30 seconds at maximum speed, discarding flow through. Buffer RPE (500 μL) was added to each column twice; the first spin was for 30 seconds at maximum speed, while the second spin was for two minutes. The empty columns were spun for a further minute to dry the columns. To elute the RNA, RNA-free H_2O (50 μL) was added to the centre of each column, incubated for two minutes at room temperature, and then centrifuged for one minute at maximum speed. A small portion of RNA (3 to 5 μl) was placed in a new tube for testing, and the remainder was stored at -80°C. RNeasy® purified RNA was analysed for concentration and quality via NanoDrop.

2.11.4. Synthesis of cDNA Using Reverse Transcriptase

Synthesis of cDNA products was performed using GoScript™ Reverse Transcription System (Promega). The RNA and a random hexamer primer were combined (reaction components and volumes are listed in **Table 2.9**) in 0.2 mL PCR tubes and incubated for five minutes at 70°C to denature any RNA secondary structures, then chilled on ice for five minutes. Reverse transcription reaction mixtures for cDNA were prepared and added to the primer/RNA mix. For the reverse transcription reaction, 15 μL of reverse transcription reaction mixture was added to each 5 μL of first strand synthesis mixture, then the combined reaction mixture was incubated for five minutes at 25°C (annealing), for one hour at 42°C (extension), and for 15 minutes at 70°C (inactivation). Extended cDNA fragments were stored at -20°C. Negative controls, which included reverse transcription reactions with no template and, for each stress condition, a sample with no reverse transcriptase added, were run concurrently.

Table 2.9. – Reverse Transcription Reaction Components and Quantities

Reaction Components	Quantity
RNA and Primer Mixture	
RNA Template	x μL (100 to 300 total ng)
Random hexamer Primers (10 μg)	1 μL (0.5 μg)
Nuclease-Free H_2O	up to 5 μL
	5 μL Total
Reverse Transcription Reaction Mixture	
Nuclease-Free H_2O	7 μL
GoScript™ Reaction Buffer (5x)	4 μL (1x)
$MgCl_2$ (25mM)	1 μL (1.25 mM)
Nucleotide Mix (10 μg)	1 μL (0.5 mM)
RNasin® Ribonuclease Inhibitor	1 μL (20 units)
GoScript™ Reverse Transcriptase (20x)	1 μL (1x)
	15 μL Total (per reaction)

2.11.5. Quality Control of cDNA Products

To confirm successful reverse transcription and that the RNA samples lacked genomic DNA contamination, a portion of each cDNA product (2 μL) was used as a template for two consecutive PCR reactions with Phusion® High-Fidelity PCR Polymerase Mastermix (New England Biolabs; PCR components and conditions are listed in **Table 2.5** and **Table 2.6**. The housekeeping ribosomal gene, *rpsL*, was used as an internal control. All samples were amplified separately by PCR using the *carD* prJS03 primer pair or the *rpsL* prJS04 primer pair (all primers are listed in **Table 2.1**). PCR control reactions with no reverse transcriptase, no template, or with *B. henselae* gDNA, were run concurrently. PCR products were run on agarose electrophoresis gels alongside Hyperladder I and analysed under UV light.

2.11.6. qPCR Quantification of *B. henselae carD* Expression

For qPCR reactions, KAPA SYBR® FAST Kit (KAPA Biosystems) was used in a CFX96™ Real-Time PCR Detection System machine (Biorad). For each *B. henselae* stress condition, two qPCR reactions were performed in duplicate. The first used *carD* gene-specific pJS03 primers to determine the level of *carD* expression, and the second used *rpsL* housekeeping gene pJS04 primers as a baseline control. qPCR control reactions with no reverse transcriptase (for each stress condition), and a no template control were run concurrently with both *carD* and *rpsL* primers.

Reaction mixtures for qPCR were prepared as below, first thawing components on ice, and then combining in the order listed in **Table 2.10**, in 0.2 mL PCR tubes with optically clear caps. Samples were run using the qPCR cycling conditions listed in **Table 2.11**, and the results analysed using CFX Manager™ 3.0 (Biorad) and using GraphPad Prism Version 6.0.

Table 2.10. – Standard qPCR Components and Quantities

Reaction Components	Quantity
qPCR Reaction Mixtures	
Nuclease-Free H_2O	4 μL
KAPA SYBR FAST Mastermix (2x)	10 μL (1x)
cDNA Template (5 ng/μL)	2 μL (10 ng Total)
Forward Primer (2 μM)	2 μL (200 nM)
Reverse Primer (2 μM)	2 μL (200 nM)
	20 μL Total

Table 2.11. – Standard qPCR Conditions

qPCR Conditions	Temperature	Hold Time	Cycle Number
qPCR Conditions			
Pre-heat	95°C	3 Minutes	
Denaturation	95°C	3 Seconds	
Annealing/Extension	58°C	20 Seconds	40 cycles
Dissociation Curve	60 to 95°C in 0.5°C increments	0.05 Seconds per increment	

Chapter 3. Construction of a *Bartonella henselae carD* Markerless Deletion Strain

3.1. Introduction

The aim of this chapter was to generate a B. henselae ΔcarD mutant strain to compare the growth rate to that of the B. henselae wild type strain under stress conditions.

CarD plays an important role in *Mycobacterium tuberculosis, Myxococcus xanthus,* and *Thermus thermophilus* bacteria*,* in global regulation of mRNA and in DNA repair; in these bacteria *carD* is up-regulated in response to DNA strand breaks caused by endonucleases and genotoxic stresses[212]. The CarD amino acid sequence homology between *B. henselae*, *M. tuberculosis*, *M. xanthus,* and *T. thermophilus* is significant; a NCBI Blast query showed that *B. henselae* CarD (NCBI: WP_011181290.1) shared 30.0%, 35.0%, and 36.0% sequence similarity with *M. tuberculosis* CarD (NCBI: WP_057133327.1), *M. xanthus* CdnL (carD equivalent; NCBI: 2LWJ_A) and *T. thermophilus carD* (NCBI: WP_011172978.1) respectively (for amino acid sequence alignments see **Appendix Figure 8.1**). Due to the level of sequence similarity between well studied bacterial species and *Bartonella* CarD, we hypothesised that CarD carries out similar functions in *Bartonella* species.

A common method of analysing specific gene function is by generating a mutant bacterial strain through targeted gene deletion. Though this method is not viable in *Mycobacterium* and *Myxococcus* as *carD* is required for growth, this does not appear to hold true for *carD* in *Bartonella birtlesii*[176], and potentially other *Bartonella* species. As such we aimed to generate a *B. henselae* Δ*carD* strain.

3.2. Results

To generate a *B. henselae* ΔcarD bacterial strain, we opted to use a targeted approach similar to those previously published for *Bartonella*[228,235,236]. Briefly, genomic sequences flanking the *B. henselae carD* gene were amplified and then fused together by PCR. The *carD* PCR fusion product was inserted into a suicide plasmid, lacking an origin of replication that functions in *Bartonella*, and transferred into *B. henselae* through either bi-parental conjugation or electroporation. The *carD* flanking sequences were long enough to enable homologous recombination between plasmid and *B. henselae* DNA, the result of which was the integration of the plasmid into the chromosome next to *carD*, resulting in a merodiploid gene (for a diagram of recombination, see **Figure 3.1**). *B. henselae* cells with the integrated plasmid were selected for by antibiotic selection. The integrated plasmid encodes a *sacB* gene, which enables negative selection and loss of the integrated plasmid, following growth on selective media containing sucrose[228,238]. The loss of the plasmid, by a second homologous recombination event, may either result in restoration of the wild type *carD* gene, or complete loss of the gene, resulting in a null mutant. The goal of this project was to assess the susceptibility of the resulting Δ*carD B. henselae* mutant strain to environmental stresses, including low temperature (28°C), haemin exposure, genotoxic stresses (ciprofloxacin and hydrogen peroxide), and nutrient starvation.
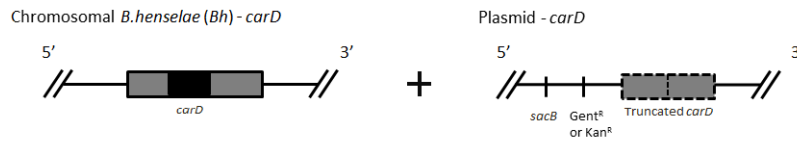
Figure 3.1. – Mutagenesis and selection of the *carD* mutant (a.) Successful transfer of the plasmid through electroporation or conjugation leads to the presence of *carD* in both the suicide plasmid and in *B. henselae* chromosomal DNA. (b.) Through merodiploid positive selection with antibiotics, only *B. henselae* with an integrated plasmid grows. Chromosomal *carD* and truncated *carD* have a crossover and recombination event that leads to chromosomal insertion of the plasmid alongside *carD*, as the plasmid cannot persist alone in the cell. (c.) Through merodiploid negative selection, only *B. henselae* that have lost the *sacB* gene can grow. The gene was lost via one of two possible *carD-carD* recombination events (see c, dotted lines, area covered by arc is lost) resulting in the loss of the plasmid (and *sacB*). Both resolutions lack both *sacB* and genes conferring resistance to gentamicin or kanamycin, although resolution 1 has resulted in a Δ*carD* mutant while resolution 2 results in restoration of a wild type *carD*.

3.2.1.　Generation of the Mutagenic Plasmid pJS01

In selecting an appropriate candidate for the starting plasmid to construct our *B. henselae* mutagenic plasmid, several criteria needed to be met. For conjugation to be successful, the mutagenic plasmid needed to possess an origin of transfer (*oriT*), and a set of mobility genes[239]. The resulting plasmid must also have the requisite genes for selection of bacterial colonies, including an antibacterial resistance gene that functions in *Bartonella* and a sucrose-selective *sacB* gene. The plasmid pEX18gm[226] met all the above criteria, and was selected to be the base of our mutagenic pJS01 plasmid.

To generate the pJS01 plasmid, several successive stages of PCR were utilised. For the first stage, flanking regions of the *B. henselae carD* gene were amplified by PCR. These flanking regions were of sufficient size to allow homologous recombination within these regions to occur. The flanking regions were fused via a subsequent PCR step, resulting in an in-frame deletion of the majority of the *carD* gene as shown below (for a diagram of the generation of *carD* fusion products, see **Appendix Figure 8.2**). The fusion product resulted in loss of 347 of 585 base pairs of the *carD* gene, leaving 258 base pairs of the *carD* gene after accounting for the addition of primer overhangs used to fuse the 3' and 5' flanking products (for the *carD* gene sequence see **Appendix Figure 8.3**). No frame shift in amino acid sequence was observed, with 86 amino acids predicted to remain in the mutant. The *carD* PCR fusion product was inserted into the pEX18gm plasmid by restriction digest and ligation, to yield the pJS01 plasmid. The resulting plasmid was verified by restriction digest, as shown in **Figure 8.2**, and sequencing (for pJS01 sequence alignments see **Appendix Figure 8.4**). The plasmid was then transformed into S17-1, and the *E. coli* strain was then unsuccessfully used for bi-parental conjugations.
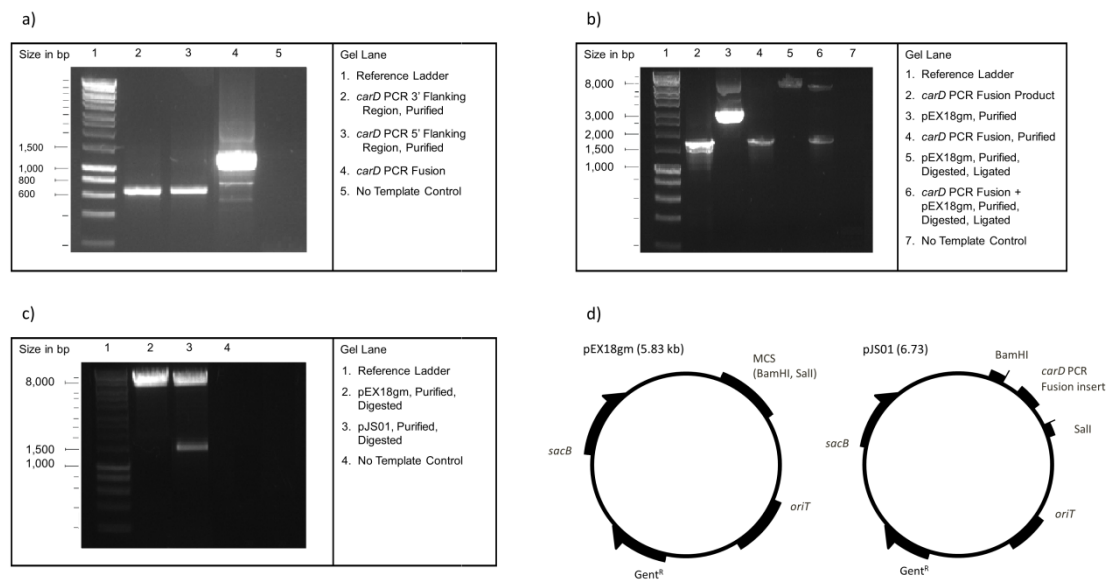
Figure 3.2. – pEX18gm and pJS01 Agarose Electrophoresis Gels and Bacterial Plasmid Maps a) Products from the first two rounds of PCR. The 5' and 3' flanking regions were amplified, purified and used as the template for the fusion PCR. Both PCR products were estimated to be ~650 base pairs, while the *carD* fusion product was estimated to be ~1,300 base pairs in size, as seen above. Products of non-specific DNA amplification were removed from the PCR fusion sample by gel purification. b) The PCR fusion and the pEX18gm products were purified, digested and ligated together. c) Ligated PCR fusion and pEX18gm products were transformed into an *E. coli* cloning strain, then purified and digested, confirming that pJS01 had the correct insert. d) Plasmid maps of pEX18gm and pJS01. Sites of interest in pEX18gm include: *oriT* (origin of transfer), *sacB* (sucrose sensitivity), Gent[R] (gentamicin resistance), and a multiple cloning site (MCS) containing both BamHI and SalI restriction sites. The pJS01 plasmid contains the same sites of pEX18gm, with the addition of the *carD* gene sequence insert (*carD* PCR fusion insert) at the MCS, to enable homologous recombination and allelic replacement of the *carD* gene.

3.2.2. Generation of the Mutagenic Plasmid pJS02

As transformation with pJS01 into *B. henselae* did not yield colonies, we chose to explore other options. Though pEX18gm met all the criteria of our *B. henselae* transformation and mutation experiments, this plasmid had not previously been used for *B. henselae* mutagenesis; therefore, a second mutagenic plasmid was constructed, starting with a plasmid, pJM05, that was derived from the *Bartonella* mutagenesis plasmid pRS14, and that had been previously used to successfully mutagenise *Bartonella*[227,228,240]. A sample of pJM05 plasmid DNA was kindly provided by Dr Jane Koehler (UCSF, California), and used to construct our mutagenic plasmid, pJS02.

The methodologies of constructing the pJS01 and pJS02 plasmids were similar, but due to the differences in restriction sites between pEX18gm and pJM05, minor adjustments were required. Unlike pEX18gm, pJM05 lacks a SalI restriction enzyme site within its multiple cloning site; the *carD* PCR fusion product used to generate pJS01 could not be used to generate pJS02. The *carD* PCR fusion product was remade essentially as before, but with different restriction enzyme sites.
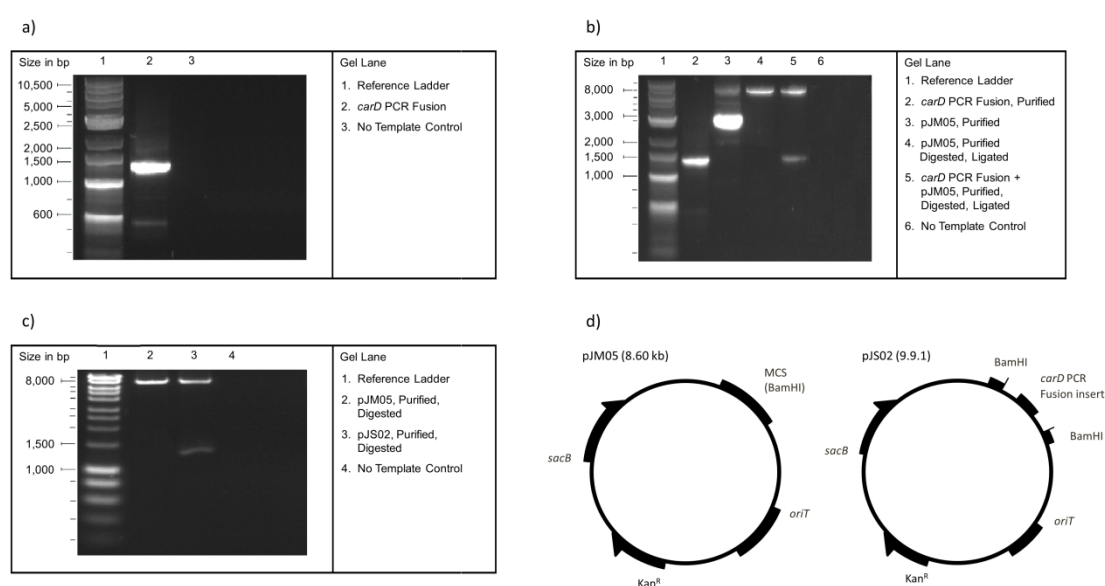
Figure 3.3. – pJM05 and pJS02 Agarose Electrophoresis Gels and Bacterial Plasmid Maps a) The *carD* fusion product used to generate pJS01 was used as a template to form the new *carD* PCR fusion product, both of which were estimated to be ~1,300 base pairs in size, as seen above. b) The purified PCR fusion product and pJM05 were digested with BamHI and ligated together. As just the BamHI enzyme was used, only the new *carD* PCR fusion product is integrated into pJM05. c) The resulting pJS02 plasmid was purified and analysed by restriction digest. d) Plasmid maps of pJM05 and pJS02. Sites of interest in pJM05 include: *oriT*, *sacB*, Kan[R] (kanamycin resistance), and a multiple cloning site (MCS) containing a BamHI restriction site. The pJS02 plasmid contains the same sites as pJM05, with the addition of the *carD* gene sequence insert (*carD* PCR fusion insert) at the MCS.

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3.2.3. Transformation of *B. henselae*

For transformation of *B. henselae*, we turned to the *Bartonella* literature for protocols. The two techniques commonly used in *Bartonella* mutagenesis protocols to transform a plasmid into the bacteria are bacterial conjugation and electroporation.

Bi-parental conjugation is a method of horizontal gene transfer via a pilus extended from the donor to the recipient cell, wherein the plasmid is linearized and transferred as single-stranded DNA into the recipient bacteria. For a successful conjugation, the RP4 conjugal transfer genes must be expressed in the donor strain. For a donor strain, we used S17-1 λ *pir E. coli*, which has these genes integrated onto the chromosome[241,242]. In preparation for the transformation, both pJS01 and pJS02 were seperately transferred into the S17-1 *E. coli* donor strain via electroporation. However, we observed that the resulting S17-1 strain with the plasmid had a reduced growth rate. We normally observed that S17-1 strains, carrying other plasmids, will reach an optical density of 1.0 after about one and a half hours, following the 1:10 dilution of an overnight culture. However, S17-1 with pJS01 or pJS02 would typically reach a lower OD_{600} after overnight growth, and typically required 4 hours or more to reach an OD_{600} of 1 when 1:10 dilutions were made the following morning. The significance of this slow growth rate is not fully understood, although it may be due to latent transcription of the *carD* gene.

In the *Bartonella* literature, bi-parental mating has been reported[230,243]. Briefly, cultures of S17-1 containing the pJS01 plasmid were grown, washed to remove antibiotics, and added to confluent *B. henselae* plates; *E. coli* and *B. henselae* cells were incubated together to allow for conjugation, and then the exconjugants were harvested and re-plated on fresh selective chocolate agar plates to select for transconjugant merodiploid

cells. After incubation, no resulting merodiploid colonies were found (attempted variations to the stated protocol are listed below). When repeated conjugations with plasmid pJS01 did not result in colonies, we opted to try conjugations using pJS02, a mutagenic plasmid constructed from pJM05, which has been used successfully to make *B. quintana* and *B. henselae* mutants numerous times[228,240]. Despite repeated attempts, no transconjugant merodiploid colonies appeared after multiple iterations using either mutagenic plasmid, or following introduction of any of the variations listed.

When the pJS01 and pJS02 plasmids were constructed and transformed into S17-1 for biparental conjugation, we observed that the resulting S17-1 strains containing the plasmids had a slow growth rate; though pJS01 growth was never compared against the base pEX18gm plasmid, growth of S17-1 carrying pJS02 was found to be significantly slower when compared to S17-1 carrying pJM05 or other plasmids (data not shown). This indicates that our pJS01 and JS02 plasmids may have detrimental effects on *E. coli* growth, which could interfere with transformation efficiency. It has been observed in other labs that cultures of S17-1 containing a mutagenic plasmid can exhibit a slowed growth rate, yet still be viable for conjugation (Dr Jane Koehler, personal communication).

Table 3.1. – Bi-Parental Conjugation Experimental Variations Each experimental step that was varied above was performed in conjunction with the regular protocol steps, and performed in duplicate alongside the regular conjugation protocol. When all options were exhausted with the pJS01 plasmid, the pJS02 plasmid was created and used in all future conjugation experiments; some, but not all of the above variations were tested using the pJS02 plasmid.

Experiment Step	Regular Protocol	Variation
S17-1 Culture Preparation	S17-1 cultures were washed three times with M199S at 10,000 x g	Washed as before, with 1x PBS instead of M199S
Confluent *B. henselae* Culture Preparation	*B. henselae* were unwashed and left on the confluent plate before addition of S17-1	Bacteria were harvested and washed once in M199S Medium at 10,000 x g, then re-plated onto fresh chocolate plates alongside S17-1
S17-1 Conjugation Concentration	S17-1 culture (in M199S at an OD_{600} of 1) was diluted by 1:50 before conjugation	Cultures are diluted by 1:30 and 1:70 dilutions
Conjugation Incubation Length	*B. henselae* and S17-1 conjugation mixtures were incubated for five hours in a 5% CO_2, 37°C incubator	Conjugation incubations were extended to eight hours or overnight
Exconjugant Incubation Length	Harvested cultures were plated and incubated on fresh selective media for 10 to 14 days	Cultures were prepared as before, incubation time was increased, 14 to 20 days
Exconjugant Incubation CO_2 Conditions	Culture were incubated in a 5% CO_2, 37°C Incubator	Selective plates were sealed inside either a 5% CO_2 GENbox, a 5% CO_2 GENbag, or a candle extinction jar, in a 37°C incubator
Exconjugant Incubation Temperature	Cultures were incubated in a 5% CO_2, 37°C incubator	Incubation temperature for selection was lowered to 35°C
Conjugation Mutagenic Plasmid	The pJS01 plasmid was transferred to S17-1 and used in the conjugation	As noted above, the pJS02 plasmid was constructed used for the conjugation
Bacterial Strain	S17-1 *E. coli* were prepared for electroporation, and used as outlined above	Fresh S17-1 *E. coli* (Biomedal) were ordered and used as before

In addition to conjugations, electroporation transformation techniques were attempted with both plasmids when no transconjugant colonies could be obtained. Electroporation protocols in *B. henselae* are frequently reported[235,236], although as transformation by electroporation may be damaging to bacteria, conjugation was our first preference. In the *Bartonella* literature, most electroporation procedures are largely similar, with only minor variations in media and recovery time[243,244]. In the literature, recovery of electroporated cells was done using a supplemented RPMI broth that included rabbit blood lysate; we were not able to obtain animal blood in time for our experiments, so Schneider's Medium containing FCS was used as a substitute.

Though wild type *B. henselae* were found to grow under these conditions, no merodiploid colonies were found using electroporation. Additionally, after the conclusion of this study, other members of our lab carried out further *B. henselae* electroporation using sheep blood lysate and similarly did not obtain merodiploid colonies. All variations attempted are listed in the table below.

Table 3.2. – *B. henselae* **Electroporation Experimental Variations** Variations listed in above were performed as for bi-parental transformations, in duplicate and beside the regular electroporation protocol.

Experiment Step	Regular Protocol	Variation
B. henselae Culture Preparation	Two confluent *B. henselae* plates were suspended in M199S, and then washed via centrifugation	Either one or three confluent plates were harvested and washed
Plasmid Electroporation Concentration	~400 ng of total plasmid DNA was treated with drop dialysis and mixed with *B. henselae* cells on ice for electroporation	~200, 300, 500, 600 ng of total plasmid DNA were used for electroporation
Recovery Incubation Length	Electroporated cells were immediately recovered in pre-warmed 37°C Schneider's Medium containing FCS, and then incubated overnight in a 5% CO_2, 37°C incubator	Electroporated *B. henselae* were recovered for seven hours, the streaked onto selective plates
Recovery Incubation CO_2 Conditions	Recovered cultures were incubated overnight in a 5% CO_2, 37°C incubator	Cultures were sealed in either a 5% CO_2 GENbox, a 5% CO_2 GENbag, or a candle extinction jar, in a 37°C incubator
Recovery Incubation Temperature	Cultures were incubated overnight in 5% CO_2, 37°C incubator	Cultures were incubated at 35°C.
Transformation Plasmid	Electroporation was performed with purified pJS02 plasmid DNA	pJS01 was used for electroporation with *B. henselae*
Bacterial Strain	S17-1 *E. coli* were prepared for electroporation, and used as outlined above	Fresh S17-1 *E. coli* (Biomedal) were ordered and used as before

3.3. Discussion

The goal of this experiment was to generate a ΔcarD *B. henselae* mutant, with the aim of comparing growth of the mutant to wild-type *B. henselae*. To determine the reason why merodiploid *B. henselae* colonies were not recovered, each experimental step must be analysed.

Transformation experiments in *Bartonella* are commonly reported in the literature, yet despite the multiple variations tested in this study, no transformed merodiploid colonies were obtained. Due to the slowed growth rate of our S17-1 *E. coli* strains, we have reason to suspect that our mutagenic *carD* plasmids had a negative effect on our transformations.

First, regarding our conjugations, the delayed growth of S17-1 containing our pJS01 or pJS02 plasmids suggests that these plasmids interfered with normal *E. coli* growth; this delay may extend to other, non-essential functions as well, e.g. pilus formation. This would thus lower the chances of a successful transformation. Second, transformation of our plasmids through electroporation does not require an intermediate S17-1 *E. coli* host, although merodiploid colonies were still not recovered. This suggests that either *B. henselae* growth is also affected by the *carD* fragment, or that the electroporation failed. Although no arcing was observed during the delivery of the pulse, electroporation is known to be damaging to cells, and we were unable to obtain merodiploid colonies. Additionally, other labs have reported difficulty with Bartonella electroporation protocols (Dr Jane Koehler, personal communication).

Potential toxicity of the *carD* mutagenic plasmid could be explained in several ways. A mutation in the pJM05 plasmid backbone, affecting, for example, the kanamycin

resistance gene, has previously been observed to impact growth to a similar degree to that which we have observed in our pJS02 strain (Jane Koehler, personal communication). This would explain why we observed slower growth with pJS02, compared to pJM05. However, this would not explain why we observed a slow growth rate with pJS01. Sequencing of the pJS02 plasmid, in addition to the MCS and the *carD* flanking region junction, would confirm if this is the case.

Alternatively, possible toxicity of the mutagenic plasmid could be due to latent expression of the truncated *carD* gene. Although around 60% of the *carD* open reading frame is deleted in our plasmid constructs, expression of the 85 remaining CarD amino acids on the pJS01 sequence plasmid is possible as no frame shift was introduced. The start codon is still present, and transcription of a truncated CarD protein is possible. The pJS01 hypothetical CarD protein has an amino acid sequence that is homologous to the N-terminal region of *M. tuberculosis* CarD and *M. xanthus* CdnL. The N-terminus region includes the β-sheets that make up the Tudor-like RNA polymerase binding domain of the proteins; although the hypothetical *B. henselae* CarD protein is heavily truncated, it has amino acid homology to *M. tuberculosis* β1 and β2 folds (a possible truncated β3 fold) and *M. xanthus* β1, β2, and β3 folds (with a possible truncated β4 fold (see **Appendix Figure 8.1**)[215,217]. As our plasmids are high copy number, even a small level of expression from each plasmid could lead to an expression sufficient to impact growth. While the target for *B henselae* CarD is unknown, both *Mycobacterium* and *Myxococcus* CarD homologues target the β-subunit of RNA polymerase, while both *B. henselae* and *E. coli* DksA target the secondary channel of RNA polymerase[209,211,219]. Though the target site on RNA polymerase differs between CarD and DksA, CarD fragment expression could potentially disrupt biological function in

E. coli and *B. henselae* by blocking the RNA polymerase binding site of CarD or DksA, or by interfering with transcription[212].

In conclusion, several follow-up experiments could be performed to determine why the transformations did not result in colonies, and to successfully generate a *carD* deletion strain. A positive transformation control reaction could be carried out, introducing a broad host range plasmid into *B. henselae*, to confirm that the transformation techniques are working. The mutagenic plasmid could be also remade, including a *carD* flanking region insert that lacks any of the *carD* open reading frame and contains only intergenic sequence flanking *carD*. Such a plasmid would likely reduce toxicity in S17-1 by eliminating any CarD fragments, and allow formation of a merodiploid strain. This has previously been carried out for the *M. xanthus cdnL* gene (*Bartonella carD*-homologue), which is an essential gene[245]. Although *M. xanthus cdnL* deletion mutants were not recovered, the merodiploid strain could be constructed; subsequent negative selection for loss of the integrated plasmid resulted exclusively in restoration of the wild type for all colonies screened. Lastly, though *B. henselae* grows in a 5% CO_2, 37°C stationary incubator, the optimum artificial conditions for bacterial growth are in a candle extinction jar and on heart infusion agar rabbit blood (HIAR) plates; these conditions could be used as the primary growth method if further experiments with *B. henselae* are carried out.

Chapter 4. Growth and Stress Response Assays

4.1.   Introduction

The aim of this chapter was to identify the level of stress exposure for each B. henselae stress condition to be used in our subsequent qPCR experiments.

Throughout the *Bartonella* life cycle, *B. henselae* encounters a range of environmental conditions. Though CarD has been shown to be involved in responding to specific bacterial stresses in other bacteria[212,223], little is known about the role of *B. henselae* CarD. To investigate the role that B. henselae CarD plays in responding to specific stresses, we examined expression of *carD* under a range of common *Bartonella* stress conditions.

To accurately quantify *B. henselae carD* expression levels in response to stress, we needed to first ascertain the conditions under which the bacteria were experiencing stress but were still viable. To this end we performed stress response assays that determined the level of stress that elicits expression of stress genes while avoiding excessive cell death. This information can be compared to stress sensitivity described for other *Bartonella* and bacterial species, and will be used as a starting point for our *carD* quantification studies in *B. henselae*.

4.1.1.   Stress Conditions

The stress conditions chosen all represent stresses that *B. henselae* is likely to encounter during infection and transmission, and a subset of these have been previously found to induce *carD* expression in other bacteria. We chose five stress conditions to test: low

temperature, haemin exposure, ciprofloxacin treatment, hydrogen peroxide exposure, and nutrient starvation.

B. henselae has previously been shown to grow in liquid Schneider's Drosophila Medium, supplemented with 10% fetal calf serum (FCS)[7]; all *B. henselae* stress assays, apart from the starvation assay, were performed using this medium. *B. henselae* cultured in Schneider's Drosophila Medium containing FCS, at 37°C, was used as the control for *B. henselae carD* qPCR expression under non-stressed conditions.

Though *Bartonella* species grows optimally at 35-37°C, the temperature of the mammalian host, *B. henselae* and *B. quintana* are exposed to temperatures of 28°C in the arthropod gut, and are known to replicate under these conditions, albeit slowly[108,246]. Temperature has been hypothesised to be a key transcriptional regulator in adaptation to the *Bartonella* arthropod vector, with expression of 5% of *B. quintana* genes experiencing a change during temperature shift from 37°C to 28°C[246]; the *carD* homologue is moderately up-regulated in *B. quintana* at 28°C, along with the stringent response gene *dksA*; the stringent response has been proposed to play a role in regulating expression and survival in the arthropod vector.

Temperature is also a factor in regulating expression of *ltpA*, the *carD* homologue in the blood borne pathogen *Borrelia burgdorferi*, the causative agent of Lyme disease[224]. *B. burgdorferi* belongs to the Spirochaetes phylum but has a life cycle that resembles that of the alphaproteobacteria *Bartonella* species, alternating between a mammalian host (*e.g.*, white-tailed deer) and a hematophagous arthropod vector (*e.g.*, *Ixodes* ticks). It should be noted that LtpA is a homologue to the *M. xanthus* CarD protein, while *Mycobacterium* and *Bartonella* CarD are homologues of the *M. xanthus* CdnL protein.

Haemin is a derivative of the haem B molecule (one of the four subunits of haemoglobin), which is derived from porphyrin IX (see **Figure 4.1**). Haemin is an essential nutrient for *Bartonella*, as a source of iron and porphryin, but is toxic at high levels[247-249]; unlike many other bacterial species, *Bartonella* lack de novo porphyrin or haem biosynthesis pathways, so external iron-containing porphyrin is instead scavenged from the environment[21]. Haemin is found in high concentrations in the arthropod midgut, where the mammalian erythrocytes are lysed, and low concentrations are found in the mammalian circulatory system. The bacterial response to haemin has not been fully elucidated, but *B. quintana* control the cellular use of haemin through the haemin utilisation locus and removal and sequestration of excess haemin though the haemin binding protein family. *B. quintana* also encodes three iron response regulators – rhizobial iron regulator A (rirA), ferric uptake regulator (fur), and iron response regulator (irr)[230,250], which may play a role in the response to haemin.

The mechanism of haemin toxicity for bacteria is not fully understood; studies indicate that the presence of haemin leads to an increase in free-radicals (due to the Fenton reaction) that can cause oxidative damage to lipids, proteins and DNA (see **Figure 4.2**)[251,252]. Though the haemin concentration in the arthropod midgut reaches a level that is toxic to most other bacterial species, *Bartonella* are unusually resistant to haemin; while the haemin utilisation locus is down-regulated at high haemin concentrations, haemin binding proteins are up-regulated by low temperature and haemin[230,250]. *B. henselae* haemin binding proteins are also involved in protection against oxidative stress via H_2O_2[230,250,253-255]. No studies into the relationship between *carD* and haemin have been previously performed, to the best of our knowledge. Yet, in its role as a global transcriptional regulator and in the stringent response, it may be

that CarD is involved in the *B. henselae* response to haemin through regulating

transcription of haemin binding proteins.
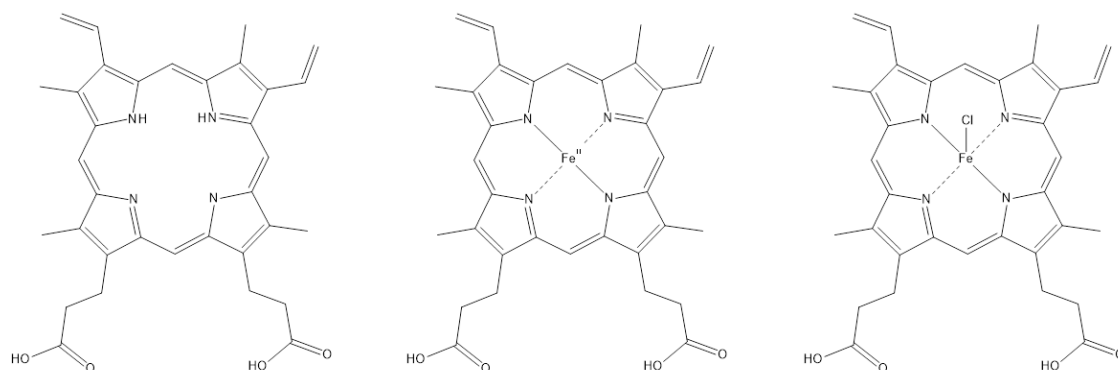
Figure 4.1. – Chemical Structure of Porphyrin IX versus Haem B versus Haemin Porphyrin IX is on the left, Heam is in the center with an Fe^{2+} molecule, and haemin is on the right with a chloride ion attached. Diagram was generated using ChemDraw Prime (Perkin Elmer Informatics).
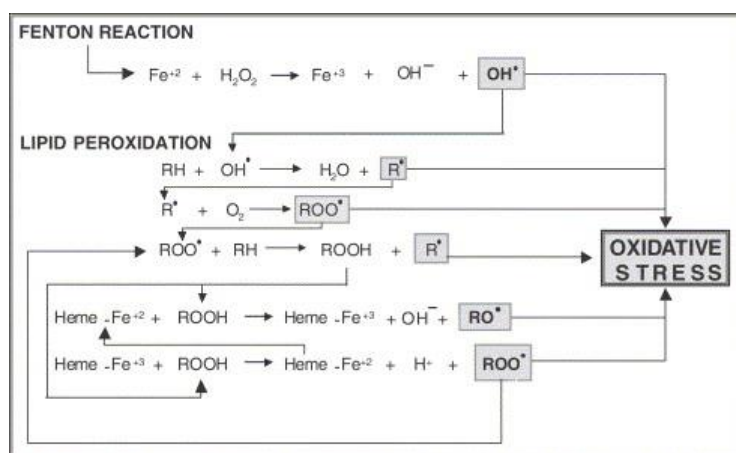
Figure 4.2. – Hypothetical Pathway of Haemin/Haem (Heme) Breakdown into Oxidative Products via the Fenton Reaction Haemin/haem is broken down via the Fention reaction to generate free radicals. Figure has been reproduced from: Insect Biochemistry and Molecular Biology, 36/4, Graça-Souza et al, Adaptations against Heme Toxicity in Blood-Feeding Arthropods, page 322, 2006, with permission from Elsevier[252].

The antibiotic ciprofloxacin and the reactive oxygen species hydrogen peroxide (H_2O_2)

are both genotoxic agents that have been previously studied in *B. henselae*,

B. bacilliformis, and in *M. tuberculosis*[212,230,253,256]. Ciprofloxacin is a fluoroquinolone

class antibiotic which targets DNA topoisomerase and DNA gyrase proteins, resulting in halted transcription. Ciprofloxacin is hypothesised to release DNA ends, due to dissociation of the topoisomerase/gyrase complex from broken DNA[190,194,212,257]. Although previously used to treat *Bartonella* infections, it has been shown that other antibiotics are more effective[256,258,259]. Hydrogen peroxide is an important reactive oxygen species (ROS) used in cellular defence, notably by macrophage and neutrophil immune cells. As shown in **Figure 4.2**, H_2O_2 can be broken down into a number of oxidative agents, which cause damage to lipids, DNA, RNA, and proteins, through electron transfer[260]. Hydrogen peroxide has also been described in the arthropod midgut along with other reactive species[252]. The presence of haemin binding proteins have been described to play a role in *Bartonella* defence against H_2O_2[253,254]. In *M. smegmatis*, not only did the presence of both ciprofloxacin and H_2O_2 lead to an up-regulation of *carD*, a bacterial strain with artificially depleted *carD* had decreased resistance to both of these agents[212].

Starvation is a distinct threat to all bacterial species, and is involved in regulation of *B. henselae* pathways, where the starvation-mediated stringent response plays an important role in both the arthropod vector and the mammalian host. Increased starvation conditions are likely to be encountered within the vector host midgut. Cat fleas who have previously taken a blood meal can survive for four days between blood meals, yet the majority of the protein gained during the meal will be lost after 12 hours[261,262]; thus *B. henselae* are likely to experience a degree of nutrient deprivation inside the flea vector between meals. Along with responding to nutrient starvation, the bacterial stringent response also detects small nutritional and environmental changes to mediate transcriptional adaption to new environments. Due to the large environmental shift from the mammalian to the arthropod host, we hypothesise that *carD* could play

an initial role during adaptation to the *B. henselae* arthropod vector. The stringent response is a critical mediator of bacterial adaptation to the arthropod vector of *B. burgdorferi*[263]. Rel$_{Bbu}$ (a RelA/SpoT homologue) and (p)ppGpp levels were greatly increased during bacterial exposure to starvation conditions, and mediated a global transcriptional response to starvation in the *B. burgdorferi* arthropod vector.

Within the mammalian host, DksA and SpoT, two mediators of the *B. henselae* stringent response, have been shown to detect changes in environmental nutrients to initiate cellular invasion. In low-nutrient conditions, the up-regulation of the stringent response (DksA/SpoT) initiates VirB/D4 T4SS (mediating invasion of endothelial cells), while high-nutrient conditions down-regulate the stringent response to initiate Trw T4SS (erythrocyte invasion)[207].

Though the role of CarD in the *B. henselae* stringent response is hypothetical, CarD directly mediates (p)ppGpp accumulation in *M. smegmatis*[182,212]; expression of the *carD* gene was up-regulated in response to ciprofloxacin and hydrogen peroxide, and under nutrient-deprived conditions. Transient *carD* depletion in *M. smegmatis* lowered bacterial resistance to all these conditions, including starvation[212], and *Mycobacterial* CarD and *E. coli* DksA act in similar roles during the bacterial response to stress. We therefore decided to investigate a possible link between *carD* and the bacterial stress response in *B. henselae*.

4.2. Results

Initially our stress exposure assays were optimised with the intention of carrying out growth-rate comparisons between the wild-type and the Δ*carD* deletion strain; if *B. henselae* lacking the *carD* gene had difficulty growing in a stress condition, we could infer that *carD* played a role in protection against that stress. As no merodiploid *carD*

strain was obtained, the stress assays were instead used as the foundation of our qPCR experiments, although *B. henselae* survival in the presence of stress provided important information about the bacteria. The conditions determined here may be used in future experiments to study the role of specific *B. henselae* genes in stress response.

For each stress condition, the survival and the response to the stress, described in the literature, was investigated for *Bartonella* and other bacterial species, to determine a starting point for our exposure assays. By applying and varying key conditions, including stressor concentration and exposure times, conditions that resulted in roughly 80% to 90% survival of the culture were determined by plating out *B. henselae* cultures and counting the number of viable colonies. We hypothesised that a survival rate of 80% to 90% would correspond to a condition where adaptive transcription responses were triggered, without excessive cell death in the population.

B. henselae cultures grown at a temperature of 37°C, in Schneider's Drosophila Medium containing FCS, reach a maximum OD_{600} of 0.5 after nine to 10 days (as shown below in **Figure 4.4**), at which point the bacteria reach a stationary phase where the growth rate slowed and the OD_{600} began to drop. By comparison, in *B. henselae* cultures at 28°C, a slower growth rate was observed. An OD_{600} of 0.36 was obtained after seven to eight days. Interestingly, the initial growth at both 37°C and 28°C remained comparable for three days (reaching an OD_{600} of ~0.29 at 37°C and ~0.275 at 28°C, at three days); deviation in the growth rate occurred subsequently. Bacterial adaptation to new conditions occurs very rapidly; therefore, for our optimised qPCR control and low temperature conditions, we assumed that an exposure time of two hours would allow for *B. henselae* to adapt to the new conditions. However, for conditions where bacterial viability rapidly decreased, shorter exposure times were used.
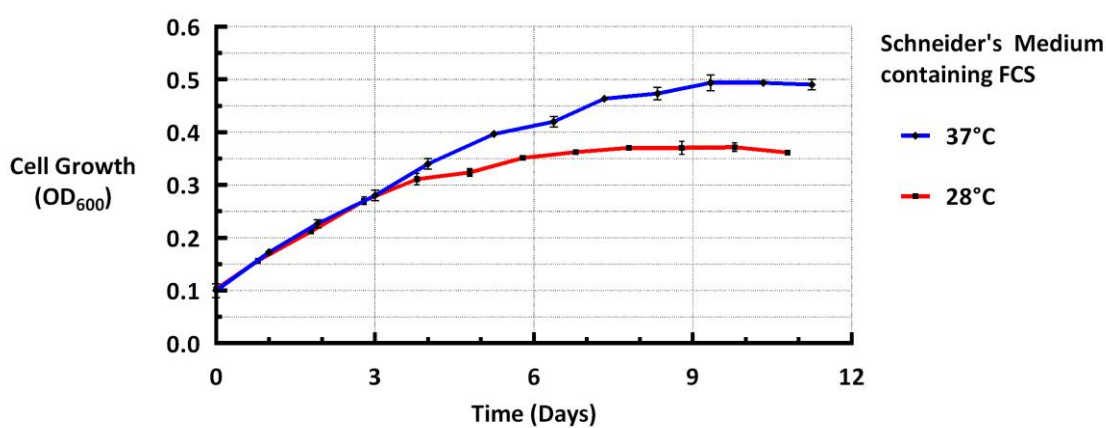
Figure 4.4. – Growth Curve of *B. henselae* at 37°C and 28°C, Measured in OD_{600} over 11 Days As the majority of our stress response assays were performed in Schneider's Drosophila Medium containing FCS at 37°C, we confirmed that the bacteria grow under these conditions and those of 28°C.

Haemin exposure assays were performed for haemin at a concentration of 0.1 mM (as depicted below in **Figure 4.5**). A decrease in survival was observed when our *B. henselae* culture was exposed to 0.1 mM of haemin. The bacteria survive exposure for up to 24 hours, though with a steady decrease in viability throughout. Initially, we cultured *B. henselae* with 0.5 mM haemin; however, viability rapidly declined and no colonies were recovered after four hours of exposure, so a lower concentration was used.
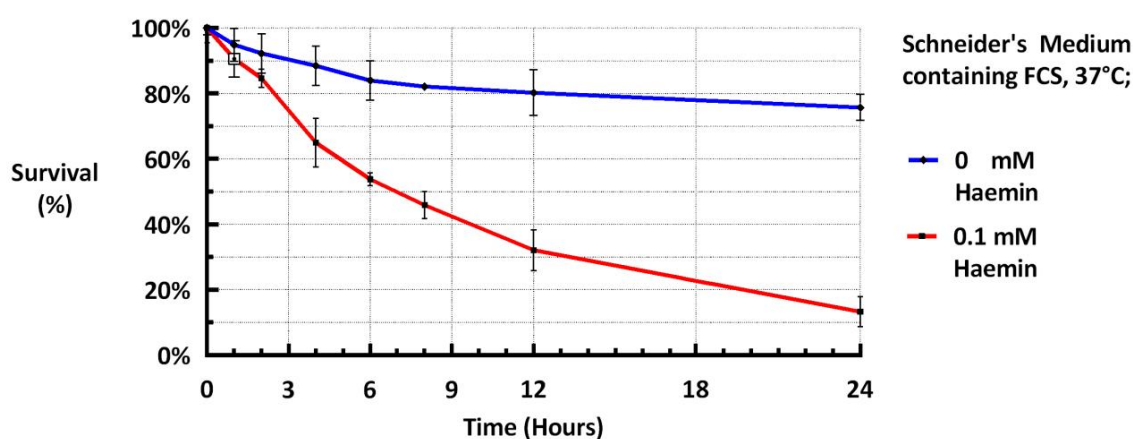
Figure 4.5. – *B. henselae* Haemin Stress Exposure Assay, in Percentage Survival, over 24 Hours Percentage survival was determined, first by plating dilutions of each time point, growing the plates for 14 days. The resulting colonies were counted to give the CFU, and then by comparing the CFU of each time point against the CFU of the number of initial colony forming units at a time point of 0 (CFU$_{tx}$/CFU$_{to}$ x100)

Ciprofloxacin stress exposure assays were performed with two antibiotic concentrations, 10 µg/mL and 25 µg/mL. A steady decrease in viability was observed at 10 µg/mL (as shown in **Figure 4.6**). The percentage survival was similar for the two concentrations of antibiotic, suggesting a degree of saturation of antibiotic within the solution. An optimum stress exposure time of 10 µg/mL for one hour was chosen (as denoted by an open square in our exposure assay).
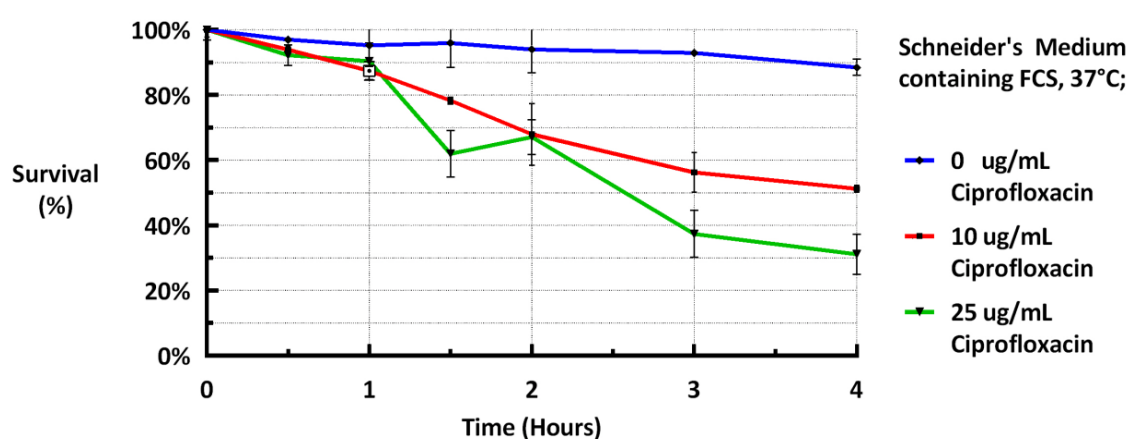
Figure 4.6. – *B. henselae* Ciprofloxacin Stress Exposure Assay, in Percentage Survival over Four Hours
Percentage of survival relative to the initial number of colonies, was determined by exposing the bacteria over four hours. Dilutions were plated, incubated for 14 days, and then the colonies were counted as for haemin.

Hydrogen peroxide exposure assays using 0.1 and 1 mM H_2O_2 were performed, as shown in **Figure 4.7**. At a lower concentration of 0.1 mM H_2O_2, viability decreased steadily over the 40 minutes of stress exposure, while at a concentration of 1 mM H_2O_2, a sharper decrease was observed in the first 10 minutes of exposure. Both concentrations elicited a quick drop in viability, yet a small part of the population appeared to persist at high concentrations of H_2O_2, with colonies obtained for both concentrations over the extent of our exposure assay. Exposure to 0.1 mM H_2O_2 for 10 minutes was chosen for our qPCR H_2O_2 stress condition (as denoted by an open square in our exposure assay).
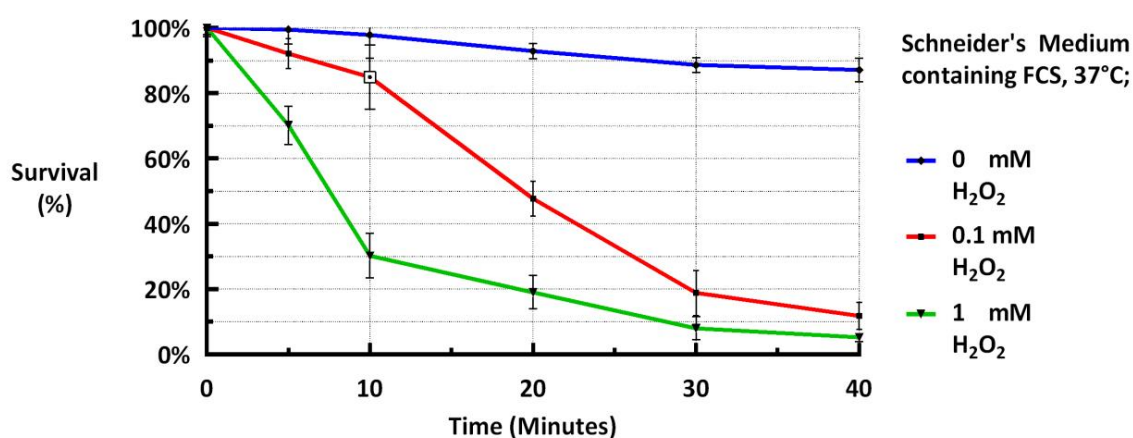
Figure 4.7. – *B. henselae* H_2O_2 **Stress Exposure Assay, in Percentage Survival over 40 Minutes** Percentage survival was determined by exposure bacteria over 40 minutes. Dilutions were plated, incubated for 14 days, and then the colonies were counted as for haemin.

For our starvation exposure assay, *B. henselae* cultures in 1x PBS were observed to have a steady decrease in percentage survival over the time course tested, compared to the non-stressed control in Schneider's Medium containing FCS (as shown in **Figure 4.8**). An optimum starvation exposure time was identified of 1x PBS for 30 minutes at 37°C (as denoted by an open square in our exposure assay).
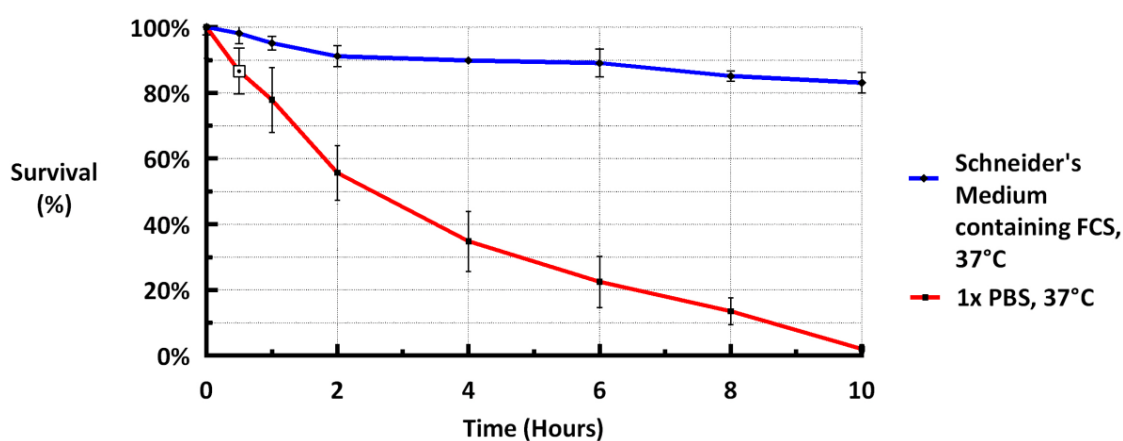
Figure 4.8. – *B. henselae* Starvation Stress Exposure Assay, in Percentage Survival over 10 Hours Percentage of survival was determined exposing the bacteria over a period of 10 hours. Dilutions were plated, incubated for 14 days, and then the colonies were counted as for haemin.

4.3.   Discussion

The overall aim of this experiment was to determine the susceptibility of *B. henselae* to a range of stresses, and to identify conditions that enabled an 80% to 90% survival rate, to test in our qPCR experiments. However, we can compare these experiments to others performed in the literature, to assess the susceptibility of *B. henselae* to each respective stress relative to other bacteria, and to consider why variations in bacterial resistance are observed.

4.3.1.   Schneider's Medium Control (37°C) and Low Temperature (28°C)

B. henselae has previously been shown in the literature to grow in liquid culture, in Schneider's Drosophila Medium supplemented with 10% fetal calf serum, in a 5% CO_2, 37°C 60 rpm shaking incubator by Riess[7]. Though *B. henselae* was observed to grow in both shaking and non-shaking incubators, a faster growth rate and overall OD_{600} was observed with shaking (four to five days to reach an OD_{600} equal to 0.7 with shaking versus nine to 10 days to reach an OD_{600} equal to 0.5 without shaking). Despite these observations, incubation times for our stress assays did not exceed three hours (including acclimatisation time), and we therefore did not subject the cultures to shaking.

The arthropod midgut and mammalian host environments differ greatly, both in environmental stresses present and available nutrient levels; multiple transcriptional changes are required for adaptation of *B. henselae* to the appropriate host environment. Change is often mediated by the various bacterial stress responses, including the general stress response and the stringent response.

One of the key differences between arthropod and mammalian conditions is temperature, which undoubtedly is a key mediator of adaptation of *Bartonella* to new host environments. *B. henselae* and *B. quintana* have been described to grow at 28°C within their respective arthropod vectors, although at a decreased rate compared to 37°C (see **Figure 4.4**)[108,246]; from these conditions we chose an exposure time (two hours at either 37°C and 28°C) for our optimised qPCR stress conditions. Such a temperature shift from the mammalian host to the arthropod vector has shown to change the transcriptional profile of *B. quintana* grown at 28°C (5.0% of tested genes were temperature sensitive)[246]. Up-regulation of alternative sigma factor regulators, RpoE and PhyR (key regulators of the general stress response), and haemin binding proteins was described at 28°C[246,264].

The increase in expression of stress response genes may be responsible for the difference in growth rate of *B. henselae* at 37°C and 28°C; during the bacterial stress response, an increase in survival is often matched by a decrease in growth[182,189]. In the *B. quintana* temperature transcriptional study, the change in gene expression occurred over several days, with stress gene expression increasing from day three to seven. This is an interesting trend, considering that when comparing the results of our 37°C and 28°C assays, growth only deviates after three to four days. The observed difference in *B. henselae* expression could be due to a delayed expression of stress genes, in a similar manner to that seen in *B. quintana* at 28°C. Although a large difference in expression will no doubt be observed between different *Bartonella* species, the two studies also differ in the media used, with solid media used in the *B. quintana* 28°C study and liquid culture used in our *B. henselae* 28°C study. Our *B. henselae* RNA samples for qPCR were collected two hours after the temperature shift, to analyse the initial bacterial

response to stress; however it would be interesting to investigate the trend of *carD* and other stress gene expression over a longer period of time.

4.3.2. Haemin

Along with low temperature, another key stress in the arthropod midgut is an increase in haemin concentration. *Bartonella* species are known to be more resistant to high haemin and haem concentrations than other bacterial species. The growth of the pathogenic bacteria *Staphylococcus aureus* and *Haemophilus influenzae* is limited in concentrations of 10 μM and 0.5 μM of haemin respectively[249,265], while *Porphyromonas gingivalis* can survive in 50 μM of haemin[266]. Reports in the literature show that haemin resistance varies among *Bartonella* species; *B. quintana* viability has been described in concentrations of 10 mM of haemin (>35.0% survival at 28°C for 24 hours), compared to the upper bounds of 5 mM of haemin in *B. henselae* (<0.5% survival at 28°C for 24 hours)[230,264]. Five haemin binding proteins are found in *B. henselae*, while eight are found in *B. quintana*[230,248,267]; differences in expression have also been described between *B. henselae* and *B. quintana* haemin binding proteins[230,255]. Variations in the number and expression of haemin binding family members between *B. henselae* and *B. quintana* are likely due to adaptations to the respective arthropod hosts; direct comparisons between *P. humanus* and *C. felis* have not been performed, yet their feeding habits are known to differ in frequency and quantity[268,269].

In our haemin exposure assay, we repeatedly observed a steady decrease in growth of *B. henselae*, in 0.1 mM of haemin over 24 hours (see **Figure 4.5**). However our results did not correspond to the levels of *B. henselae* haemin resistance reported in the

literature; *B. henselae* has previously been grown on blood agar (containing 1 mM haemin), and in Brucella broth, supplemented with 0.2 or 0.1 mM haemin[247,270]. Another, more recent study described *B. henselae* (the Marseille strain, as opposed to Houston-1) growth over six days by OD_{600}, in Borrelia media supplemented with 0, 0.01, 0.05, and 0.1 mM of haemin; cultures grew best at 0.05 mM, followed closely by 0.01 and 0.1 mM[270]. Differences between our observed *B. henselae* response to haemin and those that have been described in the literature, could be explained by the media used. Haemin was the sole source of iron in the liquid media used by Sander et al and Muller et al[247,270], as Brucella broth required ≥6 μM of haemin to support bacterial growth. By contrast, Schneider's Medium containing 10% fetal calf serum supported growth without additional iron supplementation, as the media contains free iron (0.24 μg/mL), haemoglobin (<25 mg/mL), and ferritin (0.23 μg/mL). Variations in resistance can also be seen due to differences between lab strains, protocols, and techniques. Despite the difference between *B. henselae* haemin resistance described in the literature and our results, our exposure assay demonstrated that the *B. henselae* culture was experiencing stress due to haemin, resulting in reduced viability over time; we therefore selected exposure to 0.1 mM haemin for one hour, for analysis of *carD* transcription.

For our *B. henselae* haemin exposure assay, decreased viability over 24 hours was assumed to be a symptom of stress. Such a death rate was thought to mimic the high level of haemin stress found in the midgut of the arthropod vector after a blood meal. However, the results of the study by Sander et al[247] suggested a different interpretation of our results, that what we interpreted as a *B. henselae* stress response to haemin could instead be an acclimatisation period, allowing *B. henselae* to subsequently grow in the

presence of haemin in liquid media. Sander et al grew *B. henselae* cultures in Brucella broth medium, supplemented with a range of haemin concentrations, and described growth by OD_{600} and counting viable colonies over 14 days[247]. The OD_{600} of *B. henselae* cultures grown in 0.1 and 0.2 mM of haemin showed almost no growth for the first seven days of liquid culture, but over the next seven days, these cultures attained the highest numbers of bacteria among the tested samples. No data was provided above 0.2 mM haemin concentration.

In our hands, a haemin concentration of 0.1 mM results in a substantial reduction of viability in *B. henselae*, enabling us to use qPCR result to assess the *B. henselae* response to haemin. As the haemin response pathway has not been fully deduced in *B. henselae,* several *B. quintana* studies can give us some insight into this pathway. In *B. quintana*, an increase in haemin concentration from 0.15 mM to 1.0 mM induced a down-regulation in expression in all five haemin binding proteins (by roughly 2-fold)[255]. However, a further increase in haemin concentration to 2.5 mM and 5 mM induced an up-regulation of expression in the majority of haemin binding proteins studied (between 1.5-fold to 10-fold). Additionally, another study suggests that the opposite may be occurring with *B. quintana* haemin utilisation locus genes (involved in the uptake and use of haemin molecules)[250]. During overexpression of different haemin response regulators, haemin utilisation locus genes were up-regulated in conditions of 0.15 mM of haemin and down-regulated at 2.5 mM haemin.

These values suggest that haemin concentration during an arthropod blood meal could reach levels of 2 to 5 mM. At this haemin concentration, *B. henselae* likely up-regulates haemin binding proteins and down-regulates haemin utilisation locus genes to prevent excess haemin uptake. However, between blood meals, the concentration of haemin that

B. henselae experiences could very well fall below 1 mM, where additional haemin uptake is required, with haemin binding proteins down-regulated and the haemin utilisation locus proteins up-regulated. The level of haemin concentration we used in our haemin response assay could therefore mimic the low haemin concentration of the vector midgut during starvation. In our assay, *B. henselae* cultures are transitioned from regular growth in Schneider's Medium containing FCS to media with comparatively high levels of environmental haemin; this transition could be temporarily detrimental to growth of the bacteria until they adapt through gene expression and protein synthesis.

4.3.3. Ciprofloxacin

Ciprofloxacin has previously been used to treat *Bartonella* infections; however, resistance to the drug has been frequently reported in clinical *Bartonella* infections[256,258,259]. The *Bartonella* response to ciprofloxacin and other fluoroquinolones has been assessed, using minimum inhibitory concentrations (MIC) calculated by the Etest assay[256,259,271]. The majority of fluoroquinolone resistance was judged to be the result of one of several *gyrA* point mutations (Asp-87 and Ser-83, using the *E. coli* notation); these mutations have been described in *B. henselae*, *B. quintana*, *B. bacilliformis* (where a single mutation increased resistance to ciprofloxacin by 16-fold), *E. coli*, and *M. tuberculosis* (multiple mutations increased ciprofloxacin resistance by >100-fold)[271-275]. Ciprofloxacin resistant *B. bacilliformis* strains (*gyrA*) appeared after five passages with ciprofloxacin treatment, while no gentamicin or doxycycline resistant strains appeared after 16 passages.

For our initial *B. henselae* ciprofloxacin exposure assay, a lawn of bacteria was obtained at 1 μg/mL after an hour exposure time; the ciprofloxacin concentration was therefore

increased to 10 and 25 μg/mL over several hours, at which point we observed a steady decrease in viability (50.0% survival after four hours exposure to 10 μg/mL; see **Figure 4.6**). Exposure to 10 μg for one hour was judged to elicit a stress response suitable for our qPCR experiment. The ciprofloxacin concentrations used in our experiments were well above the MIC values described for *B. henselae*, but were chosen to reduce viability of the bacteria in a short time frame (hours versus days).

Ciprofloxacin stress exposure assays have previously been performed in *M. smegmatis*, comparing the wild type to a strain with reduced *carD* expression (10 μg/mL for two hours); reduced growth was demonstrated for all strains, although reduced *carD* expression significantly reduced bacterial resistance to ciprofloxacin[212]. *Mycobacterium* is more resistant to ciprofloxacin than *Bartonella* species; *M. tuberculosis* has been described to have an MIC of 2 μg/mL, compared to 0.38 μg/mL for *B. henselae*[256,276].

4.3.4.   Hydrogen Peroxide

A high level of H_2O_2 resistance of *Bartonella* species is to be expected considering the intracellular niches the bacteria encounter, e.g. immune cells such as macrophages and in the midgut of the arthropod[254,277]. Hydrogen peroxide exposure has previously been described for *B. bacilliformis* and *B. henselae*; *B. bacilliformis* is completely resistant to H_2O_2 at a concentration of 1 mM, while *B. henselae* is susceptible to H_2O_2 at concentrations of 1 mM and 10 mM[254,278]. By comparison, our *B. henselae* H_2O_2 exposure assay was performed for two concentrations, 0.1 mM and 1 mM H_2O_2 for 30 minutes, with a rapid reduction in viability (see **Figure 4.7**). The discrepancy between our results and those reported for *B. henselae* in the literature are not

insignificant; after exposing *B. henselae* cultures to 1 mM H_2O_2 for 30 minutes we observed a 10.0% survival rate, while Liu et al observed a roughly 50.0% survival rate after exposure to 1 mM H_2O_2, and a >15.0% survival rate after 10 mM H_2O_2 exposure, both for 30 minutes[253,254]. Differences in H_2O_2 resistance between *B. bacilliformis* and *B. henselae* may be explained by the overall differences between the two species; however, we currently have no explanation as to why variation in H_2O_2 resistances were observed between the *B. henselae* assays performed in this study and those in the literature. Though both studies used *B. henselae* Houston-1 strains, in the literature the H_2O_2 exposure took place in 1x PBS and performed two post-exposure washes[254], as opposed to an H_2O_2 exposure and no wash step in our study; it is possible that H_2O_2 was carried over during our dilutions, which negatively affected *B. henselae* growth. However, it is clear from both assays that *B. henselae* can survive exposure to different levels of H_2O_2, and that a stress response has been elicited in our assay.

Growth of *M. smegmatis* was not negatively impacted during exposure to 10 mM of H_2O_2, and bacterial survival was lowered following exposure to 35 mM H_2O_2 (although colonies were recovered). Interestingly, *M. smegmatis* strains with reduced *carD* expression had greatly reduced growth during exposure of the culture to ≥ 10 mM of H_2O_2[212]. *Mycobacterial* species commonly infect macrophages for extended periods, and are likely resistant to high H_2O_2 concentration, in contrast to other bacterial species[197].

4.3.5. Nutrient Starvation

The stringent response has been shown to be crucial in bacterial adaptation to nutrient stress in innumerable bacterial species; for example, growth of *Borrelia burgdorferi* and *Brucella suis* during nutrient starvation has been previously described to require the SpoT-homologues Rel_{Bbu} and Rsh respectively[263,279]. Similarly, *B. burgdorferi* Rel_{Bbu} was also shown to mediate bacterial adaptation to its arthropod vector, the *Ixodes* tick, via global transcriptional regulation.

No studies have been carried out on the role of CarD in adaptation to nutrient starvation in *B. henselae* or other *Bartonella* species, to the best of our knowledge. However, the process has been studied in other bacterial species. During our starvation exposure assay, we observed a steady decrease in *B. henselae* viability, with no colonies recovered after 10 hours (see **Figure 4.8**). A similar *M. smegmatis* starvation exposure assay has been carried out alongside *carD* depleted strains over 24 hours[216]. A large number of *M. smegmatis* colonies were recovered over the entire period, while the *carD* depleted strains experienced increased sensitivity to starvation.

Along with detecting and controlling bacterial survival in nutritional starvation conditions, the stringent response also detects and controls adaptations to fine changes in the environment. An example of this is the regulation of *B. henselae* DksA and SpoT in the mammalian host. In early host invasion, it is proposed that the decrease in nutrients in the extracellular fluid environment up-regulate DksA/SpoT, which in turn positively regulate the VirB/D4 T4SS system; in the nutrient rich bloodstream, DksA/SpoT are down-regulated, de-repressing expression of the Trw T4SS, allowing invasion of erythrocytes. Although the nutrient level in the extracellular fluid is lower than that of the bloodstream, a large number of salts, ions, proteins, and nutrients are

still present, in contrast to the more complete nutrient deprivation in our assay; therefore *B. henselae* likely do not lose viability *in vivo* as rapidly as we observed.

4.3.6. Conclusion

For each of the *B. henselae* stress conditions outlined above (low temperature [28°C], haemin supplementation, ciprofloxacin treatment, hydrogen peroxide exposure, and nutrient deprivation), we have tested and identified the conditions that result in viability of 80% to 90% of *B. henselae* cells. These culture conditions were selected for PCR analysis of the *B. henselae carD* gene, to determine if the gene is regulated in response to stress.

Few studies have been carried out in *B. henselae*, or in other *Bartonella* species*, for the precise stress conditions tested here. However, similar studies of the bacterial response to stress in other bacterial genera were used to approximate the resilience of *B. henselae* to stress. *B. henselae* stress resistance was in line with what has been previously reported, and where it differs from what was expected (i.e., haemin and hydrogen peroxide), we have provided possible explanations to explain the lower *B. henselae* stress resistance reported in this study.

Chapter 5. qPCR Analysis of carD Expression under Stress Conditions

5.1. Introduction

The aim of this chapter was to investigate through qPCR whether B.henselae carD expression increased in response to exposure to environmental bacterial stresses.

The overarching goal of this project was to elucidate the role that the *carD* gene plays in the *B. henselae* response to common stress conditions. Initially we sought to answer our research question through the analysis of a null mutant compared to the wild type. However, as no merodiploid or Δ*carD* mutants were obtained, we turned to quantitative PCR (qPCR) to examine the expression of *carD*. qPCR is a routine molecular biology technique to determine gene expression levels in both prokaryote and eukaryote cells. RNA can be reverse transcribed into cDNA, which acts as the template for a qPCR reaction using fluorescent DNA markers; thus the fluorescent signal of a qPCR reaction is linked to the abundance of specific transcripts. A qPCR target reaction signal can be used to calculate the relative DNA abundance, or relative gene expression, between baseline and treatment conditions. A change in relative DNA expression may suggest that the gene is up- or down-regulated in response to external stimuli.

B. henselae wild type cultures were harvested and exposed to each stress condition as described in Chapter 4 (low temperature, haemin, ciprofloxacin, H_2O_2, and starvation), then *carD* expression was analysed through qPCR; the resulting qPCR reaction signals were compared against a reference housekeeping gene and a non-stressed *B. henselae* control to calculate relative *carD* expression.

5.2.   Results

When running a qPCR, several assumptions are made to enable the comparison of qPCR reactions. First, we assumed that for each qPCR reaction the efficiency was 100%, in that the target DNA was doubled every qPCR cycle (hence a log of 2 is used to calculate the fold change). We also assumed that the observed fluorescence in a qPCR reaction is proportional to the initial cDNA quantity. The last assumption was that the reference housekeeping gene, *rpsL*, was expressed approximately equally between each of our bacterial cultures.

To analyse gene expression by qPCR, for each condition a minimum of two qPCR reactions (with replicates and controls) were concurrently run using the same cDNA template; one qPCR reaction included primers specific to the target gene while the primers for the second reaction amplified the reference gene. Reference genes are housekeeping genes whose expression is relatively constant in the bacterial conditions being investigated; although one reference gene is commonly used, the use of multiple reference genes may improve accuracy. The reference gene used in this study was the *rpsL* gene, which encodes the 30S ribosomal protein S12; this reference gene is a commonly used reference gene, and has previously been used as a control for *B. henselae* qPCR studies [280-282].

qPCR incorporates a fluorescent probe that is activated when bound to DNA; the level of fluorescence in each qPCR reaction is measured after each cycle, and graphed (relative fluorescent units or RFU, vs cycles) to give a sigmoidal DNA amplification curve. For each qPCR reaction, the cycle threshold (Ct) is calculated from the reaction curve. The Ct is the number of cycles of qPCR taken for the RFU of a reaction to pass

above a set threshold. The threshold level is set by the user to be above the background noise, and at the beginning of the exponential phase of DNA amplification. The Ct of the reference gene (or genes) is compared with the Ct of a target gene to give the difference in DNA abundance between the reference and target genes, a value known as ΔCt (see below for equation). The ΔCt of a baseline control (that has normal expression of the target gene) is calculated and then individually compared against the ΔCt of each condition of interest; the outcome is ΔΔCt, which represents the change in the level of gene expression between the condition of interest and the baseline, normalised with the static expression of the housekeeping gene. Normalised target gene expression or ΔΔCt can be further converted into fold change in gene expression using a negative log of 2, as a lower Ct relates to an increased level of expression.

$$\Delta Ct = \text{Ct (target gene)} - \text{Ct (reference gene)}$$

$$\Delta\Delta Ct = \Delta Ct \text{ (treated)} - \Delta Ct \text{ (baseline)}$$

$$\text{fold change} = 2^{-\Delta\Delta Ct}$$

For the non-stressed condition and each stress condition (low temperature [28°C], haemin, ciprofloxacin, H_2O_2, and starvation) *B. henselae* cultures were stressed with the optimum stressor level, as determined by our stress response assays. Total RNA was harvested from each culture and treated with RNAse-free DNAse, which was subsequently removed. RNA samples were then reverse transcribed to cDNA. To confirm our RNA samples are free of any gDNA contamination carried over from our RNA extraction, all cDNA samples were checked by PCR reactions with prJS03 (*carD* qPCR) and prJS04 (*carD* qPCR) primer pairs, and analysed by agarose electrophoresis gel as shown below (see **Figure 5.1**). Amplification of bands in the appropriate product range (~70 bp) was confirmed, and no amplification was observed in the controls that lacked reverse transcriptase.

a)

Size in bp	Gel Lane
Lanes 1–14	1. Reference Ladder
1,000 / 800 / 600 / 400 / 200	2. 37°C cDNA
	3. 37°C no Reverse Transcriptase
	4. 28°C cDNA
	5. 28°C no Reverse Transcriptase
	6. Haemin cDNA
	7. Haemin no Reverse Transcriptase
	8. Ciprofloxacin cDNA
	9. Ciprofloxacin Reverse Transcription
	10. Reverse Transcription no RNA Template Control
	11. Reverse Transcription no Reverse Transcription
	12. PCR no RNA or cDNA Template Control
	13. *B. henselae* gDNA
	14. Reference Ladder
Size in bp (Lanes 15–24)	15. Reference Ladder
1,000 / 800 / 600 / 400 / 200	16. H₂O₂ cDNA
	17. H₂O₂ no Reverse Transcriptase
	18. Starvation cDNA
	19. Starvation no Reverse Transcriptase
	20. Reverse Transcription no RNA Template Control
	21. Reverse Transcription no Reverse Transcription
	22. PCR no RNA or cDNA Template Control
	23. *B. henselae* gDNA
	24. Reference Ladder

b)

Size in bp	Gel Lane
Lanes 1–14	1. Reference Ladder
1,000 / 800 / 600 / 400 / 200	2. 37°C cDNA
	3. 37°C no Reverse Transcriptase
	4. 28°C cDNA
	5. 28°C no Reverse Transcriptase
	6. Haemin cDNA
	7. Haemin no Reverse Transcriptase
	8. Ciprofloxacin cDNA
	9. Ciprofloxacin Reverse Transcription
	10. Reverse Transcription no RNA Template Control
	11. Reverse Transcription no Reverse Transcription
	12. PCR no RNA or cDNA Template Control
	13. *B. henselae* gDNA
	14. Reference Ladder
Size in bp (Lanes 15–24)	15. Reference Ladder
1,000 / 800 / 600 / 400 / 200	16. H₂O₂ cDNA
	17. H₂O₂ no Reverse Transcriptase
	18. Starvation cDNA
	19. Starvation no Reverse Transcriptase
	20. Reverse Transcription no RNA Template Control
	21. Reverse Transcription no Reverse Transcription
	22. PCR no RNA or cDNA Template Control
	23. *B. henselae* gDNA
	24. Reference Ladder

Figure 5.1. – Agarose Electrophoresis Gels of *B. henselae* Stress Condition Reverse Transcription Reactions To determine if our RNA extraction and our cDNA reverse transcription reaction were both successful, we performed two PCR reactions using both the prJS03 (carD) and prJS04 (rpsL) primer pairs. The cDNA of each stress condition was run beside the no-reverse transcriptase controls (to test for left over gDNA from the RNA purification), to test the reverse transcription reagents for contamination, both a no template control with no RNA added, and a no reverse transcriptase control were run. For the PCR a no cDNA template control was added to test the PCR reagents and a positive gDNA control was run for *B. henselae*. Repeats of the control lanes were run for each lane a) PCR reactions using the prJS03 (*carD* qPCR primer pair) were performed and run on electrophoresis gels, for each reverse transcribed stress condition to yield a ~70 bp cDNA product (Lanes 2, 4, 6, 8, 16, and 18). Controls were performed alongside for the reverse transcription (no reverse transcriptase, nRT for each stress; no RNA template, RT nTC) and for the PCR (no template control, PCR nTC; *B. henselae* gDNA). b) PCR reactions were also performed using the prJS04 (*rpsL* qPCR primer) as for prJS03 primers. Amplification was seen as expected with the exception of *rpsL* 37°C nRT (b, lane 3) which showed faint amplification; as no amplification was seen in the corresponding *carD* 37°C nRT (a, lane 3), this was regarded as an anomaly.

To test the suitability of the qPCR primers and cycle conditions, a set of qPCR experiments were performed on the 37°C baseline condition using the prJS03 (*carD* target gene) and prJS04 (*rpsL* reference gene) primer pairs, along with the respective controls (37°C, no reverse transcriptase with the prJS03 and prJS04 primers and qPCR no cDNA template with prJS03 and prJS04). From this qPCR, contamination was revealed in the qPCR controls – both no reverse transcriptase control reactions and also our qPCR no template controls that lacked cDNA and RNA; this implies that the contamination has not been carried over from our initial RNA extraction. The cycle thresholds given by our initial 37°C qPCR reaction indicate that the level of DNA in our 37°C cDNA reactions was ~16 times greater than the contaminated controls before amplification through qPCR; as such, we judged that the qPCR reaction conditions and primers were viable, and we used the same qPCR cycle conditions and reagent concentrations throughout.

To eliminate the contamination, successive qPCR reactions were performed, each with fresh reagents; an unopened vial of SYBR qPCR mix was used, fresh prJS03 and prJS04 primer pairs were ordered, and PCR-grade water was used to re-suspend our primers and used in our qPCR reactions. Despite fresh reagents, PCR tubes, and tips, the contamination persisted. Along with fresh reagents, the work space and all pipettes and tools used were cleaned with 10% bleach alongside the regular 70% ethanol; care was taken to open one tube at a time to avoid spread of aerosols. Despite the care taken, the biosafety cabinet we used was not solely used for PCR and qPCR and heavily used by others; as such we cannot be sure that environmental contamination was completely eliminated.

As contamination was observed in every single control tube, we hypothesised that it could be due to formation of primer dimers during the reaction; although the melt/dissociation curve of each qPCR reaction indicated that a single product was present that was specific to the primer pair used. To test this hypothesis, the post-qPCR amplified products were run on an electrophoresis gel; primer dimers should be a maximum of 40 base pairs, while qPCR products should be 70 to 75 base pairs. We determined that the qPCR product was ~70 base pairs, so primer dimers were ruled out as the cause of contamination (see **Figure 5.2**).
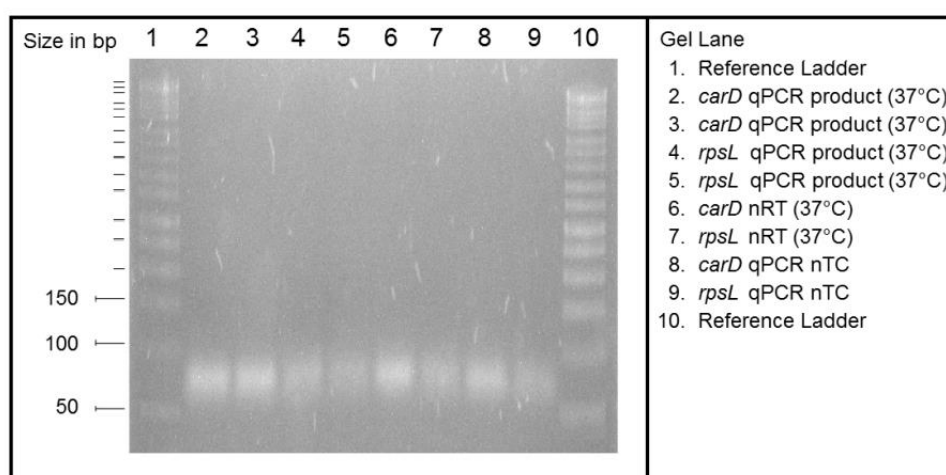
Figure 5.2. – **Agarose Electrophoresis Gel of *B. henselae* 37°C qPCR Products** As we saw contamination in all our lanes, we tested all our post-qPCR products for the formation of primer dimers. 5 μL of each amplified qPCR lane from our initial contaminated 37°C qPCR reaction were run alongside a 50 base pair DNA ladder (New England Biolabs). Lanes 2 and 3 and also lanes 4 and 5 are duplicates our qPCR. As the amplified bands are ~70 bp, no formation of primer dimers occurred.

If the contamination was not accounted for by the reagents or primer dimers, and was not the result of carryover of genomic DNA in the RNA samples, it was assumed to be from the environment; the most likely source of environmental contamination would be from mobile aerosols. As no other users of the qPCR machine reported contamination,

it was likely that contamination occurred during the preparation of the qPCR reaction mixtures. Despite the extreme care taken, the contamination persisted and the source was not found; as the low level of contamination observed, with Ct values of >30 cycles (30.60 to 33.07), and taking into consideration time constraints, we decided to continue with our final qPCR experiments with the expectation that any contamination would not significantly affect final conclusions.

The $\Delta\Delta$Ct and fold change in expression were determined for each stress condition against the 37°C baseline control, as shown below in **Figure 5.3** and in **Figure 5.4**. As expected, an increase in *carD* expression was observed under low temperature (28°C) and starvation conditions, or in the presence of ciprofloxacin or hydrogen peroxide. In contrast, a decrease in expression was observed in the presence of haemin. Against the untreated 37°C *B. henselae* control, *B. henselae* 28°C cultures experienced a *carD* fold change of 1.22; the presence of haemin resulted in decreased *carD* expression, relative to the control condition, resulting in a fold change of 0.54; *B. henselae* exposed to ciprofloxacin had an increase in *carD* expression, with a fold change of 1.27; *B. henselae* exposed to H_2O_2 had a fold change of 1.54; and *B. henselae* cultures exposed to starvation had a fold change, 1.27.
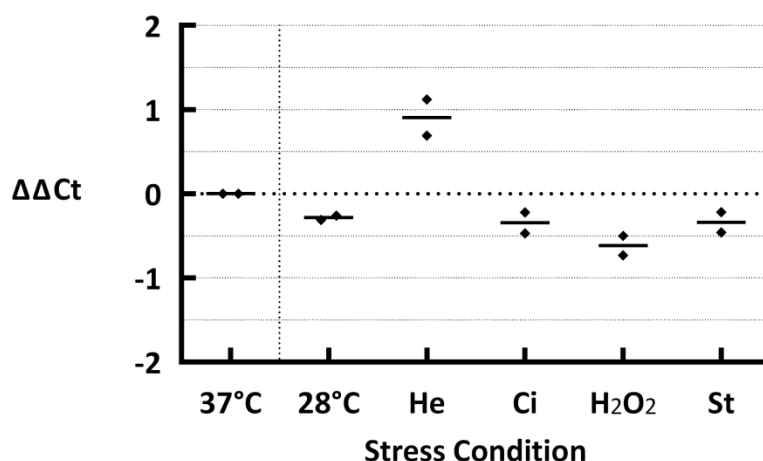
Figure 5.3. – Change in Expression (ΔΔCt) of *carD* Between 37°C non-stressed Control and other Stress Conditions using qPCR Duplicates of each stress condition were plotted and the mean marked with a line. He = Haemin, Ci = Ciprofloxacin, H_2O_2, and St= Starvation. A decrease in Ct corresponds to an increase in expression, as it indicates a greater level of initial cDNA template.
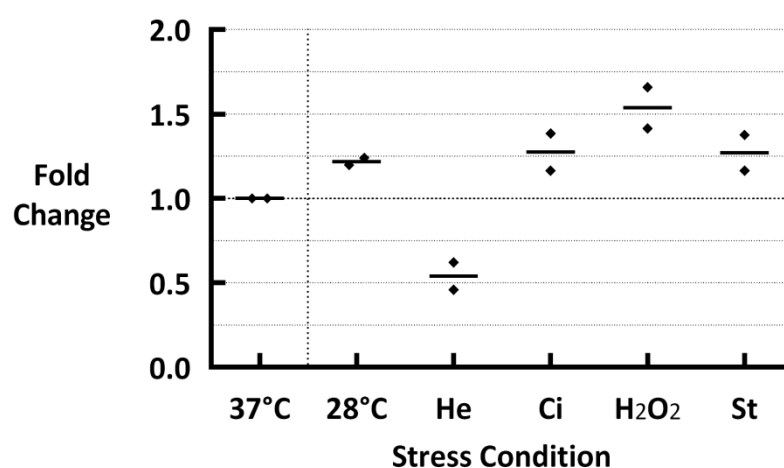
Figure 5.4. – Fold Change in Expression of *carD* Between 37°C Baseline Control and other Stress Conditions using qPCR Duplicates of each stress condition were plotted and the mean marked with a line. He = Haemin, Ci = Ciprofloxacin, H_2O_2, and St= Starvation. A fold change of 1 is equivalent to no change.

5.3. Discussion

The goal of this experiment was to determine the levels of expression of *carD* in *B. henselae* in the presence of specific bacterial stresses, and to determine fold change relative to a non-stressed control, using quantitative PCR. Our results can give us an insight into *B. henselae* transcription in key environmental niches, including the

haematophagous arthropod vector and the mammalian host. Our original hypothesis is that *carD* is a stress response gene in *B. henselae*; this has been reported in other bacterial species where *carD* has been previously described (*Mycobacterium carD* and *Myxococcus cdnL*).

For each stress environment, the level of stress exposure that we used to elicit a response may not be the same level of stress that *B. henselae* would experience in its natural environment. This is largely due to the fact that it is currently not known what precise conditions the bacteria experience during the life cycle; however, by approximating conditions of stress, we can compare changes in *carD* transcription in response to different stress exposures.

5.3.1. Low Temperature (28°C)

For our low temperature stress exposure assay (28°C for two hours), the results of our qPCR analysis showed there to be no major change in *carD* expression relative to 37°C (an increase by 1.22-fold; see **Figure 5.4**). Though the observed fold change was minimal, the transition from 37°C to 28°C might induce greater changes in *carD* expression at later time points. The global *B. quintana* expression at 28°C has previously been described (through the use of a microarray) over nine days[246]. Interestingly, *B. quintana carD* expression at 28°C, relative to expression at 37°C, increased by 1.08-fold on day three, and 2.1-fold on day seven. This is similar to what we observed for our 28°C *B. henselae* qPCR study, suggesting that *carD* performs a similar role at 28°C for both *B. henselae* and *B. quintana*. The microarray also showed at 28°C a 4-fold increase in expression of the general stress response gene PhyR (a regulator of RpoE), and a smaller increase in expression of the stringent response gene *dksA*, of 0.91-fold on day three and 2.08-fold on day seven.

Though bacterial transcriptional changes frequently occur very rapidly in response to environmental changes, the *B. quintana* microarray study showed that stress response genes, including *carD* and components of the stringent response continue to be important over longer periods of time (between three to seven days). This trend is consistent with our 37°C and 28°C growth assays, where a difference in growth rate at these temperatures was observed after three to four days (**Figure 4.4**). Similar up-regulation of *carD* and *dksA* in *B. quintana* could indicate that *carD* plays a role in the *Bartonella* stringent response.

In the tick-borne mammalian pathogen, *B. burgdorferi,* the LtpA protein has been described to regulate the transcriptional profile within the arthropod vector to promote survival[224]. The expression of this *carD*-like gene, *ltpA*, was increased 5-fold at a 23°C low temperature condition, relative to a 37°C control. However, LptA has homology to *M. xanthus* CarD, as opposed to *M. xanthus* CdnL (like *Mycobacterium* and *Bartonella* CarD); *M. xanthus* CarD and CdnL share a degree of structural homology, yet although both proteins act as global regulators, the functionality was not found to be interchangeable[215,283]. We hypothesised that despite the differences between *M. xanthus* CarD and CdnL*, B. henselae carD* could perform a similar function in *C. felis,* as *B. burgdorferi ltpA* in *I. scapularis*. Our evidence does not support this hypothesis, although further research is needed to determine the role of *B. henselae* CarD in the arthropod host.

5.3.2.   Haemin

During our *B. henselae* haemin qPCR experiments, a decrease in *carD* expression (0.54-fold relative to the control without haemin; see **Figure 5.4**) was observed.

Although this result suggests that *carD* expression is decreased in the arthropod midgut, we observed a slight increase in *carD* expression at the lower temperature associated with the arthropod.

In our *B. henselae* haemin exposure assay, results showed a large decrease in culture survival at conditions of 0.1 mM over 24 hours, which we interpreted to be toxic stress from haemin. However, reports in the literature differ as to the toxic effects of haemin. It has been shown that high concentrations of haemin (0.1 and 0.2 mM) result in a loss of viability, similar to what we observed, followed by robust growth after four days[247]. Though both studies used OD_{600}, Sanders et al (2000)[247] reported an initial decrease in viable cells at both 0.1 mM and at 0.2 mM (the effect was more pronounced at 0.2 mM), but no such decrease was seen by Muller et al (2016)[270], at 0.1 mM, although this study was conducted by assessing OD_{600}, which does not always detect loss of viability. The interpretation of these studies is that the optimal haemin concentrations may result in an initial loss of viability, after which the bacteria recover and are able to use the higher levels of haemin as a source of iron. This makes it difficult to determine which conditions are stressful for the bacteria. One interpretation of our data is that the bacteria in Schneider's Drosophila Medium are mildly stressed, due to low levels of available iron, while the media supplemented with haemin is actually more optimal. Further studies, for example analysis of a *carD* deletion mutant, will be needed to determine the role that CarD plays in adaptation to iron-depleted or iron-replete conditions.

Although the role of CarD has not been described, disruption of *carD* in the rodent pathogen *Bartonella birtlesii* prevented bacteraemia in animal hosts, suggesting it plays a role in establishing an infection in the mammalian host[176]. CarD could play a role in regulatory control of factors known to be important for invasion, such as the type 4

secretion systems, possibly through interactions with the stringent response. In *B. henselae*, the stringent response is down-regulated in the high nutrient human bloodstream; when expression of *dskA* and *spoT* is reduced, the histidine kinase component of a virulence-regulator two component system, BatS, is up-regulated, resulting in activation of Trw T4SS and invasion of erythrocytes (see **Figure 1.3**)[255].

Another consideration is that the decrease in *carD* expression observed during exposure to haemin could be explained by a decrease in overall *B. henselae* stress. When haemin is the sole source of iron in the media, *B. henselae* are able to grow, after a considerable lag period[247]; Brucella broth required a minimum of 0.006 mM (3.91 µg/mL) of haemin to support *B. henselae* growth, yet each increase of haemin up to 0.2 mM (130.33 µg/mL) increased growth. Although the level of iron in Schneider's Drosophila Medium with fetal calf serum is sufficient for *B. henselae* growth, it is possible that supplementation of haemin would increase *B. henselae* growth through an increase in iron availability. Schneider's Medium containing FCS has a small amount of free iron (0.24 µg/mL) and the iron-binding proteins haemoglobin (<25 µg/mL) and ferritin (0.23 µg/mL) provided by the fetal calf serum[284,285]. It is possible that *B. henselae* cultures in Schneider's Medium containing FCS could ultimately grow better when supplemented with 0.1 mM of haemin[249,255]. If this is the case, *carD* expression would be lower in the low-stress condition, as has been described for other bacteria. Further testing with haemin supplementation of Schneider's Drosophila Medium FCS, and experiments with a Δ*carD* mutant, would together provide clearer answers.

5.3.3. Ciprofloxacin and Hydrogen Peroxide

In our *B. henselae* qPCR experiments, results showed exposure to ciprofloxacin (10 µg/mL for 60 minutes) and hydrogen peroxide (0.1 mM for 10 minutes) lead to a minimal change in the expression of *carD* (by 1.27-fold and 1.54-fold respectively; see **Figure 5.4).** The increase in expression of *carD* suggests that CarD has a small role in the response to these conditions. Both ciprofloxacin and hydrogen peroxide are genotoxic agents, so the increase in *carD* expression is consistent with what has been reported in other bacteria.

Ciprofloxacin interferes with bacterial DNA gyrase and topoisomerase enzymes, resulting in DNA damage, while hydrogen peroxide causes damage to DNA, RNA, lipid, and protein structures via oxidisation and the formation of oxidative agents[257]. Damage to DNA can interfere with transcription, and result in stalled RNA polymerase complex. Additionally, ciprofloxacin relies on redox pumps for cellular entry, as opposed to hydrogen peroxide, which has been described in bacterial species to have a small degree of membrane permeability[286,287]. Though only a small concentration of H_2O_2 would pass into the cell, it could explain the increased *B. henselae* susceptibility to hydrogen peroxide as opposed to ciprofloxacin that we observed in our stress response assays (**Figure 4.6** versus **Figure 4.7**). During the natural *B. henselae* life cycle, exposure to H_2O_2 is found in both the midgut of the arthropod vector and from mammalian immune cells, while ciprofloxacin is only found during drug treatment[252].

A qPCR microarray study from *Mycobacterium smegmatis*[212], a close relative of *M. tuberculosis*, demonstrated that exposure to ciprofloxacin (10 µg/mL for 60 minutes) and hydrogen peroxide (10 mM for 60 minutes) increased the expression of *carD* by the

same level, an approximate 8-fold change. We observed very little change in *carD* expression under comparable conditions in *B. henselae*. However, at increased H_2O_2 exposure (35 mM for 60 minutes), viable *M. smegmatis* colonies were recovered but no change in *carD* expression was observed. A large change in *carD* expression is not surprising, considering that *carD* is known to regulate the stringent response of *M. smegmatis* in both conditions[212]. *Mycobacterium* have an almost 10-fold greater resistance to ciprofloxacin than *Bartonella* (MIC of ≤ 2 μg/mL, compared to ≤ 0.38), as well as a greater resistance to hydrogen peroxide, reflecting the importance of macrophages in the pathogenesis of *M. tuberculosis*[276]. The high levels of induction of *carD* in *Mycobacteria*, relative to what we observed in *B. henselae,* highlight the possibility that *Bartonella* CarD plays a very different role from that of *Mycobacterium* CarD.

5.3.4.   Nutrient Starvation

When we exposed *B. henselae* to complete nutrient starvation in PBS, our qPCR analysis results showed essentially no major change in *carD* expression (an increase of 1.27-fold; see **Figure 5.4**). In the *M. smegmatis carD* qPCR study, starvation conditions (1x PBS supplemented with the detergent Tween 80 for four hours), resulted in 20-fold increased *carD* expression[212]. Interestingly, an extended starvation exposure time (1x PBS with Tween 80 for 24 hours) showed a smaller increase in *carD* expression, with a 4-fold change. This is further evidence that *B. henselae* CarD is playing a role that is distinct from that reported for *Mycobacterium* CarD. Although it would be good to test additional time points for *B. henselae* in starvation conditions, the different roles played by CarD in two different pathogens likely reflects the many differences between

these bacteria, including variations in pathogen biology, life cycle, host colonisation sites, and mechanisms of transmission.

5.3.5.   Conclusion

Our initial hypothesis was that *carD* played a role in adaptations to stress, similar to that described for *Mycobacterium* CarD. However, there are many biological differences between these bacteria, and they likely experience and are adapted to different stresses; protein homologues may therefore diverge and acquire distinct functions. Additional evidence that the function of *Mycobacterium* CarD differs from that of *Bartonella* is that the former is an essential gene, while *carD* mutants have been noted in a rodent *Bartonella* species.

It was unexpected that overall our *B. henselae* stress exposure conditions resulted in a minimal increase in *carD* expression. The interesting exception to this was the response to haemin, though the decrease in expression was not expected. This result does indicate that *carD* expression is sensitive to changes in nutrient availability, though the expression was actually increased in the relatively iron-poor condition (Schneider's Drosophila Medium containing FCS). It is possible that CarD may undergo a post-transcriptional change under stress conditions. This could explain why such a small transcriptional change was found, although a change in CarD expression was observed in *B. quintana* studies. Additional studies are needed to shed light on whether the role of CarD is one of adaptation to nutrient limitation, or of adaptation to the potential toxicity of nutrients such as haemin.

Previous transcriptional studies in *B. quintana* at 37°C and 28°C gave us an insight into the role of *carD.* The showed similarly low levels of *carD* and *dksA* expression over the

first three days, with an increase in expression of both genes observed at seven days[264]. As *carD* expression is up-regulated over time, other known stringent response genes are also up-regulated. In contrast, the general stress response of *B. quintana* is highly up-regulated, especially PhyR. Similarly, in *B. henselae* cultures at low temperature, or the presence of haemin and/or hydrogen peroxide, the general stress response has been described to be up-regulated[230,247,252-254,264].

Interaction of *B. henselae* DksA and CarD has not been shown, but reports of Δ*dksA* and Δ*carD* suggest functional similarity. *dksA* mutants were shown to have limited endosome formation during *in vitro* endothelial cell invasion, possibly due to the crucial role of DksA in mediating the VirB/D4 T4SS[207,208]. Δ*carD B. birtlesii* strains were viable on lab media, yet could not establish bacteraemia in a mouse infection model[176]. If CarD also mediates *B. henselae* expression of the VirB/D4 T4SS, this could explain why the lack of *carD* prevented bacteraemia in a mammalian host.

Our observed *carD* qPCR stress results are very distinct from what has been reported in *Mycobacterial* species for ciprofloxacin, hydrogen peroxide, and starvation[212]. This result suggests a novel role for CarD in *Bartonella* species, but further studies are needed to better define this role.

Chapter 6. Research Motivations, Key Findings, and Future Directions

6.1. Research Motivation

CarD/CdnL proteins have previously been characterised in the literature, most thoroughly in *Mycobacterium* (CarD) and *Myxococcus* (CdnL), where they have been reported to act as global transcriptional regulators[212,215,219]. *Mycobacterial* CarD functions by binding to RNA polymerase and to DNA, stabilising the open promoter complex during transcription[218,220,223]. Additionally, *Mycobacterium* CarD regulates the stringent response, stabilising the (p)ppGpp-RNA polymerase complex; this potentiates (p)ppGpp activity in a similar manner to *E. coli* DksA, albeit through a different RNA polymerase binding site[210-212]. *Myxococcus* CdnL similarly binds to RNA polymerase and stabilises the open promoter complex during transcription, although the protein does not bind to DNA[215]. The difference in protein function may be due to the fact that while *Mycobacterium* only have the CarD protein, *Myxococcus* have functional CdnL and DksA proteins. *M. xanthus* CarD and DksA proteins are essential for bacterial viability, although their functions during the *Myxococcus* stress response are not yet fully understood.

Bartonella, as for *Myxococcus,* have both DksA and CarD proteins, although neither has been shown to be essential for growth through targeted deletion. *B. henselae* DksA is a key regulator of the stringent response during mammalian host cell invasion[207]. Despite the important regulatory role that CarD has been shown to play in the literature, studies on *Bartonella* CarD structure or function have not been carried out. In a study of the rodent pathogen *Bartonella birtlesii*, a genetic screen was carried out to identify factors important for invasion of erythrocytes; one of the mutants identified in the initial screen

had *carD* disrupted by a transposon. The *B. birtlesii carD* transposon mutant could not establish bacteraemia in an *in vivo* mouse infection model, but was not found to play a role in erythrocyte invasion[176]. This study, in combination with our own bioinformatic analyses of the *B. henselae* and *M. smegmatis carD* and *M. xanthus cdnL* genes, shows that a reasonable degree of homology is found between the three bacterial genera. We therefore hypothesised that *carD* plays an important role in regulating *Bartonella* response to stress.

Our research aimed to fill an important gap in the knowledge of *Bartonella* biology; to this end, we aimed to investigate whether CarD played a significant role during the initial *B. henselae* response to stresses that are commonly encountered by the bacteria.

6.2. Key Findings

Our initial hypothesis was that *carD* acted as a stress response gene in *B. henselae*, due to amino acid similarities between CarD in *Bartonella* and CarD in other bacterial genera. We investigated the expression levels of *carD* in the presence of common *B. henselae* stress conditions, including low temperature (28°C), haemin supplementation, ciprofloxacin treatment, H_2O_2 exposure, and nutrient starvation.

To this end, we initially attempted to assess the role of CarD in the *B. henselae* bacterial stress response by comparing growth of the *B. henselae* wild type strain against a *B.henselae* Δ*carD* strain in stress conditions. However, we could generate neither the *B.henselae* Δ*carD* mutant strain, nor the intermediary *B. henselae* merodiploid *carD* mutant strain. An observable decrease in bacterial growth was observed for S17-1 *E. coli* with our mutagenic plasmids (either pJS01 or pJS02); we believe that this was due to

toxicity of the truncated *carD* gene fragment, and could explain why transformations were not successful.

Instead, for each stress condition, a quantitative PCR reaction was performed to determine the relative expression levels of the *B. henselae carD* gene, between the non-stressed control and the stress condition. Overall, our *B. henselae* stress exposure conditions (with the exception of haemin) showed a minimal increase in *carD* expression, with a trend of a small increase in *carD* expression under stress conditions. However, our qPCR results neither prove nor disprove our hypothesis that *carD* is a stress gene in *B. henselae* due to the small nature of the fold change in *carD* expression. It is possible that CarD plays a role in the stress response to conditions that we did not test. Similarly, the unexpected lower expression of *carD* in the presence of haemin points to a role for CarD in either nutrient deprivation or detoxification of nutrients such as haemin. Additional experiments are needed to elucidate the role of CarD in *B. henselae* growth and response to haemin.

6.3.   Future Directions

6.3.1.   Continuation of our *B. henselae* Δ*carD* Experiments

6.3.1.1.   Generation of a *B. henselae* ΔcarD Mutant Strain

Initial attempts to generate a *B. henselae* Δ*carD* mutant using the pJS01 and pJS02 mutagenic plasmids did not yield *carD* merodiploid colonies. One possible reason for this is that expression of truncated CarD protein products interfered with normal *E. coli* growth. Another possible reason is that the *carD* gene is essential in *B. henselae*.

To elucidate the essential nature of *M. xanthus cdnL,* Gallego-García et al used a similar two-step merodiploid-intermediate mutation protocol as we have described in this study[215]. To enable homologous recombination, our mutagenic *carD* plasmid included a small segment of our *carD* gene; however, the mutagenic plasmid used by Gallego-García et al included only 3' and 5' *cdnL* intergenic flanking sequences, and no actual *cdnL* open reading frame sequence. By redesigning our *carD* mutagenic plasmid to remove any remaining *carD* sequence, we can avoid expression of the gene, and enable transformation and subsequent homologous recombination. Successful generation of the *B. henselae carD* merodiploid and Δ*carD* mutant strains would provide confirmation that *Bartonella carD* is not an essential gene.

Even if *B. henselae carD* turns out to be an essential gene, however, merodiploid colonies should still be recovered, as these isolates have one copy of the intact, wild type gene. In the case of an essential gene, however, negative selection of the merodiploid strain may only result in reversion to wild type; this was documented by Gallego-Garcia in their studies of *M. xanthus cdnL*[215]. If *carD* did turn out to be

essential in *B. henselae*, another approach would be to generate a plasmid that allows for controlled conditional knockdown of the c*arD* gene, under tetracycline control, as previously described for *M. smegmatis carD*[212].

6.3.1.2. Growth Analysis of *B. henselae* Wild-type and ΔcarD Strains during Stress Exposure

Though we observed a small increase of *carD* expression in our qPCR experiments, we do not know if the *carD* gene is enabling a transcriptional change that defends *B. henselae* against stress. If a *B. henselae* Δ*carD* mutant were obtained, we could carry out our original experimental plan to determine how important the *carD* gene is in the *Bartonella* stress response, similar to *carD* assays performed in *M. smegmatis*[212]. We have already determined the optimal conditions for growth of wild-type *B. henselae* cultures during exposure for each of our stresses of interest, although the purpose of these conditions are to elicit a stress response while retaining overall culture viability in the range of 80 to 90%. The growth of wild-type and Δ*carD* mutant strains could be compared, using spectrophotometry and viable colony counts to estimate the percent survival at various time points.

6.3.2. Confirmation of the *B. henselae* Haemin Response and Further Haemin Condition Analysis

As discussed in Chapters 4 and 5, the haemin supplementation concentration we used for our *B. henselae* haemin exposure assay (0.1 mM) may be too low to mimic the initial haemin concentration of the arthropod host vector during a blood meal[247]. To confirm this, the haemin resistance assay could be extended, with time points assessed for up to 14 days. If the *B. henselae* culture recovers and robust growth is confirmed, higher haemin concentrations would be needed to replicate the conditions in the arthropod gut.

This experiment could be followed by a *carD* qPCR assay to determine the stress response, as described for the other stress conditions.

To mitigate the initial loss of *B. henselae* viability following haemin exposure, the bacteria could be gradually exposed to increasing levels of haemin, to enable a gradual acclimatisation. Finally, the expression levels of *carD* could be assessed at higher, toxic concentrations of haemin.

6.3.3.   Expansion of the scope of our *B. henselae carD* qPCR assays

6.3.3.1.   Targeting an Increased Range of *B. henselae* Stress Genes

We cannot conclude that the *B. henselae carD* gene is involved in the *Bartonella* stress response from our *carD* qPCR assays. To better determine the role of *carD* in the stress conditions, we could also assess the expression of *B. henselae* genes that are known components of the stress response. qPCR primers can be designed that amplify the *B. henselae dksA* (or *spoT*) for the stringent response and *phyR* (or *rpoE*) for the general stress response; when used in conjugation with our *carD* primers, we could use these to compare the *carD* response against defined stress genes.

6.3.3.2.   Increase the Scope of our *B. henselae* carD qPCR Stress Assay

During culture of *B. quintana* in 28°C (against a 37°C control)[264], the initial fold change of *carD* and *dksA* gene expression was low after three days (<1.5-fold), yet an increased fold change was observed after seven days of exposure to 28°C (>2-fold). The *B. quintana* 28°C study concluded that RpoE (a general stress sigma factor) played a role in temperature mediated adaptation to the arthropod vector[264]. Additionally, the

B. henselae stress response described for haemin and H_2O_2 resulted in an increase in PhyR expression, highlighting the role of the general stress response in the initial exposure to stress in *Bartonella*[230,247,252-254,264], while the stringent response (possibly through *carD*) may mediate later adaptation to stress.

We may have observed a small *B. henselae carD* fold change in our qPCR studies because CarD plays a role in later stress adaptation. Increasing the exposure time of each stress response assay and measuring *carD* expression over a time course would verify whether this trend holds true in *B. henselae* and for multiple stresses.

6.3.3.3. Testing Stress Conditions that Mimic the *B. henselae* Environment

If *carD* expression changes are detected during our modified qPCR stress experiments, follow up experiments combining multiple stress signals could be carried out. For example, to mimic the arthropod gut, we could simultaneously test haemin exposure and 28°C, including individual stress assays as controls. It would also be interesting to see if a similar *carD* response to starvation is found in defined media, as opposed to the complete nutrient starvation assay as described here; minimal supplemented media might better replicate the natural conditions encountered by *B. henselae*.

6.3.4. Studies into the Role of *carD* during Regular Cellular Function

Often bacterial stress genes play important roles in mediating the regular bacterial growth cycle. This has been observed for *dksA* in *E. coli*[288], and in *cdnL* in *M. xanthus*[245]. It would be interesting to see if *carD* plays an similar role in *B. henselae*.

To determine this, we could attempt to quantify the expression levels of *carD* in *B. henselae* over time in culture, by investigating the levels of the gene in wild-type

cultures as they progress through the cell cycle. However, while we could attempt to use *B. henselae* as a model for this kind of cell cycle research, few genetic tools currently exist for *Bartonella* species.

Caulobacter crescentus is a free-living, Gram-negative alphaproteobacteria that lives in aquatic environments, and has CarD and DksA homologues, although their functional role has not been elucidated. *C. crescentus* undergoes asymmetric cellular division, resulting in two distinct cellular morphologies: a sessile mother cell with a stalk and a mobile daughter cell with a flagellum[289,290]. This allows cell cycle synchronisation, and accurate determination of the timing of gene expression; stalk cells are in S phase, flagellated cells in G1, and the dividing cells in G2. The organism is also highly genetically tractable, with a large number of molecular tools available. The role of the endogenous *C. crescentus carD* gene in the cell cycle could be analysed by gene deletion and mutant analysis. Finally, cross-complementation studies could be carried out between the *B. henselae carD* and *C. crescentus*, to determine if the *B. henselae carD* gene can functionally replace the *C. crescentus carD* gene. Research tools, such as transcriptional profiling or protein localisation, are available for *C. crescentus*, and these could be used to carry out a detailed analysis of the role of *B. henselae carD* during growth, the cell cycle, and stress response.

6.4. Concluding Remarks

Though CarD has been well-studied in other bacterial genera, the experiments reported here are the first experimental analysis that solely focuses on the CarD in any *Bartonella* species.

We observed through quantitative PCR reactions a trend of increased expression of *carD* in our *B. henselae* cultures under stress conditions, although this change was not found to be substantial. Further experimentation is underway, to shed light on the role of *carD* in the *Bartonella* stress response.

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Chapter 8. Appendices

8.1.   CarD Amino Acid Sequence Alignments

Alignments were performed using Clustal Omega, a multiple sequence alignment program[234].

CarD family transcriptional regulator (*Bartonella henselae*)
NCBI Reference Sequence: WP_011181290.1

CarD family transcriptional regulator (*Mycobacterium tuberculosis*)
NCBI Reference Sequence: WP_057133327.1

CdnL, CarD family transcriptional regulator (*Myxococcus xanthus*)
NCBI Reference Sequence: 2LWJ_A

pJS01 CarD is a hypothetical truncated CarD protein encoded by our pJS01 plasmid. This was obtained by analysing the Macrogen sequencing results.

```
B. henselae       8    FSTSEYIVYPTHGVGQIIAIEDQEVAGHKLKLFVIHFAKDKMDVKVPIAKAISVGMRKL
M. tuberculosis   3    FKVGDTVVYPHHGAALVEAIETRTIKGEQKEYLVLKVAQGDLTVRVPAENAEYVGVRDV
M. xanthus        14   FKTGDKAVYPGQGVGEVMGIEHTEVAGQRQSFYVLRILENGMRIMIPINKVGSVGLREI
                       *. .: .*** .*    : .** . : *.:... *:...:  : :..:*   .  **:*.:.


B. henselae            SAVDSVERALKILHGKARV-KRTMWSRRAQEYDAKINSGDLICIAEVVRDLFRSDLQPEQ
M. tuberculosis        VGQEGLDKVFQVLRAPHTE-EPTNWSRRYKANLEKLASGDVNKVAEVVRDLWRRDQERGL
M. xanthus             ISEEDVKQVYSILKEKDISVDSTTWNRRYREYMEKIKTGSVFEIAEVLRDLYLLKGDKDL
                        .: :.:. .:*:        . * *.** :     *: :*.: :***:***: . :   .


B. henselae            SYSERQLYIAALERMAREIAVINSLSETEAINLIEMHLS      170
M. tuberculosis        SAGEKRMLAKARQILVGELALAESTDDAKAETILDEVLAAA    161
M. xanthus             SFGERKMLDTARSLLIKELSLAKDCSEDEIESDLKKIFNLA    167
                       *.*:::    * . :  *::: :. .: :   . :.   :


-             -                -             -                    -


pJS01 CarD        13   FSTSEYIVYPTHGVGQIIAIEDQEVAGHKLKLFVIHFA-----TSLPKAKASI
B. henselae       8    FSTSEYIVYPTHGVGQIIAIEDQEVAGHKLKLFVIHFAKDKMDVKVPIAKAISVGMRKL
                       *************************************      .* ***
```

Appendix Figure 8.1. – ClustalOmega Amino Acid Sequence Alignments All sequences were sourced from the NCBI online gene database and then aligned with Clustal Omega with the exception of pJS01 CarD, which was not included in the initial sequence alignment. The pJS01 truncated CarD sequence shown above is a hypothetical expressed protein segment as determined from our Macrogen sequencing data. *M. tuberculosis* sequence highlighted in yellow denote β1, β2, and β3 sheets and *M. xanthus* sequence highlighted in green denote β1, β2, β3, β4, and β5 sheets[215,217]. The CarD/CdnL binding to RNA polymerase is through the N-terminal Tudor-like folds of β sheets. Though the pJS01 hypothetical CarD protein is heavily truncated, it still includes the *M. tuberculosis* β1 and β2 folds (a possible truncated β3 fold) and *M. xanthus* β1, β2, and β3 folds (with a possible truncated β4 fold).

8.2.   *carD* PCR Fusion Reaction Figure
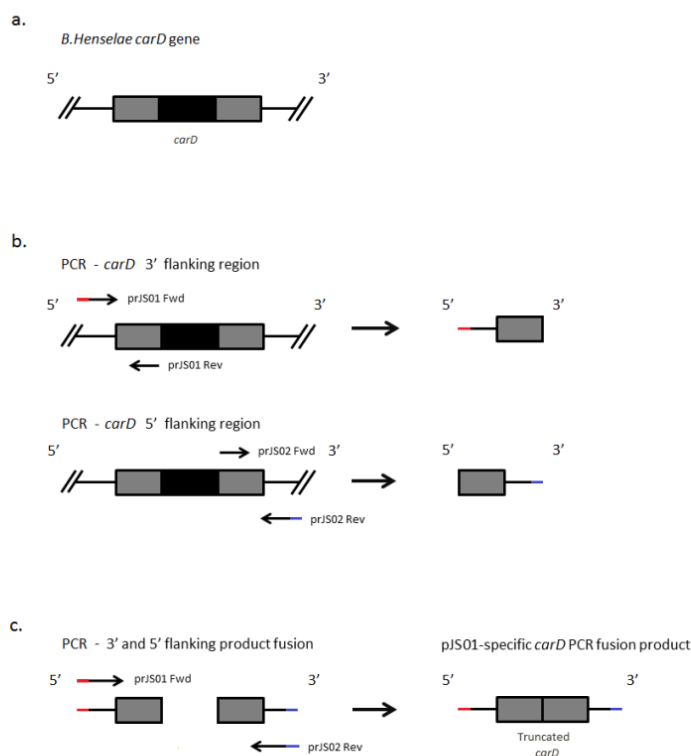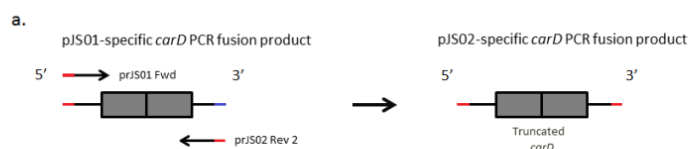
Appendix Figure 8.2. – Synthesis of the *carD* PCR fusion products for pJS01 and pJS02 1) (a.) Representation of the *B. henselae carD* gene on purified genomic DNA. The segment of *carD* coloured in black represents the section that will be removed to form a truncated *carD* gene, i.e. the pJS01 *carD* fusion product. (b.) Concurrent PCRs using *B. henselae* gDNA as a template with prJS01 and prJS02 primer pairs to yield the *carD* 3' and 5' flanking region products. Arrows indicate direction of primer extension; primer sections coloured red and blue indicate overhangs encoding BamHI and SalI restriction sites respectively. (c.) Fusion PCR using *carD* flanking region products as template DNA to yield the pJS01 *carD* PCR fusion product insert. The forward prJS01 and reverse prJS02 primers were used. 2) (a.) The pJS01 *carD* PCR fusion product was used as a DNA template for a PCR to yield the pJS02 *carD* PCR fusion product. The forward prJS01 and reverse 2 prJS02 primers were used. As before, primer sections coloured red indicate overhangs encoding BamHI.

8.3.   *B. henselae carD* Gene Sequence

```
TTGCGTTGAAGTTTGCCCTGTTGATTGTTTTTATGAAGGTGAAAACATGCTTGTCATTCATCCCGATGAATGTATCGATTGTGGTGT
GTGTGAACCAGAATGCCCTGCAGAAGCCATTAAACCGGATACAGAGCCGGGGCTAGAAAAATGGCTAGAGCTCAATCTTCAATATGC
AAACAAATGGCCTAATTTAACCACCAGAAAAGATCCTCTTCCTCAAGCAAAAGAAATGGATGGCATTCCGGATAAATTAGAAAAGTA
TTTTTCAGAAAATCCAGGAAGCGGAGAATAATAAGCATAGGACATACATCATAAGCTTTTAAAAATTGAAATCATTTCTTTGTAAAA
ATAAAAATTCCTCAGTGAAATGATTGATTCTTTTGACATTTGATGTTAATGTTATTTTAACATGATCATCTTTCTAGGTATTTTTCT
TGCTTTCTCCTTGTAAAGGAAGGTATTTTTTGTTTGTTTTAAAAGTACTCAACTTTTATGCACATTAAATTCGTATAATATACTTGG
TTAAAGAACATTCAACGTTCAGGCTTTTAGCCTGTTTTCCGTTACATTTGCTGGTCACTAAAGGGAGTAAACTAAAAT**ATGGCCAAC**
**ATGGAACGTCCTCCAATGCTAAAGGATTTTCAACTTCTGAATATATTGTCTACCCTACCCATGGAGTAGGGCAAATTATAGCAATTG**
**AAGATCAAGAAGTTGCAGGACATAAATTAAAGCT**TTTCGTCATTCATTTTGCGAAAGATAAAATGGATGTTAAAGTGCCAATTGCAA
AAGCCATTTCTGTTGGAATGCGCAAATTATCTGCTGTCGATTCCGTTGAACGTGCATTGAAAATCTTACATGGAAAAGCGCGAGTTA
AACGAACCATGTGGTCACGCCGCGCTCAAGAATATGATGCGAAAATTAATTCAGGAGATCTTATTTGTATCGCTGAAGTCGTACGCG
ATCTTTTTCGTTCAGATCTACAGCCTGAACAATCTTATTCCGAGCGTCAACTCTATATCGCTGCACTTGAAAGGATGGCCCGTGAGA
TTGCCGTTATTAATAGTCTTTCTGAAACAGAGGCTATCAATCTCATAGAAATG**CATCTCTCCCAAAAGCCAAA**GCGTCAATTTAAAA
**CTAAGAGAGATGAGGTAAATGCAAACAGCGAAGCTGTCTATGCCGCATAA**TAAAGTGCAAAATCTTTTGATCATAAAATAAAACCAG
CGAAATGCTGGTTTCGTTTTTTTATCAAGGCCCTTCAATAATTAGAAGGATATTTGGTCAGAGATTCATCCATTCTTCCTATAAATT
CCCCATGCAAATAATATTGGTGTTGAACACAATATAAGTGAAATGAGGTGTGGAGCCTTATCTCTTCACATCCTGTCATAGATGACA
TTGATCTTCTACTTCTGCCCTTATGCCTGTCTTTCCAAGAGACTTTTAAGAACACTATGAAGGAAAACATTTCTTTACCGTGCTCTT
TCATTGAGAGCCGATAAAAAAAGACAACCTTTACAGTCGCTTCACATAAGCACACTGAAAACAAAGCGCACAGGCAAACGTCACCTG
TTAAGATAACAAAAATACAGAAGAAAAACTAAGAGCTTCATCTCCTCTCTGAGAAAGAGAAGAGAAAAACAAAACCTCTGCGCACAT
CCCATTCACCGCATAGATTCATTTGTCTCTGAATCAAATGAATCCATCTCTGGTAGAGAGATAAAAATCTCAATCTTTAGCAGGCAA
CACTGAACGTCCGCGATACATTCCGGTTTTCAAATCGATGT
```

Appendix Figure 8.3. – *B. henselae carD* sequence +/- 600 basepairs The sequence was sourced from the online NCBI genbank. Bold sequence denotes the *carD* gene. Highlighted sequence denotes primer sites. Blue highlighted sequence denotes sequence removed to form the *carD* PCR fusion product.

8.4.   pJS01 Plasmid Confirmation by Sequencing

All sequencing was performed by Macrogen, South Korea, using universal M13 primers. Sequence readouts were analysed using Geneious bioinformatics software program 8.1.7[233], multiple sequence alignment tool.

```
pJS01 Calculated   GGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCA
pJS01 Forward      ------------------------------------------------------------
pJS01 Reverse      ------------------------------------------------------------

pJS01 Calculated   GTGCCAAGCTTGCATGCCTGCAGGTCTCCTGTGAACCAG-AATGCGGATACAGAGCCGGG
pJS01 Forward      -------------------------------AGCCATTAAACCGGATACAGAGCCGGG
pJS01 Reverse      --------------------------------------ATTAAACCGGATACAGAGCCGGG

pJS01 Calculated   GCTAGAAAAATGGCTAGAGCTCAATCTTCAATATGCAAACAAATGGCCTAATTTAACCAC
pJS01 Forward      GCTAGAAAAATGGCTAGAGCTCAATCTTCAATATGCAAACAAATGGCCTAATTTAACCAC
pJS01 Reverse      GCTAGAAAAATGGCTAGAGCTCAATCTTCAATATGCAAACAAATGGCCTAATTTAACCAC

pJS01 Calculated   CAGAAAAGATCCTCTTCCTCAAGCAAAAGAAATGGATGGCATTCCGGATAAATTAGAAAA
pJS01 Forward      CAGAAAAGATCCTCTTCCTCAAGCAAAAGAAATGGATGGCATTCCGGATAAATTAGAAAA
pJS01 Reverse      CAGAAAAGATCCTCTTCCTCAAGCAAAAGAAATGGATGGCATTCCGGATAAATTAGAAAA

pJS01 Calculated   GTATTTTTCAGAAAATCCAGGAAGCGGAGAATAATAAGCATAGGACATACATCATAAGC-
pJS01 Forward      GTATTTTTCAGAAAATCCAGGACGCGGAGAATAATAAGCATAGGACATACATCATAAGCT
pJS01 Reverse      GTATTTTTCAGAAAATCCAGGACGCGGAGAATAATAAGCATAGGACATACATCATAAGCT

pJS01 Calculated   TTTAAAAATTGAAATCATTTCTTTGTAAAAATAAAAATTCCTCAGTGAAATGATTGATTC
pJS01 Forward      TTTAAAAATTGAAATCATTTCTTTGTAAAAATAAAAATTCCTCAGTGAAATGATTGATTC
pJS01 Reverse      TTTAAAAATTGAAATCATTTCTTTGTAAAAATAAAAATTCCTCAGTGAAATGATTGATTC

pJS01 Calculated   TTTTGACATTTGATGTTAATGTTATTTTAACATGATCATCTTTCTAGGTATTTTTCTTGC
pJS01 Forward      TTTTGACATTTGATGTTAATGTTATTTTAACATGATCATCTTTCTAGGTATTTTTCTTGC
pJS01 Reverse      TTTTGACATTTGATGTTAATGTTATTTTAACATGATCATCTTTCTAGGTATTTTTCTTGC

pJS01 Calculated   TTTCTCCTTGTAAAGGAAGGTATTTTTTGTTTGTTTTAAAAGTACTCAACTTTTATGCAC
pJS01 Forward      TTTCTCCTTGTAAAGGAAGGTATTTCTTGTTTGTTTTAAAAGTACTCAACTTTTGTGCAC
pJS01 Reverse      TTTCTCCTTGTAAAGGAAGGTATTTCTTGTTTGTTTTAAAAGTACTCAACTTTTGTGCAC

pJS01 Calculated   ATTAAATTCGTATAATATACTTGGTTAAAGAACATTCAACGTTCAGGCTTTTAGCCTGTT
pJS01 Forward      ATTAAATTCGTATAATATACTTGGTTAAAGAACATTCAACGTTCAGGCTTTTAGCCTGTT
pJS01 Reverse      ATTAAATTCGTATAATATACTTGGTTAAAGAACATTCAACGTTCAGGCTTTTAGCCTGTT

pJS01 Calculated   TTCCGTTACATTTGCTGGTCACTAAAGGGAGTAAACTAAAAT`ATGGCATCCCAACATGGA`
pJS01 Forward      TTCCGTTACATTTGCTGGTCACTAAAGGGAGTAAACTAAAATATGGCATCCCAACATGGA
pJS01 Reverse      TTCCGTTACATTTGCTGGTCACTAAAGGGAGTAAACTAAAATATGGCATCCCAACATGGA

pJS01 Calculated   `ACGTCCTCCAATGCTAAAGGATTTTCAACTTCTGAATATATTGTCTACCCTACCCATGGA`
pJS01 Forward      ACGTCCTCCAATGCTAAAGGATTTTCAACTTCTGAATATATTGTCTACCCTACCCATGGA
pJS01 Reverse      ACGTCCTCCAATGCTAAAGGATTTTCAACTTCTGAATATATTGTCTACCCTACCCATGGA

pJS01 Calculated   `GTAGGGCAAATTATAGCAATTGAAGATCAAGAAGTTGCAGGACATAAATTAAAGCTTTTC`
pJS01 Forward      GTAGGGCAAATTATAGCAATTGAAGATCAAGAAGTTGCAGGACATAAATTAAAGCTTTTC
pJS01 Reverse      GTAGGGCAAATTATAGCAATTGAAGATCAAGAAGTTGCAGGACATAAATTAAAGCTTTTC

pJS01 Calculated   `GTCATTCATTTTGCGACATCTCTCCCAAAAGCCAAAGCGTCAATTTAAAACTAAGAGAGA`
pJS01 Forward      GTCATTCATTTTGCGACATCTCTCCCAAAAGCCAAAGCGTCAATTTAAAACTAAGAGAGA
pJS01 Reverse      GTCATTCATTTTGCGACATCTCTCCCAAAAGCCAAAGCGTCAATTTAAAACTAAGAGAGA

pJS01 Calculated   `TGAGGTAAATGCAAACAGCGAAGCTGTCTATGCCGCATAA`TAAAGTGCAAAATCTTTTGA
pJS01 Forward      TGAGGTAAATGCAAACAGCGAAGCTGTCTATGCCGCATAATAAAGTGCAAAATCTTTTGA
pJS01 Reverse      TGAGGTAAATGCAAACAGCGAAGCTGTCTATGCCGCATAATAAAGTGCAAAATCTTTTGA

pJS01 Calculated   TCATAAAATAAAACCAGCGAAATGCTGGTTTCGTTTTTTTATCAAGGCCCTTCAATAATT
pJS01 Forward      TCATAAAATGAAACCAGCGAAATGCTGGTTTCGTTTTTTTATCAAGGCCCTTCAATAATT
pJS01 Reverse      TCATAAAATGAAACCAGCGAAATGCTGGTTTCGTTTTTTTATCAAGGCCCTTCAATAATT

pJS01 Calculated   AGAAGGATATTTGGTCAGAGATTCATCCATTCTTCCTATAAATTCCCCATGCAAATAATA
pJS01 Forward      AGAAGGATATTTGGTCAGAGATTCATCCATTCTTCCTATAAATTCCCCATGCAAATAATA
pJS01 Reverse      AGAAGGATATTTGGTCAGAGATTCATCCATTCTTCCTATAAATTCCCCATGCAAATAATA
```

```
pJS01 Calculated    TTGGTGTTGAACACAATATAAGTGAAATGAGGTGTGGAGCCTTATCTCTTCACATCCTGT
pJS01 Forward       TTGGTGTTGAACACAATATAAGTGAAATGAGGTGTGGAGCCTTATCTCTTCACATCCTGT
pJS01 Reverse       TTGGTGTTGAACACAATATAAGTGAAATGAGGTGTGGAGCCTTATCTCTTCACATCCTGT

pJS01 Calculated    CATAGATGACATTGATCTTCTACTTCTGCCCTTATGCCTGTCTTTCCAAGAGACTTTTAA
pJS01 Forward       CATAGATGACATTGATCTTCTACTTCTGCCCTTATGCCTGTCTTTCCAAGAGACTTTTAA
pJS01 Reverse       CATAGATGACATTGATCTTCTACTTCTGCCCTTATGCCTGTCTTTCCAAGAGACTTTTAA

pJS01 Calculated    GAACACTATGAAGGAAAACATTTCTTTACCGTGCTCTTTCATTGAGAGCCGATAAAAAAA
pJS01 Forward       GAACACTATGAAGGAAAACATTTCTTTACCGTGCTCTTTCATTGAGAGCCGATA------
pJS01 Reverse       GAACACTATGAAGGAAAACATTTCTTTACCGTGCTCTTTCATTGAGAGCCGA--------

pJS01 Calculated    GACAACCTTTACAGTCGCTTCACATAAGCACACTGAAAACAAAGCGCACAGGCAAACGTC
pJS01 Forward       ------------------------------------------------------------
pJS01 Reverse       ------------------------------------------------------------
```

Appendix Figure 8.4. – Macrogen Sequence Alignments using Geneious pJS01 Calculated is an estimate of the pJS01 sequence, constructed from pEX18gm, *carD* sequences. pJS01 Forward and Reverse sequences are the forward and reverse sequence reads of purified pJS01 plasmids that were sent to Macrogen. Sequence highlighted in green denotes the shortened *carD* sequence insert.