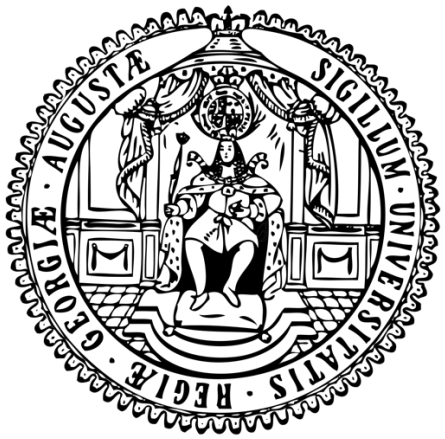
Characterization of genes differentially regulated after bile acid exposure in *Campylobacter jejuni*



Dissertation
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Submitted by

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Born in Florianópolis, Brazil

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“Um homem precisa viajar. Por sua conta, não por meio de histórias, imagens, livros ou TV. Precisa viajar por si, com seus olhos e pés, para entender o que é seu. Para um dia plantar as suas próprias árvores e dar-lhes valor. Conhecer o frio para desfrutar o calor. E o oposto. Sentir a distância e o desabrigo para estar bem sob o próprio teto. Um homem precisa viajar para lugares que não conhece para quebrar essa arrogância que nos faz ver o mundo como o imaginamos, e não simplesmente como é ou pode ser. Que nos faz professores e doutores do que não vimos, quando deveríamos ser alunos, e simplesmente ir ver”

Amyr Klink

"A man needs to travel. On your own, not through stories, pictures, books or TV. You need to travel by yourself, with your eyes and feet, to understand what is yours. To one day plant your own trees and give them value. Knowing the cold to enjoy the heat. And the opposite. To feel the distance and the unsettled, to be well under the own home. A man must travel to places he does not know to break this arrogance that makes us see the world as we imagine it, not simply as it is or can be. That makes us teachers and doctors of what we did not see, when we should be students, and simply go and see"

Amyr Klink

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ABSTRACT

In the past decades *Campylobacter* has raised as the main cause of bacterial gastrointestinal infection worldwide. *Campylobacter* causes gastrointestinal infection that can vary from asymptomatic, mild to a severe diarrhea. Annually, approximately 246,000 confirmed cases of *Campylobacter* enteritis are reported in Europe, and 74,000 just in Germany (EFSA 2017). *C. jejuni* and *C. coli* are the main species related to human infections (Dasti et al. 2010). Consequently, *C. jejuni* is recognized as an important public health issue which pronounces the importance of pathogenesis studies of this organism. In this study, novel pathogenicity factors involved in the ability of *C. jejuni* to adapt to the bile acid rich environment of the human gut are aimed to be identified by the generation of knockout mutants. Candidate genes were mainly chosen from proteomics data generated in our lab that resulted in the identification of differentially expressed proteins after exposure to sublethal concentrations of seven bile acids (Masanta et al. 2018). Ten knockout mutants were generated by the insertion of a kanamycin resistance cassette into the target gene via homologous recombination. Various phenotypic parameters were assessed such as adhesion and invasion into two different host cell types, soft agar motility, autoagglutination, biofilm formation and stress resistance. Surprisingly, from our ten knockout mutants six of them showed a strong coupled phenotype with an unstable motility behavior, an increased adhesion and invasion to Caco2 cell and increased biofilm formation. These phenotypic changes can be interpreted as adaptation processes that prepare the bacteria to better survive stress situations by hiding inside host cells or by biofilm formation. Although the six genes are involved in completely different cellular processes, their deletion seems to mimic at least in parts the effects seen after bile acid exposure, which might be explained by the activation of a common genetic program that prepares the organism to stress situations.

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ABBREVIATIONS

AI-2 – Autoinducer-2

CA – Cholic acid

Caco2 – Cancer coli-2, Human colon carcinoma

CDCA – Chenodeoxycholic acid

C-di-GMP – Bis-(3'-5') cyclic dimeric GMP

CFU – Colony forming unit

COS – Columbia blood agar

DCA – Deoxycholic acid

DGC – Diguanylate cyclase

DMEM – Dulbecco Minimal Essential Medium

EPS – Extracellular polymeric substances

FCS – Fetal Calf Serum

HBSS – Hank's Balanced Salt Solution

IBD – Inflammatory bowel disease

GBS – Guillain-Barré Syndrome

GCA – Glycocholic acid

GI – gastrointestinal

GPA – Gentamycin protection assay

LB – Luria-Bertani

LCA – Lithocholic acid

LPS - Lipopolysaccharides

MCP – Accepting chemotaxis proteins

MFS – Miller Fish Syndrome

MH – Mueller Hinton

MOI – Multiplicity of infection

NEA – Non essential amino acid

OD – Optical density

PBS – Phosphate-buffered saline

PDE – Phosphodiesterase

PCR – Polymerase chain reaction

QS – Quorum sensing

RR – Response regulator

SSR – Simple sequence repeats

TCA – Taurocholic acid

TCS – Two-component regulator system

Tlp – Transducer-like protein

TTC – 2,3,5-Triphenyltetrazolium chloride

UDCA – Ursodeoxycholic acid

WHO – World health organization

WT – Wild type

1. Introduction

1.1 Overview

Foodborne diseases are infections of the gastrointestinal (GI) tract usually caused by food or beverages containing pathogenic microorganisms or chemicals (Elgamoudi 2016). It is considered an important health issue and its economic and social impact remains unknown. Jones et al. (2008) estimate that in the last 60 years 30% of all infections were foodborne. The World Health Organization (WHO 2018) estimates annually an incidence of 4.5 billion cases of human diarrheal disease, of which 1.8 million are fatal.

Campylobacter has emerged in latest years as the principal cause of foodborne diarrheal disease in humans worldwide (Nguyen et al. 2012). Annually, 20 to 150 cases of Campylobacteriosis per 100.000 individuals have been reported in developed countries, which is above the reported levels for other foodborne pathogens such as *E. coli* and *Salmonella* sp. (Olson et al. 2008).

In the past years, the incidence of cases of *Campylobacter* infections have increased in industrialized countries. In developing countries (considered as endemic regions) epidemiological data are still incomplete.

The transmission route of *Campylobacter* is assumed to be foodborne via undercooked meat (mainly poultry and turkey), raw or unpasteurized milk and its products, but also through contaminated water and ice (EFSA 2017; WHO 2018).

Symptoms of Campylobacteriosis are watery to bloody diarrhea, with fever, nausea, vomiting and abdominal pain. It can be fatal to vulnerable individuals (Trigui et al. 2017). Of the 26 species of *Campylobacter* described, *C. jejuni* and *C. coli* cause more than 90% of all human Campylobacteriosis cases (Dasti et al. 2010).

1.2 Campylobacter characteristics

Campylobacter is a Gram-negative bacterium with spiral, curved or rod-shaped format. *Campylobacter* is capnophilic and an obligate microaerophilic (microaerobic) organism, surviving and growing best in an environment characterized by a low oxygen concentration of 5% O_2, 10% CO_2 and 85% N_2. However, there are four species (*C. gracilis*, *C. hyointestinalis*, *C. showae*, and *C. sputorum* bv. *faecalis*) that grow under anaerobic conditions (Lastovica, On, and Zhang 2014). The tolerance for oxygen (3 – 5%) can differ between species. Most *Campylobacter* strains do not grow in presence of normal atmospheric conditions, however there are a few strains that may grow under oxygen rich conditions. *Campylobacter jejuni*, for instance, has the ability to adapt to aerobic environments due to its capacity to form biofilms.

A typical *Campylobacter* is non-fermentative, catalase-negative and oxidase-positive (Lawson et al. 2001). Numerous studies have demonstrated that *C. jejuni* is sensitive to acids such as formic, acetic, ascorbic and lactic acids (Chaveerach et al. 2002).

Table 1. Phenotypic characteristics of *C. jejuni*. Adapted from Levin (2007).

Gram negative	Nitrate reduced to nitrite (+)
Growth at 42°C	Nitrite reduced (-)
Microaerophilic	DNAse production +
Catalase production (+)	Cephalothin resistant
Urease production (-)	Nalidixic acid sensitive
Hippurate utilization (+)	Cytochrome oxidase positive
Sensitive to nalidixic acid	No growth below 30°C
Carbohydrates not utilized	No growth with 3.5% NaCl
Alk. phosphatase production (+)	Reduction of triphenyltetrazolium chloride
Citrate utilization (+)	H_2S production (-)
Succinate utilization (+)	Indoxyl acetate utilization (+)

Campylobacter belongs to the Family Campylobacteraceae, in the Order Campylobacterales, Class Epsilonproteobacteria and Phylum Proteobacteria. There are currently 26 recognized species, with 9 subspecies (Fitzgerald 2015; Table 2).

Table 2. Currently described *Campylobacter* species (Fitzgerald 2015).

***Campylobacter* Species**	**Known Sources**	**Human Disease Associated**
C. jejuni* subsp. *jejuni	Poultry, cattle, sheep, wild birds, pigs	Gastroenteritis, meningitis, septicemia, Guillain-Barre syndrome
C. jejuni* subsp. *doylei	Humans	Gastroenteritis, septicemia
C. coli	Pigs, poultry, sheep, wild birds, cattle	Gastroenteritis, septicemia, meningitis
C. lari* subsp. *lari	Wild birds, poultry, dogs, cats	Gastroenteritis, septicemia
C. lari* subsp. *concheus	Shellfish	Gastroenteritis
C. fetus* subsp. *fetus	Cattle, sheep, reptiles	Gastroenteritis, septicemia
C. fetus* subsp. *venerealis	Cattle, sheep	Septicemia
C. fetus* subsp. *testudium	Reptiles	Gastroenteritis, cellulitis
C. upsaliensis	Dogs, cats	Gastroenteritis, septicemia
C. helveticus	Cats, dogs	Gastroenteritis
C. insulaenigrae	Marine mammals	Gastroenteritis
C. peloridis	Shellfish	Gastroenteritis
C. hyointestinalis* subsp. *hyointestinalis	Pigs, cattle	Gastroenteritis
C. hyointestinalis* subsp. *lawsonii	Pigs	None at present
C. lanienae	Cattle, pigs	Gastroenteritis
C. sputorum* bv *sputorum	Cattle, pigs	Abscesses, gastroenteritis
C. sputorum* bv *faecalis	Sheep, bulls	None at present
C. sputorum* bv *paraureolyticus	Cattle	Gastroenteritis
C. concisus	Humans, domestic pets	Gastroenteritis, periodontal disease, abscesses
C. curvus	Humans	Periodontal disease, gastroenteritis
C. rectus	Humans	Periodontal disease, abscesses
C. showae	Humans	Periodontal disease, abscesses
C. ureolyticus	Humans	Gastroenteritis, septicemia, soft tissue abscesses

C. gracilis	Humans	Periodontal disease, abscesses
C. hominis	Humans	None at present
C. mucosalis	Pigs	None at present
C. avium	Poultry	None at present
C. canadensis	Whooping cranes	None at present
C. cuniculorum	Rabbits	None at present
C. subantarticus	Gray-headed albatrosses, black-browed albatrosses, gentoo penguins	None at present
C. volucris	Black-headed gulls	None at present
C. corcagiensis	Lion-tailed macaques	None at present
C. iguaniorum	Reptiles	None at present

Campylobacter species are approximately 0.2 to 0.8 by 0.5 to 5 μm in size, non-spore-forming and usually motile. Depending on the species, a single polar flagellum, a bipolar flagella (Figure 1) or no flagellum is present (Kaakoush et al. 2015). *Campylobacter gracilis*, for instance, is non-motile, while *Campylobacter showae* possesses multiple flagella (Facciolà et al. 2017).

Its spiral shape morphology seems to be an important adaptation that allows *Campylobacter* species to swim through viscous environments such as the mucus in the intestinal epithelia of the host (Ferrero and Lee 1988). Under unfavorable conditions such as normal oxygen conditions (~20% O_2), low nutrient availability, temperature or stationary phase, *Campylobacter jejuni* is observed to change its morphology to a coccoid form. This coccoid shape has been suggested to be a viable non-culturable form of *Campylobacter jejuni*. (Levin 2007; Oh, McMullen, and Jeon 2015).

The growth and survival of *Campylobacter* depends on different factors such as oxygen concentration, temperature, pH and availability of water. *Campylobacter* is able to grow at pH 6.5 to 7.5 and its optimal growth temperature is between 30°C to 42°C. *Campylobacter* does not multiply at temperatures under 30°C, but can survive for more than 80 days at 4°C in water (Trigui et al. 2015) or up to 7 months in food stored at 4°C (Lázaro et al. 1999).

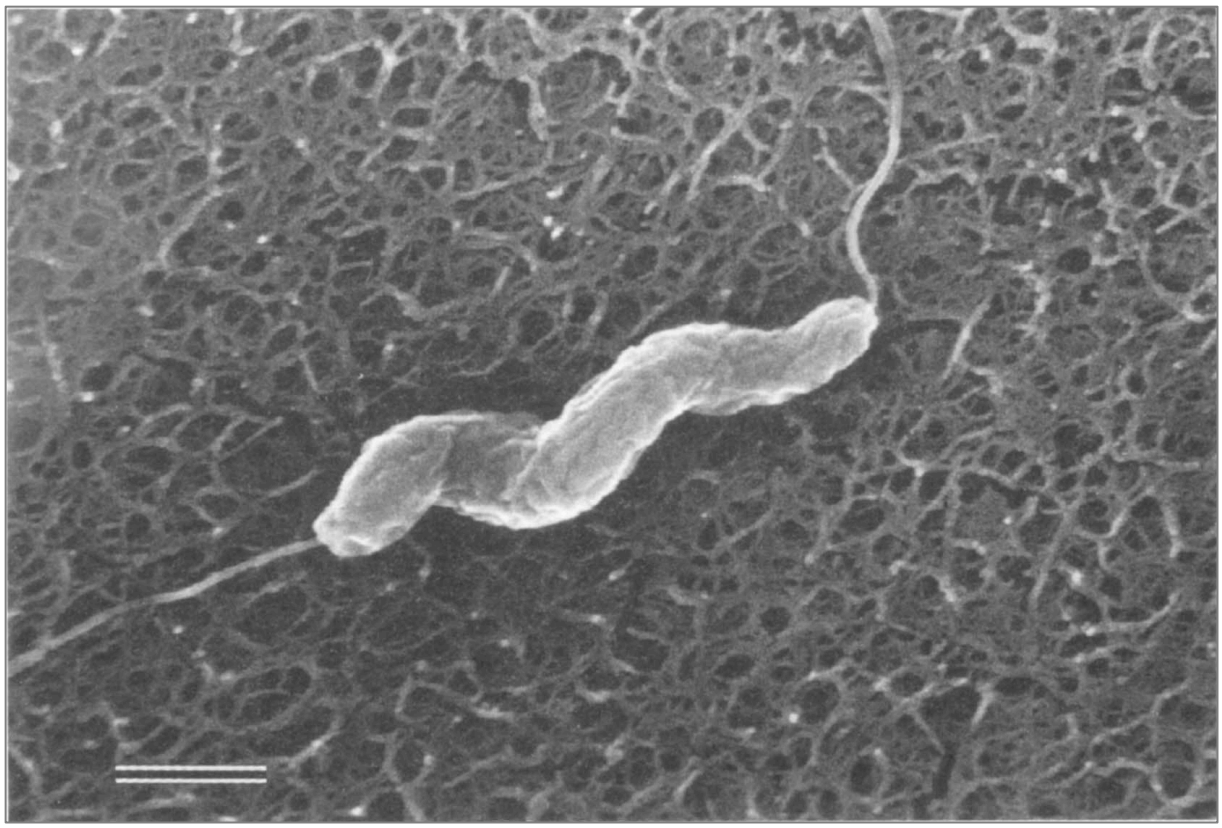

Figure 1. Scanning electron micrograph of *Campylobacter jejuni* with the flagella in both ends. Bar = 500nm. Figure reproduced from Shigematsu et al. (1998).

The size of the *C. jejuni* genome is ~1.6 megabases with hypervariable regions. *C. jejuni* is naturally competent, leading to recombination among strains, which permits the generation of even more diversity (Young, Davis, and DiRita 2007). Also, the lack of DNA-repair genes can partially explain the presence of hypervariable sequences in *C. jejuni*. Simple sequence repeats (SSR) in the *Campylobacter* genome offer rapid adaptation to different environments and provide advantage during colonization of intestinal cells by avoiding the host immune system (Parkhill et al. 2000; Bayliss et al. 2012).

1.3 *Campylobacter* in a historical view

In 1886, Theodore Escherich described for the first time a motile spiral organism isolated from infants' stool. At the time, he called it "cholera infantum". He published articles in the Weekly Munich Medical Review describing the spiral-shaped bacteria

found in 35 children suffering from intestinal disease. From 1906 to 1949, *Campylobacter* was isolated from fetal tissues, cattle, pigs and human blood and remained classified as a Vibrio-like bacterium (Butzler 2004; Skirrow 2006). However, in 1963 based on the microaerophilic growth requirements and the nonfermentative metabolism, and in order to distinguish it from *Vibrio* spp, the name *Vibrio fetus* was changed to "*Campylobacter fetus*" by Sebald and Véron, forming the type species of this genus (Olson et al. 2008; Kaakoush et al. 2015; S.L.W. On 2001). The name *Campylobacter* originates from the Greek words for curved (Campylo) rods (bacter).

Since *Campylobacter fetus* was already recognized with a pathogenic role in abortion and infectious infertility in animals, in the 70's a study by Butzler (1973) increased the attention on *Campylobacter* of both clinicians and veterinarians. Butzler demonstrated their high prevalence in human diarrhea using a filtration technique to isolate *C. jejuni* from human diarrheal stool (Butzler 2004). In 1977, Skirrow proposed a simpler method of culturing *Campylobacter* by adding the faeces directly onto blood agar containing polymyxin, trimethoprim and vancomycin (Skirrow 1977). Further studies improved the understanding in growth characteristics and isolation methods, and as a consequence, 12 new species or subspecies were described in a variety of different diseases and reservoirs from 1974 to 1988 (Vandamme and Goossens 1992).

Later, in 2000, Parkhill et al. published the first genome sequence of *Campylobacter jejuni* (NCTC11168) and described its circular chromosome of 1,641,481 base pairs with a low G+C of 30%. It was predicted to encode 1,654 proteins and 54 stable RNA species. An important finding was the hypervariable regions that might be essential for survival of the organism in the host and environment (Parkhill et al. 2000). Intragenomic mechanisms as well as genetic exchange between strains account for this large genetic variation (Boer et al. 2002).

1.4 *Campylobacter* infection

Campylobacteriosis is considered the most frequent reported bacterial infectious disease in the European Union (EU) since 2005 (Kaakoush et al. 2015; EFSA 2017) and represents almost 70% of all reported cases of zoonoses (Figure 2).

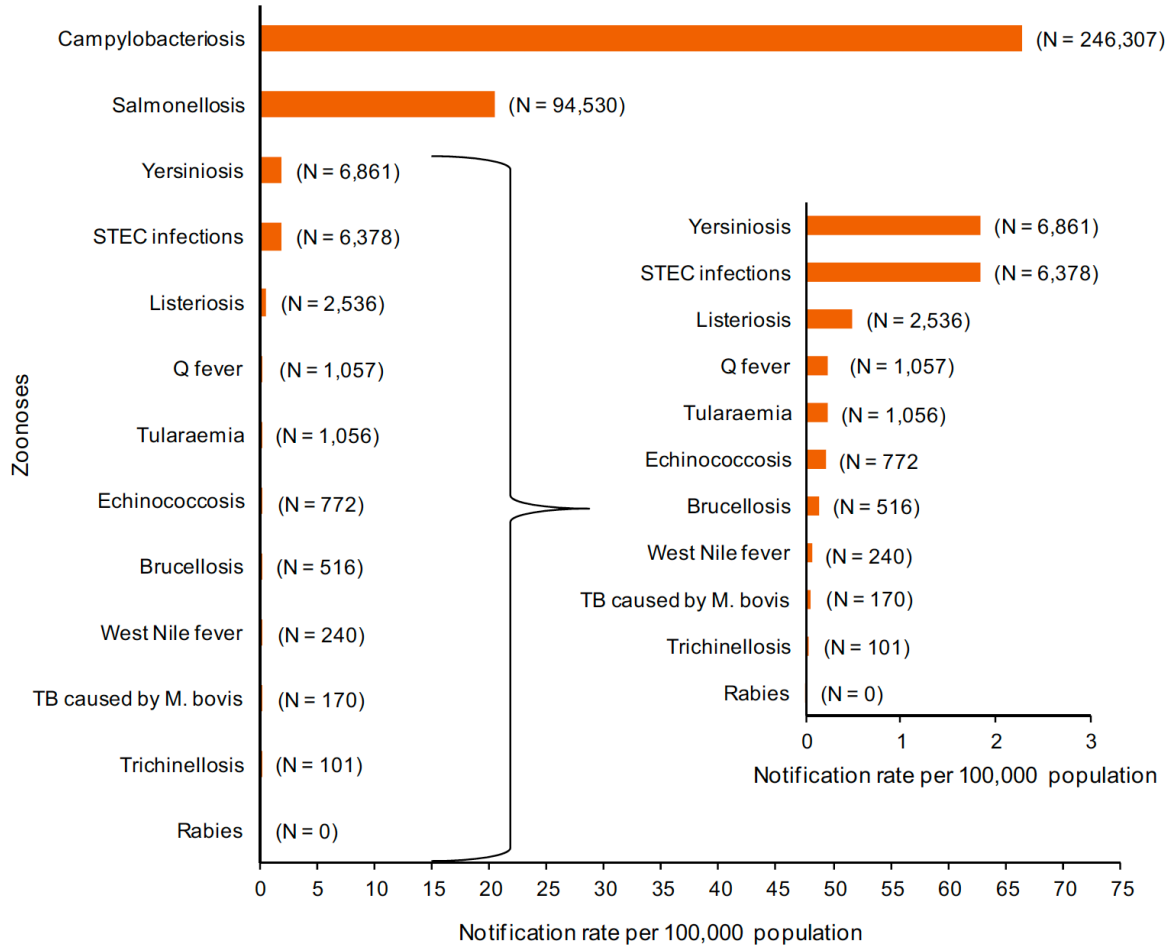

Figure 2. Reported numbers and notification rates of human zoonoses in Europe, 2016. Adapted from EFSA, 2017.

In humans, mainly two species are known to cause disease. *C. jejuni* and *C. coli* account for around 90% of all human infections (Dasti et al. 2010). Campylobacteriosis is characterized by the colonization of the small intestine and the infection can variate from asymptomatic to severe enteritis (Trigui et al. 2015).

1.4.1 Clinical manifestations of Campylobacteriosis and complications

Campylobacter infection usually is accompanied by acute abdominal pain (which can be so intense that it mimics acute appendicitis), nausea, high fever and general malaise (Blaser and Engberg 2008). Acute infection with *Campylobacter* manifests as a severe inflammatory diarrheal disease. The first symptoms start to appear within 2 to 3 days after the exposure and are gradually followed by mild or severe diarrhea. Symptoms can last up to 2 weeks (Young, Davis, and DiRita 2007). The disease is self-limiting, and the illness resolves gradually over a week without specific treatment (Blaser and Engberg 2008). However, in very serious cases, treatment with erythromycin and ciprofloxacin is recommended for adults and treatment with only erythromycin for children (Eiland and Jenkins 2008). Death in association with Campylobacteriosis is very rare, but cases in immunocompromised patients and very young children can be fatal (WHO 2018).

Campylobacter can also cause post-infection complications such as (i) Guillain-Barré Syndrome (GBS) and Miller Fish Syndrome (MFS), (ii) Reactive arthritis and (iii) Inflammatory bowel disease.

Guillain Barré syndrome is an autoimmune disease in which the immune system attacks the nervous system and it can lead to nerve inflammation causing muscle weakness and paralysis. *Campylobacter jejuni* is considered the most common pathogenic factor that triggers GBS (Ang et al. 2001). Some *C. jejuni* strains produce different surface lipopolysaccharides (LPS) that mimic the gangliosides that are present in human peripheral nerves and may act as an antigenic factor that induces GBS (Aspinall et al. 1994; Islam, Abraham, and Moran 2012). Miller Fisher syndrome (MFS) is considered a rare variant of GBS, differing in the nerve groups that are firstly affected by paralysis. In MFS, the patients are first affected in the head, while paralysis in the other forms of GBS typically start in the legs (Kozminski 2008).

Reactive arthritis is characterized by painful joints as a result from immune associated inflammation after *C. jejuni* infection (Colosimo et al. 2015). Inflammatory bowel disease (IBD) is characterized by gut inflammation that is also triggered by previous infection of *C. jejuni* or other bacteria species and viruses (Kalischuk and Buret 2010). *Campylobacter* is also found with a high prevalence in patients with Crohn's disease, a type of IBD (Mann and Saeed 2012).

1.4.2 Epidemiology

The genus *Campylobacter* includes 26 species (Table 2). Among them, the most prevalent in human infections are *C. jejuni* and *C. coli.* Other species are considered "emerging" such as *C. concisus*, *C. upsaliensis*, *C. ureolyticus*, *C. hyointestinalis* and *C. sputorum*, which have been associated with human (gastroenteritis and periodontitis) and animal infections (Liu et al. 2018).

In the past decade the incidence of Campylobacteriosis was rising worldwide. The number of cases increased in USA, Europe and Australia. *Campylobacter* is considered the most common cause of bacterial gastroenteritis worldwide. In USA, it is estimated to cause 1.3 million illnesses, 13,240 hospitalizations and 119 deaths each year (Scallan et al. 2011).

In developing countries, Campylobacteriosis is considered endemic, and asymptomatic infections are more common than in industrialized countries (Havelaar et al. 2009).

According to the European Food Safety Authority (EFSA 2017), since 2005 *Campylobacter* was the most reported gastrointestinal pathogen in the EU. In 2016, the European countries with highest notification rates were Czech Republic (228.2 cases per 100,000), Slovakia (140.5) and Sweden (111.9) (EFSA 2017). The lowest rates were reported by Bulgaria, Cyprus and Latvia ($\leq$ 4.6 per 100,000). In Germany the annual incidences of Campylobacteriosis between 2005 and 2011 ranged from 53.4 to 81.4 cases per 100,000 persons (Figure 3) (Kaakoush et al. 2015).

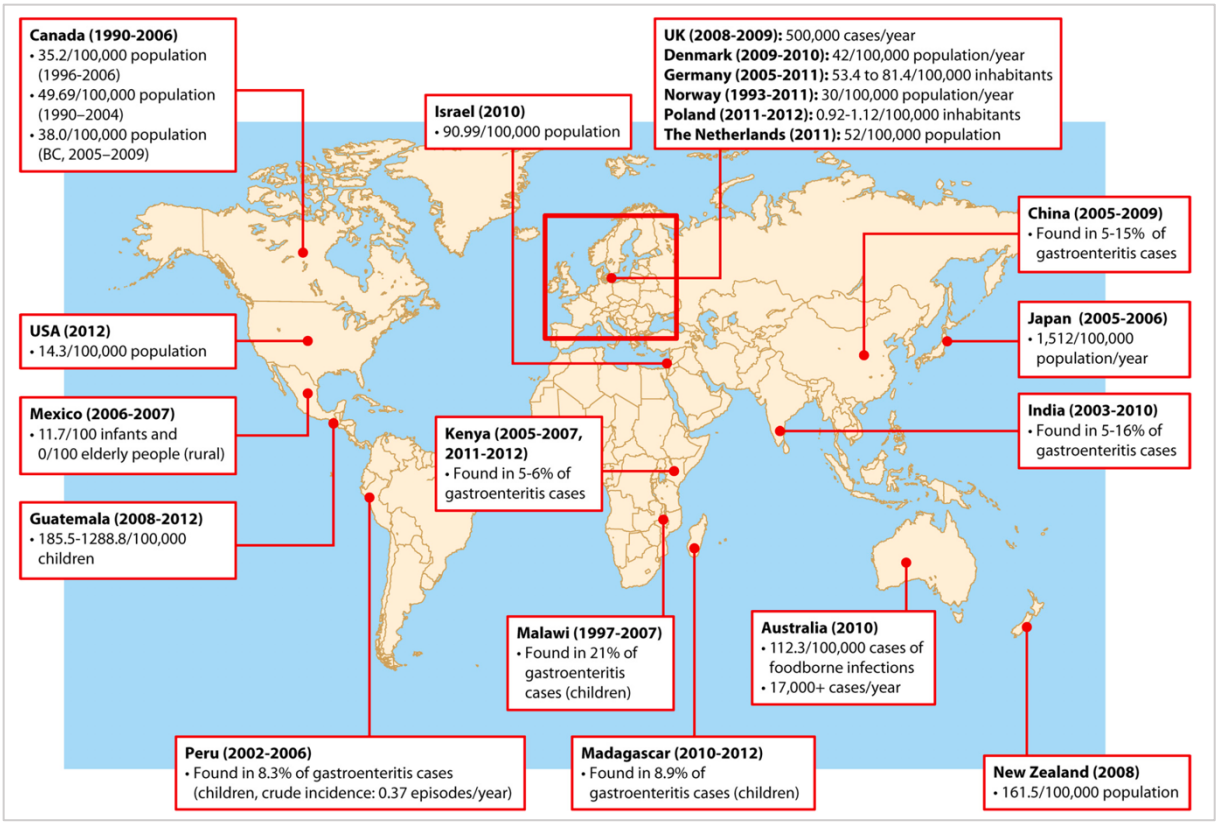

Figure 3. Incidence and prevalence of Campylobacteriosis (*C. jejuni* and *C. coli*). Data of epidemiological information from United Kingdom, Denmark, Germany, Norway, Poland, The Netherlands, Israel, China, Japan, India, Australia, New Zealand, Madagascar, Malawi, Kenya, Guatemala, Peru, Mexico, USA and Canada. Image reproduced from Kaakoush et al. (2015).

In 2013, Nielsen et al. indicated that infections with *C. jejuni* and *C. coli* usually occur in all ages, however, they are more prevalent in young children (1 to 4 years old) and in young adults (15 to 24 years old) than in other age groups (Kaakoush et al. 2015). Nevertheless, the frequency of cases among people of 60 years and older seems to be increasing (Fitzgerald 2015).

In general, *Campylobacter* infections are domestically acquired, although *Campylobacter* is also a main cause of travel-related diarrhea. According to EFSA (2017), in most European countries, more than 90% of cases were considered to be of domestic origin. Despite of that, the Nordic countries presented the highest travel-related cases – Finland (65.4%), Iceland (51.4%) and Norway (53.5%) (EFSA 2017).

In Europe and northern countries, the Campylobacteriosis cases are increased during the summer months. A sharp peak of infection with *C. jejuni* and *C. coli* is present in the summer months, and since 2011 a small annual peak during winter (January) is also observed (EFSA 2017). Epidemiology of *Campylobacter* infections in tropical and temperate countries is very different (Lastovica, On, and Zhang 2014). In tropical countries outbreaks are uncommon, although in temperate countries they are more frequent. Infections occur during the year (no seasonality) and affect mainly very young children in tropical countries. In those countries, repetitive infections may result in acquired immunity, what explains the asymptomatic infections observed in adults in developing countries (Glass et al. 1983). Nonetheless, in temperate countries such as USA and European countries, infection is observed in both adults and children, mostly during the summer months and is usually symptomatic (Glass et al. 1983; Lastovica, On, and Zhang 2014).

The actual number of cases is believed to be 9 million every year in Europe. The costs of the disease to the public health systems and the lost productivity in the EU is estimated to be around €2.4 billion a year (EFSA 2019) and in USA $1.3 billion. Several countries are investing in developing strategies to control *Campylobacter* dissemination.

1.4.3 Reservoirs and transmission of *Campylobacter*

Campylobacter species can be found in many different environmental niches such as soil, water sources, manure and are mainly found as commensals, colonizing many warm-blooded animals. In those animals, *Campylobacter* does not cause any symptoms and is disseminated via their feces (Labbé and García 2013).

Campylobacter is prevalent in animals such as poultry, turkey, cattle, sheep, pigs and is also found in their food products (e.g. dairy products and meat). Moreover, it is also present in pets such as dogs and cats (Acke 2018), wild birds and it was also found in shellfish and reptiles (Fitzgerald 2015; WHO 2018).

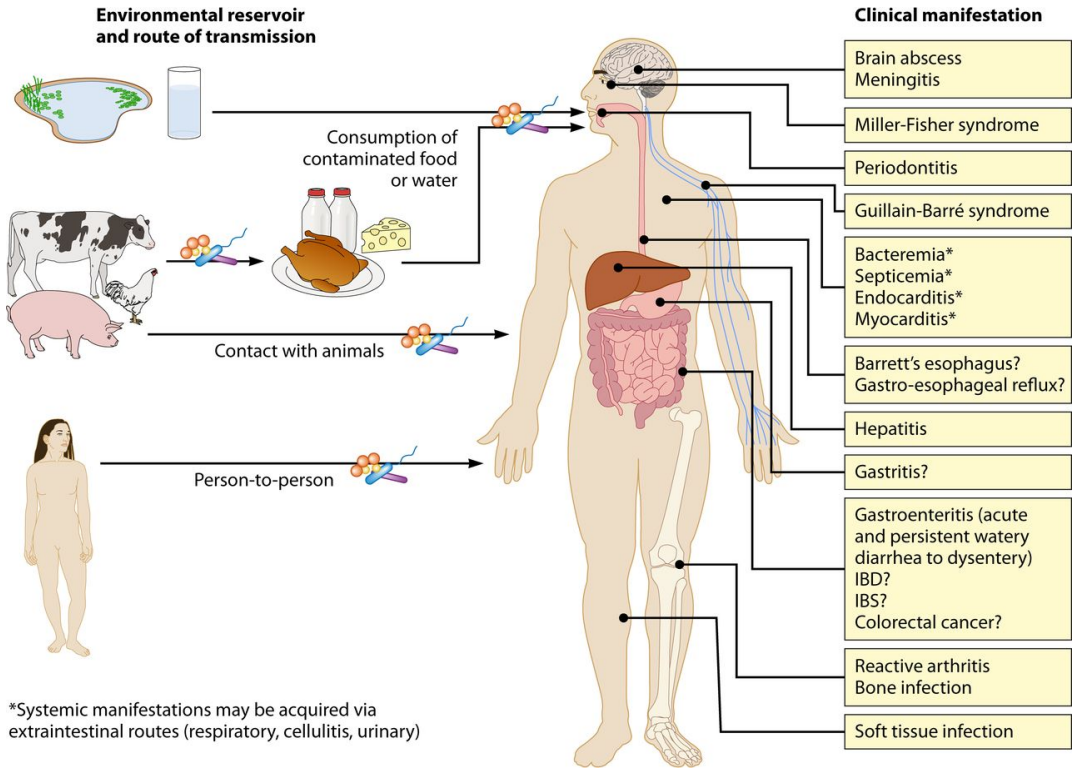

Figure 4. Environmental reservoirs, routes of transmission and clinical manifestation of *Campylobacter* species. Mainly, Campylobacteriosis can be transmitted by the consumption of untreated water, contaminated animal food and by person-to-person. Abbreviations: IBD, inflammatory bowel diseases; IBS, irritable bowel syndrome. Question marks indicate conditions for which a role for *Campylobacter* is implicated but not certain. Image reproduced from Kaakoush et al. (2015).

The warm-blooded farm animals are considered the major reservoir of *Campylobacter*, and chickens are the main source of infection in humans (Figure 4). *C. jejuni* is the most predominant infectious agent in poultry and about 10^8 colony forming units (CFU) of *C. jejuni* can be found per gram of the cecum content of chickens (Rosenquist et al. 2006).

In humans, the transmission of *Campylobacter* occurs by consumption of contaminated meat and unpasteurized milk, handling of raw meat, consumption of non-treated water and direct contact with contaminated animals (Kaakoush et al. 2015; EFSA 2017). Even though direct infection from person-to-person may occur, this transmission route has no epidemiological relevance.

To avoid *Campylobacter* dissemination, preventive measures should be taken, such as improvement of strategies to reduce cross-contamination during the food processing and slaughter, and treatment of processed food by pasteurization or high hydrostatic pressure. Another important strategy is by public education of food handling and cooking, and awareness of possible contamination and persistence of *Campylobacter* in the kitchen surfaces (Humphrey et al. 2001).

1.4.4 Treatment and antibiotic resistance

Campylobacter infections are normally self-limited, and the treatment involves rehydration to replace the electrolytes and fluids lost as a result of the diarrhea and/or vomiting (Mackenzie and Barnes 1988). Usually an antibiotic treatment is not required, but in severe cases or when the patient is immunocompromised or pregnant, drugs of choice include macrolides (e.g. erythromycin) and fluoroquinolones (e.g. ciprofloxacin) (Mamelli et al. 2003).

Antimicrobial resistance among pathogens is a global threat and *Campylobacter* are not an exception. In the past decades, a rising number of resistant *Campylobacter* isolates have developed resistance to macrolides and fluoroquinolones, but also to aminoglycosides and beta-lactams (Wieczorek and Osek 2013). The over use of antibiotics in the human population and use of antimicrobials in veterinary medicine and animal production can be correlated to the increasing number of resistant isolates of *Campylobacter* (Iovine 2013). As an alternative treatment, gentamicin and third-generation cephalosporins can be used (Aarestrup and Engberg 2001). However, *C. jejuni* and *C. coli* are nearly all resistant to penicillin, cephalosporins, rifampicin, vancomycin, sulfamethoxazole and trimethoprim (Silva et al. 2011).

1.5 Pathogenesis and virulence factors associated with *C. jejuni*

To initiate an infection, *C. jejuni* must first circumvent the host barriers in the GI tract (mechanical or immunological), besides the bile acid action. The curved morphology and high motility allow *C. jejuni* to pass across the mucus layer of the GI tract, which is the first line of defense (Jowiya 2013). The minimum infective dose of *C. jejuni* is considered low, approximately 500 CFU (Black et al. 1988), when compared to another Gram-negative enteric pathogen, *Vibrio cholerae* which requires between 10^3 to 10^8 cells for effective infection in humans. Once this first barrier has been penetrated, *Campylobacter* is able to interact with epithelial cells using adherence mechanisms and can proceed with the infection (Jowiya 2013).

The molecular pathogenesis and virulence of *Campylobacter* infections are still not well understood (Elgamoudi 2016). Virulence mechanisms of *C. jejuni* are up-regulated during the colonization in the intestine of the host (Tu, McGuckin, and Mendz 2008; Hermans et al. 2011). Sodium deoxycholate (DCA) stimulates the production of *Campylobacter* invasion antigens, the Cia proteins (Malik-Kale, Parker, and Konkel 2008). *Campylobacter* infection seems to vary depending on several factors such as genetics of the bacteria, infection dose and the gut microbiome composition (Dicksved et al. 2014; Kampmann et al. 2016).

The mice model can be a suitable colonization model; however, the colonization is atypical. It is believed that the sporadic colonization in mice is due to the resistance provided by the commensal microbiome of the mice (Bereswill et al. 2011). To overcome this problem, Bereswill et al. (2011) proposed a novel *C. jejuni* infection model with gnotobiotic mice previously treated with antibiotics to eradicate the original intestinal flora and replacing it with human gut flora. This model can be used to have a better idea of the pathogenesis of *Campylobacter* and the impact of gut flora and the host immune status.

The main factors associated with the pathogenicity of *Campylobacter* are motility and flagella-mediated protein secretion, adhesion to and invasion of host cells, chemotaxis,

capsule formation, secretion of toxin, and biofilm formation (Bolton 2015; Stephen L. W. On 2013). The role of the most considerably studied pathogenicity factors of *Campylobacter jejuni* will be considered below.

1.5.1 Flagellum

Campylobacter is a motile bacterium whose motility is enabled by its polar or bipolar unsheathed flagella. Flagella are long filaments that can be up to 20 μm long and can rotate at speeds in excess of 15,000 rpm (Rossez et al. 2015). *C. jejuni*'s motility enables the bacteria to penetrate the mucus layer in the intestinal epithelium and colonize the host. The flagellum allows the bacteria to move quickly (up to 75 μm/s) in a viscous environment and it is estimated to have a torque of 3600pN/nm, which is more than twice compared to *Salmonella* cells (Szymanski et al. 1995; Lertsethtakarn, Ottemann, and Hendrixson 2011; Beeby et al. 2016).

The flagella are required not only for the motility, but also play an important role in chemotaxis, invasion, autoagglutination, colonization and biofilm formation (Guerry 2007). Flagella are also essential in the secretion of flagellar proteins and *Campylobacter* invasion antigens (Cia) through its type III secretion system-like in the base of the flagellar structure (Michael E. Konkel et al. 2004).

The *C. jejuni* flagella are composed of proteins encoded by two genes *flaA* and *flaB*, that possess similar sequences (Alm, Guerry, and Trust 1993). Mutations in the flagellin *flaA* gene result in a very short non-functional flagellum and leads to loss in colonization capacity (Wassenaar, Bleumink-Pluym, and van der Zeijst 1991). FlaB is involved in the flagella export apparatus and flagella assembly. FlaB deficient *C. jejuni* are non-motile (Matz et al. 2002)

To create a functional flagellum, *C. jejuni* needs to regulate the transcription of *flaA* and *flaB* genes using two alternative sigma factors, σ^{28} (FliA) and σ^{54} (RpoN), respectively. In *C. jejuni,* these two alternative sigma factors regulate the expression of the flagellar genes that encode components of the flagellar organelle (Hendrixson,

Akerley, and DiRita 2001a). The σ^{28} is required in the transcription of a small subset of genes, including the expression of *flaA*, which encode the major flagellin and other filament genes (Hendrixson and DiRita 2003; Carrillo et al. 2004; Wösten, Wagenaar, and Putten 2004). σ^{54} is required for transcription of genes encoding the flagellar rod, basal body and hook components, and also a minor flagellin (*flaB*) (Jagannathan, Constantinidou, and Penn 2001; Hendrixson, Akerley, and DiRita 2001).

A functional flagellum enables the organism to swim in direction of a favorable environment, a process known as chemotaxis.

1.5.2 Chemotaxis

Chemotaxis is a mechanism by which motile bacteria either swim towards to a preferred environment (attractants) or and away from unfavorable environments (repellents). It plays an important role in commensal and pathogenic organisms (Young, Davis, and DiRita 2007). Chemotaxis has been involved in colonization and invasion of the host and is implicated in the virulence of pathogenic bacteria (Aihara et al. 2014; Bolton 2015). This process is important to the ability of *C. jejuni* to adapt to different environments and respond to them.

The typical chemotaxis requirements in *C. jejuni* are the chemoreceptors, chemosensory signal-transduction system and the flagellar apparatus (Zautner et al. 2012; Z. Li et al. 2014). *C. jejuni* is able to change directions depending on the presence of extracellular signals, such as sugars, amino acids or bile acids, by clockwise and counter-clockwise flagellar rotation. These extracellular signals are sensed by chemoreceptors named methyl-accepting chemotaxis proteins (MCP) or also termed transducer-like protein – Tlps (Z. Li et al. 2014). Mutations in Tlps such as *docB* and *docC* reduced the capacity of colonization in the chicken gut (Hermans et al. 2011). Chemotaxis in *C. jejuni* is controlled by a complex interplay of 13, or more, different chemoreceptors (Tlps) and two aerotaxis genes - Aer (Lübke et al. 2018). The chemoreceptors are divided into three subtypes: A, B and C (Table 3).

Table 3. Classification of *C. jejuni* Tlp-chemoreceptor (Zautner et al. 2012; Mund et al. 2016; Korolik 2019).

Group types	Tlp name	Name	Encoded gene
Group A	Tlp1	CcaA	cj1506
	Tlp2		cj0144
	Tlp3	CcmL	cj1564
	Tlp4	docC	cj0262
	Tlp7 mc*		cjj81176-0975
	Tlp7 m*		cj0952c
	Tlp10	docB	cj0019
Group B	Tlp9	CetA	cj1190
		CetB/Aer2	cj1189
		Aer1	cj1191
Group C	Tlp5		cj0246
	Tlp6		cj0448
	Tlp7 c*		cj0951c
	Tlp8		cj1110

* Tlp7 mc: Membrane-associated and cytoplasmic domains (*C. jejuni* 81-176 and 81116)
Tlp7 m: Membrane-associated partial receptor (*C. jejuni* NCTC 11168 and B2)
Tlp7 c: cytoplasmic partial receptor (*C. jejuni* NCTC 11168 and B2).

The group A Tlps are integral membrane proteins and include Tlp1, Tlp2, Tlp3, Tlp4, Tlp7, Tlp10 and Tlp11. Tlp7 is also classified into group C. The signal domains of Tlp2, Tlp3 and Tlp4 are identical (Parkhill et al. 2000). The Tlp group B is only represented by Tlp9 (CetA) that mediates energy taxis which leads *C. jejuni* to high redox potentials and favorable conditions for energy production (Mund et al. 2016). Two cytoplasmic ligand-binding proteins, CetB (or Aer2) and CetC (or Aer1) are also classified in group B (Figure 5) (Zautner et al. 2012; Mund et al. 2016).

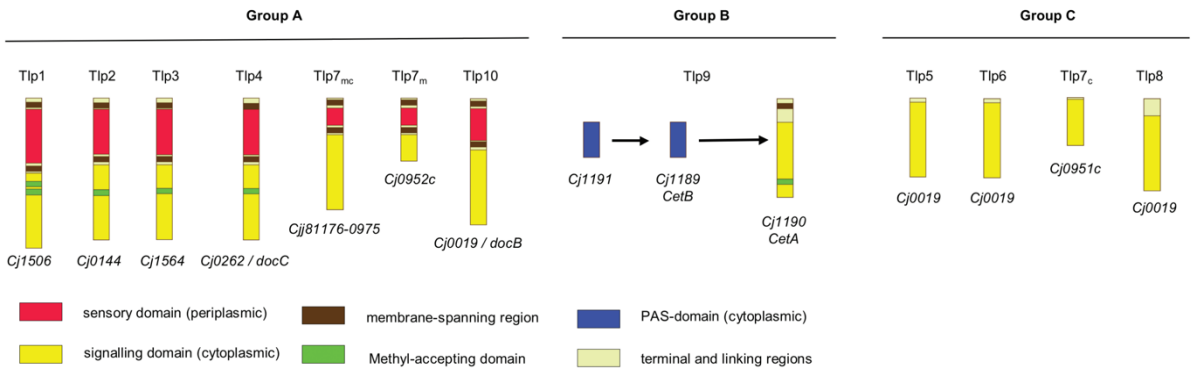

Figure 5. Domain organization of *C. jejuni* Tlp-chemoreceptor groups. Group A: receptors are anchored by membrane-spanning regions in the inner and also in the outer membrane, have a periplasmic sensory and a cytoplasmic signalling domain. Group B: represented by Tlp9 (CetA), anchored in the inner membrane, interacts with CetB triggering fumarate and pyruvate signals (Hendrixson, Akerley, and DiRita 2001). Group C: consist of a single cytoplasmic signaling domain. Figure reproduced from Zautner et al. (2012)

The group C includes Tlp5, Tlp6, Tlp7 and Tlp8, that are cytoplasmic proteins involved in detection of cytosolic signals (Lübke et al. 2018; Zautner et al. 2012). In a recent study from Lübke et al. (2018), a novel chemoreceptor gene, *tlp12* was described to encode Tlp12 chemoreceptor for glutamate and pyruvate recognition; and was present in 29.5% of the investigated *C. jejuni* strains.

1.5.3 Adhesion and Invasion

Once *Campylobacter* is ingested by the host, the organism passes through the GI tract, penetrates the mucus layer and finally colonizes its specific niche, the small intestine, by attaching or adhering to the epithelial cells. The interaction of *C. jejuni* and the host cells is a complex process involving bacterial cell surface structures and the host cell receptors (Rubinchik, Seddon, and Karlyshev 2012). Adhesion and subsequent invasion protect the organism from humoral immunity (Monteville, Yoon, and Konkel 2003).

Basically, bacteria such as *Salmonella* and *Escherichia coli* have two types of adherence structures: fimbriae or pili, and afimbrial adhesins. Unlike these organisms,

Campylobacter adhesion is not mediated by fimbriae or pili (Parkhill et al. 2000; Nougayrède, Fernandes, and Donnenberg 2003).

Tissue culture studies recognized proteins involved in *C. jejuni* adhesion to host cells such as periplasmic binding proteins Peb1 and Peb4 (Pei and Blaser 1993; Rathbun and Thompson 2009), *Campylobacter* adhesion to fibronectin, CadF (Michael E. Konkel et al. 2005), a second fibronectin-like binding protein, FlpA (Flanagan et al. 2009), a surface lipoprotein, JlpA (Jin et al. 2001), another lipoprotein, CapA (Ashgar et al. 2007), a major outer membrane protein, MOMP (Moser, Schroeder, and Salnikow 1997; Flanagan et al. 2009) and also LOS has been shown to have an important role in *C. jejuni* adhesion (Richards et al. 2013).

Once *Campylobacter* is able to adhere to the intestinal epithelial cells, its next step is invasion. Invasion is an important process in the internalization and translocation of the organism through the epithelial cell barrier of the small intestine and the main cause of enteritis (van Spreeuwel et al. 1985). *In vitro* studies of *Campylobacter* invasion into host cells have shown very low invasion rates compared to those found in clinical cases in humans (Friis et al. 2005). This might be because the high levels of oxygen, lacking mucus or other components in *in vitro* experiments influence the invasion process.

For a successful invasion in the host, *Campylobacter* secretes Cia proteins into the target cell by the type III secretion system-like present in the flagella. The Cia proteins were proven to be synthetized and secreted by *C. jejuni* upon co-culture with epithelial cells and are required for maximal cell invasion (Christensen, Pacheco, and Konkel 2009).

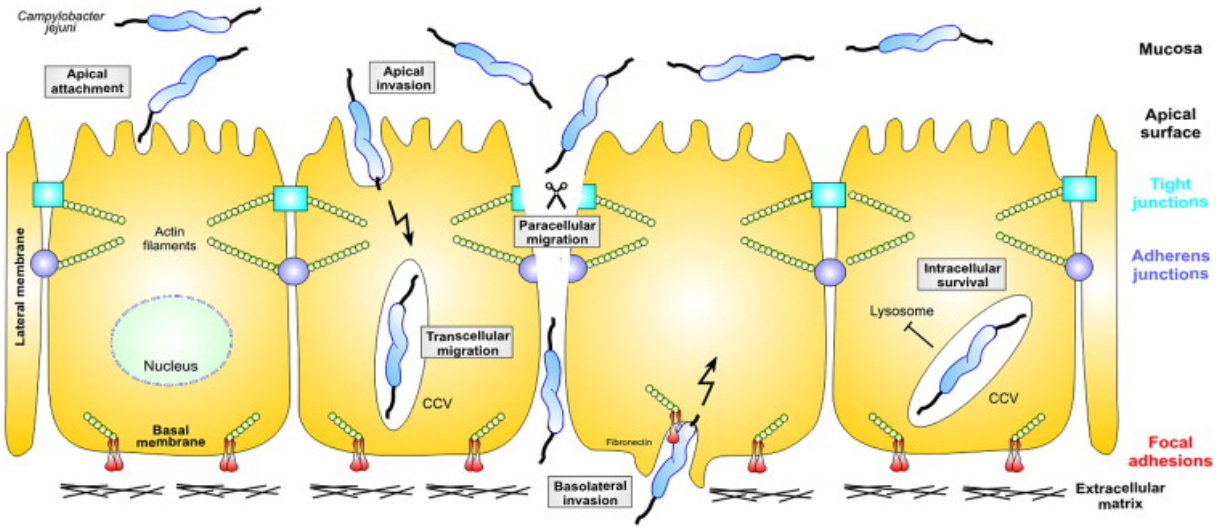

Figure 6. Hypothetical models of invasion mechanism in *C. jejuni*. Figure reproduced from (Backert and Hofreuter 2013)

Normally, healthy intestinal cells have apical basal polarity, junctional complexes and apical microvilli. *C. jejuni* can invade both polarized cells, such Caco-2 cells, and non-polarized cells, such INT407 cells (Russell and Blake 1994; Monteville, Yoon, and Konkel 2003). In order to gain access to submucosal tissues and to trigger tissue damage and finally cause intestinal diseases, bacteria have to cross the epithelial barrier of the intestine. Two main transmigration routes are described for *C. jejuni:* i) transcellular route and ii) paracellular route (Figure 6). In the transcellular route, the bacteria enter through the apical part of the epithelial cell and egress through the basal side. Bacteria that utilize the paracellular route cross the epithelial barrier over the tight and adherence junctions between the epithelial cells (M. E. Konkel et al. 1992; Bouwman, Niewold, and van Putten 2013). Once in the lamina propria, *Campylobacter* can reach different organs such as mesenteric lymph nodes, liver, spleen and vessels (Backert et al. 2013).

Other proposed invasion mechanisms for entry into host cells could be initiated by two classical signaling pathways: i) "zipper" and ii) "trigger" mechanisms (Ó Cróinín and Backert 2012). In the "zipper" mechanism, bacterial surface proteins (adhesins and invasins) bind to one or more specific host cell receptor and induce cytoskeleton and

membrane rearrangement followed by internalization, as reported for *Yersinia* or *Listeria* species (Ó Cróinín and Backert 2012). In the "trigger" mechanism the type III and type IV secretion systems of the bacterium inject effectors which mimic or hijack specific host cell factors to trigger bacterial internalization, as described in *Salmonella* and *Shigella* species (Ó Cróinín and Backert 2012).

In a recent review, Ó Cróinín and Backert (2012) present *C. jejuni* invasion by fibronectin/integrin interaction and also with the help of caveolae structures as the main invasion mechanisms. However, they do not disregard the possibility that *C. jejuni* may possess a novel entry mechanism that shares features of both "zipper" and "trigger" mechanisms, as already observed in a high resolution EM investigation (Boehm et al. 2011).

1.6 Biofilm formation

A biofilm is usually defined by a consortium of microorganisms (monospecies or multispecies) in which the cells stick to each other and often live on inert surfaces or interfaces (Kaakoush et al. 2015). These adhered cells become surrounded within a slimy and self-produced extracellular matrix that is composed of extracellular polymeric substances (EPS). The cells inside the biofilm generate the EPS constituents, that are usually a polymeric accumulation of polysaccharides (exopolysaccharides), proteins, glycoproteins, glycolipids, extracellular DNA (e-DNA) and humic substances (Flemming, Neu, and Wozniak 2007) as shown in Table 4. The biofilm plays a crucial role in bacterial survival in adverse environmental conditions, increase their antimicrobial resistance, offer protection against host defense mechanisms and serve as reservoirs for microbial contamination.

Table 4. Biofilm composition

Components	Percentage of matrix
Microbial cells	2 – 5%
DNA and RNA	< 1 – 2%
Polysaccharides	1 – 2%
Proteins	< 1 – 2% (includes enzymes)
Water	Up to 97%

Biofilms have been associated in a wide variety of microbial infections and are considered a significant problem for public health due to their resistance to antibiotics and their disease association with medical devices or other devices used in the health-care environment that are contaminated by biofilms (Jamal et al. 2015; Donlan 2001). Some characteristics of biofilms can be critical in the infection process such as: i) detachment of cells or aggregates may result in bloodstream or urinary tract infections, ii) cells may exchange resistance plasmids inside biofilms, iii) cells in biofilms present reduced susceptibility to antimicrobial agents, iv) biofilm-associated Gram-negative bacteria may produce endotoxins, and v) biofilms are resistant to host immune system clearance (Donlan 2002).

Biofilm formation is a complex process, in which the cells transform from planktonic to a sessile mode of growth in a dynamic process involving four stages: i) initial attachment to surface, ii) microcolony formation, iii) three dimensional structure formation and maturation and iv) detachment (Figure 7) (Jamal et al. 2015; Rendueles and Ghigo 2012).

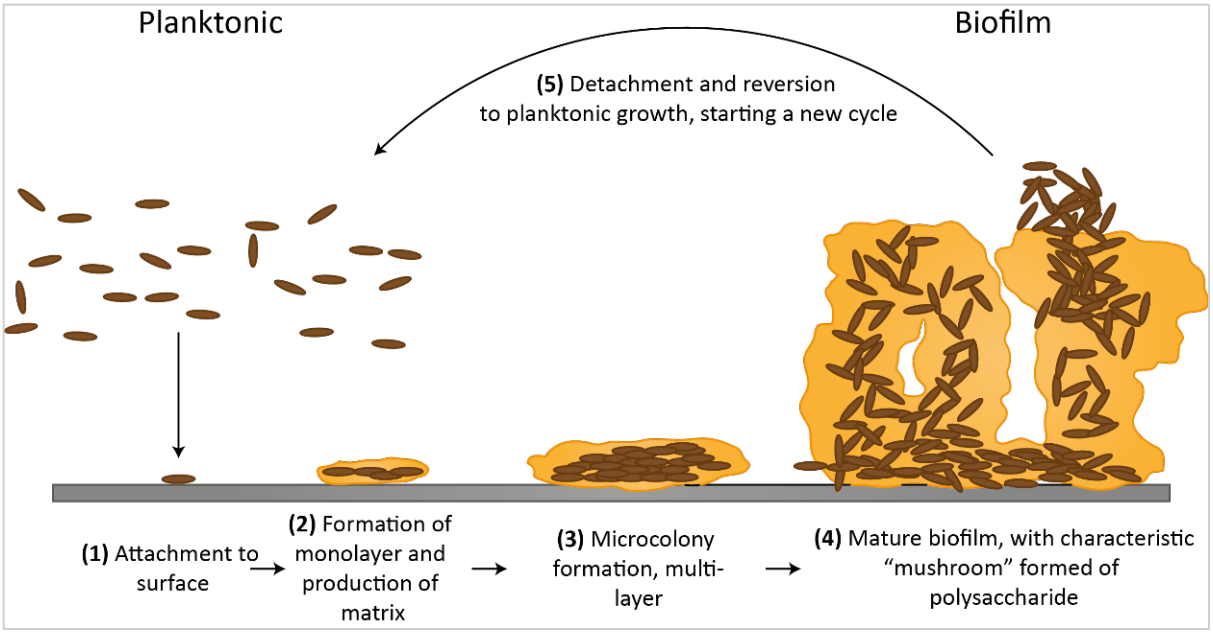

Figure 7. Representation of Biofilm formation. The biofilm formation starts with a reversible attachment of planktonic cells to the surface (1). In (2) the bacteria form a monolayer with an irreversible attachment by producing extracellular matrix. Then, multilayers are produced forming the microcolony (3), followed by later stages, when the biofilm is mature (4). This mature form is characteristic by its "mushroom" structures due to polysaccharides. Finally, in the mature biofilm can have some cells detached and dispersed in the environment (5). Figure adapted from (Vasudevan 2014).

1.6.1 Biofilm regulation

Biofilm formation is regulated by genetic and chemical signals from the environment. Current knowledge about biofilm regulation points to quorum sensing (QS), bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) and small RNA (sRNA) as the leading mechanisms of bacterial biofilm regulation, especially in Gram-negative species (Boyd and O'Toole 2012; Fazli et al. 2014).

Quorum sensing is a commonly conserved and important bacterial communication mechanism that regulates gene expression in response to fluctuations of self-generated signal molecules called autoinducers (Wolska et al. 2016). When the threshold stimulatory concentration of autoinducer is reached, a sharp alteration of gene expression occurs. Quorum sensing can regulate more than 10% of the total bacterial genome in *Pseudomonas aeruginosa* (Wagner et al. 2003). Functions such as biofilm

formation can be regulated by QS in the middle to late stages of the biofilm multilayer formation or dispersion, when the number of cells within the structure is high enough to sense the autoinducer.

Cyclic di-GMP is the second messenger of a signal transduction systems found in a variety of bacteria species. C-di-GMP binds to different cellular receptors and controls bacterial transcription, activity of enzymes and large structures (Hengge 2009). This second messenger plays an important role for the bacterial switch between been motile planktonic or in sedentary biofilm state (Hengge 2009).

C-di-GMP holds an important function in the three dimensional biofilm structure such as in the synthesis of exopolysaccharides, adhesins and adhesive pili, secretion of eDNA, and also controls the motility and cell death (Wolska et al. 2016). In general, c-di-GMP in high levels can reduce the expression and/or activity of flagella and stimulate the production of many adhesins and biofilm associated exopolysaccharides (EPS) (Hengge 2009).

In a recent review, Srivastava and Waters (2012) presented a direct correlation between QS and c-di-GMP. QS is important in sensing changes in bacterial population density and c-di-GMP is important in sensing environmental conditions. Both integrate external inputs to allow the bacteria to adapt and respond to different conditions. Srivastava and Waters (2012) proposed the integration of QS into a broader c-di-GMP signaling pathway.

Finally, the third biofilm regulation process makes use of small non-coding RNA. These have been proposed to participate in post-transcriptional regulation in bacteria, been involved in metabolic processes, pathogenesis and stress adaptation (Wolska et al. 2016). The sRNAs have their activity on various targets directly or indirectly linked to the biofilm formation. The sRNA can act as a core regulatory pathway, regulating the motility and the matrix production, and also influencing the biofilm formation and the outer membrane constitution (Van Puyvelde, Steenackers, and Vanderleyden 2013). Regulation through sRNA can occur by two mechanisms, (i) protein binding

and (ii) acting by base-pairing with other RNA (Chambers and Sauer 2013). In the protein-binding mechanism, the sRNA antagonize and sequester their related regulatory protein by mimicking several mRNA protein binding sequences (Chambers and Sauer 2013). The base-pairing mechanism occurs in cis or trans based on the base-pairing interactions and their location in the bacterial genome relative to their mRNA target (Chambers and Sauer 2013). sRNA shares extensive complementarity to their target (cis) and trans-encoded RNA shares limited complementarity in the base-pairing interaction (Chambers and Sauer 2013). The interaction between sRNA and their targets leads to changes in mRNA translation and stability, influencing the target gene expression (Chambers and Sauer 2013).

1.6.2 *Campylobacter* biofilms

Campylobacter is characterized by its low requirement of oxygen to survive. In general, *Campylobacter* does not grow in normal aerobic environment, but is still widespread in different environments. It has been proposed that *Campylobacter* survives and maintains itself in the environment with oxygen tension by forming biofilms (Joshua 2006). The biofilm allows *Campylobacter* to survive up to twice as long under normal atmospheric conditions (Asakura et al. 2007). In this mode of growth, the bacteria is protected from stressful environmental conditions such high level of oxygen, but also UV radiation, predation and desiccation (Reuter et al. 2010).

Campylobacter jejuni have been proposed to form different types of biofilm. Biofilm can form in aggregates attached to a surface, aggregates of bacteria floating in a liquid are commonly termed as flocs, and pellicles are aggregates of bacterial cells formed at the air-liquid interface (Joshua 2006). These three forms of biofilm formation resemble each other when observed by scanning electron microscopy (Joshua 2006).

The molecular regulation of *C. jejuni* biofilm formation is incompletely understood. Some genes are known to be implicated in the biofilm formation and includes genes that are responsible for motility (*flaA*) (Reuter et al. 2010), quorum sensing (*luxS*)

(Plummer 2012), cell adhesion (*cadF*), and genes involved in stress response (*cbrA*, *dnaJ*, *htrA* and *sodB*) (Oh and Jeon 2014). There is evidence that biofilm formation is flagella-mediated. Motile and flagellated strains present a higher level of biofilm formation compared to non-motile and non-flagellate *Campylobacter*. Indeed, *flaAB* mutation presented reduced biofilm formation in *C. jejuni* (Reeser et al. 2007). Similarly, strains defective in flagellar modification (cj1337) and assembly (fliS) adhere to glass surfaces poorly (Joshua 2006). A proteomic study revealed the role of the motility-associated proteins in biofilm formation, including FlaA, FlaB, FliD, FlgG, and FlgG2 (Kalmokoff et al. 2006).

C. jejuni possesses a quorum sensing related gene, luxS, that is involved in the interaction of cells, development and detachment of biofilms, but also in motility, flagellar expression, autoagglutination, oxidative stress and animal colonization (Plummer 2012). The markers involved in the stress response play a crucial role to increase the ability to form sessile cells (Oh and Jeon 2014).

Campylobacter biofilm ability improves the survival in stressful environments and promotes bacterial dispersion mainly in food processing environments (Reuter et al. 2010). A potential environmental stress for *C. jejuni* is the bile found in the GI tract during infection.

1.7 Bile acids

Once *C. jejuni* colonizes the small intestine of humans and animals, the bacteria ingested into the host will enter the small intestine where *C. jejuni* inevitably will encounter high concentration of bile acids.

Bile acids are steroid acids normally found in the bile of mammals, but also in other vertebrates. In humans, the bile acids consist of primary bile acids (cholic acid – CA, and Chenodeoxycholic acid – CDCA) and secondary bile acids (deoxycholic acid – DCA, lithocholic acid – LCA, taurocheneoxycholic acid – TCA, and glycocholic acid - GCA). The primary bile acids are synthetized from the cholesterol in the liver, and the

secondary bile acids are derived from the primary bile acids as a result of bacterial action in the colon (Hofmann 1999).

1.7.1 Function

Bile acids comprises about 80% of the organic content found in the bile. Bile acids are the final products of cholesterol metabolism in animals. Their main function is to act as emulsifying agents in the intestines to help in the digestion and absorption of fatty acids, monoacylglycerols and other fatty products (T. Li and Chiang 2009). Bile acids are produced and secreted continuously by liver cells, and further metabolism in the liver results in the formation of a conjugated form. They are conjugated via a bond between the carboxyl group of the bile acid and an amino group of glycine or taurine, resulting in the bile salt. These bile salts are then stored in the gallbladder, where they remain until they are needed in the duodenum during the intake of food (Hundt and John 2018).

It has been demonstrated that conjugated bile acids also have a second function, to inhibit the bacterial overgrowth in the small intestine as a result of its cytotoxic and bacteriostatic properties (Sung, Shaffer, and Costerton 1993). The conjugated bile acids regulate expression of host genes whose products promote innate defense against luminal bacteria (Hofmann and Eckmann 2006).

1.7.2 Bile acids and enterobacteria

The human gut carries a very densely populated and complex microbiome. The colon contains 2 to 5 $x10^{11}$ bacteria per gram of wet weight feces (Ridlon et al. 2014). To maintain the balance between an acceptable number of the intestinal flora and a healthy gut, it is believed that bile salts have a potent antibacterial activity. This antibacterial activity, for instance, is what keeps the biliary tract sterile (Sung, Shaffer, and Costerton 1993).

In the gut environment, enteropathogenic microorganisms must overcome many challenges in order to effectively establish infection in the small intestine. These challenges comprise the conditions found in the host GI tract, such as low pH in the stomach, low iron accessibility, high concentration of bile salts in the small intestine, host immune response and an already established commensal microbiome consisting of a large number of different species (Sistrunk et al. 2016). Despite the many defense mechanisms of the GI tract, enteric pathogens have evolved to survive in those conditions and to effectively colonize and start infection in the host (Sistrunk et al. 2016). Some pathogenic species have developed resistance mechanisms against the bile acids for long-term survival in the host. In fact, some bacteria use bile acids as signal to regulate virulence gene expression to either start colonization of the host or maintain the infection (Sistrunk et al. 2016). Studies have demonstrated that gut pathogens react to bile by adapting their protein synthesis, while mutations in genes encoding lipopolysaccharide, efflux pumps, regulatory networks and porins were found to affect bile resistance in enteric pathogens (Negretti et al. 2017).

1.7.3 Mode of action of bile acids in *C. jejuni*

Similar to other enteric pathogens, *C. jejuni* responds to the presence of bile acids by expressing virulence factors that allow the bacteria to survive and colonize the host. The relationship between *C. jejuni* and bile is complex. *Campylobacter* induce many physiological changes to adapt to the stress provoked by bile acids.

The CmeABC multidrug efflux pump in *C. jejuni* is known to increase the ability of the bacteria to survive in presence of antimicrobials, but also bile salts. This system is encoded by the *cmeABC* operon, that encodes a periplasmic protein, CmeA, an inner membrane transporter, CmeB and an outer membrane protein, CmeC (Sistrunk et al. 2016). This operon is also controlled by a TetR family repressor, CmeR. The presence of bile salts appear to inhibit the CmeR-*cmeABC* interaction, which results in increased expression of the efflux pump (Sistrunk et al. 2016).

Another mechanism involved in the *C. jejuni* bile response are the two-component regulatory systems (TCRSs) that sense and respond to bile salts (Sistrunk et al. 2016). In a study from 2005, Raphael et al. identified an orphan response regulator protein (*Campylobacter* bile resistance regulator, CbrR) that is involved in the bile salt response altering gene expression to cope with changing conditions. However, the CbrR-binding partners and the exact mechanism of bile resistance are still unknown.

Campylobacter jejuni also increases the expression of virulence factors after bile exposure, such as Cia proteins, biofilm formation and motility (Malik-Kale, Parker, and Konkel 2008; Sistrunk et al. 2016).

During the infection process, *C. jejuni* secrets a set of proteins directly into the epithelial cell cytoplasm through a flagellar apparatus. These proteins are known as *Campylobacter* invasive antigens (Cia). The presence of Cia in the host results in membrane modifications in signaling and intracellular trafficking, and consequently in increased bacterial uptake (Malik-Kale, Parker, and Konkel 2008; Sistrunk et al. 2016). *C. jejuni* have been found to secrete Cia proteins in response to several stimulatory substances, including the bile acids: deoxycholate, cholate and chenodeoxycholate (Michael E. Konkel et al. 1999; Rivera-Amill et al. 2001). In 2008, Malik-Kale et al. demonstrated that DCA alters the invasion kinetics, changing the required time for *C. jejuni* to be internalized from 3 hours to 15 minutes. The secretion of the Cia proteins is dependent on a functional flagellum, demonstrating that the flagella has a dual function in motility and as a type III secretion system (Malik-Kale, Parker, and Konkel 2008).

Two other virulence mechanisms in *Campylobacter jejuni* that might be regulated by bile acid exposure are the motility and biofilm formation. One study from Svensson (2014), recognized that flagella are essential to improve *C. jejuni* biofilm formation, and that bile salts exposure enhances this process. It was also observed that the expression of flagellin A is increased when *C. jejuni* is exposed to bile components, suggesting that they may induce motility and act as chemotactic attractants (Sistrunk

et al. 2016). In contrast, a study from Malik-Kale (2008) observed that DCA exposure did not alter the motility of *C. jejuni in vitro*, and that DCA did not affect adherence to epithelial cells. The divergent findings are observed for bile salt-dependent adherence to epithelial cells and motility. It is known that *C. jejuni* reacts to bile by adapting their protein synthesis to be able to survive in and colonize the host, however, future research is needed to elucidate the inevitable relationship between *C. jejuni* and bile exposure.

1.7.4 Proteomic response of *C. jejuni* to bile acid exposure

Masanta et al. (2018) compared the individual response towards seven different bile acids on a proteomic level. In the study *C. jejuni* 81-176 was exposed to a sublethal concentration of cholic acid (CA), chenodeoxycholic acid (CDCA), taurocholic acid (TCA), glycocholic acid (GCA), deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA). It was determined that DCA, CDCA and CA presented the lowest IC_{50}, which corresponds to a toxic effect in *C. jejuni*. DCA, CDCA and CA are known to be the main representative proportions of bile acid in the intestine of humans (Baars et al. 2015) and presented the strongest effect in Masanta study.

The analysis by proteome profiling by label-free mass spectrometry (SWATH-MS) revealed a strong effect for DCA and CDCA in the expression level of proteins involved in multidrug efflux transporter CmeABC, by downregulating the upstream regulatory (repressor) system CmR and CbrR. Consequently, the correlation of low IC_{50} to increased CmeABC expression matches to a direct measure of susceptibility of *C. jejuni* to bile acid stress (Masanta et al. 2018).

The flagella are involved in other functions besides motility, such as adherence, Cia proteins secretion or chemotaxis. The bile acid also leads to differentially expressed proteins involved in the flagellar structures. FlaA/B/C, FliE and MotA were up-regulated, while motor proteins FliF, FliM, FilY, and FliL were down-regulated. This

could have a potential influence on the expression of other virulence factors and on *C. jejuni* adaptation processes.

Masanta et al. (2018) also demonstrated a substantial downregulation of basic biosynthetic pathways, such as nucleotide-, protein-, lipid-, and carbohydrate-biosynthesis, additionally to a general reduction of the machinery associated in translation. To summarize, bile acids induce a complex physiological response that involve different functional mechanisms to adapt the organism to the environment.

1.8 Aims of the project

Campylobacter has merged as the main bacterial cause of gastroenteritis in the world. Its prevalence is considered high among the population and its possible complications make it very important in a socio-economic perspective. *Campylobacter* is present in different environments and is easily transmitted to humans through contaminated food and water. *Campylobacter* can persist in the environment and inside the host. The molecular mechanisms of pathogenicity and the organism adaptation to unfavorable environments such as bile exposure is incompletely understood. Previous pathogenic studies revealed that bile exposure acts as a stimulus for the regulation of many virulence mechanisms in *C. jejuni*.

Novel pathogenicity factors involved in the ability of *C. jejuni* to adapt to the bile acid rich environment of the human gut are aimed to be identified by the generation of knockout mutants. Candidate genes were mainly chosen from proteomics data generated on the previous study from Masanta (2018) that resulted in the identification of differentially expressed proteins after exposure to sublethal concentrations of bile acids.

The aim of the present study therefore was to further elucidate if these genes might be involved in either adaptation processes that result in increased stress resistance and/or play a role for the virulence of the pathogen, e.g. in motility, adhesion to, invasion and biofilm formation.

2. Material and Methods

2.1. Bacterial culture conditions, supplements and storage

2.1.1 Bacterial culture conditions

The strains of *E. coli* were grown on Luria-Bertani agar (LB) (Table 5) at 37°C. When needed, the LB plates were supplemented with ampicillin (Table 6).

The strains of *C. jejuni* were routinely grown on Columbia blood agar (Biomerieux) plates and incubated at 42°C for 48 hours, or at 37°C for 17 hours. The *C. jejuni* plates were grown under microaerophilic conditions generated by Gas-pack CampyGen 2.5L (Thermo Scientific) with 85 % N_2, 10 % CO_2, 5% O_2 in an anaerobic jar (Anaerocult). When needed, the plates were supplemented with kanamycin or chloramphenicol (Table 6).

Table 5. List of media used in this study

Media	Constituents	Manufacturer
Luria-Bertani (LB)	10g/L Tryptone 5g/L Yeast extract 5g/L NaCl up to 1L H_2O 15g/L Agar	BD BD Roth Carl Roth GmbH
Mueller Hinton (MH) broth	22g/L MH	Sigma-Aldrich
MH soft agar (0.25% and 0.4%)	22g/L MH 4g/L agar	Sigma-Aldrich Carl Roth GmbH
SOC medium	2% vegetable peptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM Glucose.	New England BioLabs
Brucella soft agar (0.4%)	28g/L Brucella 4g/L agar	BD

All media were prepared according to manufacturer's recommendations and sterilization was performed at standard conditions, 121°C for 20 min unless stated otherwise. Media was dispensed into sterile petri dishes in a laminar flow cabinet till get solidified and were stored at 5°C for up to one month.

2.1.2 Supplements

When needed, the medium was supplemented with antibiotic at the concentration shown in the Table 6. The sterile LB medium was cooled to approximately 50°C before adding antibiotics. The media was mixed thoroughly and dispensed into petri dishes under sterile conditions.

The selective Columbia blood agar (COS) plates were prepared adding 1 mL of diluted antibiotic (chloramphenicol or kanamycin, Table 6) and dried under sterile conditions in laminar flow for 30 - 45 minutes to get the entire antibiotic absorbed by the media.

Table 6. Antibiotics used for selective media

Antibiotic	Solvent	Stock concentration (mg/mL)	Final concentration (µg/mL)
Ampicillin	ddH_2O	50	50
Chloramphenicol	96% EtOH	12.5	12.5
Kanamycin	ddH_2O	50	50

2.1.3 Storage

The antibiotic stock solution was prepared with ddH_2O and filtered through a 0.45 µm filter. All aliquots were stored at -20°C.

The bacterial strains were stored in glycerol stock solution at -80°C. The *E. coli* strains were grown in 5 mL LB Broth, overnight at 37°C. Afterwards, the *E. coli* was transferred to cryo-tubes with final concentration of 30% (v/v) glycerol and stored at -80°C. *C. jejuni* colonies were removed directly from plates using a loop of 10 µl and

resuspended in a cryogenic storage beads (Viabank, Abtek Biological Ltd, England) and stored at -80°C.

2.2. Cultivation of eukaryotic cells

2.2.1 Eukaryotic cell line

Table 7. Eukaryotic cell line used in this study

Cell line	Reference
Human colon carcinoma Caco2-cell	(Fogh, Wright, and Loveless 1977)
COS-7	(Gluzman 1981)

2.2.2 Cultivation

The Caco2 (**Ca**ncer **co**li-2) and COS-7 cell lines were maintained in 75 cm^2 cell culture flasks in a total volume of 30 ml of Dulbecco Minimal Essential Medium (DMEM) supplemented with 1x Non Essential Amino acids (NEA), 10% (v/v) heat inactivated Fetal Calf Serum (FCS) and 100 U/ml penicillin and 100 µg/ml streptomycin (Table 8).

The cells were maintained in a cell culture incubator with humidified atmosphere with 5% CO_2 at 37°C. The cells were split regularly when reaching 80% confluence by removing the old medium, washing the monolayer with 5 ml EDTA, removing the cells with a short incubation with 1 ml of Trypsin at 37°C, and finally resuspending the cells in 10 ml DMEM (Lea 2015). The resuspension then can be diluted in a new flask (with 30 ml DMEM supplemented) for maintaining the cells culture and/or counted to start an experiment.

Table 8. Eukaryotic cell line media

Media	Constituents	Manufacturer
Dulbecco's Modified Eagle's medium (DMEM)	+10% Fetal Calf Serum (FCS) +1x Non-essential amino acid (100x) + 1% Penicillin/Streptomycin (10.000U/ml/10.000 µg/ml)	Biochrom Merck Merck
Hank's Balanced Salt Solution (HBSS)	With sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture	Sigma-Aldrich

2.2.3 Storage

The cells were grown in 175 cm^2 flasks until 80% confluence, harvested as usual and resuspended in 10 ml. The resuspended cells were mixed with freezing solution that consists of 20% of DMSO and 80% DMEM. The mixture with cells were then split into 1.5 ml cryovials and frozen at -80°C.

2.3. Bacterial strains and vectors

2.3.1 Bacterial strains

The knockout mutants generated in this study were generated in the reference strain *Campylobacter jejuni* subsp. jejuni 81-176.

Table 9. Bacterial strains used in this study

Bacterial strain	Ref Seq/Genotype	Reference
***C. jejuni* 81-176**	NC_008787.1	(Korlath et al. 1985)
C. jejuni* 81-176 *Δinv	$\Delta inv::kan^R$	This study
***C. jejuni* 81-176 *Δinv*-complement**	$\Delta inv::kan^R \Omega inv$	This study
C. jejuni* 81-176 *Δsas	$\Delta sas::kan^R$	This study
C. jejuni* 81-176 *Δhad2	$\Delta had2::kan^R$	This study

C. jejuni* 81-176 *Δhad2-complement	$\Delta had2::kan^R \Omega had2$	This study
C. jejuni* 81-176 *Δmaf	$\Delta maf::kan^R$	This study
C. jejuni* 81-176 *Δtgt	$\Delta tgt::kan^R$	This study
C. jejuni* 81-176 *Δtgt-complement	$\Delta tgt::kan^R \Omega tgt$	This study
C. jejuni* 81-176 *ΔtyrA	$\Delta tyrA::kan^R$	This study
C. jejuni* 81-176 *ΔtyrA-complement	$\Delta tyrA::kan^R \Omega tyrA$	This study
C. jejuni* 81-176 *ΔyajQ	$\Delta yajQ::kan^R$	This study
C. jejuni* 81-176 *ΔyajQ-complement	$\Delta yajQ::kan^R \Omega yajQ$	This study
C. jejuni* 81-176 *Δhip82	$\Delta hip82::kan^R$	This study
C. jejuni* 81-176 *Δhip82-complement	$\Delta hip82::kan^R \Omega hip82$	This study
C. jejuni* 81-176 *Δhip12	$\Delta hip12::kan^R$	This study
C. jejuni* 81-176 *Δhip12-complement	$\Delta hip12::kan^R \Omega hip12$	This study
C. jejuni* 81-176 *ΔrrF2	$\Delta rrF2::kan^R$	This study
C. jejuni* 81-176 *ΔrrF2-complement	$\Delta rrF2::kan^R \Omega rrF2$	This study
C. jejuni* 81-176 *ΔflgP	$\Delta flgP::kan^R$	This study
C. jejuni* 81-176 *ΔflgP-complement	$\Delta flgP::kan^R \Omega flgP$	This study

2.3.2 Vectors

Table 10. Vectors used in this study

Plasmid	Reference/Manufacturer
pBluescript SKII vector	Stratagene
pRRC	(Karlyshev and Wren 2005)

2.4. Instruments

Table 11. Instruments

Instruments	Model and Manufacturer
Agarose gel running system	Keutz
Anaerobic jar	Anaerocult
Bacteria incubator	Function Line, Heraeus

Bacteria incubator (with shaker)	Edmund Bühler
Cell culture incubator	Hera cell 240, Heraeus
Centrifuge bacteria (flask)	Megafuge 16R, Heraeus, Thermoscientific
Centrifuge cell culture (flask)	Megafuge 2.0RS, Heraeus
Centrifuge (Eppendorf)	5424, Eppendorf
Deep freezer (-80C)	GFL
Electro Cell Manipulator	Electro cell manipulator 600, BTX
Electrophoresis Power Pack	Standard Power Pack P25, Biometra
Electrophoresis power supply EPS 500/400	Pharmacia fine chemicals
Electrophoresis power supply standard power Pac P25	Thermo Fisher
Gel Imaging System	Gel Doc XR+, Biorad
Fume hood	Weidner
Heat Block	ThermoMixer C – 1.5mL, Eppendorf
Inverted contrasting microscope DM IL	Leica
Laminar flow	BDK
MagNA Pure LC 2.0	Roche
Microplate Reader Victor³V	MRX TC Revelation, Dynex Technologies
Microscope	Leica DMIL
Multichannel pipet	Eppendorf
Multistep dispenser pipet	Eppendorf
NanoDrop 2000c, Spectrophotometer	PeqLab, Thermoscientific
Neubauer Chamber	BRAND
pH Meter	766, Knick
Pipettes (P1000, P100, P10)	Eppendorf
Scale (0.1g)	Sartorius ENTRIS 2201-1S Balance
Scale (0.0001g)	Sartorius ENTRIS 64-1S Analytical balance
Spectrophotometer	Nanocolor VIS II, Macherey-Nagel
Thermocycler	Biometra
UV-crosslinker	NTAS
Universal shaker SM 30	Edmund Buhler

Vortex	Heidolph
Water bath	GFL

2.5. Materials

Table 12. Disposable materials

Disposable Materials	Manufacturer
Cell Culture Flask (25, 75 and 175 cm²)	Cellstar Standard Cell culture Flask, Greiner Bio-one
Cryogenic vials	Nunc
Electroporation cuvette	Pulser/MicroPulser Cuvette, Biorad
Eppendorf tubes 1.5 ml and 2 ml	Sarstedt
Falcon 15 and 50 ml	Greiner
Filter system 0.22um and 0.45um pore	Corning
Gas-pack CampyGen 2.5L	CampyGen 2.5L, Oxoid
Glass cover slip	Menzel
Glass microscopic slide 76x26 mm	Menzel
Inoculation loop 1 and 10µl	Sarstedt
Parafilm	Bemis
Pasteur capillary pipets, 230 mm	WU Mainz
Petri dishes	Sarstedt
Pipette filter Tip (1000 ul, 100ul, 10ul)	Sarstedt
pH test strpe, pH 0 - 14	Omnilabs
Syringe (1 ml, 10 ml, 30 ml, 50 ml)	Terumo
6-well plate culture plate	Greiner Bio-one
24-well plate	Cellstar, Greiner bio-one
96-well plate	Greiner Bio-one
0.2 µm Filter	500 ml Bottle Top filter, Corning

2.6. Kits, buffers, enzymes and chemicals

Table 13. List of kits, enzymes and chemicals

Kits, buffers, enzymes and chemicals	Manufacturer
Kits	
PCRBio HiFi kit	PCR Biosystems
NEBuilder HiFi DNA Assembly Cloning kit	NEB
QIAquick Gel Extraction kit	Qiagen
QIAquick PCR Purification kit	Qiagen
GenElute Plasmid Miniprep kit	Sigma-Aldrich
Buffers	
Cut Smart Buffer	NEB
Antarctic Phosphatase Buffer	NEB
50x TAE (Running Buffer)	
- 57.1 ml Acetic Acid	Merck
- 242 g Tris	Roth
- 100 ml 0.5M EDTA	Merck
Washing Buffer (electrocompetent cell)	
- 272 mM sucrose	Merck
- 15% Glycerol	Merck
Phosphate Buffered Saline (PBS) (w/o Ca^{2+} w/o Mg^{2+})	Merck
Enzymes	
Antarctic Phosphatase	NEB
BamHI	NEB
EcoRI	NEB
EcoRV	NEB
XBaI	NEB
Chemicals	
Agar-agar	Carl Roth GmbH
Agarose for DNA/RNA electrophoresis	Carl Roth GmbH
Crystal Violet	Merck
ddH_2O (0.1 µm filtered water)	Sigma-Aldrich
DMSO (diethyl sulphoxide)	Sigma-Aldrich

dNTP	Carl Roth GmbH
EDTA in PBS (1% (w/v) in PBS w/o Ca^{2+} w/o Mg^{2+})	Merck
Ethanol	Carl Roth GmbH
FCS (fetal calf serum)	Biochrom
Glycerol	Sigma-Aldrich
Lithocholic acid (LCA)	Sigma
Loading Dye (6x orange LD)	Thermo Fisher
Midori green	Nippon Genetics
Non-essential amino acid (NEA)	Merck
Sodium chenodeoxycholate (CDCA)	Sigma
Sodium cholate hydrate (CA)	Sigma
Sodium deoxycholate (DCA)	Sigma
Sodium glycocholate hydrate (GCA)	Sigma
Taurocholic acid sodium salt hydrate (TCA)	Sigma
Triton X-100	Merck
Trypsin (0.25% (w/v) in PBS w/o Ca^{2+} w/o Mg^{2+})	Merck

2.7. Oligonucleotides

Table 14. List of Oligonucleotides

Oligonucleotide	Sequence	Reference
pSK-5-flgP-F	AGGTCGACGGTATCGATAAGCTTGATATCGTAGAAAAGCA GGGCGTAATACAA	This study
Kana-5-flgP-R	TCTCGTTTTCATACCTCGGTATAATCTTACAGAAACTGTA TTCATCGGAGCAA	This study
Kana-3- flgP -F	TACTGGATGAATTGTTTTAGTACCTAGATTTTAGCAAAAA GAGCAGCGATTAC	This study
pSK-3- flgP -R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATTCAATGCTAA AGAAGTTCGAGGT	This study
CP-flgP-F	GATGTTATCGTGCAAAAAGTCGA	This study
CP-flgP-R	AACAATTCTTTCCACTTGTCTGC	This study
CO-flgP-F	AGAGGTGGTAAGGGTGTAATTTG	This study
CO-flgP-R	TTTGACATAAGTTTCGCTTTGGG	This study

Compl_pRRC_flgP_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGAGATTTAAATGAAAAAAATTTATTTTATGCTA	This study
Compl_pRRC_flgP_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATTAATAAGCAAACAATTCTTTCCACTTG	This study
pSK-5-Inv-F	AGGTCGACGGTATCGATAAGCTTGATATCGAACAATTTGCACTTGGCTCAATT	This study
Kana-5-Inv-R	TCTCGTTTTCATACCTCGGTATAATCTTACAGTTGATGTAATAACGCCAATCA	This study
Kana-3-Inv-F	TACTGGATGAATTGTTTTAGTACCTAGATTAAGGCCTTCGATACCATGAATTT	This study
pSK-3-Inv-R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATTGAGGATGTGTTTGTTTTAAATGA	This study
CP-Inv-F	TTTTAAAGCATAGCTGGGGAAGA	This study
CP-Inv-R	AGTGTAATAGGAAAAAGATAGCGA	This study
CO-Inv-F	TCCAACCCTAGCTCAAATTCTTT	This study
CO-Inv-R	GGAATTTGTGGAGTTGAAATGCT	This study
Compl_pRRC_Inv_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGAGATTTAAATGCAAAATCTTTTACTCTATAT	This study
Compl_pRRC_Inv_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATTATTTATCTTTATATATTTTTTCA	This study
pSK-5-SAS-F	AGGTCGACGGTATCGATAAGCTTGATATCGAACACTAGTAGGTCAAAGTGGTG	This study
Kana-5-SAS-R	TCTCGTTTTCATACCTCGGTATAATCTTACGCAAGTCCTAAAGCTTCAAGAAC	This study
Kana-3-SAS-F	TACTGGATGAATTGTTTTAGTACCTAGATTCTGCTAGTCTGCCTGATAAAACT	This study
pSK-3-SAS-R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATAGCCAAAGGGGAAATAAATCTCA	This study
CP-SAS-F	GCGGTTAAATTTCTTTTTGTGCC	This study
CP-SAS-R	AAGGCTCTATCATTGAAACAGCT	This study
CO-SAS-F	ATAGGTTTCATTTTAGCGGTAGC	This study
CO-SAS-R	CGCTTAAAGTTGGGAATTTCCAA	This study
Compl_pRRC_SAS_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGAGATTTAATTGAGTTCTAAATTTTCAAAAAT	This study
Compl_pRRC_SAS_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATCACTTTAAAAAAGCGGCTATCATAACTATAA	This study
pSK-5-HAD2-F	AGGTCGACGGTATCGATAAGCTTGATATCGAAATCCCGCGGTAAATCTTAACT	This study
Kana-5-HAD2-R	TCTCGTTTTCATACCTCGGTATAATCTTACCGCTTTGTTCATAGTGTTTGACA	This study
pSK-3-HAD2-F	TACTGGATGAATTGTTTTAGTACCTAGATTTGAAGGAGTGAAAGAACTTTTAGA	This study
pSK-3-HAD2-R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATTGCCAATTTCTATTAATAATTCACTCA	This study
CP-HAD2-F	ATCGATAGTGCAAATGCCATTTC	This study

CP-HAD2-R	TGGGTTCTATGCCTAAACTTACG	This study
CO-HAD2-F	TTTTTCAATGGCGCTATATCTGC	This study
CO-HAD2-R	AACTAAACGACGCGGAGTATAAT	This study
Compl_pRRC_HAD2_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGAGATTTAAATGATTAATGTATTTTTTGATAT	This study
Compl_pRRC_HAD2_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATTATAAATACTTTTGCAAAAAGCCTTTAAGC	This study
pSK-5-Maf-F	AGGTCGACGGTATCGATAAGCTTGATATCGTTGCTAAAGCGGTGATTTTACTT	This study
Kana-5-Maf-R	TCTCGTTTTCATACCTCGGTATAATCTTACTTTGGTTTTGAAAATCTTTGCCT	This study
Kana-3-Maf-F	TACTGGATGAATTGTTTTAGTACCTAGATTGAAGCTTATGAAATGCTTGCCTT	This study
pSK-3-Maf-R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATACAAGCTTGCCATTTCTATCGTA	This study
CP-Maf-F	CCGCAAAGATTGATTTTAGGCAA	This study
CP-Maf-R	CGCCTTGCCTTTATAAAGATCAT	This study
CO-Maf-F	TATCCAAGGTGTTGAAATTTGCG	This study
CO-Maf-R	TACGCGTAATAGTTCTTTCAGGG	This study
pSK-5-tgt-F	AGGTCGACGGTATCGATAAGCTTGATATCGTGCTAAAATACCGTCTATTATAATT	This study
Kana-5- tgt -R	TCTCGTTTTCATACCTCGGTATAATCTTACTCTAAAATCATCATAATATCAGAGTT	This study
Kana-3- tgt -F	TACTGGATGAATTGTTTTAGTACCTAGATTATACTTTGGGCAAAAGAGGCTAT	This study
pSK-3- tgt -R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATAGTTCTTTAGCTTTGAAAAGATGGT	This study
CO- tgt -F	TAATATTTTCAAGACGCGCTGTG	This study
CO- tgt -R	CGCATCTTTCTTGCAAGTTCAA	This study
CO-tgt-F (complem.)	ATACTTTGGGCAAAAGAGGCTAT	This study
CO-tgt-F (complem)	AAAGTCACATTGATGGGAGTCAT	This study
Compl_pRRC_tgt_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGAGATTTAAATGGAATTTAAATTAAA	This study
Compl_pRRC_tgt_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATTATTTGCCTCTTAAGTGATAAAAATTTC	This study
pSK-5-tyrA-F	AGGTCGACGGTATCGATAAGCTTGATATCGTAAGCCCCGTGATATTTATAGCG	This study
Kana-5- tyrA -R	TCTCGTTTTCATACCTCGGTATAATCTTACTTCTTTTTGTACTTCCAAGCTCT	This study
Kana-3- tyrA -F	TACTGGATGAATTGTTTTAGTACCTAGATTCTTTTTGCTCATCCTATGACAGG	This study
pSK-3- tyrA -R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATAGCCTGTTTCATCCATTCTCTTA	This study
CO- tyrA -F	ACTTCCTTCATCAAGTAGTGTGC	This study

CO- tyrA -R	CTGGCATTAAAATTTGAGGAGGA	This study
CO-tyrA-F (complem.)	CCCTGCGATTAGGTCTTATTCAT	This study
CO-tyrA-R (complem.)	AGATCCTCCAGCTAAATGAACAA	This study
Compl_pRRC_tyrA_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGA GATTTAAATGAAAATAGCAATTATA	This study
Compl_pRRC_tyrA_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATTATA AAATTTCTCTTAGAGTATTAGCCTGT	This study
pSK-5-YajQ-F	AGGTCGACGGTATCGATAAGCTTGATATCGTGTAGGAAG AGGTGGGATTATCA	This study
Kana-5-YajQ-R	TCTCGTTTTCATACCTCGGTATAATCTTACACATCAAGTT TTCCTTCACTAGA	This study
Kana-3-YajQ-F	TACTGGATGAATTGTTTTAGTACCTAGATTGAAAGTGGAG CAATGTTTCGTTT	This study
pSK-3-YajQ-R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATAGCTTGCCCT AGTTTATAAACTTCT	This study
CP-YajQ-F	TCAGCAGCTTTAGATAAGCAAGA	This study
CP-YajQ-R	TTCACCACGAATCGAAGAGTTAA	This study
CO-YajQ-F	AGTATCCACGCACCTTTAAATGA	This study
CO-YajQ-R	TGTTCAAAACCACAATCAGTTTT	This study
Compl_pRRC_YajQ_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGA GATTTAAATGGCAAGTGAA	This study
Fwd2_Compl_pRRC_YajQ	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGA GATTTAAATGGCAAGTGAACATAGTTTTG	This study
Compl_pRRC_YajQ_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATTATT TGAGATTTTTAAAACTGACATTTAACTC	This study
pSK-5-Hip82-F	AGGTCGACGGTATCGATAAGCTTGATATCGAGAACTTGAT AGAAAAAGCGGAGA	This study
Kana-5- Hip82-R	TCTCGTTTTCATACCTCGGTATAATCTTACAAGAAATTCC CGTTTTCAAGTCG	This study
Kana-3- Hip82-F	TACTGGATGAATTGTTTTAGTACCTAGATTCCATGGTAAT AGCTTTGGGAGAT	This study
pSK-3- Hip82-R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATTCTCTTTTAC TTCTTTTAAGCCT	This study
CO- Hip82-F	TAAAAGACGCACATAAATACGGC	This study
CO- Hip82-R	TGGTTATACATTTGAAGCAAGCG	This study
CO-Hip82-Compl-F	GTTTAATTCTTGCCTGTTCAGCA	This study
CO-Hip82-Compl-R	AGCGGATTTTTCAAAAGCAGATT	This study
Compl_pRRC_Hip82_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGA GATTTAAATGAAAAAATTAAGTTTAATT	This study
Compl_pRRC_Hip82_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATTATT TAATAATAGTTGGAGTAGCG	This study

pSK-5-Hip12-F	AGGTCGACGGTATCGATAAGCTTGATATCGGGAATTTGGA CTTGCATTATAGCT	This study
Kana-5-Hip12-R	TCTCGTTTTCATACCTCGGTATAATCTTACATAGTCATGA CTCATCATACCCG	This study
Kana-3- Hip12-F	TACTGGATGAATTGTTTTAGTACCTAGATTAGTGATAGAA CTTTCACAATACC	This study
pSK-3- Hip12-R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATTGCATGGCA GTCATTAAATTTTCT	This study
CO- Hip12-F	TTGGCCTTGGGTGTAGATTTAAT	This study
CO- Hip12-R	GACCCCACTAAGTCCAAGTTTTA	This study
CO-Hip12-F (complem)	ATGCAACAAAGCGTTTTAAATGC	This study
CO-Hip12-R (complem)	CACTGATGAGATATTTTGCACCG	This study
Compl_pRRC_Hip12_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGA GATTTAAATGAAAAAAGTGTATTTTTA	This study
Compl_pRRC_Hip12_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATCATA GTCTCTCTCCTTCTAA	This study
pSK-5-RrF2-F	AGGTCGACGGTATCGATAAGCTTGATATCGTCTGTAAGTT CTGCAATTCTTGC	This study
Kana-5-RrF2-R	TCTCGTTTTCATACCTCGGTATAATCTTACAAACCACCTT TTGCCCCTTTAAA	This study
Kana-3-RrF2-F	TACTGGATGAATTGTTTTAGTACCTAGATTTGGTGGCACT TGTCCAAATAATA	This study
pSK-3-RrF2-R	GCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCTTCGCTT AAAATCATCCTTG	This study
CO-RrF2-F	CCCCGTAAAAAGGGCTTAAAAAT	This study
CO-RrF2-R	CAATCACGCCAATGACCATATTT	This study
CO-RrF2-F (complem.)	TGCTATTTACCAAAGCTAGCGAA	This study
CO-RrF2-R (complem.)	ACTAACATAGGCATTAAGGTGCA	This study
Compl_pRRC_RrF2_Fwd	GAATTCTGCAGGTACCCGGGATCCACTAGTTCTAGAAGGA GATTTAAGTGCTATTTACCAAA	This study
Compl_pRRC_RrF2_Rev	AGACTTATTACTTTGTACTCTAGGGCCGCTCTAGATTATT TTTTGCCATTATTTTTCATAATATCTTC	This study
CO-Kan-R	TGGTAGCTTTTTAAATATGGCGC	This study
CO-Kan-F	TCAAGCCTGATTGGGAGAAAATA	This study
pRRC rev	CAAGAATCAATTGAGTTTATATATTGAA	This study
ak231	CTGGAACTCAACTGACGCTAAG	(Karlyshev and Wren 2005)
ak232	CTCTTGCACATTGCAGTCCTAC	(Karlyshev and Wren 2005)
ak233_81176	GCAAGAGTTTTACTTATGTTAGCGC	(Lübke et al. 2018)

ak234	GAAATGGGCAGAGTGTATTCTCCG	(Karlyshev and Wren 2005)
ak235	GTGCGGATAATGTTGTTTCTG	(Karlyshev and Wren 2005)
ak237	TCCTGAACTCTTCATGTCGATTG	(Karlyshev and Wren 2005)
M13	TGTAAAACGACGGCCAG	Sequencing primer (SeqLab, Göttingen)
M13r	CAGGAAACAGCTATGAC	Sequencing primer (SeqLab, Göttingen)

2.8 Software and web services

Table 15. List of software and web services

Software and Web services	Website
BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Fiji - ImageJ	https://fiji.sc/
Geneious 2019.1.3	https://www.geneious.com/
Gretl	http://gretl.sourceforge.net/
LateX	https://www.latex-project.org/
R studio	https://www.rstudio.com/
Microsoft Office 2011	https://www.office.com/
PubMed NCBI	https://www.ncbi.nlm.nih.gov/pubmed/
Statistica 13.3	http://www.statsoft.com/Products/STATISTICA-Features

2.9 Molecular biology methods

2.9.1 Genomic DNA Extraction

The genomic DNA extraction was performed by automated isolation and purification in the MagNA Pure instrument (Roche).

2.9.2 PCR

For the Polymerase chain reaction (PCR) a High Fidelity (HiFi) DNA polymerase (PCR Biosystems) was used following the manufacturer's instructions, in order to avoid errors in cloning genes from *E. coli* and *C. jejuni*. Taq DNA polymerase (Roche) was used for testing primers and clone confirmations.

The HiFi PCR reaction was performed in 50 µl total volume with 10 µl of 5x PCRBIO reaction buffer, 5 µl of 5 µM of forward and reverse primers, 0.5 µl HiFi polymerase, <100 ng DNA template (Table 16) and ddH_2O. The Taq PCR reaction was also performed in 50 µl total volume with 5 µl of 10x PCR reaction buffer, 1 µl dNTP (PCR Grade Nucleotide Mix, Roche), 1 µl Taq polymerase (Roche), <100 ng DNA template and ddH_2O. Pure ddH_2O (0.1 µm filtered water, Sigma) was used for the PCR reaction and also for DNA and primers dilutions.

Table 16. Standard PCR reaction mix for Hifi and Taq polymerase

PCR Hifi – Components	**PCR reaction**
Buffer 5x	10 µl
Forward Primer (5 µM)	5 µl
Reverse Primer (5 µM)	5 µl
HiFi Polymerase	0.5 µl
H_2O	up to 50 µl
Template DNA	< 100 ng
PCR Taq – Components	**PCR reaction**
Buffer 10x	5 µl
Forward Primer (5 µM)	5 µl
Reverse Primer (5 µM)	5 µl
Taq polymerase	1 µl
H_2O	up to 50 µl
Template DNA	< 100 ng

The amplification was carried out in Thermocycler (Biometra) following the general conditions shown in Table 17.

Table 17. General conditions for PCR

Step	Temperature (HiFi/Taq)	Time	Cycle	Description
1	95 - 98°C	3 – 5 min	1x	First template denaturation
2	95 - 98°C	30 sec	29x	Template denaturation
3	50 – 70°C	15 sec		Primer annealing
4	72°C	60 sec/kb (Taq) or 30 sec/kb (Hifi)		Primer extension time
5	72°C	5 min	1x	Final extension
6	4°C	…	1x	Cooling

2.9.3 Quantification of DNA

The DNA concentration was determined by NanoDrop ND 1000 spectrophotometer according to the manufacturer's instructions.

2.9.4 Plasmid DNA extraction

Plasmid DNA was isolated from *E. coli* by a Plasmid Purification kit (Sigma-Aldrich) following the manufacturer's instructions. The plasmid was resuspended in 50 µl elution buffer and the concentration was measured by NanoDrop.

2.9.5 Sequencing

The fragments and the plasmids were sequenced directly by the Company SeqLab (Göttingen, Germany). The primers used for the knockout construct sequencing were M13 and M13r or gene specific primers "CP" (confirmation primer) and "CO" (confirmation primer) shown in the Table 14. The same primers were used for confirmation of the complementation mutants.

2.9.6 Enzymatic modification of DNA

BamHI and *EcoRI* were used to digest the pSKII vector, and *XbaI* to digest the pRRC vector (used for complementation). The digestion was performed following the manufacturer's instructions. 5 µg of cloning vector was incubated with 1x Cut Smart buffer (NEB), ddH_2O (up to 50 µl), 1 µl *EcoRI* – HF (NEB) and 2 µl *BamHI* (NEB), where the enzyme is up to 10% of total volume in the reaction. The mixture was incubated for 3 hours at 37°C.

Afterwards the vector was dephosphorylated with Antarctic Phosphatase (NEB) following the manufacturer's instructions.

2.9.7 DNA and PCR products purification

The DNA extraction from agarose gel was performed using the QIAgen PCR Purification kit. After gel running, the DNA fragment was excised with a sharp blade from the agarose gel. The piece of gel was weighted and 3 volume of QG buffer to one volume of gel was added and incubated at 50°C for 10 min. Then the dissolved agarose was transferred to a QIAquick spin column and centrifuged at 13.000 rpm for 1 min, washed with 750ul of PE buffer and dried. Then the DNA was eluted by adding 30 - 50 ul of elution buffer.

The DNA concentration was measured by NanoDrop and when needed, a small volume was used to run a confirmation gel.

2.10 Knockout mutant construct generation

2.10.1 Primer design

The primers were designed using Geneious software with the genome sequence of *Campylobacter jejuni* 81-176. Eleven genes were selected to generate the knockout mutant as shown in Table 18.

Table 18. Chosen genes to knockout

	Gene	Uniprot	Gene ID
1	Inv	A0A0H3P9Z9	CJJ81176_0708
2	Sas	A0A0H3PA18	CJJ81176_0942
3	HAD2	A0A0H3PI47	CJJ81176_1247
4	Maf	A1VYL9	CJJ81176_0535
5	tgt	A1VZZ8	CJJ81176_1028
6	tyrA	A0A0H3PAH1	CJJ81176_0165
7	YajQ	A1VY95	CJJ81176_0398
8	Hip82	A0A0H3PBG0	CJJ81176_1382
9	Hip12	A0A0H3P9A5	CJJ81176_0112
10	RrF2	A0A0H3PDG2	CJJ81176_0891
11	FlgP	A0A0H3PCP8	CJJ81176_1045

The primers were designed using 30 bp overlapping regions for the cloning vector and the kanamycin cassette (Figure 8). For one knockout mutant generation, 4 different primers were needed to amplify the fragments. The first pair of primers with 5'-forward specific sequence of the gene plus 30 bp of pSKII vector and a 5'-reverse primer (in the middle of the gene) plus 30 bp of the kanamycin cassette. The other primer pair consists of a 3'-forward primer in the middle of the gene, but with 5 - 100 bp distance to the 5'-reverse primer (to delete part of the original gene), plus 30 bp overlapping for kanamycin cassette, and 3'-reverse primer at the end plus 30 bp specific sequence of the pSKII vector.

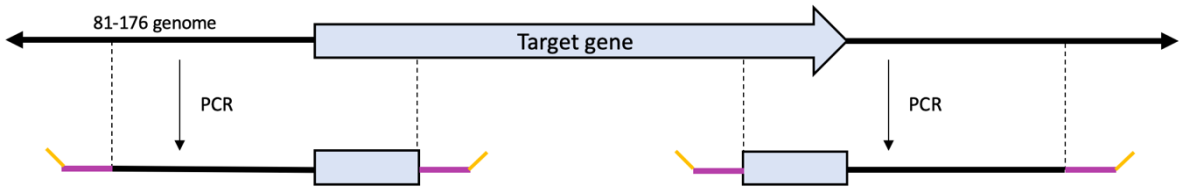

Figure 8 Amplification scheme of the target genes with flanking regions. Target gene in light blue, primers in pink and overlapping region in orange.

2.10.2 Plasmid assembly

The DNA fragments with approximately 500 bp were amplified following the Table 16 and Table 17 settings for Hifi polymerase. After amplification the PCR product was run in a 1% agarose gel electrophoresis with 100V for 1 hour. The bands with the correct size were cut out using a blade and purified using the PCR purification kit (QIAquick) as described previously (2.9.7).

The backbone for the knockout construct was provided by the pBluescript SK(+) vector containing Ampicillin resistance for selection. The vector was digested with *BamHI* and *EcoRI*.

The two fragments of the target gene, the kanamycin cassette and the digested vector were assembled using the NEBuilder Hifi DNA Assembly Cloning kit following the manufacturer's instructions (Figure 9) in a single tube reaction.

The assembled product was transformed by heat shock into *E. coli* following the manufacturer's protocol. After the transformation the *E. coli* strain was plated in selective media containing Ampicillin and incubated overnight at 37°C. Single colonies were picked, re-plated and inoculated into selective LB broth for further plasmid isolation. The plasmid construct was sent to Microsynth SeqLab – Göttingen for confirmation by sequencing using the primers M13 and M13r (Table 14).

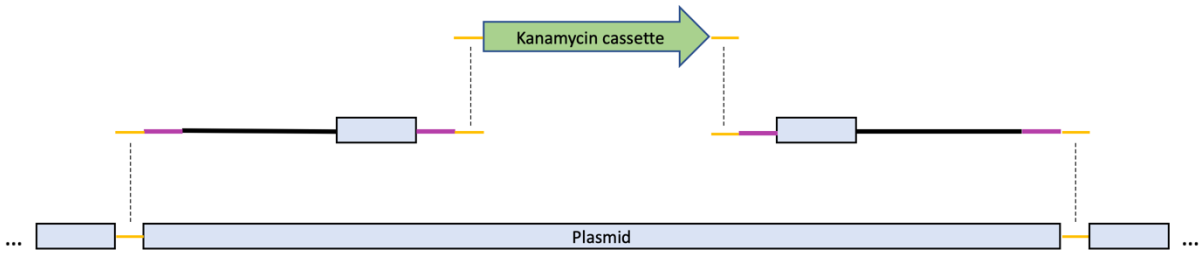

Figure 9 Assembly of gene-specific fragments, kanamycin cassette and backbone plasmid for construction of the knockout targeting vector.

2.11 Transformation by electroporation

2.11.1 Preparation of electrocompetent cells

In order to prepare competent *C. jejuni*, bacteria were grown in Columbia blood agar for 16 – 18 hours at 42°C under microaerophilic conditions. The cells obtained were resuspended in ice-cold washing buffer composed of 272 mM sucrose and 15% glycerol. Bacteria were washed 3 times with washing buffer and centrifuged at 5.000 g at 4°C for 10 min. The pellet was resuspended in 400 µl washing buffer, separated in aliquots of 100 µl and stored at -80°C.

2.11.2 Electroporation

Electroporation was performed using 2 – 3 µg (maximum volume of 10µl) of the plasmid vector mixed to 100 µl electrocompetent cells and transferred to a pre-cooled cuvette (Gene Pulser/Micropulser Electroporation Cuvette 0.1 cm gap, Bio-Rad). The electroporation was performed with a resistance of 2.5 kV, capacitance timing of 25 µF and resistance timing of 186 Ω in the BTX electro Cell Manipulator. After pulse, 100 µl of SOC medium was added to the electroporated bacteria, plated on non-selective COS plates and grown under microaerophilic conditions overnight at 37°C. On the following day, the cells were resuspended and transferred onto a selective Columbia blood agar supplemented with kanamycin. The plates were incubated at 42°C for 48 hours under microaerophilic conditions.

2.11.3 Screening for mutants

Colonies that were observed on the plate, were re-plated into selective media for genomic DNA extraction and further analysis.

The bacteria were resuspended in 300 µl buffer (MagNA Pure LC Total Nucleic Acid Isolation kit, Roche) for genomic DNA extraction and genomic DNA was isolated by MagnaPure (Roche). Then a PCR was performed using specific primers flanking the

outer gene region for confirmation of correct integration of the targeting construct (Table 14).

2.12 Growth curve

To perform the growth curve, the bacterial cultures were firstly grown on COS plates for 17 hours at 37°C under microaerophilic condition. Bacteria were resuspended in MH liquid media and inoculated in 20 ml of a 100 ml Erlenmeyer flask as a pre-culture and grown overnight at 37°C under microaerophilic conditions with 150 rpm shaking. The optical density of the pre-culture was then adjusted to OD_{600} 0.05 as a start point for the growth curve. The measurements were done every 4 hours in a total of 48 hours. Growth curves were performed in 2 biological triplicates, respectively.

2.13 Motility assays

The motility capacity of the bacteria was established by (i) inoculation in soft agar medium (Tareen et al. 2010), by (ii) 2,3,5-Triphenyltetrazolium chloride (TTC) soft agar assay, and (iii) by microscopic observation of living bacteria.

2.13.1 Soft agar motility

The strains were grown on COS plates for 17 hours at 37°C under microaerophilic conditions. Afterwards bacteria on plates were resuspended in MH liquid and the OD_{600} adjusted to 0.025. The bacterial suspension was stabbed into a 0.25% and 0.4% Mueller-Hinton agar plate using a 1 µl inoculation loop, then incubated at 37°C under microaerophilic conditions. The diameter of swarming zones was measured after 48 hours of incubation.

2.13.2 TTC assay

The compound 2,3,5-Triphenyltetrazolium chloride (TTC) is a water-soluble dye that changes color from white into red after reduction. 15 ml falcon tubes were filled up with Brucella broth with 0.25% agar and supplemented with 100 µg/ml TTC.

C. jejuni strains were grown on COS plates for 17 hours at 37°C under microaerophilic conditions. Bacteria on plates were resuspended in PBS and the OD_{600} adjusted to 1. The resuspended bacteria were inoculated by dropping 50 µl on the top of the medium and placed in the microaerophilic container with the lid of the falcon tube open. The measurement and pictures were taken after 24 hours incubation under microaerophilic conditions at 42°C.

2.13.3 Motility after invasion

Motility was also assessed after invasion performed by gentamycin protection assay. The GPA was carried as described in section 2.16, the bacteria recovered after 48 hours incubation was counted and then resuspended in MH liquid and the OD_{600} adjusted to 0.025. The bacterial suspension was stabbed into 0.4% MH agar plate using a 1 µl inoculation loop, then incubated at 37°C under microaerophilic conditions. The diameter of swarming area was measured after 48 hours of incubation.

2.13.4 Microscopic observation of motility

The bacterial strains were grown on COS plates for 17 hours at 37°C under microaerophilic conditions, then resuspended and inoculated in Muller Hinton broth and incubated overnight at 37°C. The bacteria were adjusted to OD_{600} of 0.05. An aliquot was added on a glass slide and covered with a cover slide. For the observation of the living bacteria, an inverted microscope (Leica) and camera Nikon D7100 was used. The classification for bacterial movement were:

- **motile**, when the bacterium could move freely (swim/run);

- **tumbling**, when the bacterium moves in a single direction for a given time before randomly changing direction; and
- **nonmotile**, when the bacterium doesn't move.

2.14 Biofilm formation assay

2.14.1 Crystal violet biofilm assay

The strains were grown for 17 hours on COS plates, resuspended in Mueller Hinton broth and adjusted to OD_{600} of 0.05. 100 µl of the bacterial suspension per well was added in a 96 well plate (Microplate, F-bottom, Greiner Bio-one) and incubated at 37°C and/or 42°C for 48 hours without shacking under microaerophilic conditions. After the incubation time, the plates were rinsed with sterile water once (gently) and dried at 60°C for 30 min. 100 µl of 0.1% crystal violet solution in water was added to each well for 15 min. Then the plates were rinsed with sterile water two times and dried at 60°C for 30 min. To quantify the Biofilm formation, 100 µl of dissolving solution (20% acetone and 80% ethanol) was added to the wells and incubated for 15 min at RT. After incubation, 80 µl of the dissolved crystal violet was transferred to a new 96 well plate for absorbance measuring (540 nm) using the Microplate Reader (Victor^{3}V, Wallac 1420).

The Biofilm formation was performed in five biological replicates and for each strain in four technical replicates. Mueller Hinton broth was used as a negative control for biofilm formation (Reeser et al. 2007).

Biofilm formation in the presence of DCA was performed as described above, however, the strains were diluted in the MH broth supplemented with 1.5 mM DCA. The assay to analyze the effect of oxygen on biofilm formation was performed in two plates, one incubated at microaerophilic conditions and the other in normal atmosphere, both at 37°C for 48 hours.

2.14.2 Microscopic analysis of biofilm formation

The observation of biofilms by microscopy was performed in two ways: (i) observation of the first 96 well plate after the 80 µl removal, and (ii) a new biofilm assay as described in the previous section (2.14.1) except the dissolving treatment.

The biofilms were observed in inverted microscope under 63x and 100x magnification.

2.15 Autoagglutination assay

Bacteria, grown for 17 h, were inoculated into PBS to an OD_{600} of 1 and incubated at 37°C without shaking. The optical density of the supernatant was measured after 24 hours and compared to the starting OD.

2.16 Invasion and adhesion

Invasion and adhesion were investigated by the gentamycin protection assay (GPA). In both techniques the Caco2 cell line was used. COS-7 cells were used in invasion assays only. The assays were performed in at least three biological triplicates.

2.16.1 Invasion - Gentamycin protection assay (GPA)

$2x10^5$ of $Caco_2$ cells were seeded in a volume of 1 ml into a 24 well plate and incubated overnight (DMEM + 10% FCS + 1x NEA). The *C. jejuni* strains were grown on COS plates for 17 hours at 37°C, resuspended in DMEM (without supplements) and the OD_{600} was adjusted to 0.0007 (corresponding to ~$2x10^6$ CFU/ml for the WT 81-176) for a multiplicity of infection (MOI) of 10. 1 ml of the bacterial suspension was added to the Caco2 cells, centrifuged at 600 g for 5 minutes and incubated for 2 hours at 37°C. A small volume of the bacterial suspension was kept to be plated in serial dilutions for the determination of the real number of viable bacteria added to each well (input). After the 2 hours incubation, the bacterial suspension was removed and the Caco2 monolayer was washed three times with DMEM (without supplements). 1 ml of 100 µg/ml gentamycin was added, and the plate was incubated for further 2 hours to

eliminate the extracellular bacteria. Subsequently, the wells were washed three times with DMEM (without supplements) and the cells lysed with 100 µl of 0.1% Triton X-100 in DMEM for 10 minutes to release the intracellular bacteria. After 10 minutes incubation in Triton X-100, the lysate was diluted (1:10) with DMEM and afterwards each well was plated in serial dilutions for determination of viable bacteria. The serial dilution plates were incubated at 42°C for 48 hours under microaerophilic conditions and CFU counting performed afterwards. The percentage of invasion was calculated by determination of the ratio between the number of invaded bacteria and the number of viable bacteria that were added to the wells.

2.16.2 Adhesion - Gentamycin protection assay (GPA)

The adhesion was performed in the same way as described for invasion, but without the gentamycin treatment. Adhesion determination was performed by subtracting the number of intracellular bacteria from the number of adhered cells.

2.17 Stress assay

The stress assay was performed by exposing the strains to adverse conditions such as high temperature, to bile acids and in sterile water.

2.17.1 Temperature

The strains were grown on COS plates for 17 hours and bacteria were resuspended in Mueller Hinton broth and adjusted OD_{600} of 0.05. The adjusted bacterial suspension was diluted in 10-fold serial dilutions (up to 10^{-6}) in single Eppendorf tubes. All dilutions were incubated for 1 hour at 52°C in a ThermoMixer block (Eppendorf). One serial dilution control was not treated. After heat treatment the bacterial suspension was plated on COS plates by adding 3 µl drops of each dilution. The plates were incubated for 24 hours at 42°C under microaerophilic condition. The experiment was performed in two biological duplicates.

2.17.2 Bile acids

C. jejuni strains grown for 17 h were resuspended in Mueller Hinton broth, the optical density was adjusted to 0.05 and the culture was incubated overnight at 37°C by shacking under microaerophilic condition. After incubation, the strains were diluted to an OD_{600} of 0.1. The same volume of a solution of 3 mM DCA diluted in MH was added, the final concentration was OD_{600} 0.05 and 1.5 mM DCA. The 10 ml mixture was placed in 25 ml Erlenmeyer and incubated for 24 hours at 37°C by shacking under microaerophilic condition. The final OD was measured and the difference between the non-treated and DCA-treated sample was calculated. The experiment was performed in two biological triplicates.

2.17.3 Water survival

The strains were grown on COS plates for 17 hours under microaerophilic conditions at 37°C. The strains were resuspended in double distilled sterile water and washed once by centrifuging at 3.000 g for 5 min and adjusted to OD_{600} of 0.0007 in double distilled sterile water. The tubes containing the strains diluted in water were kept at 5°C without shaking for up to 14 days and plated every 24 hours. The water survival assay was performed in three biological triplicates.

2.18 Complementation

In order to reestablish the original phenotype observed in the WT, the mutants were complemented as described by Karlyshev and Wren (2005). PCR was carried out to amplify the genes that are going to be reinserted into the electrocompetent mutants. The PCR products were purified with the QIAquick PCR purification Kit (QIAGEN). The complement vector, pRRC, was opened with XbaI (New England Biolabs) and the open ends dephosphorylated with Antartic Phosphatase (New England Biolabs), following the manufacturer instructions. The PCR fragments were ligated into the digested vector pRRC using the NEBuilder® HiFi DNA Assembly Cloning Kit (New

England BioLabs), according to the manufacturer instructions. After the assembly, the correct complementation construct was confirmed by sequencing. With the confirmed constructs, the transformations were carried out by electroporation and afterwards, selection on Columbia blood agar plates supplemented with chloramphenicol (12.5 µg/ml) was performed. Successful complementation of the knockout mutants was verified by PCR with gene specific primers (Table 14).

2.19 Statistical analysis

The statistical analysis was performed by two-sided, unpaired Student T-tests (unless stated otherwise) using the Excel Software, Statistica 13.3 and R (boxplot).

3. Results

3.1. Selection of *C. jejuni* genes for targeted gene disruption

3.1.1 Background

Based on the previous study from Masanta (2018) and de Vries (2017) we selected potential genes to generate knockout mutants. Our main focus was on genes that presented corresponding proteins differentially regulated after sublethal concentrations of bile acid exposure. The premise of our work is that these genes might be involved in either adaptation processes that result in increased stress resistance and/or play a role for the virulence of the pathogen, e.g. in motility, adhesion and invasion.

3.1.2 Selection of genes

We used three criteria to select the target genes: (i) the corresponding protein is regulated by bile acid exposure with a focus on downregulated proteins (Masanta et al. 2018), (ii) the regulation occurs preferentially in response to Deoxycholic acid (DCA) and/or Chenodeoxycholic acid (CDCA), and (iii) target genes should belong to multiple functional groups, e.g. transporter, surface proteins, signaling factors, enzymes, etc.

Since there is a lack of information about the function of proteins from bacterial pathogens that are down-regulated after bile acid exposure, we selected eight genes, whose corresponding proteins displayed significantly lower expression levels after exposure to at least one of the bile acids CA, CDCA, DCA, GCA, TCA and UDCA (Table 19). However, we also selected a protein (RrF2) that was up-regulated by an extraordinary high number of bile acids (CA, DCA, LCA, CDCA and UDCA) as shown in Table 19. Additionally, we selected a gene without bile acid regulation (*tgt*), that was associated to different physiological processes in a Transposon (Tn) gene inactivation study (de Vries et al. 2017). The gene *tgt* showed a high attenuation in the water survival assays at 4°C and in invasion assays.

Seven of the ten selected genes were regulated by DCA and/or CDCA, which are the bile acids with the highest concentration in the human intestine (20 and 35%, respectively; Baars et al. 2015).

The chosen target genes belong to various functional groups and two of them are annotated as hypothetical proteins (named in this study as Hip82 and Hip12).

Table 19. List of genes to knockout

N°	Name	Gene - ID	BA up: > 1.7x	BA down:> 1.7x	Function
1	Inv	CJJ81176_0708		DCA, GCA	Invasion phenotype protein
2	Sas	CJJ81176_0942		GCA	Sodium symporter
3	HAD2	CJJ81176_1247		TCA, UDCA	HAD-superfamily hydrolase, subfamily IA, variant 1 family protein
4	Maf	CJJ81176_0535		CA, DCA, CDCA, GCA	Maf-like protein CJJ81176_0535
5	tgt	CJJ81176_1028			Queuine tRNA-ribosyltransferase
6	tyrA	CJJ81176_0165		CDCA	Prephenate dehydrogenase
7	YajQ	CJJ81176_0398		DCA, CDCA	UPF0234 protein CJJ81176_0398/YajQ family cyclic di-GMP-binding protein
8	Hip82	CJJ81176_1382		DCA, CDCA	Hypothetical protein CJJ81176_1382
9	Hip12	CJJ81176_0112	LCA, TCA, UDCA	DCA, CDCA	Hypothetical protein CJJ81176_0112
10	RrF2	CJJ81176_0891	CA, DCA, LCA, CDCA, UDCA		RrF2 family protein, putative
C	FlgP	CJJ81176_1045	DCA	CA, TCA	Multicomponent flagellar system/Lipoprotein

The gene *flgP* was chosen for deletion in order to generate a knockout mutant that can serve as control for motility and invasion experiments. FlgP is a component of the flagellar motor and known to be required for motility although is not involved in the flagellar biosynthesis (Sommerlad and Hendrixson 2007). A *flgP* knockout mutant is thus expected to be non-motile and non-invasive.

3.2. Generation of knockout and complementation mutants

3.2.1 Generation and confirmation of knockout in *C. jejuni*

The knockout mutants were generated by double homologous recombination in *C. jejuni* 81-176 by disrupting the target gene through the insertion of a kanamycin resistance cassette.

The kanamycin resistance cassette flanked with 5' and 3' fragments of the target gene was inserted into the digested (*BamHI* and *EcoRI*) pBluescript SKII vector by NEBuilder HiFi DNA Assembly Cloning kit (NEB). The knockout constructs were confirmed by sequencing (SeqLab, Göttingen) using M13 and M13r primers. The confirmed constructs were electroporated into the wild type *C. jejuni* 81-176 and the homologous recombination resulted in the insertion of the kanamycin cassette into the target gene and consequently the disruption of the gene function.

The generated knockout mutants were confirmed by Polymerase chain reaction (PCR) using three different primer combinations, i) forward and reverse primers outside the target gene (Figure 10, A and D), ii) forward primer outside of the target gene and reverse primer inside the kanamycin cassette (Figure 10, A and C) and iii) forward primer inside the kanamycin cassette and reverse primer outside of the target gene (Figure 10, B and D).

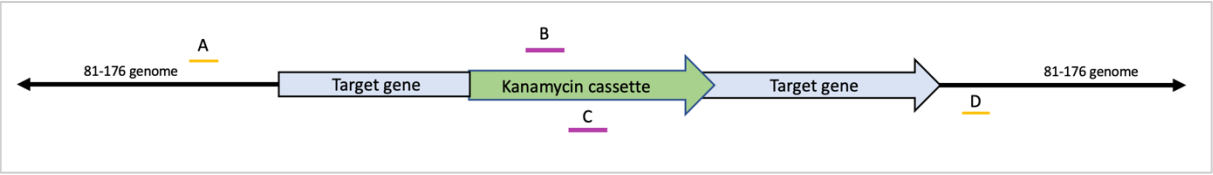

Figure 10. Scheme of primers used for knockout mutant confirmation by PCR. Forward primers A and B, reverse primers C and D. Three combination of primers were used: AD, AC and BD.

The confirmation by PCR was performed using the genomic DNA of the wild type *C. jejuni* 81-176 (Figure 11, lanes 1, 5 and 6) in comparison with the knockout mutants (lanes 2, 3 and 4). The knockout mutants presented a band of increased size using

primer pair AD, due to the insertion of the kanamycin cassette (lane 2). Primer pairs AC and BD produced bands of the expected size.

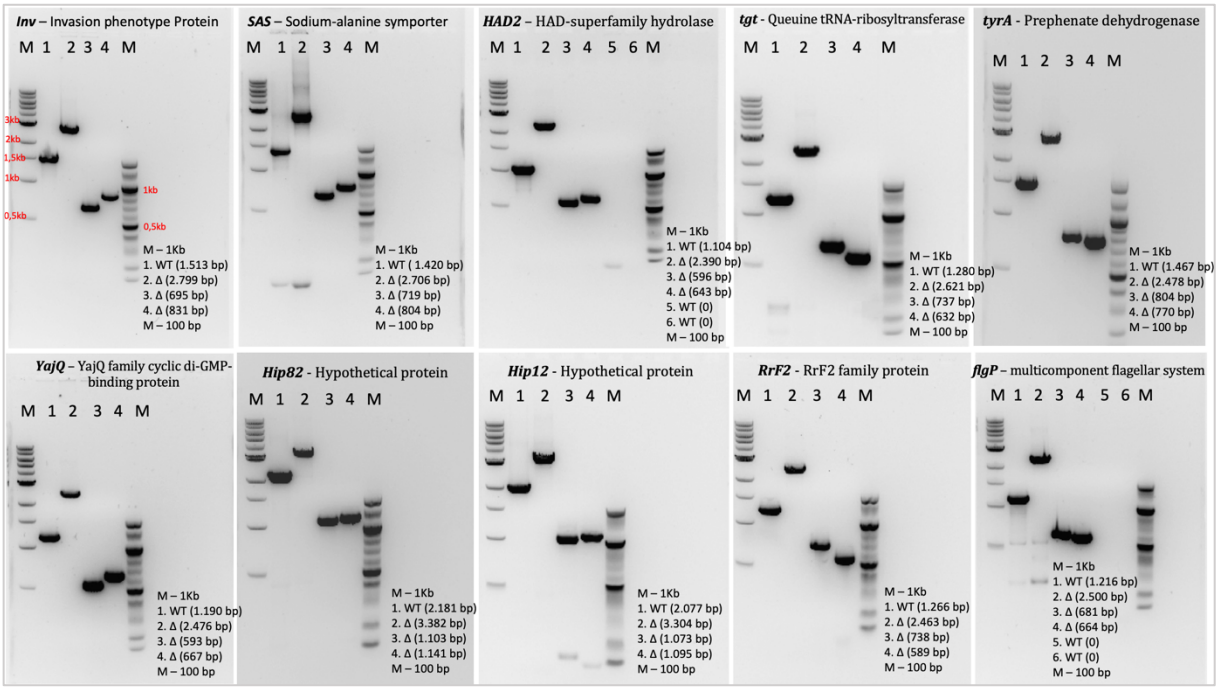

Figure 11. PCR confirmation of knockout mutation. In the sequence from the left to the right, *Δinv, Δsas, Δhad22, Δtgt, ΔtyrA, ΔyajQ, Δhip82, Δhip12, ΔrrF2* and the control mutant *ΔflgP*. DNA ladder of 1 kb (M, left side) and of 100 bp (M, right side) were used as size controls. Lanes 1: fragments amplified from genomic wildtype DNA with gene specific primers "CO_gene-name-F" and "CO_gene-name-R" (outside the target gene, see table 12). Lanes 2: fragments from knockout mutant genomic DNA, amplified with gene specific primers "CO_gene-name-F" and "CO_gene-name-R" (outside the target gene). Lane 3: knockout mutant genomic DNA amplified with primers "CO_gene-name-F" and "CO-Kan-R". Lane 4: knockout mutant genomic DNA amplified with primers "CO-Kan-F" and "CO_gene-name-R". Lanes 5 and 6: wildtype genomic DNA amplified with primers "CO-F" and "CO-Kan-R", and "CO-Kan-F" and "CO-R", respectively. The primers are listed in Table 14.

3.2.2 Complementation

The complementation of the knockout mutants was performed using the pRRC vector, by a double recombinational insertion of an exogenous coding sequence (target gene) linked to a chloramphenicol resistance cassette into one of the three conserved rRNA loci in the knockout mutant.

The complementation confirmation in *C. jejuni* was performed by several PCRs. In the first instance, the primers "CP_gene-name-F" and "CP_gene-name-R" were used, which generated a double band, one is the disrupted allele with kanamycin cassette (larger band), and the second is the novel, intact allele, inserted into the rRNA loci (smaller band with the original gene size). The near identical sequences at the three rRNA loci in *Campylobacter* permit the pRRC-construct plasmid to recombine with any of the three rRNA sites. Since the genes upstream the 16S rRNA are different, the confirmation of the exact location is made by the three forward primers ak233, ak234 and ak235, with the reverse primer ak237. The target gene can be inserted in one or two rRNA loci, as shown in the example in Figure 12.

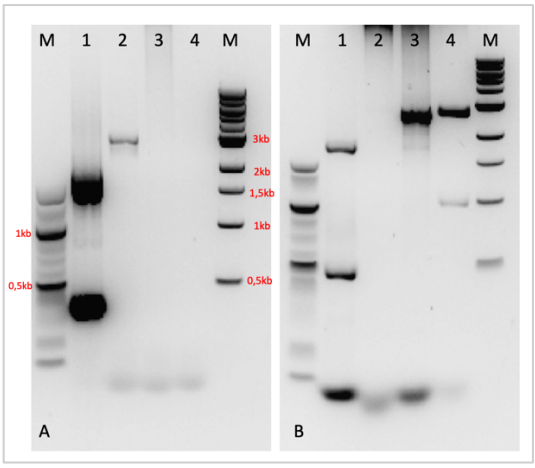

Figure 12. Confirmation PCR for complementation. A) flgP complementation confirmation. B) tyrA complementation confirmation. M - ladder markers of 100 bp and 1 kb; Lane 1 primers "CP-flgP-F" and "CP-flgP-R" (for ΔflgP) and "CP-tyrA-F"and "CP-tyrA-R" (for ΔtyrA); Lane 2 primers ak233 and ak237, Lane 3 primers ak234 and ak237 and Lane 4 primers ak235 and ak237.

From the 10 knockout mutants, eight were successfully complemented, however it was not possible to complement two mutants *Δsas* and *Δmaf*.

3.3. Characterization of knockout mutants

The strains *C. jejuni* 81-176 (reference strain) and the 10 knockout mutants were phenotypically characterized and compared to each other.

3.3.1 Growth curve

The growth curve of all 10 knockout mutants were analyzed in comparison with the parental wild type strain by performing growth kinetics in Mueller Hinton broth (Figure 13). Other parameters such as hours to peak and maximum OD are shown in Table *20*.

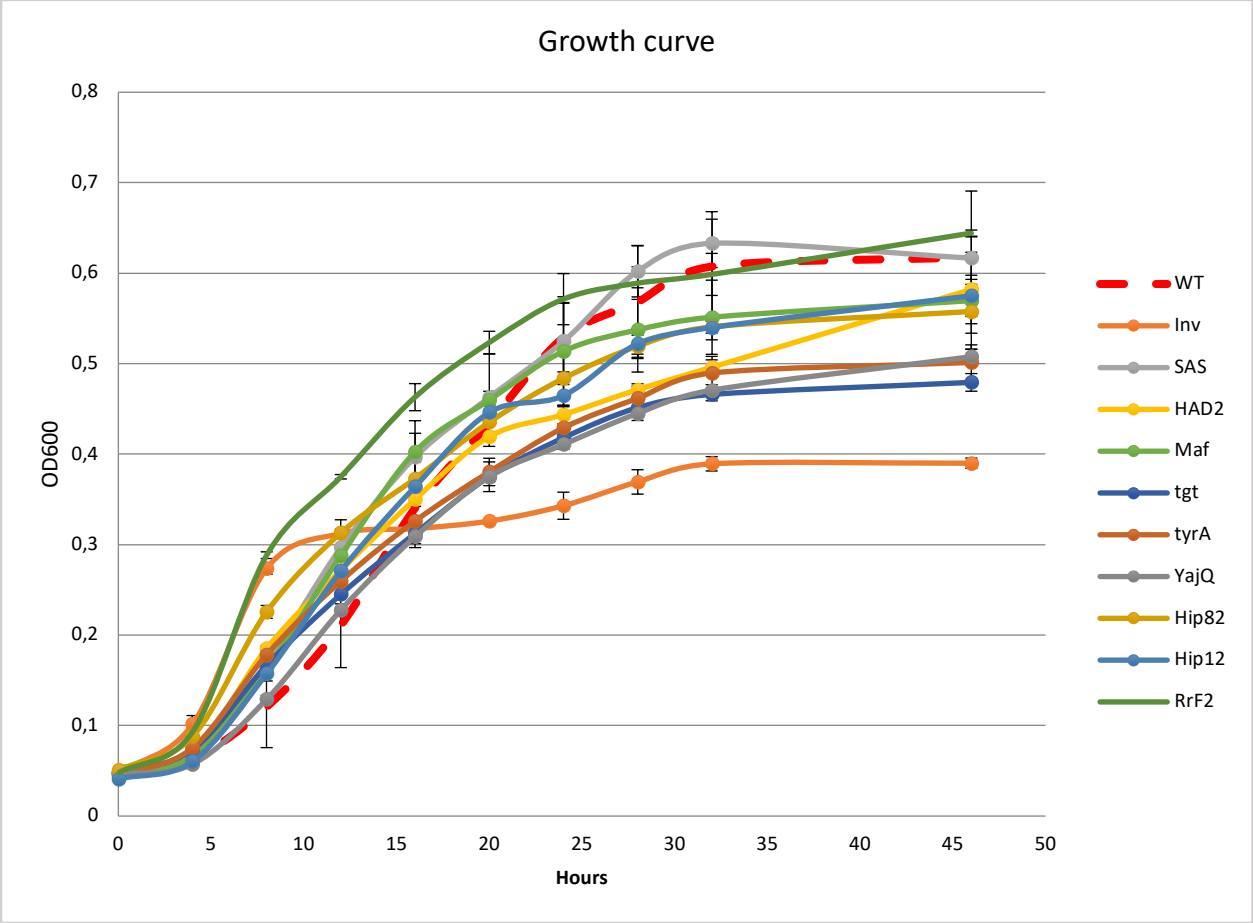

Figure 13. Growth curve. The growth kinetics were performed in MH broth at 37°C, under microaerophilic conditions and 150 rpm shaking. The time points were measured every four hours. The data points represent the means and the standard deviations of two biological triplicates.

The peak of the maximum OD_{600} ranged between 0.39 (*Δinv*) and 0.64 (*ΔrrF2*) in 32 and 46 hours, respectively.

Table 20. Growth curve: hours to peak and maximum OD

Strain	Hours	Max OD
WT	32	0.61±0.06
Δinv	32	0.39±0.008
Δsas	32	0.63±0.03
Δhad2	46	0.58±0.006
Δmaf	32	0.55±0.04
ΔtyrA	32	0.49±0.02
ΔyajQ	46	0.51±0.008
Δhip82	32	0.54±0.007
Δhip12	32	0.54±0.01
ΔrrF2	46	0.64±0.003

3.3.2 Virulence related phenotypic assay

3.3.2.1 Motility

3.3.2.1.1 Soft agar assay

The motility in *C. jejuni* is considered essential in the colonization and establishment of disease in humans. This organism is characterized by its fast movements mediated by bipolar flagella. To analyze the mutant's motility phenotype, we performed the motility soft agar assay to assess the capacity of movement of the knockout mutants compared to the parental strain *C. jejuni* 81-176. The motility assays were performed in Mueller Hinton 0.25% and 0.4% agar, Brucella 0.25% agar (with TTC) and 0.4% agar. Motility was quantified by measuring the diameter of the grown area after 48 hours under microaerophilic conditions (as represented by Figure 14 B).

Functional flagella and motor machinery are required for swarming under every condition tested. First, we analyzed the motility of the control mutant *ΔflgP*, that showed, as expected, a complete loss of motility compared to the wild type 81-176

(Figure 14). Complementation of the knockout mutant *ΔflgP* showed restoration of 64% of the original phenotype (p=0.00013) as shown in Figure 14 A.

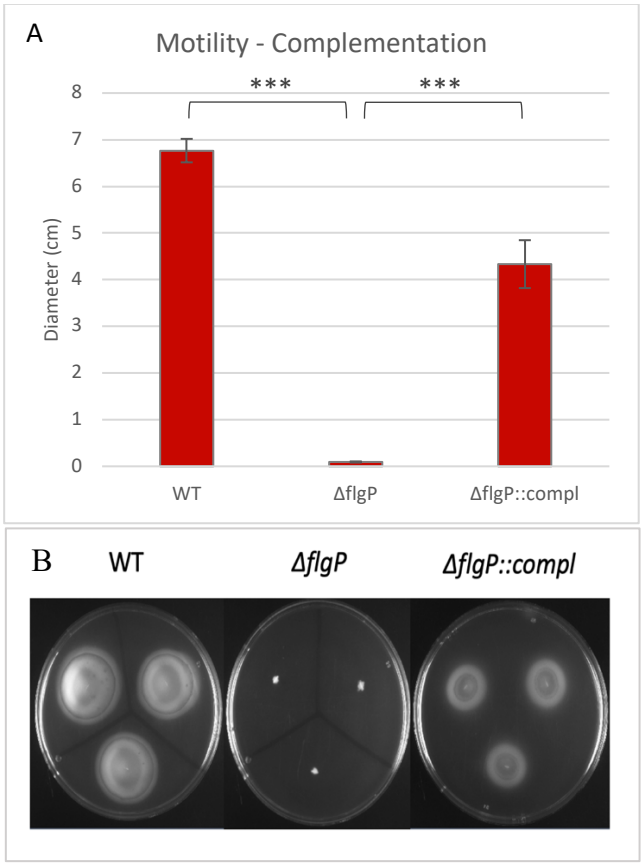

Figure 14. Motility of the *C. jejuni* 81-176 wild type strain and its Δ*flgP* knockout mutant and the Δ*flgP* complementation mutant (A and B). Motility assay performed in Mueller Hinton 0.4% agar plates with the strains grown for 17 hours. In A, the bars represent the diameter (cm) mean ± standard deviation of three technical replicates. In B, representative pictures of the motility grown zones for WT, Δ*flgP* and Δ*flgP*::compl.

The motility assay performed with the knockout mutants were done in two different agar concentration, 0.25% and 0.4% (Figure 15). Moreover, motility in two different media, Mueller Hinton and Brucella was compared (Figure 16). Mutants were considered non-motile when displaying less than 1 cm of grown area. The different agar concentrations and also the different media did not have a major influence on the motility. Almost all the mutants did not show statistically significant differences between agar concentration (WT, p=0.06258) and media (WT, p=0.23666), however,

some mutants were close to the threshold of 1 cm and were considered non-motile. Six knockout mutants, *Δinv*, *Δhad2*, *Δtgt*, *ΔtyrA*, *ΔyajQ* and *ΔrrF2*, presented low (below 1 cm) or none motility in the standard motility assay with 0.4% agar (Figure 15 in blue). However, the six non-motile mutants in MH 0.4% agar presented slight motility (above 1 cm) in other agar concentration and media.

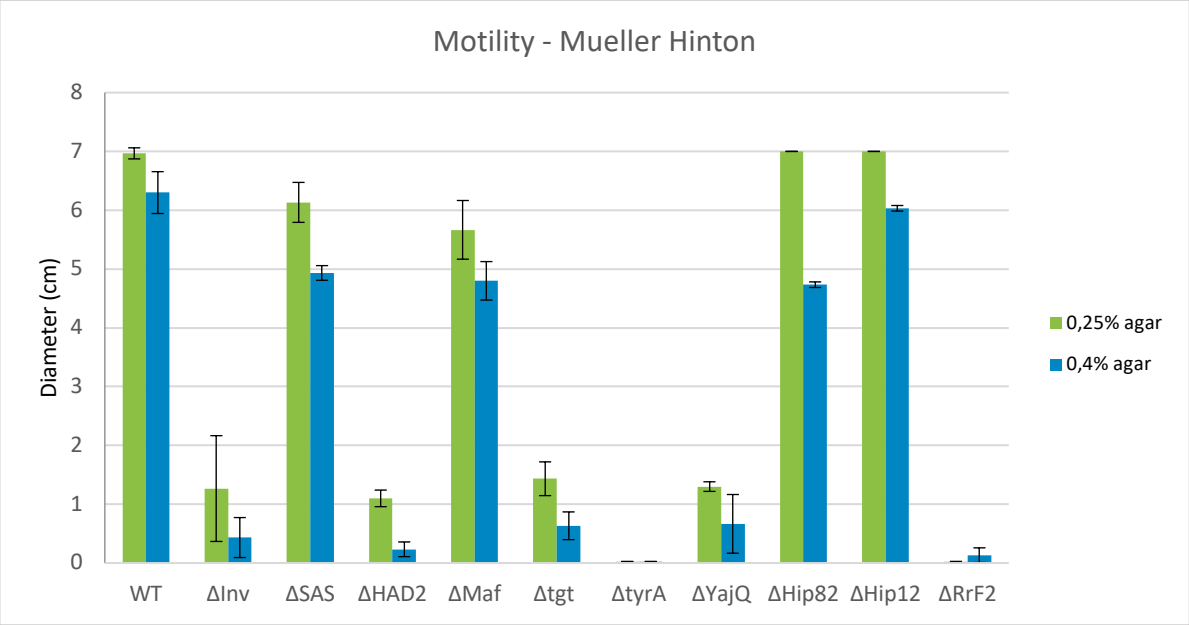

Figure 15. Motility assay with two different agar concentrations (0.25% and 0.4%) in Mueller Hinton grown under microaerophilic conditions for 48 hours. The bars represent the diameter (cm) mean ± standard deviation of three technical replicates.

In Mueller Hinton 0.4% agar (Figure 15), the six mutants that showed pronounced motility phenotype were *Δinv* with 93.1% motility reduction (p= 7.258E-05), *Δhad2* with 96.3% reduction (p= 2.211E-05), Δ*tgt* with reduction of 89.9% in motility (p= 4.740E-05), *ΔtyrA* with reduction of 99.7% (p= 1.532E-05), *ΔyajQ* with 89.4% motility reduction (p= 0.00020) and *ΔrrF2* with motility reduction of 97.9% (p= 2.072E-05) compared to the wild type *C. jejuni* 81-176. The other four mutants, three of them presented a slight motility reduction of 21.7% in *Δsas* (p=0.00686), 23.8% reduction in *Δmaf* (p=0.01176) and 24.9% reduction in *ΔHip82* (p=0.0350). The only knockout

mutant that did not present statistically significant motility reduction was *ΔHip12* (p=0.352).

Five knockout mutants, *Δinv*, *Δhad2*, *Δtgt*, *ΔtyrA* and *ΔyajQ* that were considered non-motile in MH 0.4% (Figure 15) showed a discrete motility in Brucella 0.4% (Figure 16), indicating a possible phenotype related to the media.

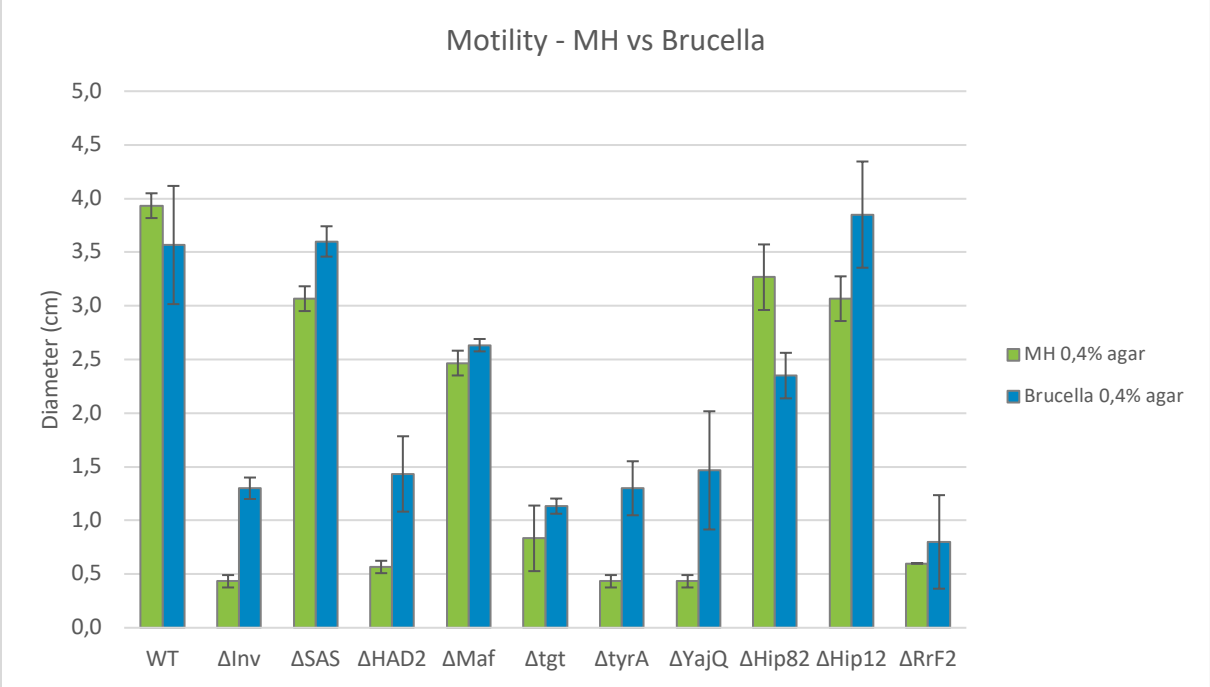

Figure 16. Motility assay performed with two different media (Mueller Hinton and Brucella) with 0.4% agar concentration. The strains were grown for 17 hours under microaerophilic condition and were diluted to OD_{600}=0.025 and stabbed into the plates and incubated for 48 hours under microaerophilic conditions. The bars represent the diameter (cm) mean ± standard deviation of three technical replicates.

C. jejuni needs to survive in different ecological niches and it might be possible that the extent of motility is influenced by the specific conditions of the respective habitat. It was subsequently investigated whether *C. jejuni* mutants that passed a recent intracellular period within Caco2 cells, displayed an altered motility. A motility assay was thus performed from *C. jejuni* that successfully invaded Caco2 cells and that were recovered by lysis of this cell line. (Figure 17). This motility assay displayed differences in four knockout mutants, when compared to the previous, standard motility assay.

Two mutants regained motility, namely Δ*tyrA* and *ΔrrF2*. The motility zone for the ΔtyrA mutant was increased from 0.3 cm to 2.6 cm increment of 8.7-fold; p=3.757E-06) and the *ΔrrF2* mutant from 0.4 cm to 4.3 cm (increment of 10.75-fold, p=4.78E-05). Two mutants presented a reduction of motility after invasion. The motility zone for the *Δhip82* mutant was reduced in average from 3 cm to 0.57 cm (reduction of 81%, p= 5.3E-05) and that of the *Δhip12* was reduced from 4.53 cm to 1.2 cm in average (reduction of 73.5%, p=0.00032).

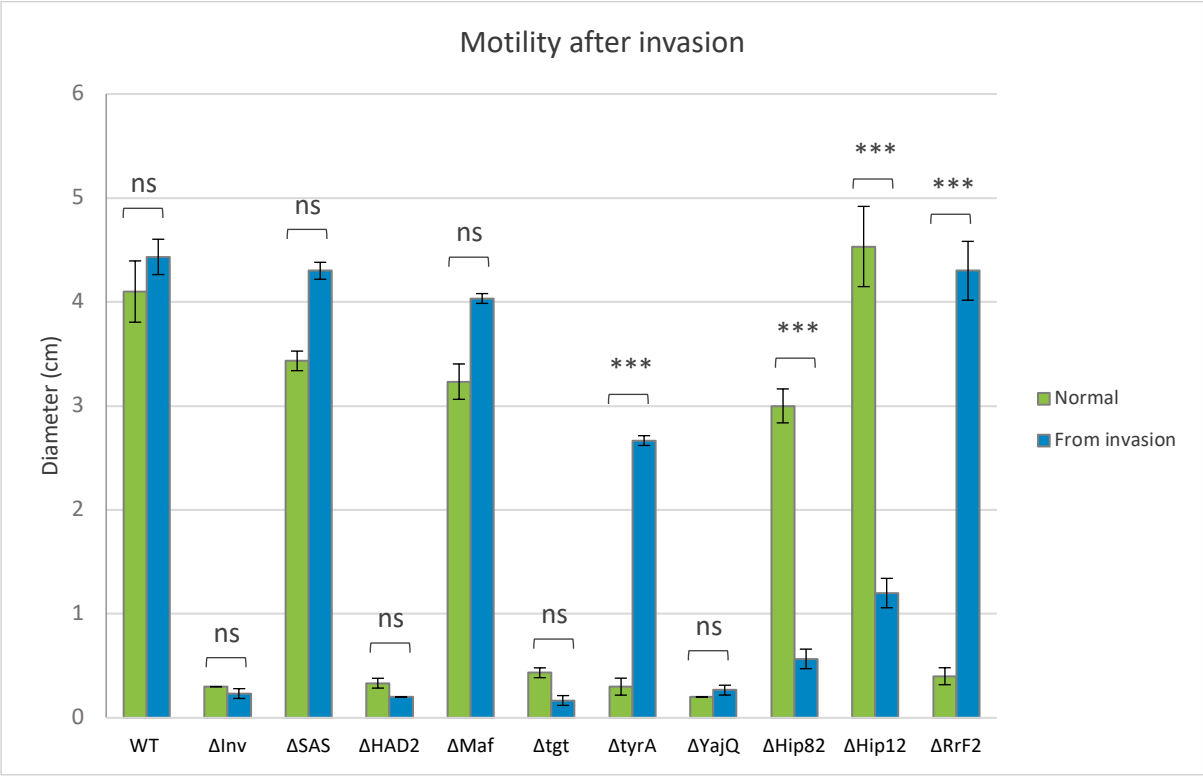

Figure 17. Motility assay performed with strains recovered after Invasion assay. Motility performed in Mueller Hinton 0.4% agar concentration. The strains grown after invasion, were resuspended and diluted to OD_{600}=0.025 and stabbed into the plates and incubated for 48 hours under microaerophilic conditions. The bars represent the diameter (cm) mean ± standard deviation of two biological triplicates. Two-sided unpaired Student t-test, ns: not significant; *p≤0.05; **≤0.01 and ***≤0.001.

From the six knockout mutants that presented pronounced reduced motility phenotype, only in the *ΔyajQ* mutant the original phenotype was restored after complementation with an increase of 85.3% of the wild type level (p=0.0059), as shown

in Figure 18. Except for *Δinv-compl* (p=2.303E-05), the other complemented mutants did not present significantly increased motility when compared to the respective knockout mutants. When both, mutant and complementation difference, are compared to the WT, *Δinv* showed an increase of 16.9%, *Δhad2* with an increase of 3% (p=0.0723), *Δtgt* and *ΔtyrA* with 6.5% increased (p=0.103 and p=0.183, respectively), and *ΔrrF2* with an increase of 3.5% (p=0.142).

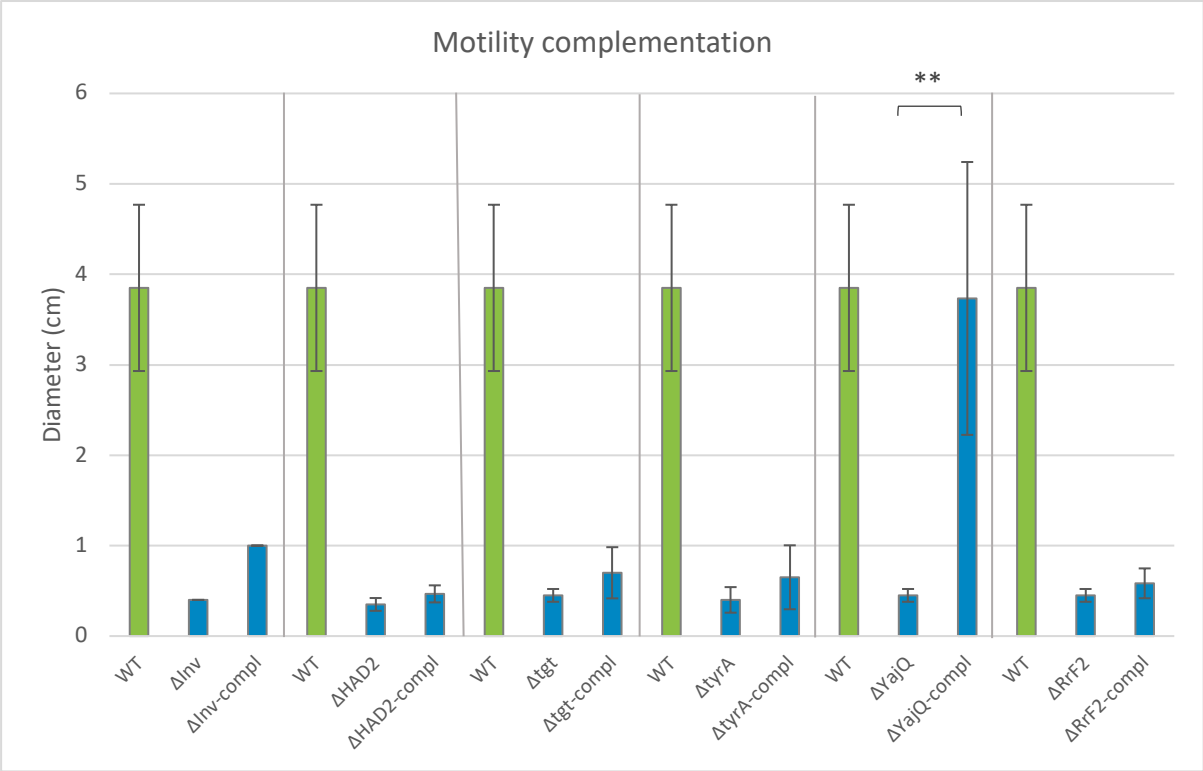

Figure 18. Motility assay for six knockout mutant complementation. In green the wild type and in blue the knockout mutant and its complementation. Assay performed in Mueller Hinton 0.4% agar concentration. The bacteria were grown for 17 hours under microaerophilic condition and were diluted to OD_{600}=0.025 and stabbed into the plates and incubated for 48 hours under microaerophilic conditions. The bars represent the diameter (cm) mean ± standard deviation of three technical replicates. Two-sided unpaired Student t-test, **≤0.01.

3.3.2.1.2 TTC motility assay

A tube-based motility assay that uses TTC as a staining technique to visualize the metabolic activity of bacteria was also applied to monitor the motility phenotype of the mutants. The TTC motility assay was performed in 15 ml Falcon centrifuge tubes filled with Brucella 0.25% agar, supplemented with TTC.

The TTC motility assay showed increased motility for two knockout mutants that previously presented lower motility in soft agar, *Δtgt* and *ΔrrF2* (Figure 19). The *Δtgt* mutant represents 37.5% of the wild type motility and the *ΔrrF2* mutant represents 70.5% of wild type motility level. In the previous motility assay, the *Δtgt* displayed only 10% and *ΔrrF2* 2.1% of wild type motility level (Figure 15, 0.4% agar).

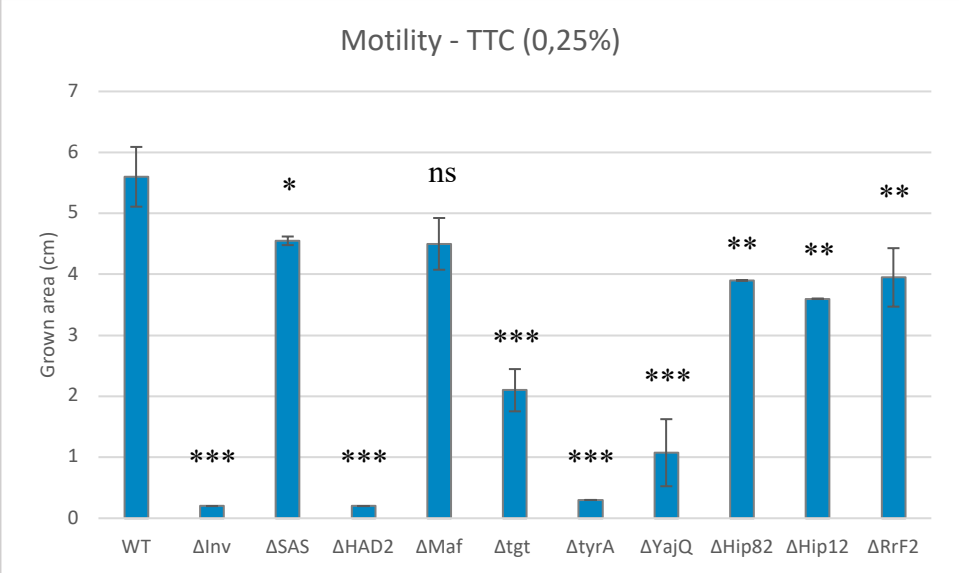

Figure 19. TTC motility performed in 15 ml tubes with Brucella 0.25% agar supplemented with 100 µg/ml TTC. The strains were grown for 17 hours under microaerophilic condition and were resuspended, and optical density adjusted to OD_{600}=1. 50µL of the bacterial suspension was added to the top of the medium and incubated for 48 hours under microaerophilic conditions with lid open. The bars represent the diameter (cm) mean ± standard deviation of three technical replicates. Two-sided unpaired Student t-test, ns: not significant; *p≤0.05; **≤0.01 and ***≤0.001.

3.3.2.1.3 Microscopy based motility assessment

In addition to the previously described assays, motility was also assessed by phase contrast microscopy. The knockout mutants were qualitatively compared to the wild type strain and to the non-motile *ΔflgP* control mutant. Wild type *C. jejuni* were motile and presented fast movement in liquid media, however, the *ΔflgP* mutant that has a mutation in an important motility component is totally static. The knockout mutants

were classified in non-motile ("N", such *ΔflgP*), intermediate (represented with M- or M, in Table 21) and motile (M+, such WT).

Table 21. Microscopic motility assessment of knockout mutants in MH broth.

	Microscopy
WT	M +
Δinv	M +
Δsas	M -
Δhad2	M
Δmaf	M
Δtgt	M +
ΔtyrA	M +
ΔyajQ	M +
Δhip82	M +
Δhip12	M -
ΔrrF2	M
ΔflgP	N

All the six knockout mutants that presented a pronounced reduction in the motility showed low or normal motility under microscopic assessment. The mutants *Δinv*, *Δtgt*, *ΔtyrA* and *ΔyajQ* presented similar motility and speed as the wild type. However, the mutants *Δsas* and *Δhip12* that presented normal or a minor motility reduction in soft agar, showed reduced motility in the microscopic assay.

A B C

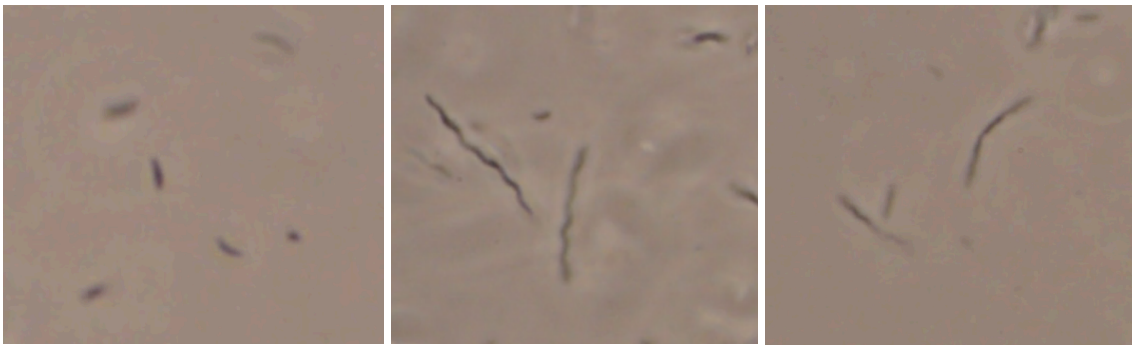

Figure 20. Phase contrast microscopy of the knockout mutants. Magnification of 63x. A) wild type; B) *Δhip12* mutant and C) *ΔrrF2*.

During the microscopic motility assessment an increased size of the mutant *Δhip12* and *ΔrrF2* was detected. These two knockout mutants showed a double or triple size bigger when compared to the WT (Figure 20).

All the motility phenotype observed in the applied motility assays are summarized in Table 22.

Table 22. List of mutants with motility phenotype.

Strains	0.4% agar (M.H.)	0.4% agar (Brucella)	0.25% agar (M.H.)	0.25% agar (Brucella)	Microscopy
ΔInv	N	M-	M-	N	M +
ΔHAD2	N	M-	M-	N	M
Δtgt	N	M-	M-	M-	M +
ΔtyrA	N	M-	N	N	M +
ΔYajQ	N	M-	M-	M-	M +
ΔRrF2	N	N	N	M	M
ΔflgP	N	N	N	N	N

3.3.2.2 Gentamycin Protection assay

3.3.2.2.1 Invasion

To establish the invasion protocol, we tested different mediums, DMEM and HBSS, with and without centrifugation step, using multiplicity of infection (MOI) of 10, 20 and 30. The media, centrifugation and MOI did not show any significant influence on the wild type invasion level, not exceeding more than 2% difference (data not shown). The invasion was performed by gentamycin protection assay (GPA) in DMEM, with centrifugation after the bacterial inoculation into the eukaryotic cell line and with a MOI of 10.

The control mutant *ΔflgP* showed as expected very low invasion into Caco2 cell of 0.005% compared to the WT with 0.56% (p=3.716E-06) (Figure 21). The complementation of *ΔflgP* restored the invasion phenotype to 1.3% (p=0.00012).

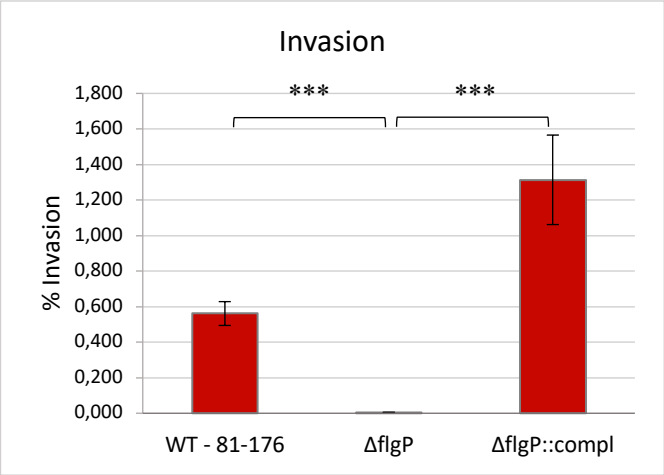

Figure 21. Invasion assay for control mutant *ΔflgP* and its complementation. The invasion assay was performed on Caco2 cells at 37°C and with a multiplicity of infection (MOI) of 10. The invasion values were calculated as a percentage of the CFU of recovery bacteria that were added to the experiment. The bars represent the means ± standard deviation of two biological triplicates. Two-sided unpaired Student t-test, ***≤0.001.

Interestingly, the previous six mutants that showed a strong motility phenotype and in addition *Δhip82*, also presented a highly increased invasion phenotype (Figure 22, red arrows). *Δinv* showed a mean of 14.1% (p=0.0001), *Δhad2* showed a mean of 15.8% (p=0.0033), *Δtgt* had a mean of 11.2% (p=0.0011), *ΔtyrA* presented a mean of 11.1% (p=0.0016), *ΔyajQ* presented a mean of 12.8% (p=0.000283), *Δhip82* presented a mean of 9.6% (p=0.0245) and *ΔrrF2* presented a mean of 11.7% (p=5.951E-05).

Δmaf and *Δhip12* did not present statistically significantly increased invasion rates, with the mean of 1.02% (p=0.789) and 1.07% (p=0.976), respectively. The only mutant that presented lower invasion rate was *Δsas* with a mean of 0.3% invasion (p=0.000144).

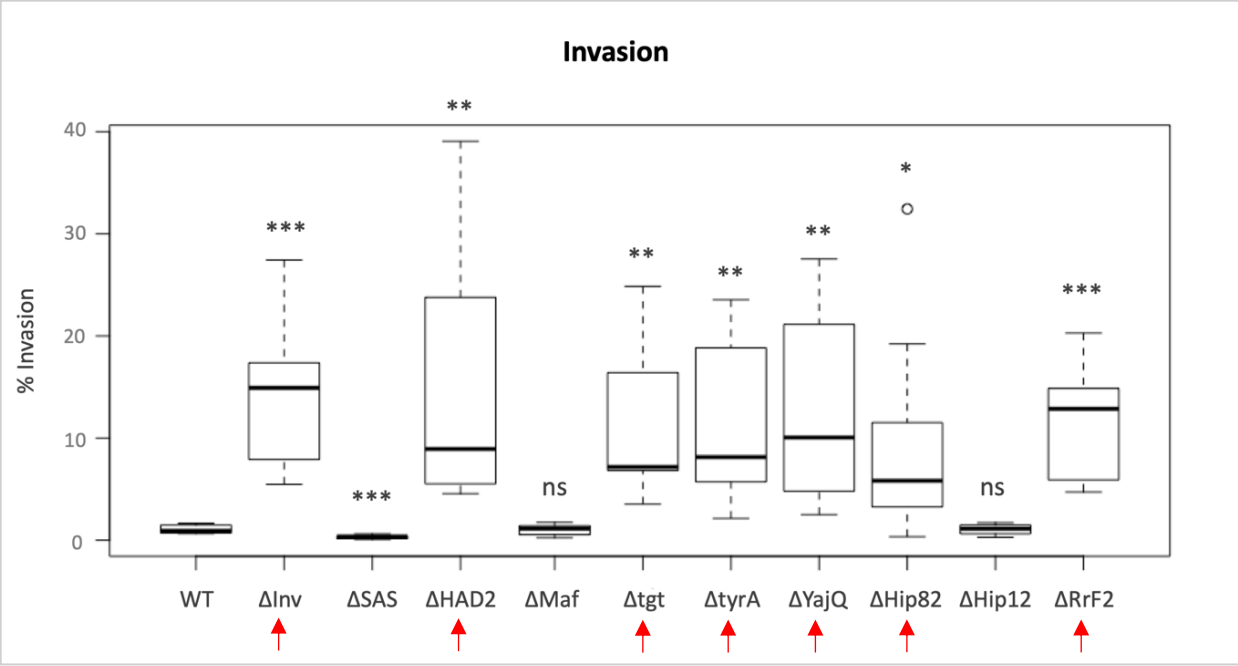

Figure 22. Invasion assay by Gentamycin protection assay. The invasion assay was performed on Caco2 cells at 37°C and with a multiplicity of infection (MOI) of 10. The invasion values were calculated as a percentage of the CFU of recovery bacteria that were added to the experiment. The bars represent the means ± standard deviation of nine biological triplicates. Two-sided unpaired Student t-test, **ns**: not significant; *p≤0.05; **≤0.01 and ***≤0.001. Red arrows indicate the knockout mutants with high invasion phenotype.

3.3.2.2.2 COS-7 cell line

The invasion assay was also performed with a second eukaryotic cell line, COS-7, to exclude phenotypes related to the cell line used (Caco2). The COS-7 line is a fibroblast-like cell line, derived from monkey kidney tissue.

The wild type presented higher invasion rates in COS-7 (2.9%) compared to Caco2 cells (1.1%). The same is observed for *Δinv* and *ΔyajQ* with 32.6% and 25.3% invasion rates, respectively in COS-7, instead of 14.1% and 12.8% in Caco2 cells. However, *Δhad2* and *ΔrrF2* presented similar invasion levels in COS-7 (13.7% and 13.8%, respectively) compared to Caco2 cells, with 15.8% and 11.7%. Once the differences between COS-7 and Caco2 were not significant for the WT, we decided to perform the adherence and invasion assays with Caco2 cell line further on.

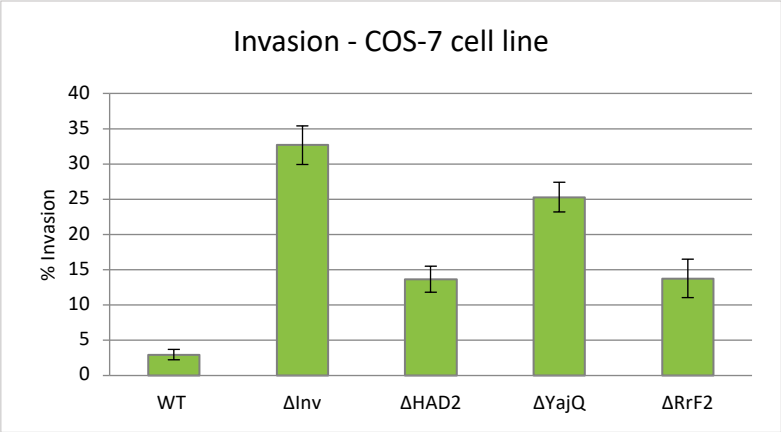

Figure 23. Invasion assay by Gentamycin protection assay performed with COS-7 cell line. The invasion values were calculated as a percentage of the CFU of recovery bacteria that were added to the experiment. The bars represent the means ± standard deviation of three technical replicates.

3.3.2.2.3 Adhesion

The adhesion capacity to Caco2 cell was performed in parallel to the invasion assay, since the adhesion needs the invasion percentage to be calculated.

The adhesion presented similar behavior compared to the invasion (Figure 24). The same seven high invasion phenotype mutants also presented high adhesion rates in Caco2 cell. The knockout mutants *Δinv* and *Δtgt* revealed higher adhesion rates compared to the invasion rate. The *Δinv* mutant showed 34% adhesion and 16.3% invasion (p=0.0092), and although statistically not significant, *Δtgt* showed 34.4% for adhesion and 24.9% for invasion (p=0.058).

Comparing the adhesion of the knockout mutants to the reference strain, all except *Δmaf*, presented statistical significance. The seven mutants that presented high adhesion rates were *Δinv* with 34% adhesion (p=0.0002), *Δhad2* with 16.1% (p=0.0014), *Δtgt* with 34.3% (p=0.005), *ΔtyrA* with 8.5% (p=0.0003), *ΔyajQ* with 19.6% (p=0.0046), *Δhip82* with 3.9% (p=0.023) and *ΔrrF2* with 11.9% of adhesion (p=0.0019). The mutants *Δsas* and *Δhip12* presented reduced adhesion of 0.6% (p=0.003) and 0.7% (p=0.027), respectively compared to the WT with 1.8%.

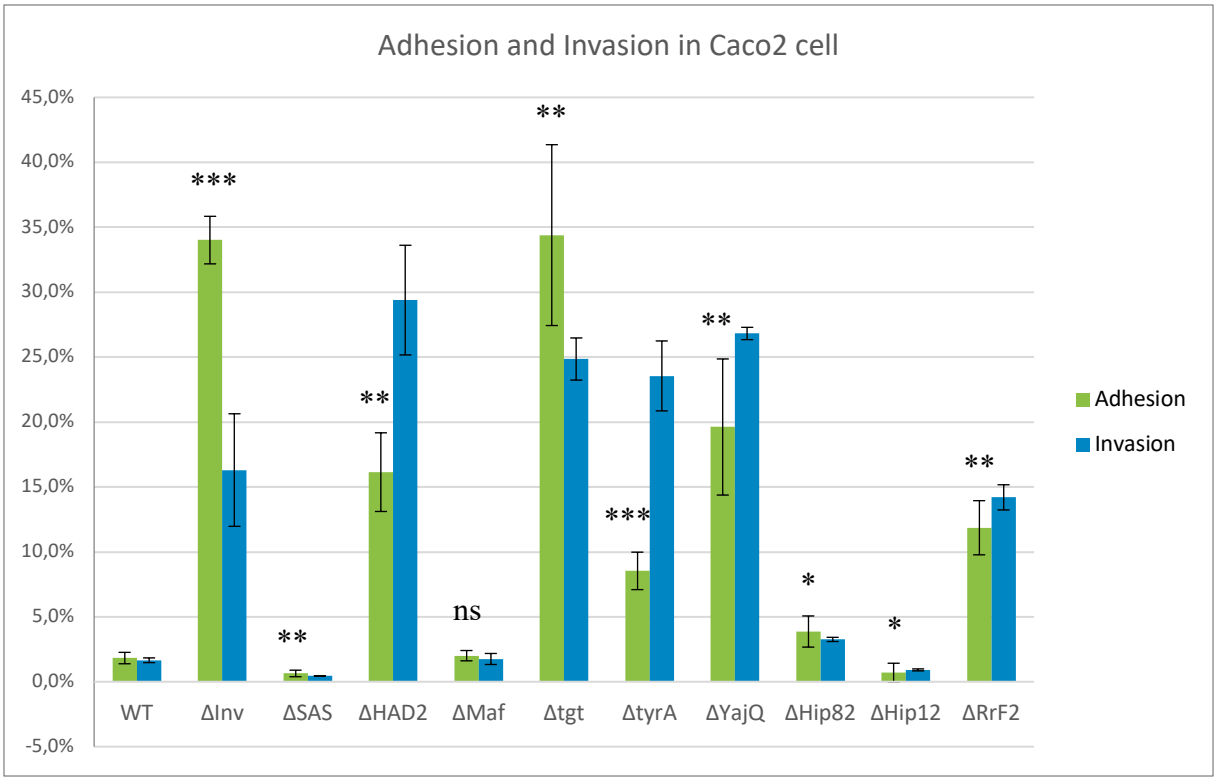

Figure 24. Adhesion and invasion by Gentamycin protection assay. The adhesion and invasion assays were performed on Caco2 cells at 37°C with a multiplicity of infection (MOI) of 10. The adhesion values were calculated as a percentage of the recovered bacteria that were added to the experiment subtracted by the invasion percentage. The bars represent the means ± standard deviation of two biological triplicates. Two-sided unpaired Student t-test, **ns**: not significant; *p≤0.05; **≤0.01 and ***≤0.001, statistical information for adhesion compared to the WT.

3.3.3 Autoagglutination

The autoagglutination assay was performed for all knockout mutants and it was measured by the difference between the input OD_{600}=1 to the OD_{600} after 24 hours in PBS. The wild type presented an autoagglutination of 85% and only *ΔrrF2* presented a statistically significant reduction of 59% autoagglutination (p=0.00168) (

Figure **25**).

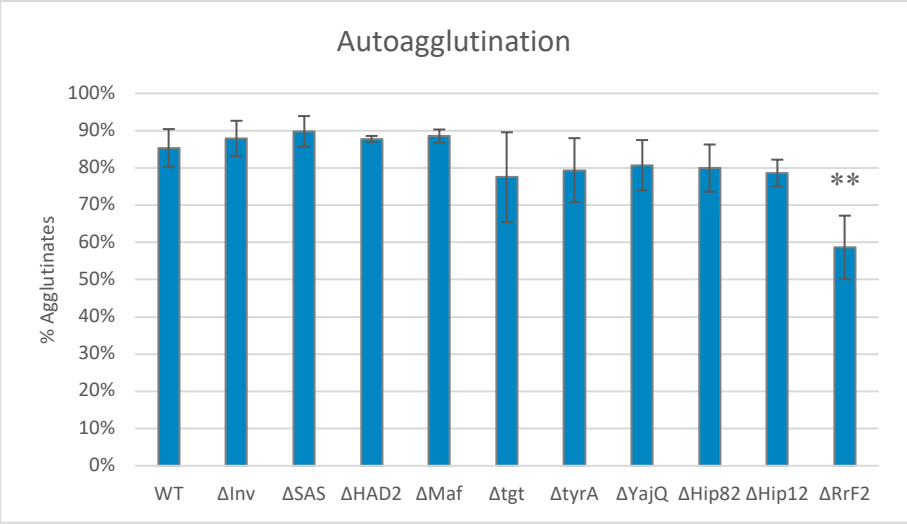

Figure 25. Autoagglutination assay, represented by the autoagglutination percentage of input OD and supernatant OD after 24 hours. The bars represent the means ± standard deviation of three biological quadruplicates. Two-sided unpaired Student t-test, **≤0.01.

Additionally, the autoagglutination of the complementation mutant for the knockout mutant *ΔrrF2* was tested. The complementation could restore the original phenotype from 65% to 76% (p=8.771E-08).

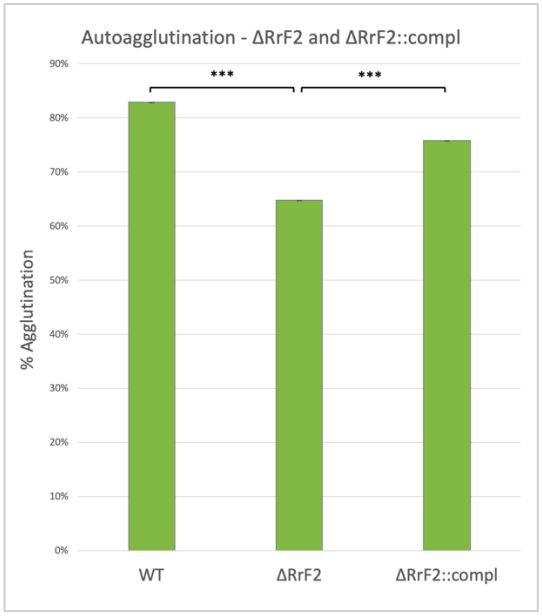

Figure 26. Autoagglutination of *ΔRrF2* and its complementation. The bars represent the means ± standard deviation of two biological quadruplicates. Two-sided unpaired Student t-test, ***≤0.001.

3.3.4 Biofilm formation

3.3.4.1 Crystal violet

To analyze variations on biofilm formation, we tested the biofilm formation capacity to polystyrene (96 well plates) by crystal violet staining. The quantitative analysis of biofilm formation of the knockout mutants showed that the control mutant *ΔflgP* presented a low biofilm formation with a crystal violet absorbance from 0.016 (WT) to 0.0014 (p=0.049), corresponding to a reduction of 91.25% (Figure 27). The complementation restored the original phenotype with a 19.3-fold increase compared to the *ΔflgP* mutant (p=0.0385).

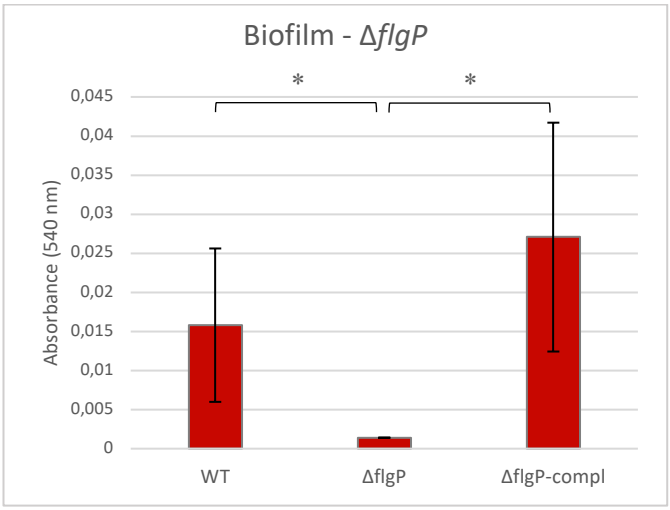

Figure 27. Biofilm formation for the control mutant ΔflgP and its complementation. The bacteria were incubated for 48 hours in 96 well plates under microaerophilic conditions. The biofilms were stained with crystal violet and the absorbance at 540nm was measured. Bars represent the means ± standard deviation of three biological triplicates. Two-sided unpaired Student t-test, *p≤0.05.

The quantitative measurement of biofilm formation revealed eight knockout mutants with an increased biofilm formation (*Δinv*, *Δhad2*, *Δmaf*, *Δtgt*, *ΔtyrA*, *ΔyajQ*, *Δhip82* and *ΔrrF2*). Two mutants showed low levels of biofilm formation (*Δsas* and *Δhip12*). When compared to the reference strain, *ΔInv* showed 5.2-fold higher levels of biofilm formation (p=5.095E-09), *Δhad2* showed 4.2-fold levels (p=4.435E-20), *Δmaf* 1.7-fold

(p=0.0019), *Δtgt* 4-fold (p=6.749E-14), *ΔtyrA* 5.7-fold (p=4.498E-22), *ΔyajQ* 4.1-fold (p=2.438E-07), *Δhip82* 7.4-fold (p=1.686E-24) and *ΔrrF2* 3.6-fold (p=2.345E-11).

In contrast, *Δsas* and *Δhip12* were not able to consistently form biofilms and were easily removed during the washing step. Compared to the WT, *Δsas* showed a reduction of 12% (p=0.204) and *Δhip12* showed 1.8-fold increment, however values were statistically not significant (p=0.079) (Figure 28).

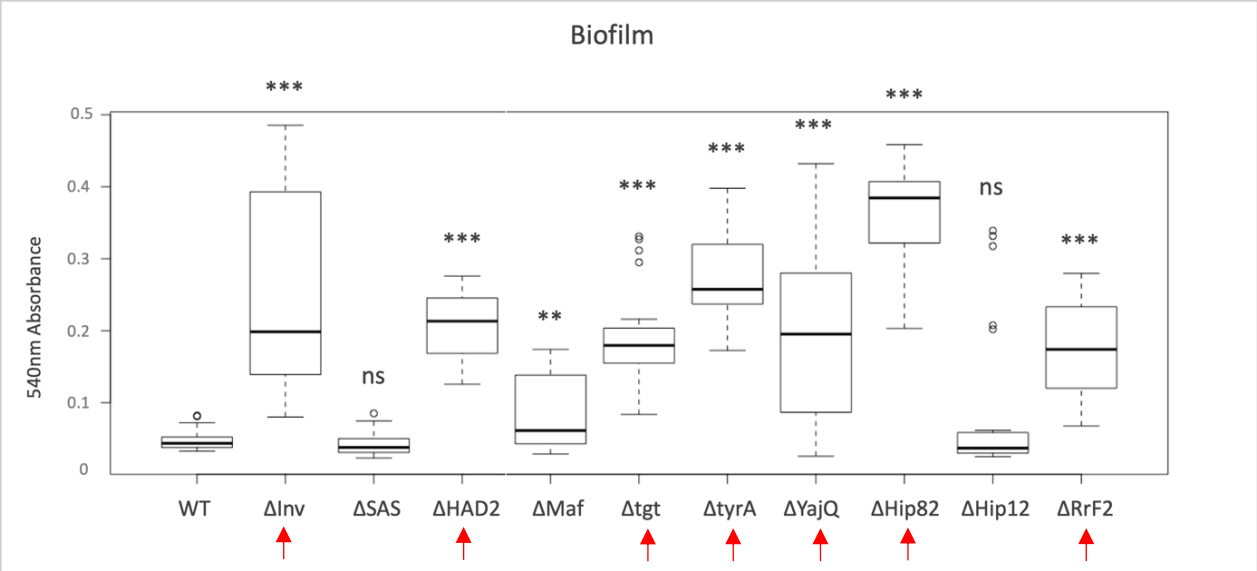

Figure 28. Biofilm formation for knockout mutants. The strains were incubated for 48 hours in 96 well plates under microaerophilic conditions. The biofilms were stained with crystal violet and the absorbance was measured at 540nm Bars represent the means ± standard deviation of four biological quadruplicates. Two-sided unpaired Student t-test, **ns**: not significant; *p≤0.05; **≤0.01 and ***≤0.001.

A qualitative microscopic analysis of biofilm formation after 15 minutes incubation in crystal violet confirmed the robust capacity of the eight mutants to strongly attach to the polystyrene (Figure 29). Corroborating to the quantitative measurements, the Figure 29 shows the reference strain, the control mutant *ΔflgP*, the *Δsas* and *Δhip12* with low biofilm attachment to the polystyrene surface and subsequently low crystal violet staining.

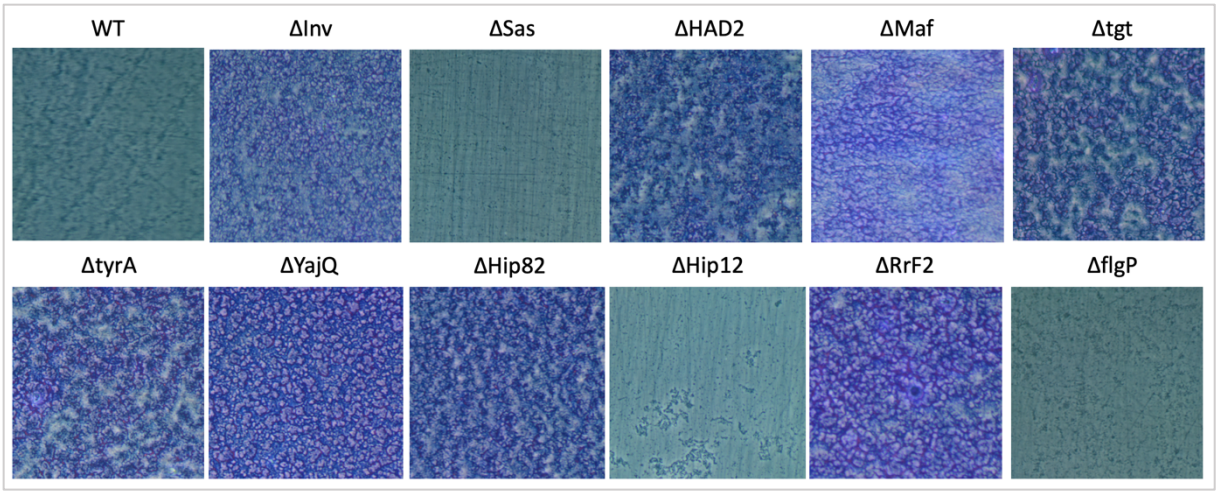

Figure 29. Imaging of biofilm attached to the polystyrene surface. At 10x magnification. The pictures were taken after the 15 minutes incubation in crystal violet and two washing steps.

3.3.4.2 Bile acid effect on biofilm formation

To investigate the effect of bile acids on the biofilm formation in the knockout mutants, the assay was performed with an addition of 1 mM DCA in the incubation media.

Except for the wild type, all mutants presented a reduced biofilm forming capacity when exposed to DCA during the incubation of 48 hours. Any strain tested presented statistical significance, however, *Δinv*, *Δsas* and *Δmaf* presented $p<0.07$. When exposed to DCA the reference strain presented an increment of 4.5-fold ($p=0.13$). In contrast, we observed a reduction for *Δinv* (95%, $p=0.068$), *Δsas* (88%, $p=0.057$), *Δhad2* (94%, $p=0.182$), *Δmaf* (88.7%, $p=0.063$), *Δtgt* (93.7%, $p=0.168$), *ΔtyrA* (29.9%, $p=0.53$), *ΔyajQ* (72.6%, $p=0.2$), *Δhip82* (66.5%, $p=0.153$), *Δhip12* (87.5%, $p=0.12$) and *ΔrrF2* (32.3%, $p=0.56$).

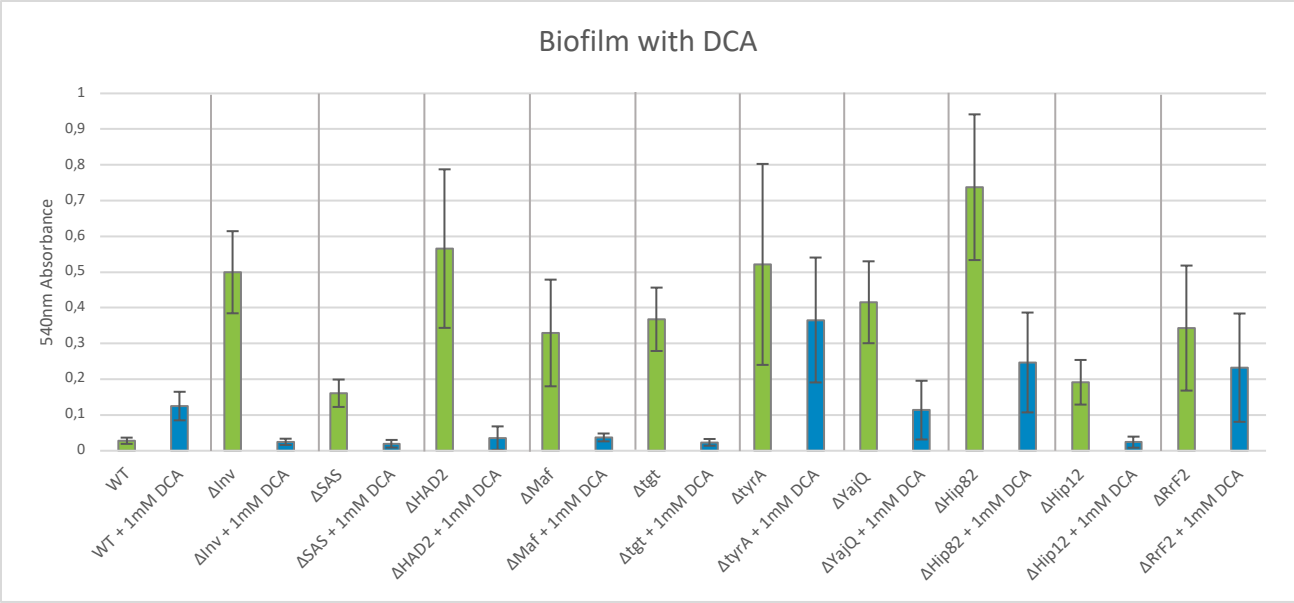

Figure 30. Biofilm formation with effect of the bile acid DCA. The strains were incubated for 48 hours with 1 mM DCA in 96 well plates under microaerophilic conditions. The biofilms were stained with crystal violet and the absorbance was measured at 540nm. Bars represent the means ± standard deviation of two biological quadruplicates. Two-sided unpaired Student t-test.

3.3.4.3 Effect of oxygen on biofilm formation

A biofilm is considered to be a persistent state that forms in challenging environments. Normal levels of oxygen can affect *Campylobacter* behavior and the biofilm might be a mechanism of survival under these conditions. To assess the effect of oxygen on the biofilm formation, we incubated the bacterial suspension on 96 well plates under normal atmosphere.

The biofilm formation in presence of regular oxygen levels was higher in the wild type with an increase of 1.4-fold (p=0.0328). The same increment was observed, but without significance in *Δtgt* (1.1-fold, p=0.758), *Δhip82* (1.4-fold, p=0.129), *ΔrrF2* (1.2-fold, p=0.301) and with significance in *Δhip12* with 2.6-fold (p=0.0183). In contrast, we observed a decrease in biofilm formation without significance in the mutants *Δinv* (0.8-fold, p=0.260), *ΔtyrA* (0.8-fold, p=0.388), *ΔyajQ* (0.9-fold, p=0.869), and with significance in the mutants *Δsas* (0.7-fold, p=0.015), *Δhad2* (0.6-fold, p=0.026) and *Δmaf* (0.5-fold, p=0.0013).

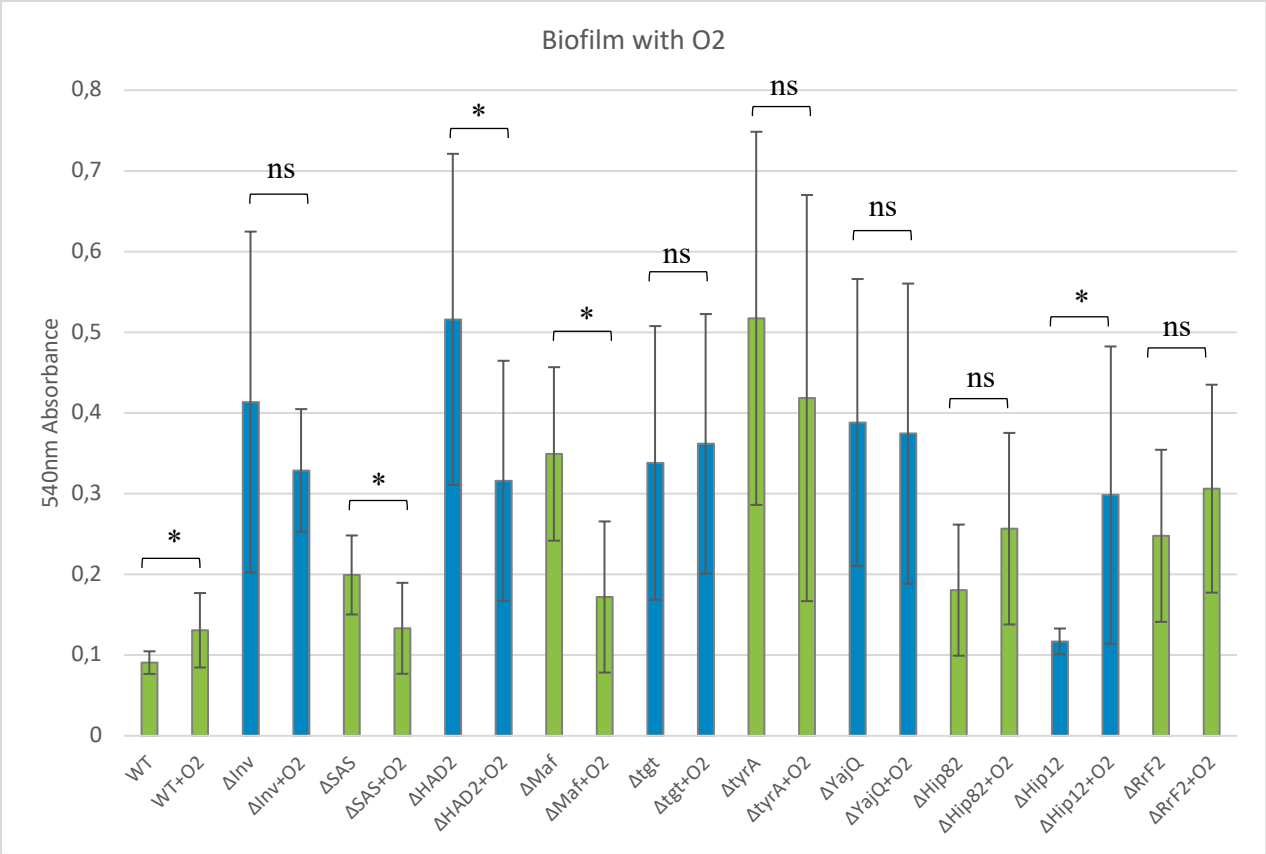

Figure 31. Effect of normal atmospheric conditions on Biofilm formation. The strains were incubated for 48 hours in 96 well plates under normal and microaerophilic conditions. The biofilms were stained with crystal violet the absorbance was measured at 540nm. Bars represent the means ± standard deviation of two biological quadruplicates. Two-sided unpaired Student t-test, **ns**: not significant; ***p≤0.05.

3.3.5 Stress assays

3.3.5.1 Temperature

To analyze the effect of increased temperature in the knockout mutants, we exposed the strains at 52°C for 1 hour.

This temperature let to a moderate CFU decrease in the wild type. Mutants that displayed a larger effect than the WT were *ΔyajQ* and ΔrrF2 (Figure 32).

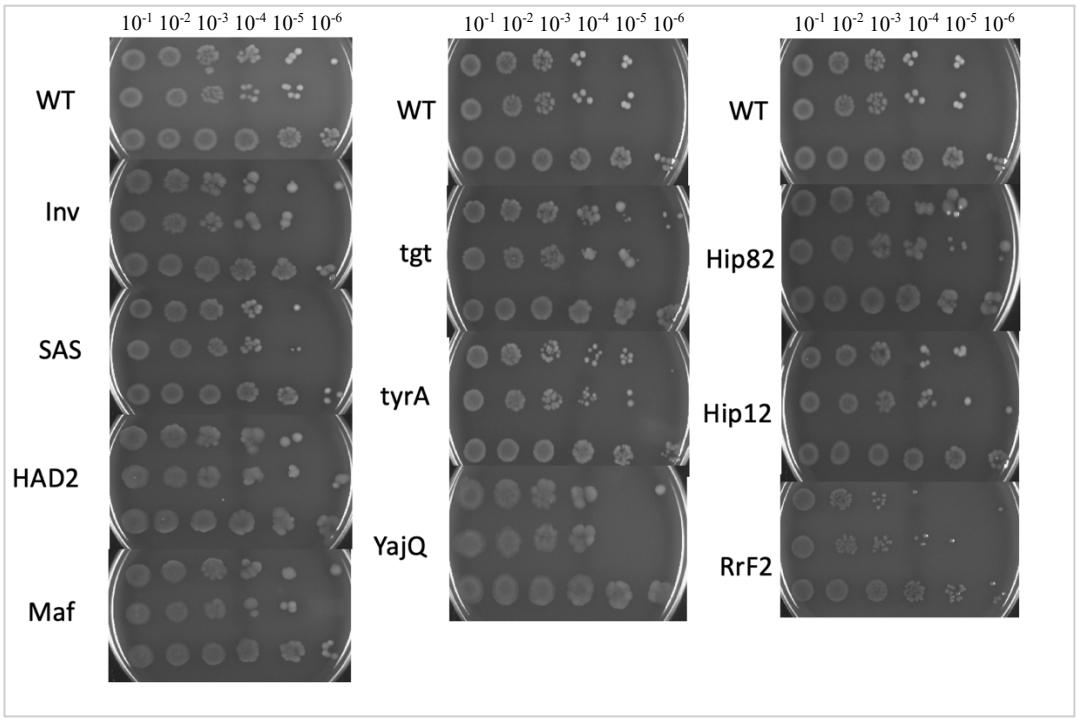

Figure 32. Temperature stress. The strains were incubated for 1 hour at 52°C and then plated by spot dilutions in log10 dilutions. The spots with the treated strains are assigned in the first and second rows as duplicates. The third row contains the control incubated for 1 hour at RT.

3.3.5.2 Water survival

To analyze the survival rate in sterile water at 4°C, we performed a water survival assay, a quantitative measurement by counting the colony forming units (CFU) plated daily for 2 weeks. The knockout mutants chosen for water survival consist in mutants significantly attenuated in de Vries et al. (2017) in sterile water at 4°C.

The reference strain presented a survival period up to 11 days (in the 7th day counting 47.3 CFU), and the same period of survival was observed for *ΔtyrA* (with an average of 128.1 CFU in the 7th day). *Δhip12* showed a longer survival period of up to 13 days (with an average of 5.8 CFU in the 11th day). Moreover, the knockout mutant *Δtgt* showed the highest survival with an average of 41.7 CFU in the 14th day.

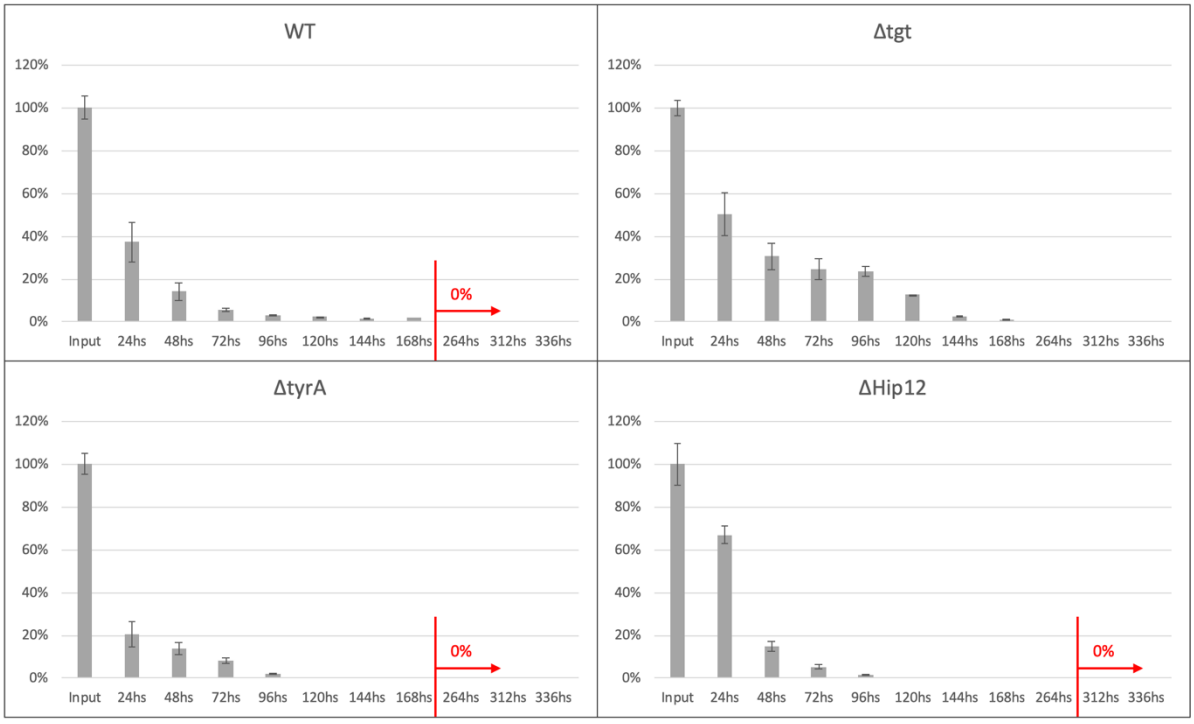

Figure 33. Water survival in sterile water kept at 4°C for up to 14 days. The number of CFU was calculated by serial dilutions made daily. The bars represent the means ± standard deviation of two biological triplicates.

3.3.5.3 Bile acid

During colonization in the human gut, *C. jejuni* will encounter different concentrations and composition of bile acids. The main bile acids found in the human small intestine consists of primary bile acids (cholic acid – CA, and chenodeoxycholic acid – CDCA) and secondary bile acids (deoxycholic acid – DCA, lithocholic acid – LCA, taurocheneoxycholic acid – TCA, glycocholic acid – GCA and ursodeoxycholic acid - UDCA).

Previously, Masanta et al. (2018) showed that three of those seven bile acids presented low IC_{50}, which confers them the most toxic effect to *C. jejuni*. DCA, CDCA and CA had IC_{50} values of 1.45, 2.41 and 3.48mM, respectively. Since DCA represents 20% of the bile in humans with the lowest IC_{50} and also with strong effect in protein regulation, we used DCA to test the knockout mutant's survival.

To analyze the survival after DCA exposure, the mutants were added to MH with a final concentration of 1.5mM DCA and incubated for 24 hours under microaerophilic conditions.

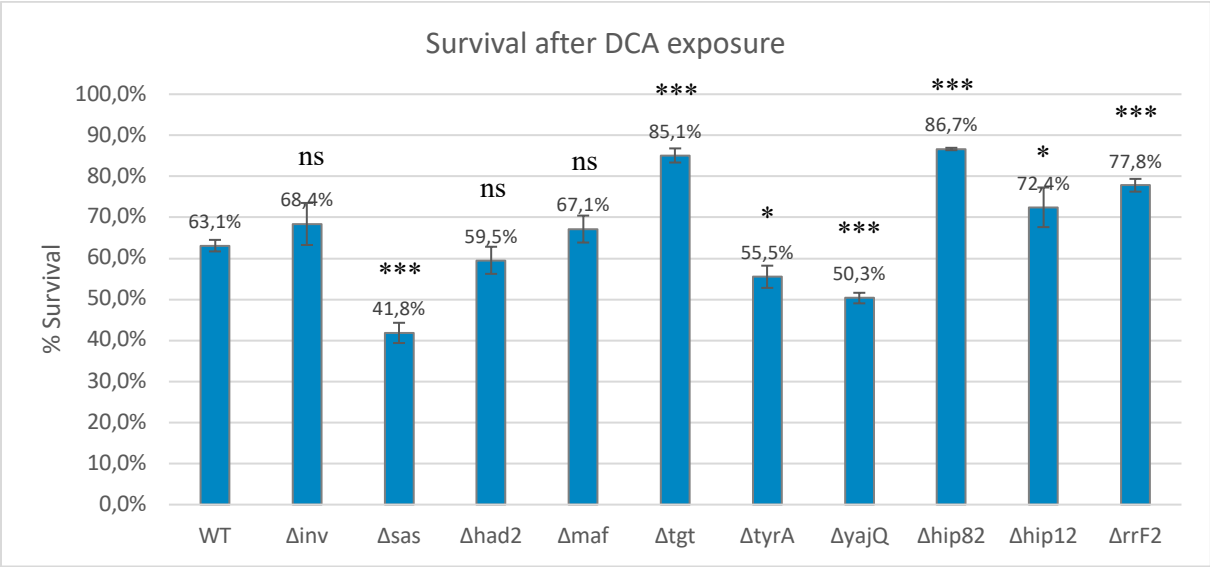

Figure 34. Survival after DCA exposure. The bars represent the means ± standard deviation of two biological triplicates. Two-sided unpaired Student t-test, **ns**: not significant; *p≤0.05 and ***≤0.001.

The percentage represents the ratio between the non-treated and DCA-treated samples. In Masanta et al. (2018), the WT 81-176 showed 50% of survival when cultured with DCA 1.45mM. In our experiment the same strain showed a similar survival rate of 63.1% in 1.5mM DCA. Three knockout mutants *Δsas* (p=0.0002), *ΔtyrA* (p=0.012) and *ΔyajQ* (p=0.0003) showed a statistically significant reduction in survival after 24 hours with 1.5mM DCA. Four mutants presented increased survival, namely *Δtgt* (p=6.8E-05), *Δhip82* (p=9.1E-06), *Δhip12* (p=0.03) and *ΔrrF2* (p=0.0002).

4. Discussion

Campylobacter jejuni as a foodborne bacterium, faces a variety of stress in the environment and needs to respond accordingly to persist. The environmental stress such as the exposure to bile in the intestine has been found to provoke adaptive responses in this bacteria, which need to adjust their protein synthesis to survive (Negretti et al. 2017). The ability of *C. jejuni* to respond to bile is considered complex and is still poorly understood. With the purpose to better understand the bile acid influence in the physiological adaptation processes of *C. jejuni*, we generated knockout mutants in genes encoding proteins differentially regulated after bile acid exposure, with a focus on proteins, which are down-expressed after bile acid exposure.

Unexpectedly, phenotypical analysis with our ten knockout mutants revealed a strong coupled phenotype in six of them. The mutants *Δinv*, *Δhad2*, *Δtgt*, *ΔtyrA*, *ΔyajQ* and *ΔrrF2*, showed similar patterns of phenotypic changes in motility, adhesion to, invasion and biofilm formation.

4.1 A motility phenotype was environmental condition-dependent in six mutants

In order to infect and cause disease in the host, *Campylobacter* must reach a suitable environment for growth and colonization by using its filamentous tail known as flagellum. *C. jejuni* motility is considered a key factor during colonization, as well as in pathogenesis, playing an important role in the development of the disease (Vliet and Ketley 2001). Interestingly, in our study, we observed an unusual motility phenotype that was not expected. All genes selected for knockout generation were not, in the first instance, associated with flagella, chemotaxis nor any motility related functions. Six knockout mutants, *Δinv*, *Δhad2*, *Δtgt*, *ΔtyrA*, *ΔyajQ* and *ΔrrF2*, presented low (below 1 cm of swarming area) or none motility in the standard motility assay with MH 0.4% agar. However, the same six mutants, which will be termed as “unstable motility

phenotype", showed a discrete motility above 1 cm in different agar concentration (0.25%), media (Brucella) and in liquid medium (microscopy) (Table 22). The other four mutants showed normal motility phenotype compared to the WT in all conditions. The knockout mutant *Δflg*P which does not produce a functional flagellum was generated to serve as a negative control for motility assay (Figure 14). This mutant was completely motility defective under all conditions (agar concentration and media) tested.

Normally, *Campylobacter* display straight swimming periods and eventual tumbling behavior. This behavior can be altered when the viscosity of the media is increased to simulate the intestinal mucus. In this viscous environment, *Campylobacter* enhances its motility velocity and shows longer straight swimming periods followed by pauses instead of tumbles (Szymanski et al. 1995). The increased viscosity obtained by the agar concentration in our motility assays presented the opposite effect, almost all mutants were more motile in the lowest agar concentration (MH 0.25%) and in Mueller Hinton broth (microscopy). However, the knockout mutants *Δinv*, *Δhad2*, and *ΔtyrA* showed lower motility in Brucella 0.25%. In addition, the type of medium also influences the motility phenotype of the "unstable motility phenotype" mutants. Brucella medium showed diverse phenotypes, mostly with reduced motility (however more than 1 cm of swarming area) in both agar concentrations (Table 22). Differences in media composition might explain the phenotype observed. Mueller Hinton consists in less components than Brucella. Mueller Hinton contains beef extract, acid hydrolysate of casein (peptone) and starch (its hydrolysis yields dextrose), while Brucella contains tryptone, peptic digest of animal tissue (peptone), sodium chloride, yeast extract, dextrose and sodium bisulfite. The richer components of Brucella might be recognized as a chemoattractant by *C. jejuni* mutants and/or provide more energy for motility. However, MH 0.25% and MH broth without agar (for microscopy) also presented modest or normal motility. The motility performed in test tubes with TTC (Figure 19) had a different setup comparing to the normal soft agar assay. In TTC

assays performed in 0.25% agar, the motility was measured by the colored zone of growth along the tube, while in the plate soft agar, the grown zone was horizontally measured. In the TTC test, the bacteria need to swim heading down through the media reaching more nutrients and this direction is supported by the gravity, what might be an additional explanation to the small increased motility observed in the mutants *Δtgt* and *ΔrrF2*.

The "unstable motility phenotype" mutants change their behavior in lower viscosity and richer media, presenting a modest increment in motility when compared to the standard soft agar assay (MH 0.4% agar). Interestingly, some bacteria species present diverse swimming abilities such to cross through viscous environments (e.g. GI mucus) in which others are incapable to move. *Campylobacter* species are known to generate one of the highest flagellar motor torque and with a fast swimming speed in high viscosity environment (Beeby et al. 2016). Chaban et al. (2018) studied the torque evolution in *C. jejuni* and other three different organisms from the same class Epsilonproteobacteria, that are also present in similar environments where *C. jejuni* is found. The authors state that the higher torque motor is associated to the bacterial cell shape, since the helical shape of *C. jejuni* facilitate the movement in viscous environment. The flagellar motor is a periplasm-spanning rotary motor and its torque is generated by proton flux through inner membrane stator complexes (MotA/B) and is constituted of a conserved core of ~20 structural proteins (Beeby et al. 2016). To maintain a functional high torque structure as flagella in *C. jejuni*, the organism has a high energy consumption. The six "unstable motility phenotype" mutants might have an energy generation in a sub-optimal level or are associated to some metabolic disfunction that would result in the lower motility observed in different viscosity and nutrient availability. *C. jejuni* can only afford this high energy cost given that it is consistent to the habitat in the gut, which provides a good nutrient availability. Other organisms that inhabit in lower viscosity and lower nutrients are more efficient with a simpler motor (Chaban, Coleman, and Beeby 2018). This high cost energy of having a

potent flagellum motor and a sub-optimal energy generation might be correlated to the singularities observed in the "unstable motility phenotype" mutants in different media. Composition and availability of nutrients can affect the chemotaxis behavior in *C. jejuni*. Sensing certain substrates might modulate motility, induce biofilm formation, and increase resistance to stress and minimize energy expenditure in different niches (Chandrashekhar, Kassem, and Rajashekara 2017). *C. jejuni* regulates its motility by chemotactic signaling systems, which allow the bacteria to follow favorable environment/nutrient or scape from a harmful condition (Lertsethtakarn, Ottemann, and Hendrixson 2011). Two-component regulator systems (TCS) mediates the chemotaxis, and includes a membrane associated histidine auto-kinase/sensor and a cytoplasmic response-regulator protein (Chandrashekhar, Kassem, and Rajashekara 2017). TCS facilitate sensing of nutrients in the environment and responding to a stimulus and consequently play an important role in the pathogenesis of enteropathogens.

Additionally, the nutritional environment can also influence the motility by quorum sensing. Quorum sensing mediated by autoinducer-2 (AI-2) is widely conserved among Gram-negative and Gram-positive bacteria and has been associated to environmental adaptation of pathogens such as *E. coli* and *Salmonella* spp. It has been demonstrated that *C. jejuni* possess in its genome the *luxS* gene and is able to produce AI-2, the only AI described in *C. jejuni*. After its discovery, consequently, many studies have confirmed that motility on soft agar is decreased in *luxS* mutant strains (Jeon et al. 2003; Holmes et al. 2009; Quiñones et al. 2009; Plummer et al. 2011). Cloak et al. (2002) demonstrated measurable levels of AI-2 in milk and chicken broth, suggesting that nutrients present in this common food source are required for AI-2 production. The swarming motility of other organisms such as *Serratia liquefaciens* is nutritionally controlled (Eberl et al. 1996). Also, in *Pseudomonas aeruginosa* the QS was shown to employ its nutritionally conditional control of biofilm formation through regulation of motility (Shrout et al. 2006). It would thus be interesting to investigate in future

whether QS and AI-2 levels are altered in the "unstable motility phenotype" mutants, which might explain the motility phenotype observed.

The only "unstable motility phenotype" mutant whose target gene product was up-regulated by bile acids in the proteomic study from Masanta et al. (2018) (Table 19) was *ΔrrF2*. RrF2 was up-regulated by five different bile acids (CA, DCA, LCA, CDCA and UDCA) and also presented the unstable motility phenotype. The reduced motility observed in MH 0.4% agar for the *ΔrrF2* mutant might be associated to transcriptional malfunction since RrF2 is a transcriptional regulator (CJJ81176_0891, Uniprot). The same knockout mutant showed reduced autoagglutination (Figure 25 and Figure 26). The role of autoagglutination in pathogenesis of *C. jejuni* has not been determined, but is strongly implicated in virulence of other species such enteropathogenic *E. coli* and *V. cholerae* (Golden 2002; Knutton et al. 1999). A recent study assessed autoagglutination in *C. jejuni* strain 81–176 and strongly associated this property with flagellar expression (Misawa and Blaser 2000), however, the genes responsible for autoagglutination in *C. jejuni* have not been identified.

Compared to *Bacillus subtilis* or *E. coli* with fourteen and seven sigma factors, respectively; *Campylobacter jejuni* was identified with only three (σ^{28}, σ^{54}, and σ^{70}). This lower number of sigma factors suggests that certain pathways may be coordinately regulated (Carrillo et al. 2004). While *rpoD* encodes σ^{70} that is involved in the expression of housekeeping genes, *rpoN* (σ^{54}) and *fliA* (σ^{28}) are associated to a number of flagellar genes (Jagannathan, Constantinidou, and Penn 2001). The flagellar production requires significant energy expenditure, consequently, an adequate regulation of flagellar genes is important to avoid unnecessary energy outflow. A misbalanced or interference in this complex coordination could result in an unstable phenotype in *C. jejuni*.

4.2 The mutants with "unstable motility phenotype" display high invasion and adhesion to Caco2 cells

Campylobacter pathogenesis includes some important stages: i) motility to reach its specific niche, ii) adhesion to the intestinal epithelium and iii) invasion of the target cells and development of the disease. The symptoms associated to Campylobacteriosis include bloody and inflammatory diarrhea, caused by bacterial disruption and invasion into the intestinal epithelium (Mills et al. 2012).

The strain *C. jejuni* 81-176 was first isolated from an outbreak in 1985 associated to raw-milk consumption and is proven to be highly invasive compared to other strains (Korlath et al. 1985). The invasive properties provide the possibility to study the molecular basis of this virulence mechanism used by *Campylobacter*. In the absence of an appropriate animal model that mimics human disease (Newell 2001), invasion has been largely studied using *in vitro* cell culture. Coote et al. (2007) demonstrated that different *C. jejuni* isolates adhered and invaded more efficiently to human colonic Caco2 cells than other cellular types. Considering that, we used Caco2 cells in our experiments to investigate *Campylobacter* invasion. However, due to the unexpected phenotypes observed in the same six "unstable motility phenotype" mutants, we also used a second cell line, COS-7, to confirm the high invasion phenotype observed. The invasion rate determined by GPA with COS-7 cell line also confirmed the increased invasion phenotype in *Δinv*, *Δhad2*, *ΔyajQ* and *ΔrrF2*, when compared to the WT strain. As expected, the control mutant *ΔflgP* (with paralyzed flagella) showed reduced adhesion (data not shown) and invasion rates, both phenotypes were successfully restored to WT level after complementation (Figure 21).

Surprisingly, our gentamycin protection assay results (3.3.2.2) revealed an increased invasion phenotype in the six "unstable motility phenotype" mutants and in *Δhip82*. Previous studies already proved the close relationship between motility and events such adhesion and invasion (Szymanski et al. 1995) and this association might play a role

between the unstable motility phenotype and adhesion and invasion phenotype observed in those six mutants.

The complex flagellar structure plays a role in motility but is also closely associated to secretion factors involved in the invasion of epithelial cells. Numerous studies have demonstrated that the *C. jejuni* flagellum functions as a secretory organelle by a type III secretion system-like transport mechanism and is needed for Cia protein export (Konkel et al. 2004; Barrero-Tobon and Hendrixson 2014). The secretion of Cia proteins demands a functional flagella basal body and hook, and at least one filament protein. The expression of *flaA* gene is necessary for maximal invasion in eukaryotic cells and for translocation of *C. jejuni* across polarized cells (Grant et al. 1993; Wassenaar, Bleumink-Pluym, and van der Zeijst 1991). The correct flagella formation is important for the extracellular delivery and for translocation to the intracellular environment for some proteins, such as Cia (CiaB, CiaC, CiaD, CiaI), FlaC and FspA. Masanta et al. (2018) showed an up-expression of the external structures of the flagellar apparatus *flaA* (by DCA, CDCA and GCA), *flaB*, *flaC*, *fliE* and *motA* (DCA and CDCA), and proposes that those structures also have other functions besides motility, such as cell adherence. Masanta et al. (2018) suggest that chemotaxis mediated flagellar motility by DCA, CDCA and GCA are also involved in adherence to epithelial cells. From our increased adherence phenotype mutants (in the six "unstable motility phenotype" mutants), three of them: Inv, tyrA and YajQ, were proteins down-expressed in the WT, while RrF2 was up-expressed; by DCA and/or CDCA and/or GCA.

It has been demonstrated that motility is switched with changes in gene expression during stationary growth phase, reducing motility in mid-stationary phase (Wright et al. 2009); however, the expression of flagellar genes is up-regulated during this phase and in presence of bile acids (Wright et al. 2009). The up-regulation includes genes that putatively encode proteins responsible for the hook, rod and P-ring, and those structures are maintained during the late-stationary phase, even with reduced motility (Carrillo et al. 2004; Parkhill et al. 2000). Konkel et al. (2004) described that mutants

in different components of the flagellar apparatus failed to secret the Cia proteins. It is tempting to speculate that even with lower motility phenotype, the flagellar structures might be maintained during the stationary phase to fulfill a secretory function needed during invasion. The "unstable motility phenotype" mutants showed a condition-dependent motility and an increased invasion phenotype, that fits to the above stationary phase behavior and consequently to an expected Cia protein excretion. Chloramphenicol is a selective inhibitor of bacterial protein synthesis and can be used in sublethal concentration to selectively inhibit protein synthesis without killing the organism (Konkel and Cieplak 1992). In future analysis for the increased invasion phenotype observed in our study, a potential experiment adding a sublethal concentration of chloramphenicol previously and during contact to the Caco2 cells to the GPA would inhibit the Cia protein synthesis. And consequently, reveal a possible association of increased invasion and Cia production.

Intriguingly, the observed increased adhesion and invasion phenotypes associated to instability in the motility is a new phenomenon not described in the literature so far. Particularly lower or absence of motility is associated to lower invasion rates (Szymanski et al. 1995; Michael E. Konkel et al. 2004) as observed in our control mutant *ΔflgP*. Additionally, the high adhesion and invasion rates are also not common phenotype observed in knockout mutants. Du et al. (2016) described a virulence-associated gene by knocking out the *cj0371* gene. The invasion and colonization investigations showed an increased invasion phenotype in the knockout mutant, and suggested that the gene might play a negative role in pathogenicity, which is expected to be suppressed during the infection (Du et al. 2016). Similar increased invasion phenotype was also observed by Lübke et al. (2018) in knockout mutant in the transducer-like protein - Tlp12 generated in *C. jejuni* A17.

During the invasion assays of this work, the Caco2 cells and also the *C. jejuni* added to the experiment were incubated for 2 hours in the cell incubator in a humidified atmosphere with 5% CO_2. The higher concentration of O_2 during the incubation time

could trigger adaptation genes and consequently modulate the invasion phenotype. As WT, *Δmaf*, *Δhip12* and control mutant *ΔflgP* did not present such effect, the oxygen regulation might be potentialized in the mutants *Δinv*, *Δhad2*, *Δtgt*, *ΔtyrA*, *ΔyajQ* and *ΔrrF2*. Once the bacteria are internalized, the effect of oxygen *in vitro* and bile acids *in vivo* are greatly diminished.

The fact that the target genes that were disrupted in our knockout mutants showed an increased adhesion and invasion phenotypes possess diverse cellular and metabolic functions, highlights that adhesion and invasion are a multifaceted phenotype, implicating different pathways in these complex mechanisms.

4.3 Cyclic-di-GMP might be involved in the unstable motility phenotype

To survive and be successful in diverse and continuously changing environments, bacteria engage many strategies to sense and adapt to their surroundings. One such system is the bis-(3'-5') cyclic dimeric GMP (c-di-GMP) signaling network. C-di-GMP controls several behaviors and processes including motility, biofilm formation, virulence, differentiation and cell cycle progression (Hengge 2009). C-di-GMP is produced by diguanylate cyclase (DGC) from two GTP, and is hydrolyzed into a linear 5'-pGpG or two GMP molecules by a c-di-GMP specific phosphodiesterase (PDE) (Sisti et al. 2013). The DGC activity is conferred by the conserved GGDEF functional domain, while PDE activity is performed by conserved EAL or HD-GYP domains (Sisti et al. 2013). Remarkably, individual bacterial genomes commonly encode numerous GGDEF and EAL/HD-GYP proteins (Galperin 2005), indicating that the c-di-GMP network is a very complex and tightly regulated system.

Bacteria normally use c-di-GMP to transmit environmental signals to downstream receptors that modulate many important cellular processes for survival. An essential feature of c-di-GMP regulation is the capacity of this second messenger to control the lifestyle transition between motile to sessile (planktonic to biofilm form). It has been described that elevated levels of intracellular c-di-GMP promote sessile lifestyle and

stimulate the production of extracellular polymeric substance (EPS) matrix and subsequent biofilm formation (Römling, Galperin, and Gomelsky 2013). In contrast, low levels of c-di-GMP are associated with active motility. Increased levels of c-di-GMP promote biofilm formation and reduced motility in *Pseudomonas aeruginosa* (Kuchma et al. 2015), *Salmonella* spp. (Zorraquino et al. 2013), *Bacillus subtilis* (Chen et al. 2012), *Bordetella bronchiseptica* (Sisti et al. 2013), *Shewanella oneidensis* (Gao, Meng, and Gao 2017), *Vibrio cholerae* (Kovacikova, Lin, and Skorupski 2005), and others.

Interestingly, our six "unstable motility phenotype" mutants showed non-motile phenotype (in MH 0.4% agar) and variable motility phenotypes in other conditions, and also presented increased biofilm formation (Figure 28 and Figure 29). These phenotypes correlate with high c-di-GMP levels observed in other species. It would thus be interesting to determine whether c-di-GMP concentrations are also elevated in the "unstable motility phenotype" mutants.

High intracellular levels of c-di-GMP could be due to an increased production or to an accumulation by low hydrolysis of the second messenger. It has been shown that environmental cues such oxygen, bile acid and QS autoinducers can directly regulate DGCs or PDEs in various bacteria (Koestler and Waters 2014).

C-di-GMP represses the expression of virulence factors in *Vibrio cholerae* and its intracellular concentration is low during infection, however Koestler and Waters (2014) found that bile acids increase the intracellular second messenger concentration. *V. cholerae* in response to bile acids shows significantly enhanced biofilm formation and is more resistant to the toxicity of bile acids within the biofilm (Hung et al. 2006). Indeed, in our biofilm assay performed in presence of 1 mM DCA, the wild-type strain showed a 4.5-fold increased biofilm formation, however with no statistical significance. Little is known about c-di-GMP in *C. jejuni* and its effect in the bacterial physiology and gene expression. Raphael et al. (2005) described a mutant of *C. jejuni* sensitive to DCA, the gene Cj0643 or *cbrR* (*Campylobacter* bile resistance regulator). The *cbrR*

analysis demonstrated that it contains two tandem response regulator (RR) receiver domains and a C-terminal GGDEF domain, indicating a possible link between bile resistance and second messenger production. The mechanism of bile acid resistance of *cbrR* in *C. jejuni* remains to be determined, as well as the c-di-GMP involvement in bile acid resistance and lifestyle transition in *C. jejuni*.

In 2014, An et al. published a study that identified a c-di-GMP binding protein in the plant pathogen *Xanthomonas campestris* pv. *campestris* (Xcc). They identified a protein of the YajQ family as a probable c-di-GMP receptor. With the aid of recombinant YajQ-like proteins from different bacterial human pathogens it was also possible to show the role of YajQ in virulence and its specific association to c-di-GMP. The findings from An et al. (2014) identified a new class of cyclic di-GMP effectors that regulate bacterial virulence that can also be related to our knockout mutant *ΔyajQ* from the same family.

4.4 The mutants with "unstable motility phenotype" are assigned to different functions/pathways

The target genes selected for knockout generation are annotated to different functions and pathways as listed in Table 19. In the first instance, the six mutants with the "unstable motility phenotype" were not associated to the flagellar apparatus nor any motility related function. The functions and pathways include a protein associated with invasion (Inv), a hydrolase from the HAD-family (HAD2), an enzyme involved in tRNA modification (tgt), a prephenate dehydrogenase found in the Shikimate pathway (tyrA), a putative c-di-GMP effector/binding protein (YajQ) and a transcription factor (RrF2). All those functions are not associated by any similar function that link them and the genes were located far from each other in the *C. jejuni* 81-176 genome. However, the genes *Inv* and *RrF2* were situated closely to other genes associated to the flagellar apparatus. A gene encoding a flagellar L-ring (CJJ81176_0710) was situated upstream of *Inv*, both encoding in the antisense direction. Regarding the *RrF2* gene, a

flagellar hook (*FlgL*, CJJ81176_0894) was situated upstream and *flhA* (flagellar biosynthesis protein, CJJ81176_0890) was situated downstream, all three in antisense direction. The knockout mutation in these two genes could present a polar effect on the upstream and downstream localized genes and alter their expression to a level that shows a detectable phenotype.

For our surprise, the same six "unstable motility phenotype" mutants presented other strong phenotypes associated with an increased adhesion, invasion and biofilm formation (Table 23). This coupled phenotype might be influenced by a general genetic program that link all these features together. The knockout mutants *Δinv*, *Δhad2*, *ΔtyrA* and *ΔyajQ* are knockout mutants generated from the target genes that were down-regulated after bile acid exposure in Masanta et al. (2018) study. The bile acids regulation is the common link between the five knockout mutants (except for the gene *tgt* that was not differentially regulated by any bile acid) and may have influence in the adaptation in *C. jejuni* that were observed in our assays.

Table 23. Summary of phenotypes obtained in the study.

Strains	**"UMP"**	**Adhesion**	**Invasion**	**Biofilm**
Δinv	Yes	+	+	+
Δsas		-	-	
Δhad2	Yes	+	+	+
Δmaf				
Δtgt	Yes	+	+	+
ΔtyrA	Yes	+	+	+
ΔyajQ	Yes	+	+	+
Δhip82		+	+	+
Δhip12				
ΔrrF2	Yes	+	+	+

The only mutant that showed similar phenotype observed with increased invasion and biofilm formation, except for the normal motility, is the *Δhip82* mutant (Table 23). The *Hip82* was a gene down-regulated by DCA and CDCA in Masanta et al. (2018) study. It showed the highest biofilm formation among the ten mutants, however presented normal motility phenotype comparable to the WT in most assays, except in the motility after invasion (Figure 17) that showed reduction in motility after invasion. The *Hip82* is annotated as a hypothetical protein and is situated between elongation factors and other hypothetical proteins.

The increased invasion and also the increased biofilm formation provide protection for the bacteria against the stress found in the GI tract environment. Once within the epithelial cell or inside the biofilm structure, *Campylobacter* has no bile acid pressure and can proceed with the colonization processes.

Until the date, there is no literature available that describes such phenomenon in *Campylobacter* or other organism, since knockouts from target genes that were down-regulated by any specific condition is uncommon in the literature.

The fact that our genes were classified as part of diverse metabolic pathways emphasizes that the unstable motility phenotype, as well as adhesion, invasion and biofilm formation are somehow connected and responding in a similar manner.

4.5 The transcriptional regulator RrF2 may be involved in biofilm formation, invasion and autoagglutination in *C. jejuni*.

RrF2 is annotated as "RrF2 family protein" and shows the same sequence as *cymR* in *C. jejuni* (Uniprot A0A1E7NYK4). *CymR* is a repressor that belongs to the widespread and poorly characterized RrF2 family of transcriptional regulators (Shepard et al. 2011). The *RrF2* presents 30.57% sequence identity with the *cymR* gene in *B. subtilis* (strain 168) and 29.71% identity with *S. aureus*. CymR is the master regulator of cysteine metabolism and is described to play an important role in biofilm formation, stress response and virulence in *S. aureus* (Soutourina et al. 2010, 2009), sulfur

utilization and is a cysteine biosynthesis repressor in *B. subtilis* and *S. aureus* (Commichau and Stülke 2015).

Interestingly, the *RrF2* gene was up-regulated by five bile acids (CA, DCA, LCA, CDCA and UDCA) and might regulate by repressing genes involved in the phenotypes observed for invasion, biofilm formation and autoagglutination.

In general, the RrF2 family is Incompletely characterized. In particular, for *C. jejuni* no literature is available for this gene till the date. *RrF2* has been described for the first time in *C. jejuni* in this work and might be associated to virulence in this organism. Further studies are needed to better understand and characterize its contribution in the regulation of the phenotypes observed in our study.

4.6 A transporter mutant is associated to adhesion and invasion of Caco2 cell

For some bacteria, a sodium circuit is an important link between endergonic and exergonic membrane reactions, been a common method of substrate uptake in living cells (Wilson and Ding 2001). The knockout mutant *Δsas* was the only mutant that showed a direct reduction phenotype associated to invasion, adhesion and biofilm formation (Figure 22, Figure 24 and Figure 28). This gene is annotated with a Sodium symporter function and was downregulated by GCA in the Masanta et al. (2018) study. The *Sas* gene may therefore be an important player in 81-176 adhesion, invasion and biofilm formation. Most likely to be an integral component of the membrane, the *Sas* may enable adhesion and invasion by actively facilitating the attachment of the bacterium to its host.

The growth kinetics was similar to the WT, showing no growth deficiency that could explain the low invasion, adhesion and biofilm formation observed. The growth kinetics was performed with normal MH without any difference in sodium or any other component. We were unsuccessful to generate complementation for *Δsas*, and for now

is not possible to confirm the phenotypes observed, however other complementation techniques could be applied for a future confirmation of this finding.

4.7 Stress adaptation of *C. jejuni*

C. jejuni adaptation to different environments and/or conditions is an important feature enabling the bacteria to save resources by expressing only the appropriate group of genes in specific conditions. Usually enteropathogens adapt to different environments and are able live in a free living form and also colonizing a host (Rivera-Amill et al. 2001). Environmental conditions such as temperature, nutrient starvation, oxygen levels, pH and bile acids serve as environmental signals that can lead to differentiated protein synthesis.

An evident external stress for *Campylobacter* is the bile acids. Previously, Masanta et al. (2018) showed that DCA was the bile acid with strong effect in the *C. jejuni* protein regulation and also with the lowest IC_{50}. We tested the survival rate of our 10 knockout mutants growing with 1.5mM DCA for 24 hours under microaerophilic conditions (Figure 34). Three knockout mutants showed reduced survival in DCA, *Δsas*, *ΔtyrA* and *ΔyajQ*. Those three target proteins, Sas, tyrA and YajQ, were down-regulated by GCA, CDCA and DCA/CDCA, respectively, and are probably involved in bile acid adaptation in *C. jejuni*. In contrast, three mutants showed increased survival in DCA, *Δtgt*, *Δhip82* and *ΔrrF2*. In Masanta et al. (2018) the tgt protein did not show any significant regulation by bile acid, Hip82 protein was down-regulated by DCA and CDCA, and RrF2 protein was up-regulated by CA, DCA, LCA, CDCA and UDCA.

Besides the DCA exposure, we challenged our knockout mutants to other stresses to assess their survival at increased temperature (Figure 32) and survival in sterile water at low temperature (4°C) (Figure 33).

The high temperature treatment only showed reduction of one log in the Unstable Motility Phenotype mutants *ΔyajQ* and *ΔrrF2* (10^{-4}) compared to the WT (10^{-5}). In the water survival, only the *Δtgt* and *Δhip12* mutants showed a longer survival

compared to the WT. The mutant *Δtgt* showed the longest survival rate with an average of 41.7 CFU at day 14. The tgt and tyrA transposon mutants were significantly attenuated in the Tn mutagenesis study (de Vries et al. 2017), however, in our water survival assay, the *Δtgt* mutant showed longer survival. Therefore, *tgt* could be implicated in the adaptation to lower temperatures and/or longer survival in starvation.

4.8 Proposed model

Campylobacter jejuni is normally found in the human and animal intestine, in untreated surface water and in contaminated food (raw meat, milk or dairy products). Those environments provide highly different conditions and require adaptive responses for *C. jejuni* survival. *Campylobacter* developed physiological strategies to adapt and survive inside the host for a successful colonization.

After internalization via ingestion into a human host, *C. jejuni* enters the small intestine, where it finds a low pH from the stomach and a high concentration of bile acids in the lumen. The bile concentration in human GI tract is present in a gradient from high concentration in the proximal small intestine (duodenum) to very low levels in the large intestine. In the first part of the small intestine, predominantly the primary bile acids, CA and CDCA are found. The microbiome present in the intestine express bile salt hydrolases which deconjugate the host bile acids what leads to the formation of secondary bile acids and thus creates a significant change in the host bile acid pool (Ridlon, Kang, and Hylemon 2006). About 5% of primary bile acids remains in the colon, the rest (primary and secondary) are reabsorbed participating in a negative feedback inhibiting bile synthesis. In a healthy person, approximately 5% of DCA and LCA is excreted in the feces, consequently these bile acids are present along the intestine, while the others are mostly reabsorbed (Camilleri 2014).

The presence of bile acids might serve as an indicator for the presence inside the gut lumen and trigger the expression of virulence genes. Some genes such as the multidrug

efflux pump *CmeABC* are up-regulated, while repressor genes such as *cmeR* and *cbrR* are down-regulated by bile acids (Masanta et al. 2018).

Bile acids are toxic substances for enteropathogens like *C. jejuni*. Bile acids can cause disruption of cellular membranes, protein misfolding, oxidative damage to DNA and cause a differentiated bacterial gene expression (Jia and Xie 2018; Sistrunk et al. 2016; Joyce and Gahan 2016). In order to survive, the organism reduces bile acid exposure by protecting itself inside a biofilm structure or hiding inside a host cell.

In our six "unstable motility phenotype" mutants we observed an environmental condition dependent reduction of motility, as well as an expressive increment in invasion and biofilm formation. From the six "unstable motility phenotype" mutants, four target genes (*Inv*, *Had2*, *tyrA* and *YajQ*) were down-regulated by bile acids (Masanta et al. 2018). It is possible that knocking out genes that were predominantly down-regulated after bile acid exposure (Table 19) can artificially mimic parts of the response observed in *C. jejuni* when challenged with bile acids, at least for the specific cellular process in which they are involved in.

Organisms such as *Campylobacter* react to short term external changes by reversibly adjusting their physiology to maximize resource consumption while preserving structural and genetic integrity to maintain its robustness. By disrupting a target gene, we caused a disturbance that might lead to the observed phenotypes such as the unstable motility and increased invasion and biofilm formation.

Some disturbances of metabolic flow or general healthiness of the organism might lead to the activation of a common genetic program that all in once leads to these phenotypes that we generated with the deletion of genes that are naturally down-regulated in presence of bile acids stress. This disturbance was not sufficient to show a growth alteration in our mutants, but it was clearly affecting a more complex regulation involving motility, invasion and biofilm formation that prepares the organism to better deal with stress situations. Bile acid offer kind of stress to the organism, and deletion

of that genes is also a kind of stress situation and independently of specific stress that is encountered by *Campylobacter* the outcome seems to be very similar.

5. Conclusion

5.1 Conclusions

Understanding how *Campylobacter jejuni* can adapt in the hostile environment within the host may help to create strategies to limit the bacterial impact. *C. jejuni* might serve as a model for understanding how pathogens with limited regulatory repertoires adapt to different environments.

In summary, our findings suggest a tight and complex regulation of bile acid in the adaptation inside the host. The high concentration of bile acid DCA promotes biofilm formation and consequently protection against the harmful condition. Along the small intestine, the bacteria get in contact with different bile acids that indicates the environmental changes (and its position within the host). By knocking out genes that were down-regulated by bile acids we potentially mimicked the proteomic regulation effect of bile acids. The outcome in the coupled phenotypes observed in six knockout mutants were very similar for the individual deleted genes although they participate in very different activities/functions. The phenotypic variations can be interpreted as adaptation processes that prepare the bacteria to better survive stress situations.

5.2 Suggestions for future research

Several experiments can be tested in order to validate and prove the adaptive mechanisms of *C. jejuni* over to the bile acid exposure. And we propose some of the possible specific assays that could be performed in a future research.

In order to check if the knockout mutants showed a polar effect of the deletion in the adjacent genes, it is proposed the comparison of the transcription of genes downstream and upstream of the deletion in the mutant and in the parental strain by RT-PCR.

Originally the present project did not take into account the c-di-GMP regulation during the adaptation process in *C. jejuni*. The phenotypes observed in the "unstable motility phenotype" mutants suggest a potential association of cyclic-di-GMP in the motility reduction and increased biofilm formation. Accordingly, experiments involving the second messenger detection would be beneficial for the unstable motility and increased biofilm formation confirmation. To the date, there is a lack of literature regarding *Campylobacter* second messenger regulation as well as the identification of genes involved in such signaling system.

As mentioned previously, the use of sub-lethal concentration of chloramphenicol as a selective inhibitor of bacterial protein synthesis could be used in future analysis of the association of Cia production and the increased invasion phenotype observed in our study. The addition of sub-lethal concentration of chloramphenicol to the GPA assay would inhibit Cia proteins synthesis when the mutants are in contact to the Caco2 cells. And subsequently, reveal a possible association of increased invasion and Cia production during invasion process.

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