Adaptation of respiration and metabolism in *Campylobacter jejuni*

Anne-Xander van der Stel

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Adaptation of respiration and metabolism in *Campylobacter jejuni*

Adaptatie van respiratie en metabolisme in
Campylobacter jejuni
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Anne-Xander van der Stel
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Promotor: Prof. dr. J.P.M. van Putten

Copromotor: Dr. M.M.S.M. Wösten

''It is paradoxical, yet true, to say, that the more we know, the more ignorant we become in the absolute sense, for it is only through enlightenment that we become conscious of our limitations. Precisely one of the most gratifying results of intellectual evolution is the continuous opening up of new and greater prospects.''

Nikola Tesla (1915)

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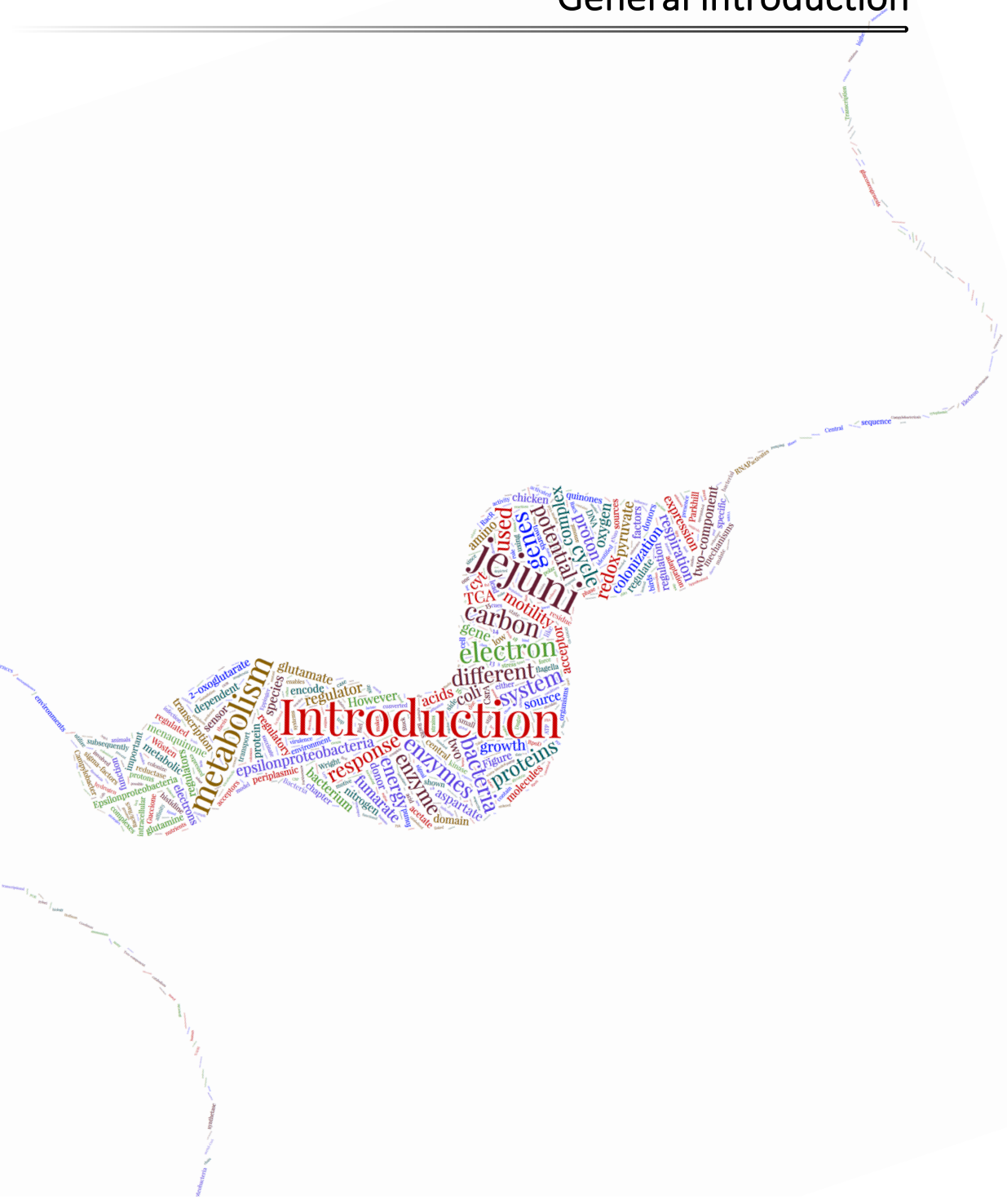
General introduction



EPSILONPROTEOBACTERIA

Epsilonproteobacteria are Gram-negative spiral-shaped bacteria that are very motile due to their polar flagella (Blaser, 1997; Parkhill et al., 2000). They colonize diverse habitats ranging from deep-sea hydrothermal vents to the intestines of animals (Eppinger et al., 2004; Nakagawa and Takaki, 2001). Over the last years, the interest in this class of bacteria is steadily increasing. Sampling of diverse environmental locations shows that epsilonproteobacteria are more abundant than previously thought, while whole genome sequencing allows the identification of more species and a more in-depth analysis of the biology of representative organisms (Eppinger et al., 2004; Keller et al., 2015; Yamamoto and Takai, 2011). Epsilonproteobacteria are also recognized as important pathogens. *Arcobacter* species are recognized as emergent human pathogen (Ramees et al., 2017). Nearly half of the human population is colonized with the ulcer-causing stomach bacterium *Helicobacter pylori*, whereas *Campylobacter jejuni* is now one of the most prevalent bacterial food-borne pathogens (Deng et al., 2016; Eusebi et al., 2014).

CAMPYLOBACTER JEJUNI

Campylobacter jejuni is a small microaerophilic bacterium. It has a curved, rod-like shape and has two polar flagella. *C. jejuni* can survive in surface waters, but is mostly found colonizing hosts (Young et al., 2007). Because *C. jejuni* has an optimal growth temperature of approximately 42°C, birds are its preferred host. *C. jejuni* colonizes the gastrointestinal tracts of birds, but also that of other warm-blooded animals. *C. jejuni* has a small and compact genome of ~1.6 Mbp, which encode for around 1,600 genes. Because of a lack of extensive DNA repair mechanisms, the genomes of epsilonproteobacteria contain hypervariable sequences (Parkhill et al., 2000) and exhibit a very low co-linearity (Eppinger et al., 2004). The polar flagella provide the bacterium with motility, which is essential for its colonization and virulence (de Vries et al., 2017; Morooka et al., 1985; Szymanski et al., 1995). Chemo- and energytaxis direct the bacteria to more beneficial environments and help find the ideal colonization niche (Zautner et al., 2012).

The main route of transmission of *C. jejuni* to humans is contaminated chicken meat. *C. jejuni* can colonize the gastrointestinal tract of an entire flock of chickens in a couple of days (Newell and Fearnley, 2003). During colonization, the ceca of the birds are colonized to high levels of 10^9 cfu/gram of gut content. *C. jejuni* colonizes the gastrointestinal tract of birds and other warm-blooded animals as a commensal bacterium, although small immunological responses are sometimes observed (Hermans et al., 2012). Humans, on the other hand, suffer from infection with *C. jejuni.* In humans the bacterium penetrates the mucus layer of the intestines and invades host cells, evoking a strong immune response (Backert et al., 2013; Islam et al., 2014). Campylobacteriosis usually leads to episodes of severe (bloody) diarrhea, which can last for up to 10 days. Campylobacteriosis is self-limiting and antibiotics are typically not prescribed in case of an infection (Blaser, 1997; Ternhag et al., 2007). Nevertheless, in rare cases (1 in ~1000), infection with *C. jejuni* can lead to the auto-immunity-based Guillain-Barré syndrome. In this case, anti-bodies directed against the lipooligosaccharide (LOS) of *C. jejuni* which cover the bacterium's

exterior, cross-react with gangliosides located on the peripheral neurons (Heikema et al., 2015; Shahrizaila and Yuki, 2016).

CENTRAL METABOLISM OF *C. JEJUNI*

C. jejuni lacks the glycolytic enzymes glucokinase and 6-phosphofructokinase and is thus incapable to utilize hexoses and pentoses as carbon and energy source (Kelly, 2001; Parkhill et al., 2000). Recently, *Campylobacter* species were identified that do encode an Entner-Doudoroff pathway and can catabolize glucose into pyruvate (Vegge et al., 2016; Vorwerk et al., 2015). On top of that, some *C. jejuni* strains harbour a genetic island that enables utilization of fucose (Stahl et al., 2011).

Tricarboxylic acid cycle

Still, most *C. jejuni* strains rely on amino acids and organic acids that feed into the tricarboxylic acid cycle (TCA cycle) as carbon- and energy source (Figure 1). Serine and lactate are both imported and subsequently converted to pyruvate (Thomas et al., 2011; Velayudhan et al., 2004). Pyruvate itself is also taken up by *C. jejuni* and catabolised to acetyl-CoA, which is either converted to acetate, or feeds into the TCA cycle (Wright et al., 2009). Aspartate is imported by *C. jejuni* and deaminated by the enzyme AspA to fumarate. The TCA cycle intermediate fumarate can be used as carbon and energy source (Guccione et al., 2008). The amino acids glutamate and proline can also be used as carbon source. These amino acids are first catabolised to aspartate and subsequently fed into the TCA cycle (Guccione et al., 2008). Some strains also contain genes that encode for the periplasmic enzymes GGT and AnsA, which enables the catabolism of glutamine and asparagine (Hofreuter et al., 2006). These amino acids are converted to glutamate and aspartate, respectively. Furthermore, *C. jejuni* imports and metabolizes the TCA cycle intermediates oxaloacetate, citrate, isocitrate, 2-oxoglutarate and succinate (Mohammed et al., 2004). *C. jejuni* prefers the utilization of the amino acids serine and aspartate, which are the first depleted carbon sources from the medium (Leach et al., 1997; Wright et al., 2009).

Anaplerotic pathways

C. jejuni has an extensive anaplerotic metabolism, which balances the TCA cycle, the lower glycolytic pathways and the pyruvate node (Velayudhan and Kelly, 2002). The TCA is replenished by conversion of pyruvate into oxaloacetate by PycAB. The Mez enzyme, which is coupled to NADP reduction, decarboxylates malate to pyruvate. However, due to the low affinity for malate, this enzyme is hypothesized to play a minor role in the metabolism of *C. jejuni*. The gluconeogenesis is fuelled by the production of phosphoenolpyruvate from oxaloacetate by the enzyme PckA. A knock-out mutant lacking of the *pckA* gene was shown to be lethal, indicating that the anabolic function of the gluconeogenesis in this assaccharolytic bacterium is very important. The presence of the enzyme Pyk, which catalyses the reaction of PEP to pyruvate is remarkable as it could lead to a futile cycle. However, Pyk activity could also function to balance the amount of PEP and hence the flux of the gluconeogenesis (Velayudhan and Kelly, 2002).

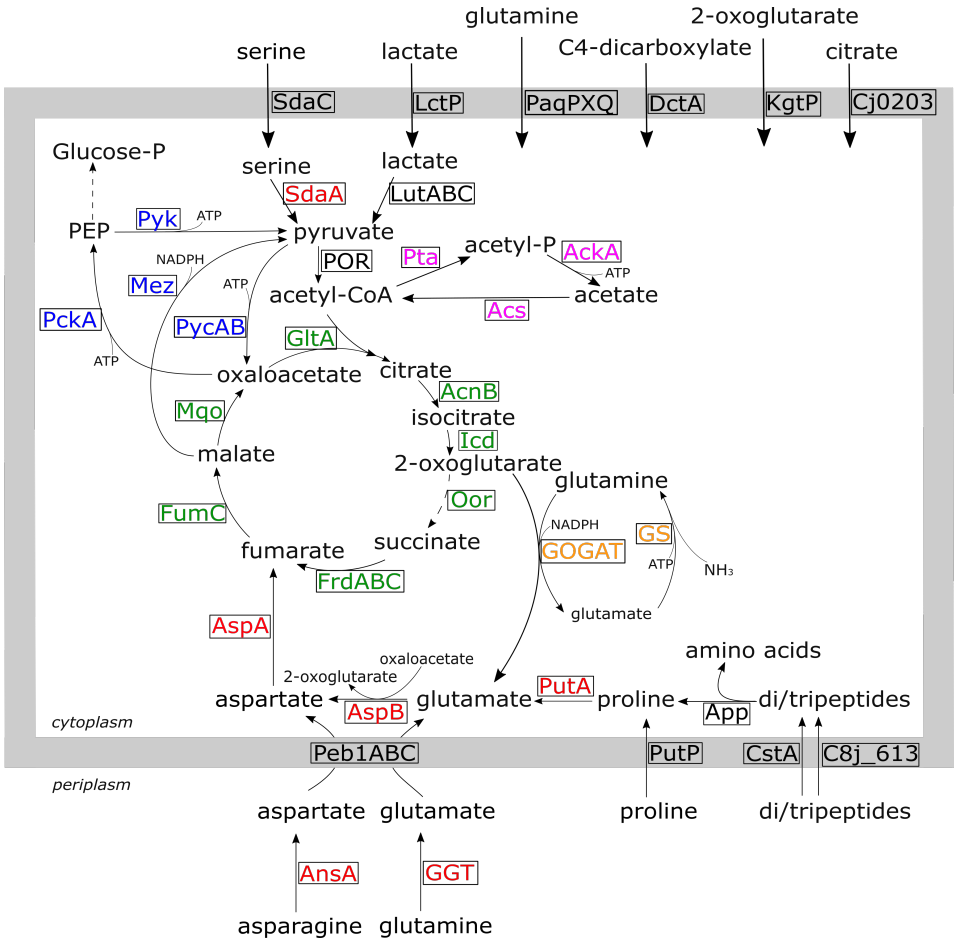


Figure 1: Central metabolism of *C. jejuni*. This model shows the central (carbon) metabolism of the molecules that *C. jejuni* uses as carbon source. Central is the tricarboxylic acid (TCA) cycle (green). The TCA cycle is fed from acetyl-CoA, or amino acids catabolized through aspartate and fumarate. Enzymes responsible for the catabolism of amino acids are depicted in red. The GS/GOGAT system is colored in orange. The acetate metabolism enzymes are in pink. The anaplerotic pathway enzymes are indicated in blue.

Nitrogen assimilation

Intracellular glutamate and glutamine are the most important nitrogen sources for bacteria (Reitzer, 2003). *C. jejuni* contains the ammonium assimilation system enzymes glutamine synthetase and glutamine-oxoglutarate-aminotransferase (GS-GOGAT). Together these enzymes yield a net reaction that consumes 2-oxoglutarate and ammonium, and produces two glutamate molecules, at the expense of NADPH and ATP (Figure 1). The glutamine synthetase of proteobacteria can be post-translationally regulated by adenylylation (Wulff et al., 1967). However, the glutamine synthetase enzymes of the epsilonproteobacteria do not have the conserved residues that are reversibly adenylylated (Garner et al., 1998). In contrast to other bacteria (e.g. *H. pylori, E. coli*), *C. jejuni* does not encode for the enzyme glutamate dehydrogenase (Gdh), which

bidirectionally converts glutamate to 2-oxoglutarate directly. Instead, the aminotransferase AspB, not only deaminates glutamate to produce 2-oxoglutarate, but also aminates oxaloacetate producing aspartate (Howlett et al., 2014). The expression of the genes coding for GS-GOGAT and Gdh are strictly regulated in other bacteria, such as *E. coli*. Expression of these nitrogen metabolism genes is based amongst others on the carbon and nitrogen status of the cell (Heeswijk et al., 2013). However, the regulators described for *E. coli* are not encoded in the *C. jejuni* genome. The regulatory cues and mechanisms that control the gene expression of these enzymes in *C. jejuni* are still an enigma (Leyn et al., 2016; Parkhill et al., 2000).

Metabolic state of the *C. jejuni* is linked to motility

C. jejuni, just like *E. coli*, excretes acetate that is later re-used as carbon source during stationary growth phase (Wright et al., 2009). In *E. coli*, extracellular acetate activates the BarA/UvrY two-component system, which activates two noncoding RNAs that regulate the activity of the post-transcriptional regulator CsrA (Vakulskas et al., 2015). *C. jejuni* also encodes CsrA. However, the CsrA protein in *C. jejuni* does not regulate the same pathways as in *E. coli*. In a *csrA* mutant strain a plethora of metabolic, virulence and motility related proteins are differentially expressed (Fields et al., 2016). Most of this regulation seems to be indirect, since CsrA only binds to a small amount of mRNA targets (Dugar et al., 2016). Moreover, CsrA activity is not regulated by an acetate activated two-component system (TCS), but rather by the amount of intracellular flagellin proteins in a growth phase dependent manner (Radomska et al., 2016). The motility of *C. jejuni* peaks around the end of the exponential growth phase in batch cultures (Wright et al., 2009). This strongly suggests that motility is intrinsically linked with the metabolic state of the cell and serves to search for more favourable environments, when nutrients are running low.

Regulation of the central metabolism

To date, no transcriptional regulators have been identified that regulate genes of the central metabolism in *C. jejuni*. Strikingly, almost all proteins known to regulate the central metabolism in other proteobacteria (e.g. CRP, ArcA, Lrp) are not found in *C. jejuni* and other epsilonproteobacteria (Leyn et al., 2016). This suggests that the metabolic regulation in this subdivision of the proteobacteria has undergone a separate evolution and might have different regulatory circuits that respond to other cues than the canonical model organisms.

ENERGY METABOLISM OF *C. JEJUNI*

To conserve energy, bacteria utilize redox reactions to build up a proton motive force. The proton motive force (pmf) is used to generate ATP, which drives most endergonic reactions in the cell and is also used to drive transport of nutrients over the membrane or to rotate the flagella, needed for motility. The proton motive force consists of the pH difference over the membrane (ΔpH) and the electrical potential over the membrane (Δψ). Specialized enzymes guide electrons from an electron donor to an electron acceptor, while also transferring charges (protons and electrons) over the inner membrane, generating a membrane potential (Mitchell, 1961; Simon et al., 2008). Some

membrane proteins of this electron transport chain (ETC) function as proton pump and can directly pump protons from the cytoplasmic side to the periplasmic side of the membrane. Another mechanism to generate a membrane potential is via a redox loop. During this process electron transfer from the electron donor- to the terminal electron acceptor complex is facilitated by membrane-associated molecules called quinones. When fully reduced, quinols carry two electrons and two protons and freely diffuse through the membrane (Söderhäll and Laaksonen, 2001). This enables electron (and proton) translocation from one side of the membrane to the other. Bacteria can synthesize different kind of quinones, with different redox properties. *E. coli* makes three different quinones: ubiquinone, demethylmenaquinone and menaquinone. These different quinones are produced dependent on the electron acceptor that is used. Ubiquinone (E'° = +110 mV) is used for oxygen respiration, but cannot be used for respiration with fumarate (E'° = +35 mV), since its redox potential is higher than that of fumarate. In this case, menaquinone (E'° = -74 mV) is used. Nitrate (E'° = +430 mV) respiration is facilitated by demethylquinone (E'° = +40 mV), which has an intermediate redox potential. *C. jejuni* and other epsilonproteobacteria only make menaquinone (MK) and a methyl-substituted menaquinone (mMK), with an even lower redox potential (E'° = -124 mV). No functional distinction has been made between these different quinones yet, although some reductases do have a higher affinity for mMK than MK (Dietrich and Klimmek, 2002; Juhnke et al., 2009).

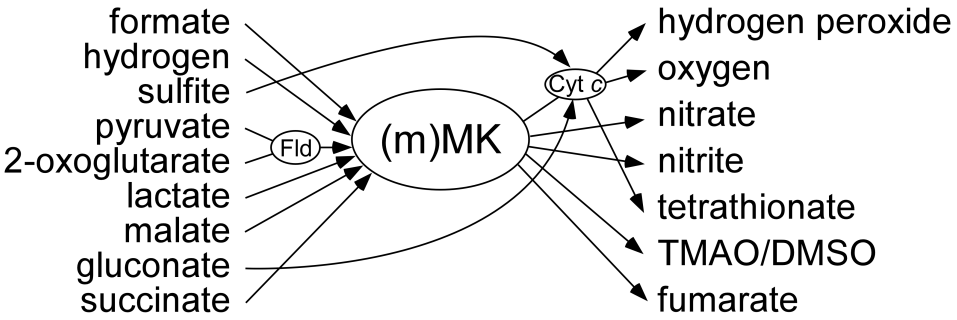


Figure 2: Overview of the *C. jejuni* electron transport chain. On the left, electron donors are ordered and listed using their respective standard electron potential, most negative on top. On the right, electron acceptors are shown, ordered using their respective standard electron potential, most positive on top. Electron carriers are depicted in ovals: Fld: flavodoxin, (m)MK: (methyl)menaquinone and cyt *c*. Arrows depict redox protein(s).

Electron acceptors

C. jejuni can utilize various molecules as electron acceptor, using different specialized enzyme complexes. Although the exposed iron-sulphur clusters of the atypical enzymes, like POR and OOR, make theses bacteria oxygen sensitive (Kendall et al., 2014), oxygen is the preferred electron acceptor for many epsilonproteobacteria including *C. jejuni* (Kelly, 2001; Sellars et al., 2002). During oxygen respiration the proton translocating cyt *bc*1 complex donates electrons to cyt *c* proteins complex, which subsequently reduces cyt cbb_3, a proton pumping oxidase (Jackson et al., 2007; Tanigawa et al., 2010, 1; Tsukita et al., 1999). Due to these two proton translocating enzyme complexes, oxygen respiration yields a pmf needed for growth. Apart from respiration with oxygen, respiration with

other compounds like fumarate, nitrate, nitrite, TMAO/DMSO or tetrathionate is possible if the appropriate reductase is expressed (Figure 2). Since most of these reductases have a catalytic subunit that is facing towards the periplasmic side of the membrane, these complexes are hypothesized to function as electron sink only (Biel et al., 2002; Guccione et al., 2010; Hermann et al., 2015; Pittman and Kelly, 2005).

Electron donors

Various carbon sources also act as electron donor in *C. jejuni*. For example, the oxidation of succinate, malate and lactate is directly coupled to menaquinone reduction (Figure 2) (Hoffman and Goodman, 1982). Epsilonproteobacteria encode for an atypical pyruvate oxidoreductase (POR) and a 2-oxoglutarate oxidoreductase (OOR), which are classically found in strict anaerobes (Hughes et al., 1998; Parkhill et al., 2000). Electrons liberated from pyruvate and 2-oxoglutarate oxidation are shuttled to the Nuo complex. The Nuo complex found in epsilonproteobacteria (εNuo) (named after its NADH:ubiquinone oxidoreductase orthologue in *E. coli*) is a proton pumping enzyme complex that reduces menaquinones. The *εnuoA-N* gene cluster is homologous to those of other organisms, except for the *nuoEF* genes. These genes are replaced with two other genes (*nuoXY*) that likely facilitate the redox coupling with the proteins flavodoxin or ferredoxin instead of the canonical NADH (Weerakoon and Olson, 2008). The functional implications of using a redox protein rather than NADH as electron shuttle to the εNuo complex, are unknown. However, some species are described to be dependent on exogenous electron donors (Gilbert et al., 2014; Macy et al., 1986). This could indicate that the εNuo complex is not able to sustain growth without another electrogenic electron donor. Formate and molecular hydrogen are used as electron donors and have a low mid-point redox potential. These kinds of organic or inorganic molecules that do not serve as carbon source, are only utilized as electron donor. Like *C. jejuni*, most epsilonproteobacteria encode at least one hydrogenase and a formate dehydrogenase (Hoffman and Goodman, 1982; Laanbroek et al., 1978; Marreiros et al.; Olson and Maier, 2002; Takai et al., 2005). The enzymes that oxidize hydrogen or formate have a similar topology. They have an active site in the periplasm and a membrane spanning subdomain that interacts with menaquinone near the cytoplasm. This makes these enzymes electrogenic, since the electrons from formate or hydrogen are either transported to an intracellular electron acceptor, or the protons are released by a periplasmic reductase (Biel et al., 2002; Jormakka et al., 2002). Both these events lead to a membrane potential due to charge separation and are therefore important in the survival of these organisms (Biel et al., 2002; Simon et al., 2008). Other molecules that are exploited as electron donor include sulfite and gluconate, which both donate their electrons to cyt *c*, bypassing menaquinones and the cyt bc_1 complex (Campbell et al., 2006; Myers and Kelly, 2005). The versatility of electron donors and –acceptors that can be used, hints at changing and variable environments that *C. jejuni* encounters.

Regulation of energy metabolism

Bacteria have evolved different strategies to adapt to the available metabolic substrates. Often an enzyme is only expressed when its substrate is present, or when all other possible substrates are exhausted (Golby et al., 1999; Unden and Bongaerts, 1997). This requires specific sensor and regulator proteins for every substrate. However, multiple

studies have shown that co-respiration takes place in epsilonproteobacteria, indicating that in these bacteria the regulation of branched electron transport chains respond to more global cues from the environment (Dahle et al., 2013; Goris et al., 2015; Lorenzen et al., 1993; Weingarten et al., 2008).
For *E. coli*, multiple global and local regulatory proteins have been identified that helps this bacterium to adapt to the presence of different electron acceptors and carbon sources. For instance, adaptation towards anaerobic conditions is regulated by ArcAB and FNR transcription factors, resulting in a complete metabolic reprogramming of the cell (Gunsalus and Park, 1994; Salmon et al., 2005). The DcuRS and NarPL two-component systems regulate the fumarate and nitrate reductase genes (Constantinidou et al., 2006; Goh et al., 2005), while the presence of different carbon sources leads to CRP dependent metabolic adaptation (Chubukov et al., 2014; Gosset et al., 2004). These and other well-studied regulators are absent from the genomes of epsilonproteobacteria. This lack of knowledge makes it difficult to predict the behaviour of this class of bacteria in different growth environments.

It has been reported that *C. jejuni* adapts its gene expression during the colonization of the chicken cecum (Woodall et al., 2005). Together with the cyt cbb_3 oxidase, a cyt *c* peroxidase and the alternative reductase complexes Nap, Nrf and Mfr are upregulated, indicative of adaptation towards low oxygen environment as exists in the chicken cecum. Furthermore, the enzyme AspA is highly upregulated, increasing the production of intracellular fumarate, which is consequently used as electron acceptor by FrdABC (Guccione et al., 2010). These observations suggest that regulation of energy metabolism does occur in *C. jejuni*. The underlying mechanism however, is still an enigma.

TRANSCRIPTION FACTORS OF *C. JEJUNI*

In prokaryotes, genes are transcribed to mRNA by RNA-polymerase (RNAP). The apo-RNAP needs to bind a sigma-subunit, which recognizes a specific nucleotide sequence on the DNA (Browning and Busby, 2004). Bacteria can express multiple sigma-factors that recognize distinct DNA sequences. Bacteria express other sigma-factors to drastically change the gene expression of many genes at once, for instance, during heat-, nitrogen- or starvation stress (Wösten, 1998).

C. jejuni encodes for only three sigma-factors: RpoD (σ^{70}), RpoN (σ^{54}) and FliA (σ^{28}). RpoN and FliA are involved in the expression of the motility genes (Jagannathan et al., 2001; Wösten et al., 2008). All other gene expression is dependent on RpoD (Dugar et al., 2013; Sharma et al., 2010). The consensus sequence recognized by RpoD consists of a extended -10 box and a periodic A/T signal, instead of a defined -35 sequence as present in other bacteria (Dugar et al., 2013; Petersen et al., 2003).

Apart from the sigma-factors, *C. jejuni* is predicted to contain 31 other transcription factors of which 7 belong to the family response regulators. Transcription factors (TF) can bind specific DNA sequences and interact with RNAP, thus promoting or preventing RNAP-DNA interaction. TFs use different mechanisms to activate or repress gene expression,

which is partially based on the location of DNA-binding relative to the transcriptional start site (TSS). For example, competitive binding of a TF to the sigma-factor binding site hinders RNAP binding and transcription. Binding on an upstream region, while attracting RNAP to the promoter region promotes transcription (Browning and Busby, 2004). Transcription factors of *C. jejuni* that do not belong to the family of response regulators have functions in heat-shock response (HrcA, HspR) (Holmes et al., 2010), iron or molybdenum homeostasis (Fur, ModE) (Taveirne et al., 2009; Vliet et al., 1998), reactive oxygen species stress (PerR, RrpAB) (Gundogdu et al., 2015; Handley et al., 2015), reactive nitrogen species stress (NssR) (Elvers et al., 2005) or heme uptake (Cj1387) (Johnson et al., 2016).

Two-component systems

A number of *C. jejuni* response regulators have been characterized in more detail. The DNA–binding response regulator together with a sensor protein (histidine kinase) form a two-component system (Ann M. Stock et al., 2000). The homodimeric sensor protein has a sensing domain that often protrudes into the periplasm, so it can probe the bacterial environment. Once a specific ligand is bound or another type of signal is sensed, an allosteric change moves the transmembrane domains relative to each other. This change is amplified by the HAMP domain, which causes the kinase domain to translocate to a new position, whereupon a conserved histidine residue (on either its own or the other dimer) is phosphorylated by the kinase domain (Gao and Stock, 2009). This phosphoryl group is subsequently transferred to an aspartate residue of the cognate response regulator. When not activated, the histidine kinase can also dephosphorylate the response regulator. Once phosphorylated, the response regulator has a higher affinity to its specific target sequence on the DNA (Stock et al., 1989). During this activated state, the response regulator behaves as a transcription factor and either activates or represses gene transcription. Two-component systems of *C. jejuni* are involved in phosphate limitation (PhosRS) (Wösten et al., 2006), motility (FlgRS) (Wösten et al., 2004), biofilm formation (CprRS, CosR) (Svensson et al., 2009; Turonova et al., 2015), and chicken colonization (DccRS, RacRS) (Brás et al., 1999; Wösten et al., 2010). Except for PhosS, the ligand or activating mechanism of these regulatory proteins has not been elucidated.

The putative two-component system RacRS (reduced ability to colonize) had been shown to be involved in adaptation towards chicken colonization (Brás et al., 1999). This system consist of a sensor protein RacS and a response regulator RacR. The sensor has an N-terminal periplasmic domain and a cytoplasmic signaling domain containing the histidine residue that is used to auto phosphorylate. The RacR protein has a helix-turn-helix motif, characteristic for DNA-binding proteins, and contains an aspartate residue that can be phosphorylated (presumably by RacS). The genes regulated by the RacRS system and how this system influence the adaptive capabilities of *C. jejuni* that enable colonization of the chicken gut, are still unknown.

1

AIMS AND OUTLINE OF THIS THESIS

Whereas for other pathogens like *Salmonella* and *Helicobacter* species, multiple virulence factors have been discovered, the pathogenic nature of *C. jejuni* remains largely enigmatic (van Putten et al., 2009). Certain is the requirement for energy to thrive and hence the search and competition for nutrients has been proposed to be a key determined in pathogenicity (Bäumler and Sperandio, 2016; Rohmer et al., 2011). Better understanding of the fundamentals of energy acquisition and its complementary regulatory structure could potentially lead to new strategies to combat bacterial colonization and disease (Barrett and Hoch, 1998; Cook et al., 2014). The metabolism and energy conservation mechanism in *C. jejuni* are poorly understood and all published studies show that *C. jejuni* has an aberrant physiology compared to other pathogenic model bacteria. To date, there are no proteins or mechanisms known that regulate these important aspects of the biology of *C. jejuni.* Therefore, the main aim of this thesis is to discover and unravel novel regulatory mechanisms of the metabolism and energy conservation pathways of *C. jejuni*. These could explain how this peculiar organism adapts and thrives in its natural environment. The starting point of this project was to fully elucidate the role of the crucial two-component system RacR/RacS in *C. jejuni;* to identify the complete regulon of RacR; to dissect the influence of genetic inactivation of RacR on the *C. jejuni* metabolism and to resolve the nature of the signal that is sensed by the sensor RacS.

In **chapter 2** of this thesis, the regulon, the stimulating conditions and the function of the unique RacR/RacS two-component system in *C. jejuni* is studied, with emphasis on the regulation of respiration. In **chapter 3** the role the RacR/RacS system in carbon and nitrogen metabolism is highlighted. In **chapter 4** experiments are conducted to elucidate the generation of the membrane potential in *C jejuni* and its impact on the motility, ATP production and growth. In **chapter 5** the regulation of the central metabolism is investigated which appeared to be non-canonical catabolite repression, dependent on intracellular succinate levels. The novel findings are discussed and put in a broader perspective in **chapter 6**.

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Chapter 2

The *Campylobacter jejuni* RacRS system regulates fumarate utilization in a low oxygen environment

Anne-Xander van der Stel[1*], Andries van Mourik[1*], Linda Heijmen-van Dijk[1], Craig T. Parker[2], David J. Kelly[3], Chris H. A. van de Lest[4], Jos P. M. van Putten[1] and Marc M. S. M. Wösten[1]

[*]These authors contributed equally

[1]Department of Infectious Diseases and Immunology, Utrecht University, Utrecht 3584CL, The Netherlands.
[2]Produce Safety and Microbiology Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Albany, CA 94710, USA.
[3]Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield, S10 2TN, UK.
[4]Departments of Biochemistry and Cell Biology, Utrecht University, Utrecht 3584CL, The Netherlands.

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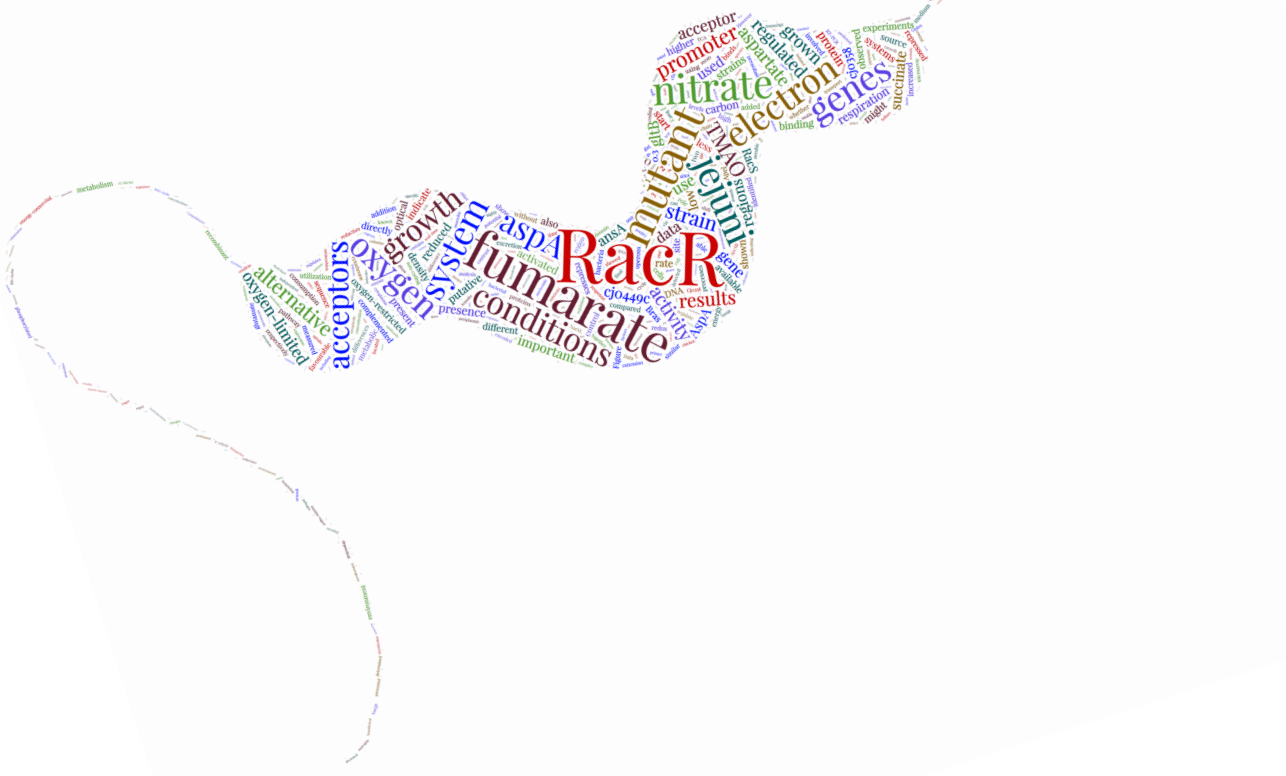

SUMMARY

The natural environment of the human pathogen *Campylobacter jejuni* is the gastrointestinal tract of warm-blooded animals. In the gut, the availability of oxygen is limited; therefore, less efficient electron acceptors such as nitrate or fumarate are used by *C. jejuni*. The molecular mechanisms that regulate the activity of the highly branched respiratory chain of *C. jejuni* are still a mystery mainly because *C. jejuni* lacks homologues of transcription factors known to regulate energy metabolism in other bacteria. Here we demonstrate that dependent on the available electron acceptors the two-component system RacRS controls the production of fumarate from aspartate, as well as its transport and reduction to succinate. Transcription profiling, DNAse protection and functional assays showed that phosphorylated RacR binds to and represses at least five promoter elements located in front of genes involved in the uptake and synthesis of fumarate. The RacRS system is active in the presence of nitrate and trimethyl-amine-N-oxide under oxygen-limited conditions when fumarate is less preferred as an alternative electron acceptor. In the inactive state, RacRS allows utilization of fumarate for respiration. The unique *C. jejuni* RacRS regulatory system illustrates the disparate evolution of *Campylobacter* and aids the survival of this pathogen.

INTRODUCTION

Metabolic activity is essential in all living organisms to conserve energy for cellular biosynthesis. A central pathway for biosynthetic processes is the tricarboxylic acid (TCA) cycle. During the main route of the TCA cycle, the oxidative direction, acetyl-CoA is converted to CO_2, reducing equivalents and biosynthetic intermediates. Carbon substrate oxidation results in the release of electrons to carriers that connect with the electron transport chain, where coupled proton translocation results in the establishment of an electrochemical proton gradient that is used to synthesize ATP. Bacteria control their energy-conserving processes at different levels, including via transcriptional regulatory systems that respond to the available carbon sources, electron acceptors and oxygen levels (Unden and Schirawski, 1997; Janausch et al., 2002; Bott, 2007; Tolla and Savageau, 2010). In *Escherichia coli,* several metabolic genes are regulated by the two-component system DcuSR, activated by extracellular C4-dicarboxylate molecules like fumarate (Golby et al., 1999). The ArcAB two-component system and the transcription factor FNR are both modulated in activity by changes in oxygen concentration. These systems activate hundreds of genes, including genes involved in carbon- and electron-flow, that control the switch between anaerobic and aerobic growth (Gunsalus and Park, 1994; Levanon et al., 2005). In other bacterial species, additional two-component systems, such as DctBD and CbrAB, regulate the sensing and utilization of nutrients to optimize metabolic processes (Reid and Poole, 1998; Nishijyo et al., 2001; Zhou et al., 2008).

To regulate the order of utilization of alternative electron acceptors in oxygen-restricted environments, many bacteria, including *E. coli* and *Pseudomonas aeruginosa*, exploit the nitrate-responsive NarXL or nitrite-responsive NarQP two-component systems (Unden

and Bongaerts, 1997; Schreiber et al., 2007). Several bacterial species also possess the two-component system TorSR to sense trimethylamine N-oxide (TMAO) and regulate transcription of genes for TMAO respiration during oxygen limitation (Bordi et al., 2004; Moore and Hendrickson, 2009). Such regulation results in a differential use of electron acceptors according to their midpoint redox potential, such that nitrate (E'° nitrate/nitrite +430 mV) is preferred over TMAO (E'° TMAO/TMA +160 mV), which is preferred over fumarate (E'° fumarate/succinate +30 mV).

Campylobacter jejuni is an oxygen-sensitive, spiral-shaped microaerophilic bacterial pathogen that shows a high metabolic versatility. The main natural habitat of *C. jejuni* is the oxygen-limited gastrointestinal tract of poultry, but the bacterium also survives in surface water and can be isolated at several points in the food chain from farm to fork. Metabolic versatility in *C. jejuni* includes a highly branched electron transport chain that allows both aerobic and anaerobic respiration (Kelly, 2008). *C. jejuni* seems to prefer aerobic respiration; however, it is also able to use alternative electron acceptors like nitrate, TMAO/ dimethylsulphoxide (DMSO), fumarate and tetrathionate (Sellars et al., 2002; Pittman et al., 2007; Liu et al., 2013). In addition, *C. jejuni* contains all the enzymes for a complete oxidative TCA cycle, central to a flexible energy metabolism (Kelly, 2008). In contrast, *C. jejuni* possesses only 37 transcription factors of which six form, together with a histidine kinase, a two-component system (Wösten et al., 2008). Furthermore, the bacterium cannot utilize sugars apart from L-fucose in some strains (Gripp et al., 2011) (Parkhill et al., 2000; Pearson et al., 2007; Stahl et al., 2011) and it seems that selected amino acids act as the major primary energy source. Serine and aspartate are the most preferred amino acids, but also glutamate, proline, asparagine and glutamine can be utilized, and some strains also have the ability to convert glutathione to glutamate (Guccione et al., 2008; Hofreuter et al., 2008).
Transcriptome analyses of *C. jejuni* grown under different environmental conditions indicate that metabolic gene expression is probably tightly regulated both *in vitro* (Gaynor et al., 2004) and *in vivo* (Stintzi et al., 2005; Woodall et al., 2005). Genes involved in the TCA cycle, the electron transport chain and intermediary metabolism are transcribed at higher levels in the chicken cecum (Woodall et al., 2005). Genes highly expressed in this low oxygen environment encode two different fumarate reductases (encoded by *frd* and the recently renamed *mfr*) (Weingarten et al., 2009; Guccione et al., 2010), an aspartase (encoded by *aspA*), and the antiport system DcuB, which takes up fumarate and secretes succinate (Guccione et al., 2008; 2010). Fumarate respiration has been shown to play an important role in chicken colonization and in *C. jejuni* intracellular survival (Weingarten et al., 2009; Liu et al., 2012). Taken together, these results suggest that *C. jejuni* must possess regulatory mechanism(s) that control the switch between metabolic pathways in response to nutrient and/or oxygen limitation. Yet, based on sequence homology, none of the above-mentioned two-component systems or transcription factors involved in metabolic adaptation are encoded in the *C. jejuni* genome (Parkhill et al., 2000; Pearson et al., 2007).

The *C. jejuni* RacRS system is a member of the OmpR/EnvZ family of two-component systems. The sensor RacS is a putative periplasmic-sensing histidine kinase, such as EnvZ containing a predicted periplasmic domain located between two transmembrane helices

and a cytoplasmic HAMP, HisKA and HATPase_c domain (Mascher et al., 2006). The RacRS system has been reported to be important in the ability to colonize chickens and to support growth above 42°C (Bras et al., 1999; Apel et al., 2012), suggesting that the system regulates genes important for *in vivo* colonization in a temperature-dependent manner. Furthermore, it has been reported that mutants in the RacRS two-component system display increased length heterogeneity, are reduced in swimming speed, and are defective for invasion of Caco-2 epithelial cells (Apel et al., 2012). So far two genes are known to be regulated by RacR, i.e. a periplasmic cytochrome *c* peroxidase homologue *cj0358c* (Bras et al., 1999) and *dnaJ* encoding a heat shock chaperone (Apel et al., 2012).

Here we report that the *C. jejuni* two-component system RacRS is not a temperature-sensitive system but is needed under oxygen-limited conditions for the cells to control fumarate metabolism, in the presence of other more energetically favourable electron acceptors. Under the oxygen-limited conditions present in the poultry gut, the RacRS system is likely to play a key role in determining optimal nutrient utilization.

RESULTS

The RacS and RacR proteins form a two-component system

To investigate whether RacS (Cj1262 / C8j1206 / YP_002344653.1) and RacR (Cj1261 / C8j1205 / YP_002344652.1) really constitute a two-component system, we performed phosphorylation and phosphotransfer assays. For these experiments, the cytoplasmic domain of RacS and the entire RacR protein were isolated as His-tagged recombinant proteins. Autophosphorylation of RacS was detected in the presence of radioactive ATP and a rapid phosphate transfer was observed when the recombinant regulator RacR was added to the phosphorylated RacS protein (Fig. 1). This phosphate transfer experiment shows that RacS and RacR communicate with each other and thus represent a classical two-component system.

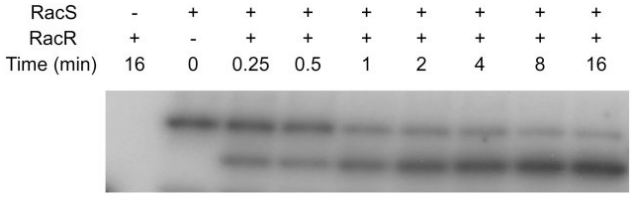

Figure 1: Phosphate transfer between the cytoplasmic domain of the sensor RacS and its cognate regulator protein RacR. In vitro phosphate transfer assay between the recombinant truncated Histidine-tagged RacS and recombinant Histidine-tagged RacR protein was visualized using 12% SDS-polyacrylamide gels and autoradiography. Autophosphorylation of the RacS protein (50 pmol) was observed after 15 min of incubation with [γ-32P]ATP at room temperature (not shown). Phosphotransfer from 32P-RacS to RacR (50 pmol) was followed at the indicated time points.

The RacRS system is required for growth under reduced oxygen levels

To address the role of the RacR–RacS two-component system in *C. jejuni* in more detail we inactivated the *racR* gene of strain 81116 (Palmer et al., 1983) by insertion of an antibiotic resistance cassette. Inactivation of *racR* in strain 81116 has previously been reported to result in a decreased growth rate at ≥ 42°C but not at 37°C (Bras et al., 1999; Apel et al., 2012). To confirm these results, we compared the growth rate of the mutant and parent strain at different temperatures. To our surprise no growth rate differences between the *racR* mutant and the parent strain were observed for strains grown under microaerophilic conditions in Heart Infusion (HI) medium either at 37°C, 42°C or at 44°C (Fig. 2A–C).

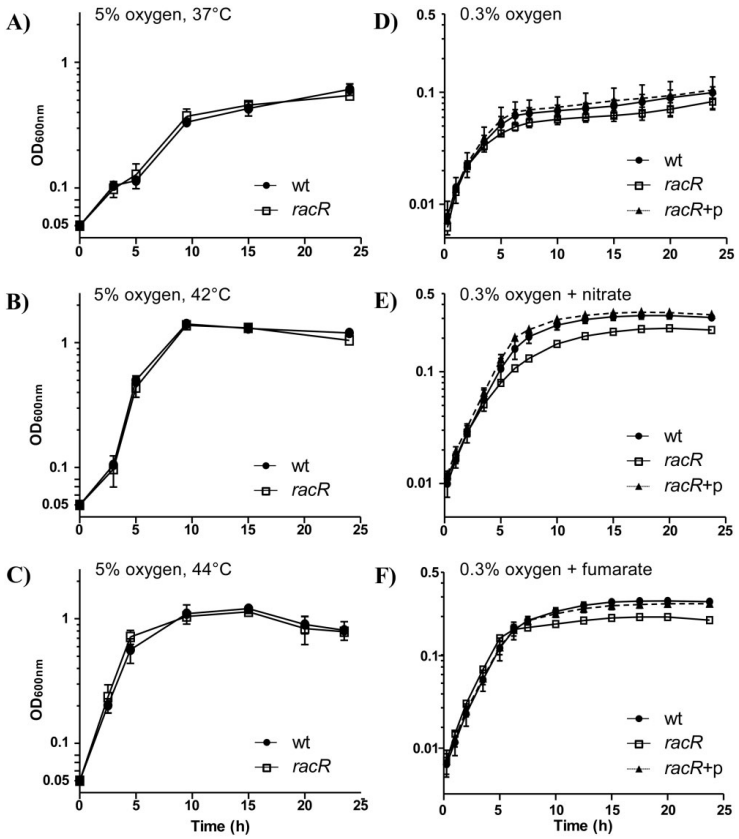

Figure 2: Growth curves of *C. jejuni* strain 81116. Growth is recorded of the wt (filled circle), the *racR* mutant (open square) and the *racR* mutant complemented with pMA1-1261-1263 (triangle up). Growth curves were generated in HI medium in a microaerophilic atmosphere (5% O_2) at: **A**. 37°C. **B**. 42°C. **C**. 44°C. **D**. Under oxygen-limited (0.3% O_2) conditions at 42°C. **E**. With 50 mM $NaNO_3$. **F**. With 50 mM sodium fumarate. Growth was determined by monitoring the optical density at 600 nm. Data are presented as mean of at least three independent experiments ± SD.

As RacR is known to be important for *C. jejuni* to colonize the (oxygen-limited) chicken gut and one of the two known genes regulated by the RacRS two-component system is a cytochrome *c* peroxidase homologue Cj0358 (Bras et al., 1999), we hypothesized that

instead of temperature, the concentration of oxygen may influence the activity of the RacRS regulon. Therefore, we measured the optical density of the wild-type (Wt) strain, the *racR* mutant and a complemented *racR* mutant grown in HI under reduced oxygen (0.3% O_2) conditions (Fig. 2D). A small but reproducible difference in the growth pattern was seen for the *racR* mutant compared with the parent strain, where the mutant cells appeared to enter stationary phase earlier than the Wt strain. This growth defect was restored after complementation of the mutant in trans with an intact copy of the *racR* and *racS* genes, indicating that the RacRS system might be important for *C. jejuni* to grow in oxygen-limited environments.

As oxygen is used as an electron acceptor, we tested if we could restore the growth defect of the *racR* mutant under low oxygen conditions by the addition of alternative electron acceptors such as TMAO, nitrate or fumarate (Sellars et al., 2002). The growth difference between the Wt and *racR* mutant increased in the presence of TMAO (50 mM) (data not shown) or when 50 mM nitrate was added as an alternative electron acceptor (Fig. 2E). When fumarate (50 mM) was added, both the Wt and the *racR* mutant had a similar growth rate during the logarithmic phase; however, in contrast to Wt, the growth rate of the *racR* mutant decreased earlier when the bacteria entered the stationary phase (Fig. 2F). Complementation in trans restored the growth defects of the *racR* mutant. The actual number of colony-forming units (cfu) per millilitre of culture at several time points are verified by serial dilution and plating (data not shown). No difference was found between the growth of the Wt and RacR mutant by OD_{600} readings versus cfu counting. These results show that the *racR* mutant grows less well compared with Wt when alternative electron acceptors have to be used.

Identification of the genes dependent on a functional RacRS system

To identify the mechanism(s) causing the RacR phenotype, we first compared the gene expression profiles of the Wt and the *racR* mutant by microarray analysis. For this purpose, total RNA was isolated from early stationary phase cultures grown under oxygen-limited (0.3%) conditions with 50 mM nitrate at 42°C. These experiments revealed that 11 genes are activated and 17 genes are repressed more than fourfold by the RacRS system (Fig. 3A, Supporting Information Tables S3 and S4). As certain ribosomal genes that we did not expect to be regulated by RacR showed more than a threefold difference between the Wt and *racR* mutant, we focused on genes that showed more than fourfold difference in transcript level. To verify the microarray results, the transcript levels of 14 of the 28 upregulated or downregulated genes comprising all single genes (except two ribosomal genes) and one gene of each putative operon were determined by real-time RT-PCR. The transcript abundance of 11 of the selected genes differed more than fivefold between the Wt and the *racR* mutant (Fig. 3B). Collectively, the microarray and real-time RT-PCR data indicate that inactivation of the *racR* gene represses the transcription of the *gltB* gene and four putative operons: *cj0175c-cj0176c*, *fldA-cj1384c*, *katA-cj1386* and *chuABCD*. Besides hypothetical proteins, these genes code for a glutamate synthase (*gltB*), iron-uptake ABC transporter (*cj0175c*), flavodoxin (*cj1382c*), ankyrin repeat-containing protein (*cj1386*) and a catalase (*katA*).

A)

wt over *racR*			wt over *racR*		
cj0007	*gltB*	4.282	*cj0029*	*ansA*	-5.001
cj0175c	*cj0175c*	5.154	*cj0068*	*pspA*	-4.037
cj0176c	*cj0176c*	4.735	*cj0069*	*cj0069*	-4.769
cj0985c	*hipO*	4.411	*cj0086c*	*ung*	-7.28
cj1382c	*fldA*	5.249	*cj0087*	*aspA*	-78.15
cj1383c	*cj1383c*	5.065	*cj0088*	*dcuA*	-12.49
cj1384c	*cj1384c*	5.714	*cj0089*	*cj0089*	-4.281
cj1385	*katA*	6.23	*cj0244*	*rpmI*	-4.048
cj1386	*cj1386*	5.147	*cj0358*	*cj0358*	-8.192
cj1615	*chuB*	4.896	*cj0437*	*mfrA*	-4.014
cj1617	*chuD*	4.358	*cj0438*	*mfrB*	-4.24
			cj0448c	*cj0448c*	-5.119
			cj0449c	*cj0449c*	-6.947
			cj0961c	*rpmH*	-4.296
			cj1491c	*cj1491c*	-4.167
			cj1492c	*cj1492c*	-4.089
			cj1493c	*cj1493c*	-8.08

B)

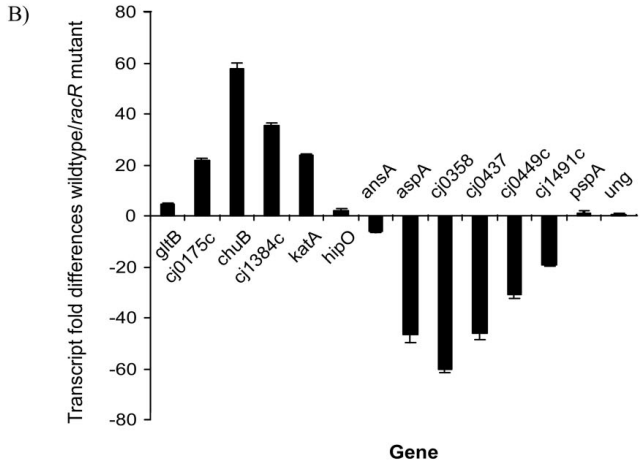

Figure 3: Identification of RacR-dependent genes. **A**. Microarray results obtained by comparing mRNA profiles of the strain 81116 and the *racR* mutant strain grown under oxygen-limited conditions with 50 mM $NaNO_3$ at 42°C. Shown are genes with a more than fourfold higher or lower transcript amount in the Wt strain compared with the *racR* mutant (left and right columns respectively). **B**. Transcript fold differences between strain 81116 and its *racR* mutant grown under oxygen-limited conditions observed for the *ansA, pspA, ung, gltB, cj0175c, chuB, cj1384c, katA, hipO, aspA, cj0358, mfrA, cj0449c* and *cj1491c* genes as estimated by RT-PCR. Each sample was examined in four replicates and experiments were repeated with at least two independent preparations of RNA. Data are presented as mean ± SD.

Inactivation of the *racR* gene leads to activation of the transcription of four putative operons: *aspA-dcuA-cj0089*, *mfrXABE* (formerly annotated *sdhABC*; Guccione et al., 2010), *cj0448c-cj0449c* and *cj1491c-cj1493c*. The *aspA* gene codes for aspartase, which converts aspartate to fumarate. This gene is located in a putative operon together with *dcuA* (an anaerobic C4-dicarboxylate transporter) and *cj0089*, coding for a hypothetical protein. The *mfrXABC* genes encode a periplasmic facing fumarate reductase enzyme complex that converts fumarate to succinate. *cj0448c* is predicted to encode an accessory colonization factor (*acfB*) as identified in *Vibrio cholerae* (Everiss et al., 1994) or a probable MCP protein Tlp6 (Hartley-Tassell et al., 2010), while *cj0449c* codes for a hypothetical protein. The proteins translated from the *cj1491c*, *cj1492c* and *cj1493c* genes might code for a putative two-component sensor, a two-component regulator and an integral membrane protein respectively. Finally, we could confirm that the cytochrome *c* peroxidase homologue Cj0358 identified by Bras and colleagues (1999) is repressed by RacR. Overall,

the data indicate that the RacRS system mainly regulates genes important for fumarate respiration, iron acquisition and oxidative stress responses.

RacR DNA binding motif

To investigate whether RacR is able to directly bind to DNA promoter regions, electrophoretic mobility shift assays (EMSA) were performed. Recombinant RacR or phosphorylated RacR (accomplished by the addition of RacScyto and ATP) was incubated with [γ-^{32}P]ATP labelled DNA fragments containing the promoter regions of *gltB*, *cj0176c*, *chuA*, *ansA*, *aspA*, *cj0358*, *mfrX*, *cj0449c*, *cj1493c*, the intergenic region between *cj1384c* and *katA* genes or the RacR-independent *phoX* gene. A band shift was only observed for the *gltB*, *aspA*, *ansA*, *cj0358* and the *cj0449c* promoter region (Fig. 4), but not for the other promoter elements. Less RacR protein was needed to observe a band shift when RacR was phosphorylated by the cytoplasmic domain of RacS. The band shifts were not present or only a part of the ^{32}P-labelled DNA fragments shifted when an excess of unlabelled DNA was added. These results show that the *aspA*, *ansA*, *cj0449c*, *cj0358* and *gltB* promoter elements are regulated directly by RacR, while the other promoter elements are probably indirectly dependent on RacR.

The complete transcriptome map, including all transcription start sites of *C. jejuni* 81116 genes, has recently been published (Dugar et al., 2013). To investigate whether these transcription start sites include the RacR-dependent 5' mRNA, we determined the 5'-mRNA start site of the RacR-dependent promoters *ansA*, *aspA* or *cj0449c* by primer extension experiments (Fig. 5A). A clear cDNA product was detected 94, 58, 39 bp in front of the *ansA*, *aspA* or *cj0449c* initiation codons respectively, but only when RNA of the *racR* mutant was used, consistent with the real-time RT-PCR and microarray data for these genes. The identified 5' mRNAs were identical to the transcriptional start sites located on the published transcriptome map of *C. jejuni* 81116 (Dugar et al., 2013). Based on data of Dugar and colleagues (2013) the 5' mRNAs of the *gltB* and *cj0358* genes starts 44 and 29 bp upstream of the start codon respectively.

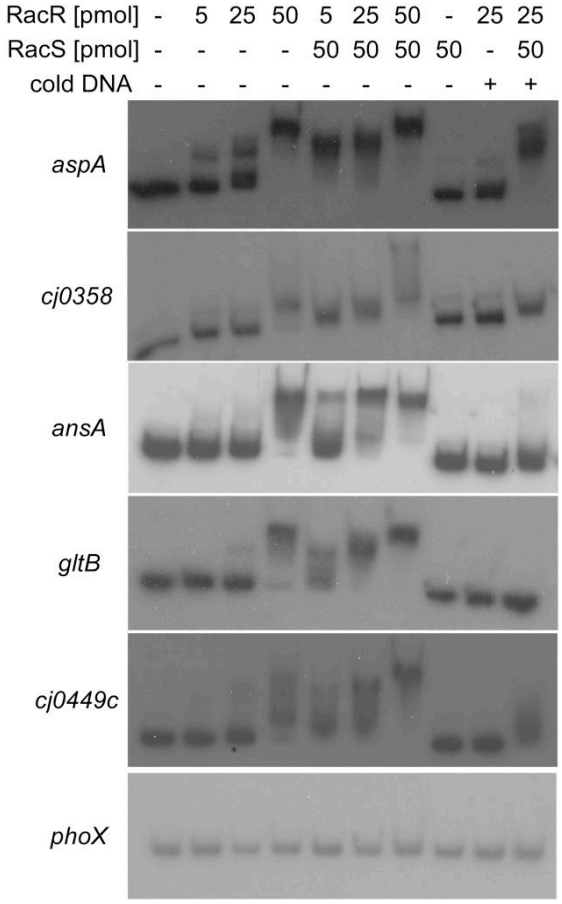

Figure 4: Identification of genes directly regulated by RacR. Electrophoretic mobility shift assays showing that recombinant Histidine-tagged RacR binds to the [γ-^{32}P]ATP labelled promoter regions (~ 25 pmol) upstream the *gltB, aspA, ansA, cj0358* and the *cj0449c* promoter regions but not to the negative control, the *phoX* promoter region. The concentration of the proteins in each reaction is indicated at the top of each lane. The specificity of the protein–DNA interaction was determined by the addition of a 10-fold excess of unlabelled promoter region DNA.

To identify whether RacR binds to a specific nucleotide sequence, footprinting experiments were performed on both strands of the *aspA*, *ansA*, *cj0449c*, *cj0358* and *gltB* promoter elements. Only when phosphorylated RacR was used did RacR protect the coding as well as non-coding regions in all five promoter elements from DNAses, except for the non-coding region of *cj0358* (Supporting Information Fig. S1). A large part of the nucleotide sequence of the coding and non-coding strands recognized by RacR overlapped each other (Fig. 5B). A sequence logo could be constructed of the nucleotide sequence of the five identified RacR binding regions (Fig. 5C) revealing that RacR binding site is very AT-rich. Based on the primer extension and footprinting results, RacR binds close to or at the putative −10 regions

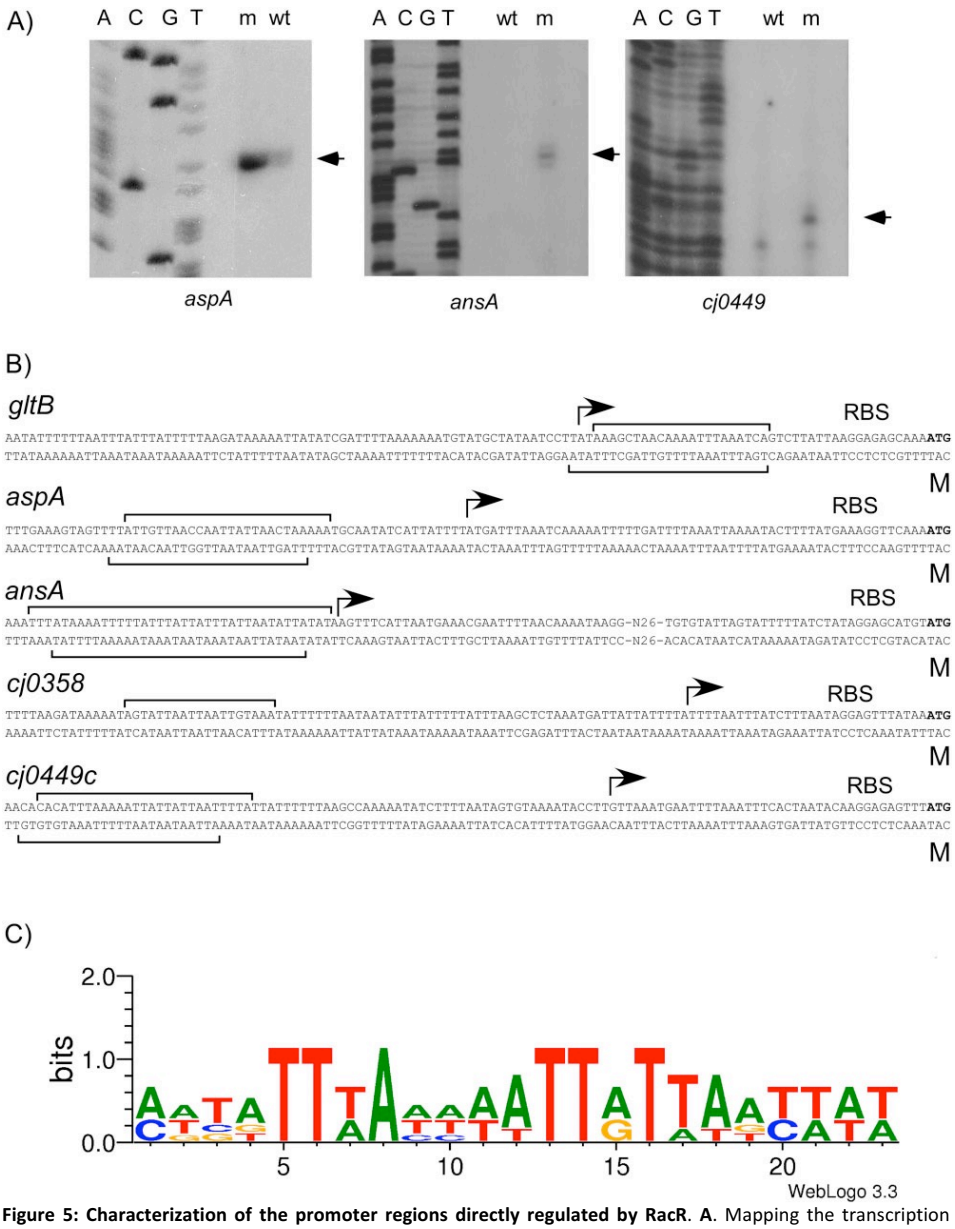

Figure 5: Characterization of the promoter regions directly regulated by RacR. **A**. Mapping the transcription start sites of the *ansA, aspA and cj0449c* promoters. Primer extension products were generated using total RNA isolated from strain 81116 (wt) or the *racR* mutant (m). Bacteria were grown in defined medium (DM) under low (0.3%) oxygen conditions containing 50 mM nitrate. The primer extension products were run on a 6% sequencing gel against dideoxy sequencing reactions primed with the same primer as used for the extension reactions. The sequence spanning the transcription start site is shown, and the transcription start site is marked with an arrow. **B.** Promoter regions of the *gltB, aspA, ansA, cj0358* and the *cj0449c* genes. The hooked arrow indicates the transcription start site. Brackets mark the DNase I-protected nucleotides by RacR of the coding and non-coding strands. **C**. Sequence logo of the DNase I-protected nucleotides by RacR of the *gltB, aspA, ansA, cj0358* and the *cj0449c* promoter regions using WebLogo software.

RacR controls AspA activity in response to the electron acceptors nitrate and TMAO

The nature of the genes regulated by RacRS system and the phenotypic characterization of the *racR* mutant predicts that the RacR/RacS signal transduction system plays a role in the recognition of the nature of available electron acceptors. To substantiate this, we measured the activity of the product of the *aspA* gene, which, as shown above, is a repressed and directly regulated RacRS-dependent gene. The activity of the AspA enzyme, which converts aspartate to fumarate, was measured for strains grown in defined medium (DM) under high (10%) or low (0.3%) oxygen conditions with aspartate as main C-source and with or without the addition of one of the alternative electron acceptors, nitrate, TMAO or fumarate. At high oxygen conditions, no AspA activity was detected in either Wt or *racR* mutant cells, while under low oxygen conditions a high activity was recorded, in agreement with the known oxygen regulation of AspA (Guccione et al., 2008). However, the AspA activity of the Wt strain was not only strongly reduced at high oxygen concentration but also significantly reduced under low oxygen conditions in the presence of nitrate or TMAO (Fig. 6). In contrast, under oxygen-limited conditions, the AspA activity in the *racR* mutant was similar in the absence or presence of nitrate, TMAO or fumarate (Fig. 6). These results show that AspA activity depends on the nature of available electron acceptors and that RacR regulates AspA activity under low oxygen conditions when nitrate or TMAO are available.

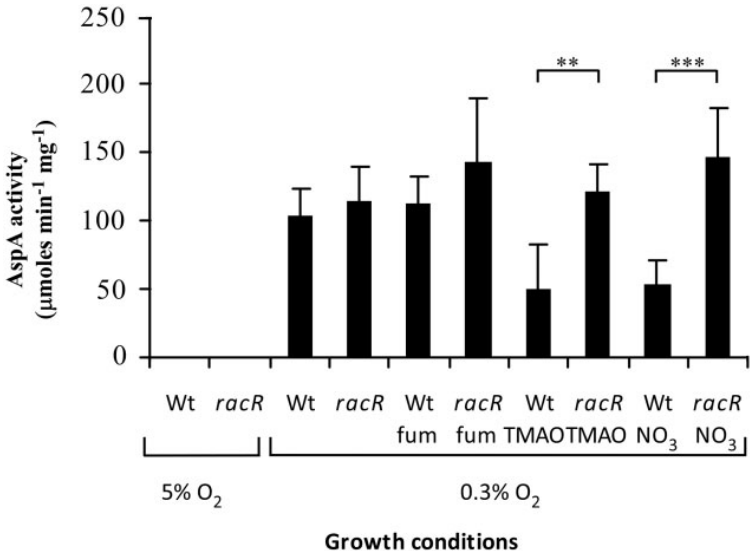

Figure 6: Influence of the RacR on AspA activity. AspA enzyme activity was measured of strains grown in DM under high (10%) or low (0.3%) oxygen conditions with aspartate as main carbon source and with or without 50 mM nitrate, 50 mM TMAO or 5 mM fumarate as alternative electron acceptors. Aspartase enzyme activity presented as µmol min^{-1} mg $protein^{-1}$ was measured at 240 nm, corresponding to the absorbance of fumarate. Data are presented as mean ± SD. Data were analysed by ANOVA with Bonferroni multiple comparison test (**P < 0.01, ***P < 0.001).

RacR is required to make optimal use of nutrients when less favourable electron acceptors are present

The AspA activity results described above imply that the RacRS two-component system acts to decrease conversion of aspartate to fumarate under low oxygen conditions when other more energetically favourable electron acceptors are available. To test this hypothesis further, we first investigated whether the *racR* mutant is able to grow in media with fumarate as sole carbon source. For this purpose, we used the defined medium (DM) of Leach and colleagues (1997), lacking serine, aspartate, asparagine, proline, glutamine, glutamate, pyruvate and 2-oxoglutarate. The Wt and the *racR* mutant were unable to grow in this minimal medium unless a carbon source was added (Fig. 7A). Both strains reached a similar final optical density under microaerophilic conditions when 15 mM fumarate was added. However, at reduced oxygen levels, the final optical density of the *racR* mutant was significantly less than that of the Wt or the complemented *racR* mutant. This indicates that the *racR* mutant grows on fumarate as sole carbon source but less well than the Wt under oxygen-limited conditions. The final optical density of the Wt and the complemented *racR* mutant strongly increased when, in addition to fumarate, 25 mM nitrate was also present. In contrast to the Wt and the complemented *racR* mutant, the optical density of the *racR* mutant under these conditions hardly increased, indicating that nitrate is poorly used by the *racR* mutant as electron acceptor. When pyruvate together with fumarate was added as carbon source the final optical density of both the Wt and the *racR* mutant was twice as high as with fumarate alone. These data suggest that the *racR* mutant is able to use fumarate as carbon source, but in contrast to the Wt, is unable to repress fumarate respiration in response to alternative electron acceptors under low oxygen conditions.

Fumarate reduction via either Frd or Mfr will result in succinate excretion into the environment. To test whether the *racR* mutant is indeed using fumarate mainly as an electron acceptor, we grew the Wt, *racR* mutant and complemented *racR* mutant with fumarate or fumarate and nitrate under reduced oxygen (0.3% O_2) conditions in DM (Leach et al., 1997) containing 20 mM aspartate instead of 20 mM serine and analysed the spent media of these strains by LC/MS/MS for the utilization of aspartate and fumarate and for the excretion of succinate. The growth curves with fumarate plus nitrate (Fig. 7B) were similar to those with nitrate alone (data not shown). Under both conditions, the *racR* mutant yielded a lower growth rate and lower final optical density than the Wt strain. No differences were observed in the utilization of aspartate and fumarate when the strains were grown without nitrate (Fig. 7C and D). However, especially in the Wt strain, the rate of consumption of aspartate was strongly reduced when nitrate was present (Fig. 7C). Although the rate of fumarate consumption by the *racR* mutant was a little less with nitrate, it was significantly higher than by the Wt grown with nitrate (Fig. 7D). So, although the *racR* mutant consumed aspartate and fumarate faster than the Wt in the presence of nitrate (Fig. 7C and D), this did not result in a higher optical density (Fig. 7B). The amount of succinate excreted by the *racR* mutant grown with or without nitrate was similar, indicating that under these growth conditions fumarate is used by the *racR* mutant as electron acceptor (Fig. 7E). Consistent with the higher rate of fumarate consumption in the *racR* mutant compared with Wt, the rate of succinate excretion by the *racR* mutant was also much higher than that of the Wt grown with nitrate. The fact that the specific

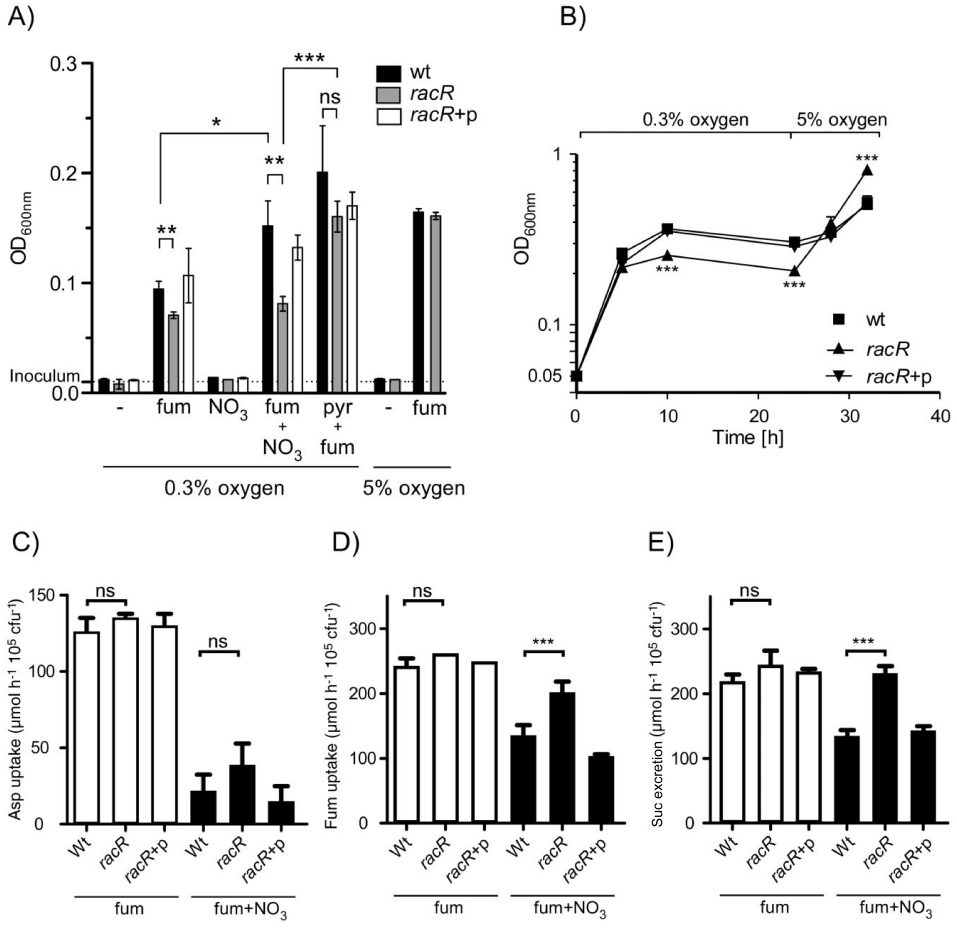

Figure 7: RacR influences nutrient utilization. The *racR* mutant is less able to make optimal use of nutrients under reduced oxygen concentrations in the presence of nitrate. **A**. Final optical density OD_{600} of the Wt and the *racR* mutant grown in minimal DM lacking, serine, aspartate, asparagine, proline, glutamine, glutamate, pyruvate and 2-oxoglutarate with or without 15 mM fumarate, 25 mM nitrate or 15 mM pyruvate under microaerophilic (5% O_2) or oxygen-limited (0.3% O_2) conditions at 42°C. **B**. Growth curves of the *C. jejuni* Wt (filled squares), the *racR* mutant (filled triangle up) and the *racR* mutant complemented with pMA1-1261-1263 (triangle down) grown for 24 h at 42°C in DM with 50 mM fumarate and 50 mM nitrate under oxygen-limiting conditions followed by 8 h of growth under microaerobic conditions. The results shown are the mean and standard deviation of three independent experiments. The consumption or secretion of aspartate, fumarate and succinate in the medium by the 81116 Wt, *racR* mutant strain and the *racR* complemented strain grown in DM under oxygen-limiting conditions with 50 mM fumarate and with or without 50 mM nitrate was measured by using LC/MS/MS. DM contains 20 mM aspartate. **C**. Aspartate consumption (µmol h^{-1} 10^5 cfu^{-1}). **D**. Fumarate consumption (µmol h^{-1} 10^5 cfu^{-1}). **E**. Succinate secretion (µmol h^{-1} 10^5 cfu^{-1}). The data shows the mean and standard deviation of three independent experiments using LC/MS/MS analysis. Data were analysed by ANOVA with Bonferroni multiple comparison test (*P < 0.05, **P < 0.01, ***P < 0.001).

rates of fumarate consumption and succinate excretion were very similar indicates stoichiometric fumarate reduction is occurring. Overall, these data indicate that even in the presence of the energetically more favourable nitrate, the *racR* mutant still uses fumarate as electron acceptor and therefore cannot optimally adjust its electron acceptor preference in the same way that Wt bacteria are able to do.

As excreted succinate should be taken up and catabolised by bacteria grown under oxygen-rich conditions, we followed changes in growth when cells were initially maintained under oxygen-limited (0.3% O_2) conditions for 24 h and then incubated for another 8 h under standard microaerobic (5% O_2) conditions (Fig. 7B). The increased availability of oxygen only slowly increased Wt growth (doubling time 10 h) but caused rapid growth of the *racR* mutant (doubling time 4 h). This would be consistent with the higher succinate concentrations excreted by the *racR* mutant during oxygen-limited growth now supporting a period of more rapid aerobic growth compared with the Wt cells.

DISCUSSION

Regulation of energy-conserving metabolic processes is crucial for bacteria to survive environmental changes and compete with other microorganisms. Currently, there is only limited information on the regulation of such energy-conserving processes in *C. jejuni*. Here we show that the RacRS two-component system prevents the use of fumarate as electron acceptor when more favourable electron acceptors are present as depicted in a simplified model (Fig. 8). In this model, we propose that the RacRS system is activated under low oxygen conditions when the electron acceptors nitrate or TMAO are present. Under these conditions, the RacRS system represses the uptake of fumarate via DcuA and the production of fumarate via the aspartate–fumarate pathway (AspA) in order to prevent the use of fumarate as electron acceptor, which might otherwise cause a loss of carbon and energy in the form of excreted succinate. Our data demonstrate that this system is needed to optimize nutrient utilization under oxygen-restricted conditions, and we showed by transcriptome analysis, EMSA and footprinting experiments that RacR directly binds to DNA thereby activating and repressing genes involved in key energy-conserving reactions.

Previous studies have demonstrated that the RacRS two-component system is important for *C. jejuni* to colonize chickens and that this system supports growth at 42°C (Bras et al., 1999) or above 42°C (Apel et al., 2012). Two genes, encoding a periplasmic cytochrome *c* peroxidase homologue Cj0358 (Bras et al., 1999) and *dnaJ* encoding a heat shock chaperone (Apel et al., 2012), have been reported to be regulated by RacR. Although temperature dependent transcription is a well-known feature in several other bacterial species (Goransson et al., 1990; Aguilar et al., 2001; Braun et al., 2007), we were unable to confirm the temperature-sensitive phenotype for the RacRS system in strains *Cj*81116 (Fig. 2B and C), *Cj*11168 or *Cj*81-176 (data not shown). These different phenotypic results may be due to temperature-dependent oxygen solubility, the use of other lab strains of

*Cj*81116 and *Cj*81176, the design of *racR* mutation, microaerobic conditions (with or without H_2) or slight differences in growth conditions (media/vessels/volume/shaking) used in different lab.

The putative cytochrome *c* peroxidase Cj0358 initially suggested to us that the RacRS system might be responding to different oxygen concentrations instead of the temperature. The *racR* mutant showed slightly reduced growth under restricted oxygen conditions (Fig. 2D), which might explain the inability of the mutant to colonize the oxygen-restricted environment of the chicken gut (Bras et al., 1999) (Weingarten et al., 2008). *C. jejuni* contains a complex network of enzymes allowing the use of alternative electron donors and acceptors (Kelly, 2008), permitting growth and energy conservation in the low oxygen environment of the poultry gut. Therefore, we tested if we could restore the growth defect of the *racR* mutant under low oxygen by the addition of alternative electron acceptors such as nitrate, TMAO or fumarate (Sellars et al., 2002). Because of differences in reduction potential between different redox couples, there is an ordered preference in the usage of these molecules (Iuchi and Lin, 1987). More severe growth differences were observed when nitrate or TMAO was used as alternative electron acceptor (Fig. 2E); however, only a minor effect was observed when fumarate was present (Fig. 2F). Similar results were obtained for the *C. jejuni* strains NCTC 11168, 81-176 and isogenic *racR* mutants (data not shown). These results indicate that the RacRS system is important under oxygen-restricted conditions for the cells to be able to use other alternative electron acceptors.

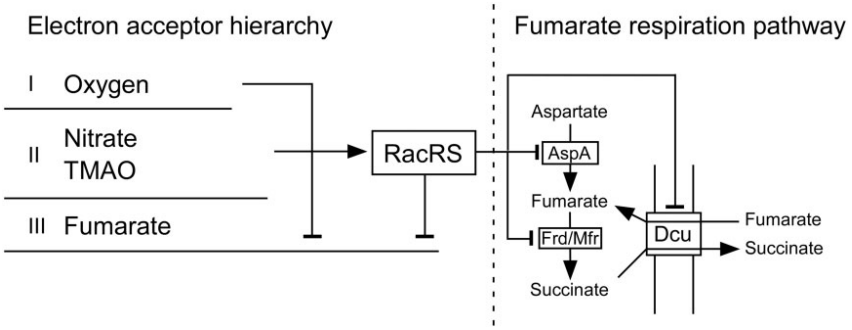

Figure 8: Role of the RacR/RacS system in *C. jejuni* strain 81116. The RacR/RacS system represses the use of fumarate as electron acceptor when more favourable electron acceptors (nitrate or TMAO) are available under oxygen-limited conditions. This is accomplished by repressing the aspartate–succinate pathway. Repressing the fumarate respiration pathway leads to less excretion of succinate in the environment.

Microarray analysis and real-time RT-PCR analyses indicate that the *gltB* gene and four putative operons, *cj0175c-cj0176c, fldA-cj1384c, katA-cj1386* and *chuABCD*, are activated, while *cj0358* and four putative operons, *aspA-dcuA-cj0089*, *mfrXABE*, *cj448c-cj0449c* and *cj1491c-cj1493c*, are repressed when RacR is active (Fig. 3). Cj0358 has previously been identified to be regulated by RacR (Bras et al., 1999). We were unable to confirm RacRS regulation of *dnaJ* (Apel et al., 2012) in our strain 81116. Interestingly, the operons that are activated by RacR are repressed by the iron-responsive transcription factors Fur (Palyada et al., 2009) or PerR (van Vliet et al., 1998). The Fur repressed operons *cj0175-*

cj0176, *chuABCD* and *fldA-cj1384c* encode respectively for a Fe^{3+}- and hemin ABC transport system and a flavodoxin. The *C. jejuni* flavodoxin is an efficient electron acceptor of OOR, a complex that plays a vital role in central carbon metabolism in the TCA cycle by decarboxylation of 2-oxoglutarate in the presence of Coenzyme-A (Weerakoon and Olson, 2008). PerR represses the *katA-cj1386* operon, which encodes a catalase that degrades hydrogen peroxide to prevent the formation of highly reactive oxygen species. Another gene activated by the RacRS system is *gltB*, a gene involved in the generation of glutamate. Glutamate is by far the most important natural source of nitrogen for bacteria and it can be used to produce aspartate (Reitzer, 2003; Guccione et al., 2008). RacR, however, represses the aspartate–fumarate pathway, as the transcription of genes that are involved in uptake (*dcuA*), generation (*aspA*, *ansA*) and reduction (*mfrXABE*) of fumarate are upregulated in the *racR* mutant. Taken together, these results indicate that the RacRS system might be important for regulating the balance between catabolism and anabolism or balancing the carbon:nitrogen ratio (Kumar and Shimizu, 2010). Some RacR-dependent genes may be directly regulated by another two-component system encoded by the *cj1491c* and *cj1492c* genes as the transcription for these genes is increased in a *racR* mutant.

The RacR protein contains a clear helix–turn–helix motif, characteristic for DNA binding proteins. Our results indicate that recombinant RacR directly binds to the promoter regions located upstream of *gltB*, *aspA*, *ansA*, *cj0358* and the *cj0449c* genes (Fig. 4 and 5). A single retarded DNA–RacR complex was detected indicating that these promoter regions contain only one RacR binding site. Phosphorylated RacR has a higher affinity for the RacR-dependent regions than unphosphorylated RacR, a phenomenon often observed for two-component regulators (Cho et al., 2001). The Fur/PerR regulated genes and the putative two-component system Cj1491c/Cj1492c are probably indirectly regulated by RacR as no RacR binding could be detected by EMSA. Footprinting analysis revealed a clear overlap of the coding and non-coding strands of the *gltB*, *aspA*, *ansA* and the *cj0449c* promoter regions confirming that only a single RacR binding site is present in each promoter region. These promoter regions have in common the RacR binding site consisting of 23 nucleotides (Fig. 5C), which is in the opposite orientation in the repressed versus activated RacR-dependent promoters, a phenomenon also observed for other two-component regulators (Unden and Bongaerts, 1997; Bezy and Kehoe, 2010). RacR does not bind to a fixed position in relation to the transcription start point. The reason for this might be that RacR can be a repressor and activator of genes at the same time. A genomic search revealed that there are 19 other potential RacR binding sites in the genome of *C. jejuni* 81116 of which the *mfr* promoter is one. This may indicate that RacR binds to a specific AT-rich DNA structure (Rohs et al., 2010) rather than an absolutely conserved nucleotide sequence as exists for the *C. jejuni* two-component systems PhosRS and DccRS (MacKichan et al., 2004; Wösten et al., 2006). Based on the microarray data and the identified RacR binding site, more genes might be directly regulated by RacR.

The phenotypic characterization and the identification of the RacR regulon indicate that this system is important under oxygen-restricted conditions for the use of alternative electron acceptors and to control energy-conserving processes. As the production of fumarate (E'° +30 mV) via AspA is repressed by RacR, the function of the *C. jejuni* RacRS

system might be to reduce the use of fumarate as electron acceptor when molecules with higher and thus more favourable midpoint redox potential are available. The alternative electron acceptors nitrate (E'° +430 mV) and TMAO (E'° +160 mV) increased the activity of the RacRS system as measured by a decreased AspA activity (Fig. 6), which leads to reduced fumarate respiration and therefore to a reduced excretion of succinate (Fig. 7). These results show that the RacRS system is important under oxygen-restricted conditions to control fumarate metabolism when higher redox potential electron acceptors are present.

In *E. coli* the usage of alternative electron acceptors is under strict hierarchal control of different transcriptional regulators (Unden and Bongaerts, 1997; Goh et al., 2005). The ArcAB two-component system modulates the expression of numerous genes in response to a shift from aerobic to anaerobic growth conditions by monitoring the redox state of the quinone pool (Bekker et al., 2010). The TorSR system is activated by TMAO under anaerobisis and induces the expression of the TMAO reductase genes in *E. coli* (Bordi et al., 2004). To use nitrate as an alternative electron acceptor under oxygen-restricted conditions, *E. coli* and *P. aeruginosa* use two dual-component regulatory systems, NarXL and NarQP, which are both activated by nitrate (Goh et al., 2005). NarXL represses the fumarate respiration pathway as NarL represses genes like *aspA*, *dcuA*, *dcuB* and *frdABCD* genes. NarQP controls reductases for the alternative electron acceptors nitrate and nitrite [respectively encoded by the *nap*- and *nrf* genes (Stewart and Bledsoe, 2003)]. The RacRS system is not a homologue of the NarXL, TorSR or ArcAB two-component systems, although functional similarities exist. RacRS and NarXL are both activated under oxygen-restricted environments in the presence of nitrate and they repress the fumarate respiration pathway (Fig. 7). However, RacRS has a broader function as it is also activated under oxygen-restricted conditions in the presence of TMAO. In *C. jejuni*, the *nap*, *nrf* and TMAO reductases are neither regulated by oxygen nor by other conditions (Sellars et al., 2002; Taveirne et al., 2013). Because no homologues exist of the NarQP, TorSR, ArcAB and the DcuSR two-component systems nor a homologue of the transcription factor FNR, the alternative electron acceptors may not be hierarchically controlled in *C. jejuni* except for fumarate as this compound can be used as either carbon source or electron acceptor. The *C. jejuni* RacRS system might have evolved to a less specific regulatory system monitoring the redox state of, for instance the quinone pool, like the *E. coli* ArcAB to allow maximal use of available nutrients. However, further work will be needed to determine exactly what RacS is sensing and how this information is transduced to RacR.

In conclusion, we show that the RacRS system is the first *C. jejuni* transcription regulatory system identified to play an important role in central energy metabolism. We show that this system regulates the uptake, synthesis and respiration of fumarate in response to the availability of alternative electron acceptors, in order to fine-tune the bacterial metabolic behaviour during restricted-oxygen conditions, as exists in its primary natural niche, the poultry gastrointestinal tract.

MATERIAL AND METHODS

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Supporting Information Table S1. *C. jejuni* strains were grown on plates containing blood agar base II (Oxoid, London, UK) and 5% horse blood (Biotrading, Mijdrecht, The Netherlands) lysed with 0.5% saponin (Sigma, St Louis, MO) or in HI broth (Biotrading) at 37°C under microaerophilic conditions (5% O_2, 10% CO_2, 10% H_2 and 75% N_2) using the Anoxomat system (MART Microbiology, Lichtenvoorde, The Netherlands). *E. coli* strains were grown on Luria–Bertani (LB) agar plates or in LB broth (Biotrading) at 37°C. When appropriate, media were supplemented with chloramphenicol (20 μg ml^{-1}), kanamycin (50 μg ml^{-1}) and/or ampicillin (100 μg ml^{-1}).

Construction of *racR* mutants

To disrupt the *racR* gene, a 2540 bp DNA fragment, containing the *racR*, racS and recR genes, was amplified from the *C. jejuni* 81116 chromosome using the primers CJ1261F and CJ1261R (Supporting Information Table S2). The PCR product was tailed with a 5′-A nucleotide using Taq polymerase (Invitrogen, Carlsbad, CA) and ligated into the pGEM-T Easy vector to obtain pGEM1261. Plasmid pGEM1261 was subsequently amplified with primers Cj1261FBamHI and Cj1261RBamHI to introduce a BamHI restriction site. The PCR product was digested with BamHI and ligated to a 0.7 kb BamHI fragment containing the chloramphenicol resistance gene of pAV35, resulting in the knockout construct pGEM1261::Cm carrying the *racR* gene with a 7 bp deletion. In the knockout construct, the Cm^R gene is in the same orientation as the *racR*/*racS* genes. To disrupt the *racR* gene in *C. jejuni* strains 81116, 11168 and 81-176, pGEM1261::Cm was introduced into these strains by natural transformation. Double cross-over recombination events were confirmed by PCR.

Construction of the *racR* complementation plasmid

To complement the *racR* mutant, the *racR*, *racS* and *recR* genes were amplified of the chromosomal DNA of *C. jejuni* 81116 with pfu polymerase (Promega) and the primers RacRKpnI and RacRXhoI. The product was digested with KpnI and XhoI and ligated into the shuttle plasmid pMA1 (van Mourik et al., 2008). The resulting complementation plasmid pMA1-1261-1263 was first transformed into *E. coli* S17 (Parke, 1990) and then conjugated (Labigne-Roussel et al., 1987) to the *racR* mutant.

Growth experiments

To generate growth curves under microaerophilic conditions (5% O_2, 10% CO_2, 10% H_2 and 75% N_2), overnight cultures (O/N) of *Campylobacter* grown in HI were diluted to OD_{550} of 0.05 in fresh 5 ml HI media in vertical standing 25 cm^2 vent capped tissue culture flasks (Corning). The cultures were shaken at 150 r.p.m. for 24 h at 37°C, 42°C or 44°C. Growth curves were generated in an anaerobic chamber (Coy Labs, MI, USA). Throughout the growth cycle, cultures remained under the indicated incubation conditions, and at the indicated times, samples were collected for analysis. To avoid residual oxygen entry in the anaerobic chamber, growth curves in HI under oxygen-limited conditions (0.3% O_2, 10% CO_2, 10% H_2, and 79.7% N_2) at 42°C were generated in a 'honeycomb' 10 × 10 well microplate using a Bioscreen C MRB (Oy Growth Curves Ab) computer-controlled

incubator placed in the anaerobic chamber. Overnight cultures of *Campylobacter* grown in HI were diluted to OD_{600} of 0.01 in fresh 200 µl HI with or without the addition of 50 mM fumarate, TMAO or nitrate. The optical density at 600 nm was measured every 15 min over a 24 h period. The number of colony-forming units per millilitre of culture at 5, 10, 15 or 24 h of growth in all above growth conditions were quantified by serial dilution and plating. Growth curves in DM (Leach et al., 1997) containing 20 mM aspartate instead of 20 mM serine with 50 mM nitrate with or without 50 mM fumarate under oxygen-limited conditions (0.3% O_2, 10% CO_2, 10% H_2 and 79.7% N_2) at 42°C were generated in jars using the Anoxomat system (MART Microbiology BV). Overnight cultures in DM were diluted to OD_{600} of 0.05 in fresh 10 ml DM. To avoid disturbing the oxygen concentrations, the growth curves were generated by using different jars for each measurement point. Growth curves in minimal DM lacking serine, aspartate, asparagine, proline, glutamine, glutamate, pyruvate and 2-oxoglutarate with or without 15 mM fumarate, 25 mM nitrate or 15 mM pyruvate at 42°C were also generated in a jar. The overnight pre-cultures in DM were washed with minimal DM and diluted to OD_{600} 0.01. All growth curves were performed in triplicate.

Construction and purification of recombinant RacR and the cytoplasmic domain of RacS

To obtain recombinant RacR and the cytoplasmic domain of RacS, the *Campylobacter* genes *racR* and *racS* were amplified by PCR using the primer combinations RacRPstI/RacRNHISNdeI and RacSPstI/RacScytoNdeI (Supporting Information Table S2) and the Pfu proofreading enzyme according to the instructions of the manufacturer. The resulting PCR fragments of 670 and 786 bp were cloned into the pGEM-T Easy vector (Promega) to form pGEM-RacR(N-his) and pGEM-RacScyto(N-his) respectively. After verification by sequence analysis, the plasmids were digested with PstI and NdeI, and the PCR fragments were cloned into NdeI and PstI sites of expression plasmid pT7.7 (Tabor and Richardson, 1985) to form pT7.7-RacR(N-his) and pT7.7-RacScyto(N-his). Finally, the protein expression plasmids were transformed into *E. coli* BL21(DE3), and expression and purification of histidine-tagged RacR and RacS proteins was performed as described previously (Wösten et al., 2004). Protein concentrations were determined using the BCA protein assay kit (Pierce).

Phosphorylation assay

In vitro autophosphorylation of purified recombinant his-tagged RacS (50 pmol) was performed at room temperature (RT) for 15 min in the presence of 10 µCi [γ-^{32}P]ATP (MP Biomedicals, The Netherlands) and 100 µl of phosphorylation buffer (50 mM Tris-HCl, 75 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, pH 8.3). After 15 min, recombinant RacR (50 pmol) was added to the mixture, in a molar ratio of 1:1. The reaction was stopped by adding SDS loading buffer to 10 µl samples taken after 0.25, 0.5, 1, 2, 4, 8 or 16 min of incubation. Samples were run on a 12% SDS–polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed.

RNA isolation

RNA was extracted from the Wt and *racR* mutant strain grown under low oxygen concentrations in HI with 50 mM $NaNO_3$ until late logarithmic (log) phase (16 h) using the

RNA-Bee kit (Tel-Test). RNA samples were treated with RNAse-free DNAse I (Invitrogen) according to the manufacturer's manual.

Microarray hybridization and analysis

Microarray hybridization and analysis was performed as previously described (Wösten et al., 2006). Microarray data have been included as Supporting Information.

Real-time RT-PCR

Real-time RT-PCR analysis was performed as previously described (Wösten et al., 2004). Primers used in this study are listed in Supporting Information Table S2. The calculated threshold cycle (Ct) for each gene amplification was normalized to the Ct value for the gene *gyrA*, amplified of the corresponding sample before calculating fold change using the arithmetic formula ($2^{\Delta\Delta Ct}$) (Schmittgen, 2001). Each sample was examined in four replicates and was repeated with at least two independent preparations of RNA. Standard deviations were calculated and displayed as error bars.

Electrophoretic mobility shift assay

The promoter regions upstream of the genes *aspA*, *cj0358*, *mfrX*, *cj1491c*, *gltB*, *dcuB*, *cj0449c, cj0175, chuU, cj1384, ansA and phoX* were amplified by PCR using the primer pairs listed in Supporting Information Table S2 and *C. jejuni* 81116 genomic DNA as template. To obtain radioactive labelled PCR fragments one of the primers from each primer pair was first labelled by [γ-^{32}P]ATP and T4 polynucleotide kinase (Invitrogen) for 30 min at 37°C according to the manufacturer's manual. The radioactive labelled PCR products, approximately 25 pmol, were incubated with 0, 5, 25, or 50 pmol of RacR and 0 or 50 pmol of RacSc-H_6 for 20 min at RT in binding buffer containing 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 50 µg ml^{-1} of bovine serum albumin, 10 µg ml^{-1} of poly-(dI-dC) and 10% glycerol. Due to the presence of ATP, the cytoplasmic domain of RacS autophosphorylates and a rapid phosphotransfer to RacR is accomplished (data not shown). For competition assays, RacR was pre-incubated for 15 min with 10 times excess of unlabelled PCR fragment. Samples were run on 6% non-denaturing Tris-glycine polyacrylamide gels at 4°C. After electrophoresis, gels were dried and autoradiographed.

DNase I footprinting

DNase I protection assays were performed for both DNA strands using the appropriate labelled primer. The radioactive labelled PCR products, approximately 25 pmol, were incubated with 0, 50, 150 pmol of RacR and 0 or 50 pmol of RacSc-H6 in a final volume of 15 µl as described for the EMSA. DNase I (100 units) (Fermentas) was added and incubated for 150 s at room temperature. The reaction was stopped by adding 1 µl of 25 mM EDTA and 5 min incubation at 65°C. Samples were analysed by denaturing PAGE (6%) by comparison with a DNA sequence ladder generated with the appropriate primer.

Primer extension experiments

Analysis of the 5′ ends of the *aspA*, *ansA and cj0449c* mRNA transcripts was performed by primer extension using [γ^{32}P]ATP labelled primers. A total of 5 pmol of primer *aspA*Rpromprex, ansA-R or Cj0449c (Supporting Information Table S2) was annealed to 20 µg of RNA extracted from *Campylobacter* grown in defined medium with 0.08 mM [Pi]. Synthesis of cDNA was performed using SuperScript II RnaseH− reverse transcriptase

(Invitrogen) according to the manufacturer's instructions. The extension products were analysed by electrophoresis on a 6% polyacrylamide, 7.5 M urea gel and compared with sequence ladders initiated with primers *aspA*Rpromprex, ansA-R or Cj0449c.

High-performance liquid chromatography–tandem mass spectrometry analysis

C. jejuni was grown in DM with 20 mM aspartate, 50 mM fumarate with or without 50 mM nitrate under oxygen-limited conditions. At 0, 5, 10, 15 and 24 h, 1 ml of the culture was centrifuged at 14,000 r.p.m. for 5 min. The supernatants were diluted in milli-Q water adjusted to pH 2.4 with formic acid and injected on a Synergi 4u Fusion-RP (150 × 2.0 mm, particle size of 4 µm) analytical column (Phenomenex, Utrecht, The Netherlands). Elution was performed isocratically with milli-Q (adjusted to pH 2.4 with formic acid) : acetonitrile [9:1 (v/v)] at a flow rate of 0.3 ml min^{-1}, and the column effluent was introduced by an atmospheric pressure chemical ionization interface, in negative mode, with an ionization current of −1 µA and a source temperature of 350°C, into a 2000 QTRAP mass spectrometer (Sciex, Toronto, ON). For maximal sensitivity and for linearity of the response, the mass spectrometer was operated in multiple-reaction monitoring mode at unit mass resolution. Peaks were identified by comparison of retention time and mass spectrum with authentic standards. Ion transitions monitored were m/z 115.0/71.0 (fumarate), 117.0/73.0 (succinate), and 132.1/88.0 (aspartic acid) at collision energies of −12, −15 and −20V respectively. Simultaneously, the three molecules were monitored by single-ion monitoring. Data were analysed with Analyst software version 1.6.1 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Enzyme assays

Aspartase activity was measured as described by (Guccione et al., 2008). In brief, bacterial cultures of Wt strain 81116, *racR* mutant and the complemented *racR* mutant were grown O/N at 37°C in DM under microaerophilic conditions. These pre-cultures were diluted to OD_{600} of 0.05 in DM with 20 mM aspartate as major carbon source and/or 50 mM nitrate, TMAO or fumarate as alternative electron acceptor and incubated under low (0.3%) or high (10%) oxygen conditions. End logarithmic phase bacteria (OD_{600} nm 0.2–0.4) were washed with PBS and lysed by sonication (6 × 5 s) on ice. Protein concentration was determined using the BCA protein assay kit. Enzyme activity was measured at 240 nm, corresponding to the absorbance of fumarate, by using the Omega FLUOstar (Isogen, BMG Labtech). Enzyme activity is presented as µmol min^{-1} mg $protein^{-1}$.

Statistical analysis

Prism software (GraphPad, San Diego, CA) was used for statistical analysis. Data was expressed as mean ± SD. Results were analysed by one-way ANOVA followed with Bonferroni post-hoc tests; $P < 0.05$ was considered statistically significant.

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AUTHOR CONTRIBUTION

AXS, AvM, JvP, DK and MW contributed to the conception of the experiments. AXS, AvM, CP, LH, CL and MW performed the experiments. Growth curves, nutrient utilization experiments were performed by AXS. AvM performed mutagenesis, enzyme assays and autophosphorylation experiments. LH performed RT-qPCR experiments. MW performed foot-printing experiments, primer extenstions and EMSA experiments. Microarray experiments were performed by CP. HPLC/MS/MS analysis experiments were performed by CL. AXS, AvM, DK and MW performed analysis of the data. AXS, AvM, JvP and MW wrote the manuscript.

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SUPPLEMENTARY MATERIAL

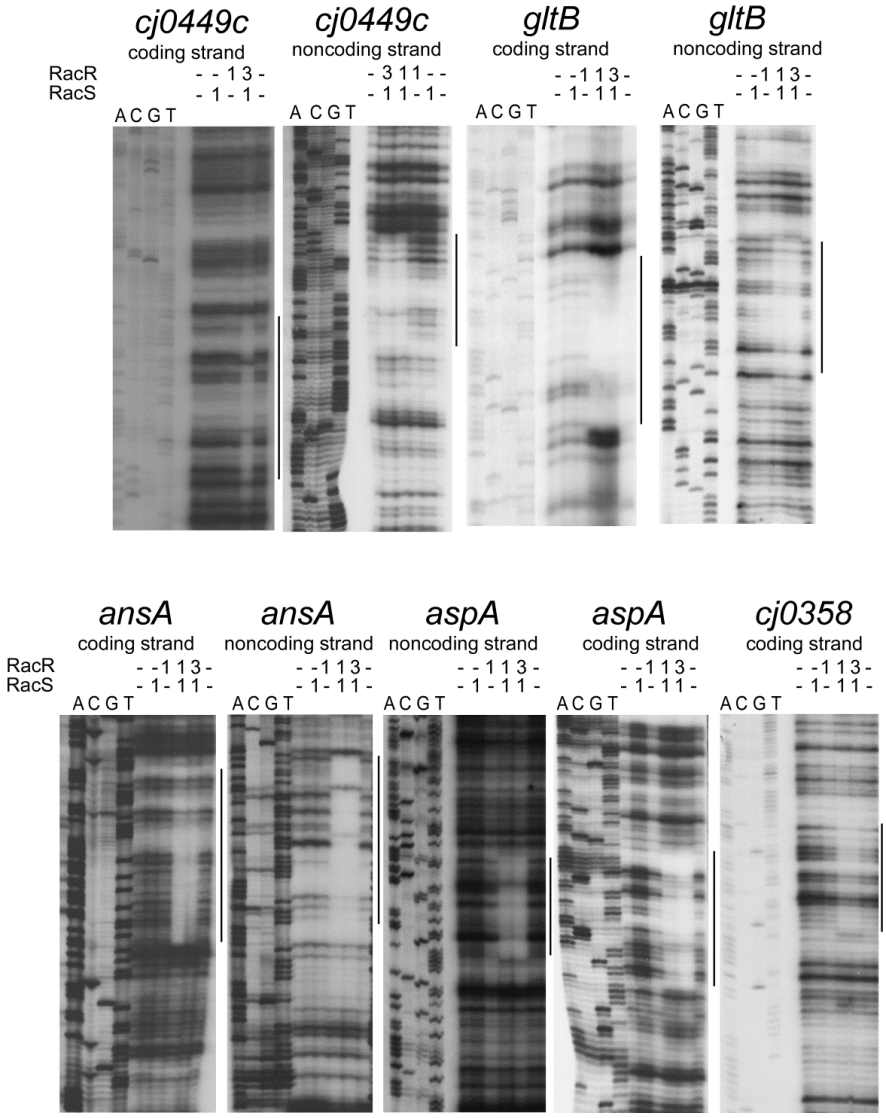

Figure S1: DNase I footprinting analysis. Foot printing of RacR with the *gltB*, *aspA*, *ansA*, *cj0358* and the *cj0449c* promoter elements performed on both the coding and non-coding strands. The amount of RacR and RacS added to the DNA fragments is indicated at the top of the figures (1 = 50 pmol; 3 = 150 pmol). Solid lines represent the RacR binding region. Samples were analysed by denaturing PAGE (6%) by comparison with a DNA sequence ladder generated with the appropriate primer. DNase I footprinting analysis of the *gltB*, *aspA*, *ansA*, *cj0358* and the *cj0449c* promoter elements performed on both the coding and non-coding strand. The amount of RacR and RacS added to the DNA fragments is indicated at the top of the figures (1 = 50 pmol; 3 = 150 pmol). Solid lines represent the RacR binding region. Samples were analysed by denaturing PAGE (6%) by comparison with a DNA sequence ladder generated with the appropriate primer.

Table S1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Origin or reference
Strains		
E. coli PC2955	relA1 _80dlacZ_M15 phoA8 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS glnV44; used for cloning	NCCB
E. coli BL21(DE3)	F^- *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3); used for protein overexpression	Novagen
E. coli S17	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* (Tmp^r Str^r); used for conjugation	(Parke, 1990)
C. jejuni 81116	Wildtype	(Palmer *et al.*, 1983)
C. jejuni 1261::Cm	C. jejuni 81116 racR::Cm	This study
C.jejuni 1261::Cm+p	*C. jejuni* 81116 *racR*::Cm with pMA1-1261-1263	This study
Plasmids		
pGEM-T Easy	Ap^R; 3.0 kb; LacZα; TA cloning vector	Promega
pBluescript II KS	Ap^R; 3.0 kb; LacZα	Stratagene
pT7.7	Ap^R; 2.5 kb; expression vector	(Tabor & RRichardson, 1985)
pAV35	pBluescript II SK containing *Campylobacter coli* Cm^R	(van Vliet *et al.*, 1998)
pMA1	Km^R; 10 kb; *E.coli/C. jejuni* shuttle vector	(van Mourik *et al.*, 2008)
Plasmids constructed for mutagenesis		
pGEM1261	Ap^R; 5.5 kb; pGEM-T easy/*C. jejuni* 1261-1263	This study
pGEM1261::Cm	Ap^R Cm^R; 6.3 kb; pGEM-T easy/*C. jejuni racR*::Cm	This study
Plasmids constructed for complementation assays		
pMA1-1261-1263	Km^R;12.5 kb; pMA1/*C. jejuni 1261-1263*	This study
Plasmids constructed for RacR and RacS overexpression		
pGEM-RacR(N-his)	Ap^R; 3.7 kb; pGEM-T easy/*C. jejuni racR-N-his*	This study
pGEM-RacScyto(N-his)	Ap^R; 3.8 kb; pGEM-T easy/*C. jejuni racS*-cytoplasmic part with N-terminal His-tag	This study
pT7.7-RacR(N-his)	Cm^R; 3.2 kb; pT7.7/*C. jejuni* RacR with N-terminal His-tag	This study
pT7.7-RacScyto(N-his).	Cm^R; 3.3 kb; pT7.7/*C. jejuni* RacS cytoplasmic part with N-terminal His-tag	This study

Table S2. Primer sequences used in this study. Sequence introduced for cloning purposes are underlined.

Name	Sequence (5'-3')
Primers used for cloning	
Cj1261R	ACTAAATTTTTGTTCTAAAAT
Cj1261F	TAGCAAAAATAAAATCAATAA
Cj1261FBamHI	CAAGGATCCTGAGTCTTATTCGTCGCAC
Cj1261RBamHI	TCAGGATCCTTGCATACATTTCTTTTGGA
RacRXhoI	ACTCGAGTAGCAAAAATAAAATCAATAA
RacRKpnI	AGGTACCACTAAATTTTTGTTCTAAAAT
RacRPstI	TTCTGCAGTCATCCTATCAGTTTATAT
RacRNHISNdeI	TACATATGCATCACCATCACCATCACATTAATGTGTTGATGATAG
Cj1261RBamHI	TCAGGATCCTTGCATACATTTCTTTTGGA
RacSpstI	TTTCTGCAGTTATTTTTCTTTATCTCCAAAGAAA ATAGA
RacScytoNdeI	ATTCATATGCACCATCACCATCATCACTCTTTAGAGCCTTTAAAA
Primers for gel mobility shift assay (Distance to the initation codon bp)	
aspAFprom (-223 bp)	AGCTTGCAAAAATATATTAATTT
aspARpromprex (+58 bp)	TAATAAACCTCATCAGAGATTTC
gltB-F (-165 bp)	TTTAGGTAGTATTATCGCCATGTT
gltBrtaq (+75bp)	TACCACAGGCATCGTGTTCG
ansA-F (-236 bp)	GCCAGAATTGAATCAAAATATTTC
ansA-R (+8 bp)	GCTTTTTTCATACATACTCC
Cj0176c (-224 bp)	AGTAAAAATAAAATTATTTTTTTCATAGAC
Cj0177 (+20bp)	CTTGCAAAAAATGAAGAAAAC
Cj1614 (-225 bp)	TTCATATGAGAAATAATGCTTTCAAAATTC
chuA (+19 bp)	AAGCTTTTTTATTTGGGTGC
Cj0449c (+17 bp)	TCTCTATATTCATGTAGCAT
Cj0450 (-230 bp)	CGCAGCTTCTACTCTAAGAA
Cj436fabF (-163 bp	AGAATGCAATTTATGAATGGAGAATT
Cj437mfrx (+58 bp)	AATCAAAGAAGATAGATGAATATAAT
Cj1493cF (-459bp)	CTCCTGAGTAGGACAAATCC
Cj1492prex (+48bp)	CATAGAATAGCAGTGTCCAAAA
Cj1384c (-193bp)	GCACCAATAACCAAAACTGA
katA4 (+23 bp	CCAAAATCGTTAGTTAATTTTTTC
Cj144F (-158bp)	GATGTTAAGAAGAAGAGGTT
CJ0145R (+19 bp)	TTAAAAACAATCTTCTTTCCAT
Cj0358F (-202bp)	CATAATATCCTTTTCAAATCAT
Cj0358R (+14bp)	AATGATTTTACTTTCATTTATAAAC
Primers for RT-qPCR	
Cj0087Ftaq	GGTCCAAAATGTGGTCTTAATGAGAT
Cj0087Rtaq	TTCAGGAATAACAGGATTTACCTTACC
sdhAFtaq	TGCAAATGGTGGAACTCTTATTACA
sdhARtaq	GCTCACCACGATTATTTAAAAGATAT
ansAtaqF	AAGGGTGTTATGGTAGCTATGAATGA
ansAtaqR	TCAGGAGAAGAGAAAGCATCAACAT
Cj0358taqF2	TGCTGAAACTGCTCCATATTTTCA
Cj0358taqR2	GCCAAGTTGCACACTACCCATT
gltBFtaq	GTGCATGGCTGTGAATATATGACA
gltBRtaq	AACAACGCCCCCACTCATAC
pspAFtaq	GCTGGAACTTTTGCAAGAGCTT
pspARtaq	GCATTTGCCCATTGATCTTTTT
ungFtaq	CAAGGAGTTTTGCTCTTAAATTCTATTCT
ungRtaq	TCTTGCCAACCCCACGAA
Cj449Ftaq	TTCCATGCTGCTAAGAGAAGTTCTAC

Cj449Rtaq	AATTAATGTCAGAGCTAAAAGGTAAAGATG
Cj1491Ftaq	TTTTTTAAGCCCCTCATCTCCAT
Cj1491Rtaq	GAGGTTAAGGCTAGAGAATCAATGATTAAT
Cj175Ftaq	GCTAATGACGGAAATAAAGAGGCTAA
Cj175Rtaq	GATCTCTATCTCCACCCTTTGGATT
chuBFtaq	GCGGTTTTGGCGTATTTGTTA
chuBRtaq	AGTCGCTATAGAAGCTTTAGAAGAAATTTT
Cj1384Ftaq	CTTGCGATTAACTCTATTTTCATTACGT
Cj1384Rtaq	GATGAAATCACTCCTATTAGAGCTGTTTT
katAFtaq	CTCCTCTTTTCCTACGCTTTTCTACT
katARtaq	TCTCACATCGCGTTCAGCAT
hipOFtaq	CCAGAAAAGGCAAAAGATCCTATTT
hipORtaq	ACATTGCGAGATACTATGCTTTGTAAA
gyrAftaq	GAACTCATGGCTAAATTCATACAAACA
gyrArtaq	GCTTTATTTCCGCTTTGCACTT
Cj0147Ftaq	CATCAATAACTGGAGCAAGCATTC
Cj0147Rtaq	GCAAAAGAAGGTGTAGCTTTAGTTGATT

Table S3: Micro array data showing genes with more than two fold higher transcript amounts in the *racR* mutant compared to Wt.

Gene	Fold Change	Common	Product
Cj0029	5,001	ansA	L-asparaginase (ansA)
Cj0040	2,306	Cj0040	conserved hypothetical protein
Cj0045c	2,186	Cj0045c	Hemerythrin domain protein
Cj0068	4,037	pspA	protease IV, putative
Cj0069	4,769	Cj0069	conserved hypothetical protein
Cj0070c	2,098		
Cj0075c	2,493	Cj0075c	Domain of unknown function (DUF224) family
Cj0076c	2,495	lctP	L-lactate permease
Cj0079c	2,156	cdtA	CdtA protein
Cj0080	2,389	Cj0080	conserved hypothetical protein
Cj0081	2,6	cydA	cytochrome d ubiquinol oxidase, subunit I (cydA)
Cj0082	2,704	cydB	cytochrome d ubiquinol oxidase, subunit II (cydB)
Cj0086c	7,28	ung	uracil-DNA glycosylase (ung)
Cj0087	78,15	aspA	aspartate ammonia-lyase (aspA)
Cj0088	12,49	dcuA	C4-dicarboxylate transporter, anaerobic (dcuA)
Cj0089	4,281	Cj0089	conserved hypothetical protein
Cj0091	3,07	Cj0091	conserved hypothetical protein
Cj0092	3,264	Cj0092	conserved hypothetical protein
Cj0095	3,547	rpmA	ribosomal protein L27 (rpmA)
Cj0096	2,158	Cj0096	GTP-binding protein Obg (obg)
Cj0100	2,134	Cj0100	ATPase, ParA family (soj)
Cj0101	2,273	Cj0101	chromosome partioning protein, ParB family (spo0J)
Cj0102	2,053	atpF'	ATP synthase B/B' CF(0) domain protein
Cj0103	2,77	atpF	ATP synthase B/B' CF(0) domain protein
Cj0104	2,197	atpH	ATP synthase F1, delta subunit (atpH)
Cj0144	3,395		
Cj0244	4,048	rpmI	
Cj0245	3,534	rplT	
Cj0289c	2,526	peb3	accessory colonization factor AcfC (acfC)
Cj0290c	2,09		
Cj0291c	2,988	glpT	glycerol-3-phosphate transporter (glpT)
Cj0293	2,081	surE	stationary-phase survival protein SurE (surE)
Cj0296c	2,402	panD	aspartate 1-decarboxylase (panD)
Cj0297c	2,625	panC	pantoate--beta-alanine ligase (panC)
Cj0298c	3,146	panB	3-methyl-2-oxobutanoate hydroxyl-methyltransferase (panB)
Cj0313	2,895	Cj0313	membrane protein, putative
Cj0350	2,785	Cj0350	conserved hypothetical protein
Cj0354c	3,97	fdxB	ferredoxin
Cj0357c	2,849	Cj0357c	conserved hypothetical protein TIGR00023

Cj0358	8,192	Cj0358	cytochrome c551 peroxidase (yhjA)
Cj0369c	2,714	Cj0369c	ferredoxin, putative
Cj0391c	2,008	Cj0391c	conserved hypothetical protein
Cj0408	3,434	frdC	Fumarate reductase respiratory complex, transmembrane subunit
Cj0410	2,862	frdB	fumarate reductase, iron-sulfur protein, putative
Cj0437	4,014	sdhA	succinate dehydrogenase, flavoprotein subunit (sdhA)
Cj0438	4,24	sdhB	fumarate reductase, iron-sulfur protein, putative
Cj0439	2,507	sdhC	Domain of unknown function (DUF224) family
Cj0441	2,16	acpP	acyl carrier protein (acpP)
Cj0442	2,004	fabF	Beta-ketoacyl synthase domain protein
Cj0448c	5,119	Cj0448c	accessory colonization factor AcfB, putative
Cj0449c	6,947	Cj0449c	conserved hypothetical protein
Cj0450c	2,614	rpmB	ribosomal protein L28 (rpmB)
Cj0451	2,348	rpe	ribulose-phosphate 3-epimerase (rpe)
Cj0458c	2,001	miaB	
Cj0459c	3,458	Cj0459c	conserved hypothetical protein
Cj0470	2,366	tuf	translation elongation factor Tu (tuf)
Cj0471	2,364	rpmG	ribosomal protein L33 (rpmG)
Cj0473	2,528	nusG	transcription antitermination protein NusG (nusG)
Cj0493	2,898	fusA	translation elongation factor G (fusA)
Cj0604	2,006	Cj0604	conserved hypothetical protein
Cj0643	2,555	Cj0643	GGDEF domain protein
Cj0671	3,162	dcuB	C4-dicarboxylate transporter, anaerobic (dcuB)
Cj0697	2,008	flgG2	Flagella basal body rod protein domain protein
Cj0853c	2,13	hemL	glutamate-1-semialdehyde-2,1-aminomutase (hemL)
Cj0864	2,213	dsbA	
Cj0884	3,387	rpsO	ribosomal protein S15 (rpsO)
Cj0887c	3,89	flaD	Bacterial flagellin C-terminus domain protein
Cj0893c	3,456	rpsA	ribosomal protein S1 (rpsA)
Cj0906c	2,448	Cj0906c	conserved hypothetical protein
Cj0909	3,696	Cj0909	conserved hypothetical protein
Cj0921c	2,56	peb1b	major cell-binding factor precursor
Cj0922c	3,418	peb1c	probable abc transporter atp-binding protein peb1c.
Cj0936	3,616	atpE	ATP synthase F0, C subunit, putative
Cj0951c	2,29	Cj0951c	methyl-accepting chemotaxis protein, putative
Cj0952c	3,585		
Cj0961c	4,296	rpmH	ribosomal protein L34 (rpmH)
Cj0965c	2,488	Cj0965c	conserved hypothetical protein TIGR00051
Cj0977	2,852	Cj0977	conserved hypothetical protein
Cj1026c	3,66	Cj1026c	conserved hypothetical protein
Cj1034c	2,348	Cj1034c	conserved hypothetical protein
Cj1070	2,194	rpsF	ribosomal protein S6, putative
Cj1071	3,121	ssb	single-strand binding protein (ssb)
Cj1096c	2,275	metK	S-adenosylmethionine synthetase (metK)
Cj1170c	2,156	Cj1170c	conserved hypothetical protein
Cj1189c	2,483	Cj1189c	methyl-accepting chemotaxis protein
Cj1190c	2,949	Cj1190c	accessory colonization factor AcfB (acfB)
Cj1191c	2,578	Cj1191c	methyl-accepting chemotaxis protein
Cj1242	5,59	Cj1242	conserved hypothetical protein
Cj1261	2,055	racR	response regulator (regX3)
Cj1359	3,177	ppk	polyphosphate kinase (ppk)
Cj1364c	3,05	fumC	fumarate hydratase, class II (fumC)
Cj1489c	2,171	ccoO	cytochrome c oxidase, cbb3-type, subunit II (ccoO)
Cj1490c	2,712	ccoN	cytochrome c oxidase, cbb3-type, subunit I (ccoN)
Cj1491c	4,167	Cj1491c	DNA-binding response regulator, putative
Cj1492c	4,089	Cj1492c	Histidine kinase-, DNA gyrase B- phytochrome-like ATPase domain
Cj1493c	8,08	Cj1493c	membrane protein, putative
Cj1540	2,185	Cj1540	conserved hypothetical protein
Cj1586	2,381	Cj1586	ferrisiderophore reductase (hmpA)
Cj1592	2,795	rpsM	ribosomal protein S13/S18 (rpsM)
Cj1631c	2,15	Cj1631c	conserved hypothetical protein
Cj1632c	2,331		
Cj1656c	2,98	Cj1656c	
Cj1658	2,459	Cj1658	hypothetical protein
Cj1701c	2,011	rpsC	ribosomal protein S3 (rpsC)
Cj1702c	2,252	rplV	ribosomal protein L22 (rplV)

Cj1703c	2,511	rpsS	ribosomal protein S19 (rpsS)
Cj1704c	2,516	rplB	ribosomal protein L2 (rplB)
Cj1705c	2,783	rplW	ribosomal protein L23 (rplW)
Cj1706c	3,293	rplD	ribosomal protein L4/L1 family (rplD)
Cj1707c	3,583	rplC	hypothetical protein
Cj1708c	3,26	rpsJ	ribosomal protein S10 (rpsJ)
HS41ORF26	2,569		
ORF00311	2,168	ORF00311	methyl-accepting chemotaxis protein, putative
ORF01561	2,021	ORF01561	vacuolating cytotoxin precursor, putative

Table S4: Micro array data showing genes with more than two fold higher transcript amounts in the Wt compared to *racR* mutant.

2

Gene	Fold Change	Common	Product
Cj0007	4,282	gltB	glutamate synthase, large subunit (gltB-1)
Cj0009	3,466	rnhB	ribonuclease HII (rnhB)
Cj0034c	2,06	Cj0034c	conserved hypothetical protein
Cj0036	2,065	Cj0036	conserved hypothetical protein
Cj0037c	3,352	Cj0037c	Cytochrome c subfamily, putative
Cj0146c	2,108	trxB	thioredoxin reductase (trxB)
Cj0168c	2,381	Cj0168c	conserved hypothetical protein
Cj0173c	2,107	Cj0173c	spermidine/putrescine ABC transporter, ATP binding subunit
Cj0174c	2,611	Cj0174c	iron(III) ABC transporter, permease protein, putative
Cj0175c	5,154	Cj0175c	iron(III) ABC transporter, periplasmic iron-compound binding
Cj0176c	4,735	Cj0176c	conserved hypothetical protein
Cj0182	2,406	Cj0182	membrane protein, putative
Cj0185c	2,044	Cj0185c	alkylphosphonate utilization operon protein PhnA (phnA)
Cj0316	2,16	pheA	chorismate mutase/prephenate dehydratase, putative
Cj0334	3,691	ahpC	antioxidant, AhpC/Tsa family
Cj0389	2,073	serS	seryl-tRNA synthetase (serS)
Cj0403	2,016	Cj0403	conserved hypothetical protein
Cj0483	2,257	uxaA'	conserved hypothetical protein
Cj0531	2,465	icd	isocitrate dehydrogenase, NADP-dependent
Cj0532	2,683	mdh	L-malate dehydrogenase, putative
Cj0533	2,775	sucC	succinyl-CoA synthase, beta subunit (sucC)
Cj0534	2,07	sucD	succinyl-CoA synthase, alpha subunit (sucD)
Cj0535	2,088	oorD	ferredoxin, 4Fe-4S-related protein
Cj0559	2,052	Cj0559	pyridine nucleotide-disulfide oxidoreductase, putative
Cj0569	2,12		
Cj0596	2,308	peb4	cell binding factor 2 precursor
Cj0613	3,239	pstS	phosphate ABC transporter, periplasmic phosphate-binding
Cj0615	2,011	pstA	phosphate ABC transporter, permease protein (pstA)
Cj0623	2,049	hypB	hydrogenase accessory protein HypB (hypB)
Cj0624	2,355	hypC	hydrogenase assembly chaperone hypC/hupF (hypC)
Cj0625	2,643	hypD	hydrogenase expression/formation protein HypD (hypD)
Cj0626	2,354	hypE	AIR synthase related protein, C-terminal domain protein
Cj0699c	2,601	glnA	glutamine synthetase, type I (glnA)
Cj0702	2,242	purE	phosphoribosylaminoimidazole carboxylase, catalytic subunit
Cj0715	2,477	Cj0715	Transthyretin precursor (formerly prealbumin) domain
Cj0716	2,854	Cj0716	phospho-2-dehydro-3-deoxyheptonate aldolase
Cj0717	2,511	Cj0717	conserved hypothetical protein
Cj0719c	2,063	Cj0719c	conserved hypothetical protein TIGR00044
Cj0721c	2,583	Cj0721c	conserved hypothetical protein
Cj0839c	2,266	Cj0839c	conserved hypothetical protein
Cj0840	2,581	fbp	fructose-1,6-bisphosphatase (fbp)
Cj0984	2,24	Cj0984	conserved hypothetical protein
Cj0985c	4,411	hipO	hippurate hydrolase
Cj0986c	3,388		
Cj0987c	3,142		
Cj1023c	2,142	asd	aspartate-semialdehyde dehydrogenase (asd)
Cj1044c	2,025	thiH	thiH protein (thiH)
Cj1161c	2,032	Cj1161c	cation-transporting ATPase, E1-E2 family, putative
Cj1162c	2,687	Cj1162c	

Cj1265c	2,158	hydC	Nickel-dependent hydrogenases b-type cytochrome subunit
Cj1266c	2,09	hydB	hydrogenase-2 large chain precursor
Cj1286c	2,116	upp	uracil phosphoribosyltransferase (upp)
Cj1360c	2,472	Cj1360c	
Cj1381	2,61	Cj1381	conserved hypothetical protein
Cj1382c	5,249	fldA	flavodoxin
Cj1383c	5,065	Cj1383c	conserved hypothetical protein
Cj1384c	5,714	Cj1384c	conserved hypothetical protein
Cj1385	6,23	katA	catalase
Cj1386	5,147	Cj1386	Ank repeat domain protein
Cj1466	2,019	flgK	flagellar hook-associated protein FlgM, putative
Cj1567c	3,453	nuoM	NADH dehydrogenase I, M subunit (nuoM)
Cj1568c	3,367	nuoL	NADH dehydrogenase I, L subunit (nuoL)
Cj1569c	2,88	nuoK	NADH dehydrogenase I, K subunit (nuoK)
Cj1570c	3,681	nuoJ	NADH dehydrogenase I, J subunit, putative
Cj1571c	3,125	nuoI	NADH dehydrogenase I, I subunit (nuoI)
Cj1573c	3,403	nuoG	formate dehydrogenase, alpha subunit, putative
Cj1613c	3,785	Cj1613c	conserved hypothetical protein
Cj1615	4,896	chuB	hemin ABC transporter, permease protein, putative
Cj1616	3,391	chuC	iron compound ABC transporter, ATP-binding protein,
Cj1617	4,358	chuD	iron compound ABC transporter, substrate-binding protein,
Cj1643	2,083	Cj1643	PDZ domain (Also known as DHR or GLGF). protein
Cj1645	2,626	tkt	transketolase (tkt)

2

Chapter 3

The *Campylobacter jejuni* RacRS two-component system activates the glutamate synthesis by directly upregulating γ-glutamyltranspeptidase (GGT)

Anne-Xander van der Stel[1*], Andries van Mourik[1*], Paweł Łaniewski[2], Jos P.M. van Putten[1], Elżbieta K. Jagusztyn-Krynicka[2] and Marc M.S.M. Wösten[1]

[*]These authors contributed equally

[1]Department of Infectious Diseases and Immunology, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[2]Department of Bacterial Genetics, Institute of Microbiology, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland

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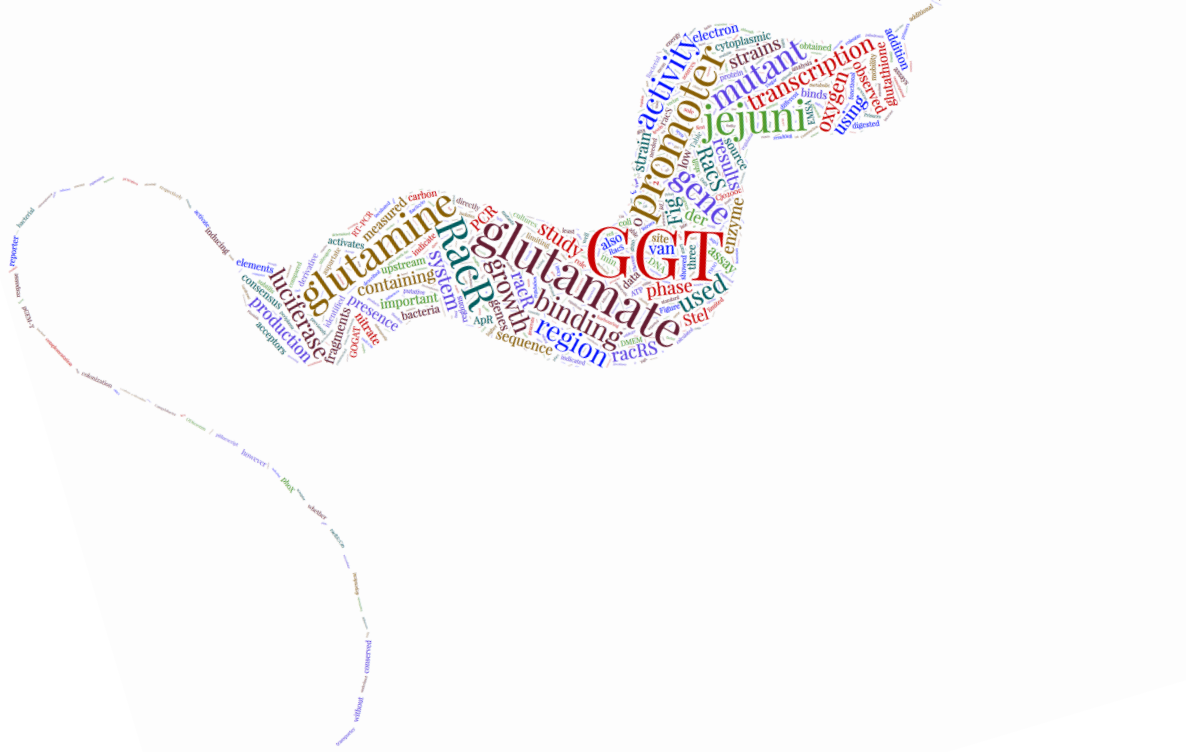

SUMMARY

The highly conserved enzyme γ-glutamyltranspeptidase (GGT) plays an important role in metabolism of glutathione and glutamine. Yet, the regulation of *ggt* transcription in prokaryotes is poorly understood. In the human pathogen *Campylobacter jejuni,* GGT is important as it contributes to persistent colonization of the gut. Here we show that the GGT activity in *C. jejuni* is dependent on a functional RacRS (reduced ability to colonize) two-component system. Electrophoretic mobility shift and luciferase reporter assays indicate that the response regulator RacR binds to a promoter region ~80 bp upstream of the *ggt* transcriptional start site, which contains a recently identified RacR DNA binding consensus sequence. RacR needs to be phosphorylated to activate the transcription of the *ggt* gene, which is the case under low oxygen conditions in presence of alternative electron acceptors. A functional GGT and RacR are needed to allow *C. jejuni* to grow optimally on glutamine as sole carbon source under RacR inducing conditions. However, when additional carbon sources are present *C. jejuni* is capable of utilizing glutamine independently of GGT. RacR is the first prokaryotic transcription factor known to directly upregulate both the cytoplasmic (glutamine-2-oxoglutarate aminotransferase (GOGAT)) as well as the periplasmic (GGT) production of glutamate.

INTRODUCTION

The enzyme γ-glutamyltranspeptidase (GGT, EC 2.3.2.2) is highly conserved among eukaryotic and prokaryotic organisms (Ong et al., 2008), where it has a key function in glutathione metabolism. In prokaryotes, GGT is produced as a proenzyme in the cytoplasm and is then translocated into the periplasm where it undergoes autocatalytic cleavage. This proteolysis yields a mature dimer which transfers γ-glutamyl moieties from extracellular glutathione and related compounds to amino acids or peptides or catalyses the hydrolysis of the glutamyl group to generate glutamate (Hanigan, 1998). GGT activity in *Escherichia coli* and *Bacillus subtilis* is maximal in stationary growth phase (Suzuki et al., 1986; Xu and Strauch, 1996). In *B. subtilis* GGT is indirectly transcriptionally regulated in response to low L-glutamate concentrations via the quorum sensing two-component system ComP/ComA (Kimura et al., 2004). In *Helicobacter pylori* expression of GGT is reported not to be growth phase dependent, but is upregulated at low pH (Wachino et al., 2010) and to be involved in acid resistance and immune stimulation (Miller and Maier, 2014; Gong et al., 2010).

The bacterium *C. jejuni* is a major foodborne pathogen in humans and colonizes the intestinal tract of many warm-blooded animals (Blaser, 1997). *C. jejuni* lacks the glycolytic enzyme phosphofructokinase and is therefore not able to use exogenous sugars as a carbon source, although some strains were shown to be able to metabolize fucose (Muraoka and Zhang, 2011; Stahl et al., 2011). Hence, amino acids (i.e. aspartate, glutamate, proline and preferentially serine) are likely to sustain the growth of *Campylobacter* in the intestine (Guccione et al., 2008). Although the genome of *C. jejuni* encodes for a functional glutamine transporter (Lin et al., 2009), only isolates containing

GGT are also able to utilize glutamine and glutathione as sole carbon/energy source (Hofreuter et al., 2006). In the periplasm this enzyme converts glutamine and glutathione to glutamate, which is subsequently taken up via the aspartate/glutamate-binding protein PEB1 (Del Rocio Leon-Kempis et al., 2006). After glutathione cleavage by GGT the remaining dipeptide cys-gly is also imported by *C. jejuni* and used as sulphur source (Vorwerk et al., 2014). The expression of *ggt* is reported to be maximal in late log phase (Hyytiäinen et al., 2012). The presence of GGT allows *C. jejuni* strains to enhance their colonization persistence in the avian gut and to colonize the intestine of mice (Barnes et al., 2007; Hofreuter et al., 2008).

The regulator RacR (reduced ability to colonize) is like GGT needed to sustain the colonization of chickens (Brás et al., 1999) and is detected in nearly all *C. jejuni* isolates (Kordinas et al., 2005; Talukder et al., 2008; Quetz et al., 2012). Recently we showed that the RacRS two-component system of *C. jejuni* is active under low oxygen conditions in the presence of alternative electron acceptors (e.g. nitrate or TMAO) (van der Stel et al., 2015). Under these conditions, RacR represses the transcription of several genes including the *aspA* gene and at the same time it activates the *gltBD* genes. The products of the *gltBD* genes form the glutamine-2-oxoglutarate aminotransferase (GOGAT) complex, which is responsible for cytosolic glutamate generation (Guccione et al., 2008). Glutamate is an important nitrogen source for bacteria as it functions as precursor for amino acid and nucleotide anabolism (Reitzer, 2003; Heeswijk et al., 2013; Hofreuter, 2014). Here we investigated whether the generation of periplasmic glutamate accomplished by GGT is also regulated by RacR.

RESULTS

RacR regulates *ggt* transcription and activity.

As the *C. jejuni* RacRS two-component system regulates the cytoplasmic glutamate production by activating the *gltBD* genes (van der Stel et al., 2015), we wondered whether GGT, responsible for the periplasmic glutamate production, is also regulated by the RacRS system. To investigate this, we measured the *ggt* transcripts in the *C. jejuni* 81116 wildtype strain, the isogenic *racR* mutant and in the complemented *racR* mutant, grown until late log phase under RacRS inducing conditions, i.e. 0.3% O_2 with 50 mM nitrate. To verify that we used RNA that was isolated under RacR inducing conditions, we also measured the transcripts of *aspA* and *gltB* genes. Using real time RT-PCR we observed that inactivation of *racR* resulted in a 55-fold increase of *aspA* mRNA, and a 6-fold decrease of the *gltB* consistent with our previous results (van der Stel et al., 2015) and confirming that RacR is induced under these conditions. A significant 5-fold decrease was observed for the *ggt* mRNA transcripts. The differences between the wt and the *racR* mutant were almost restored to wt levels by introducing complementation plasmid harbouring the RacRS operon (Fig. 1). These results suggest that RacR has a strong influence on the production of glutamate as it not only activates the transcription of the genes required for the cytoplasmic glutamate production (*gltB),* but also the periplasmic production of glutamate (*ggt)*.

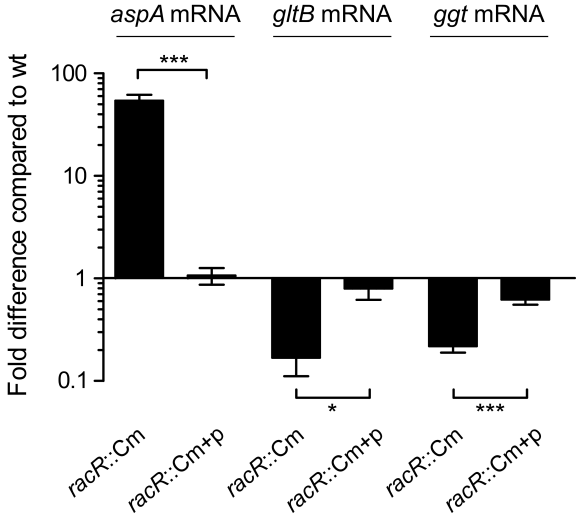

Figure 1: RacR activates *ggt* transcription. Real-time RT-PCR data showing the transcript fold difference of the *aspA, gltB* and *ggt* mRNA in the *C. jejuni racR* mutant (*racR*::Cm) and complemented strain (*racR*::Cm+p) compared to the wt. Cultures were grown in HI medium with addition of nitrate until end-log phase under oxygen limiting conditions. Fold increase is calculated using the $2^{\Delta\Delta Ct}$ method using *rpoD* as reference gene. Data represent the mean values and standard error of three independent experiments. *, p<0.05; ***, p<0.001.

To verify that the two-component system RacR/RacS also influences the GGT enzyme activity, we measured the GGT activity in *C. jejuni* stationary phase cultures grown at 0.3% O_2 with or without the addition of nitrate (Fig. 2). Only background levels of GGT activity were observed in the *ggt* mutant indicating that this enzyme is solely responsible for the production of 3-carboxy-4-nitroaniline in the GGT assay. Maximum GGT activity of bacteria grown in HI liquid medium was observed in stationary phase (data not shown). A

low GGT activity was measured in wt and *racR* mutant when the strains were grown at 0.3% O_2, however the GGT activity increased three-fold in the wt bacteria when nitrate was present. This induction was not observed in the *racRS* double mutant or the single *racR*, or *racS* mutant strains. When the *racRS, racR or racS* mutants were complemented with a plasmid harbouring the RacRS operon the GGT activity was restored to wildtype levels. These results indicate that GGT activity largely depends on a functional and activated RacR and RacS, as exists under limited oxygen condition in the presence of an alternative electron acceptor.

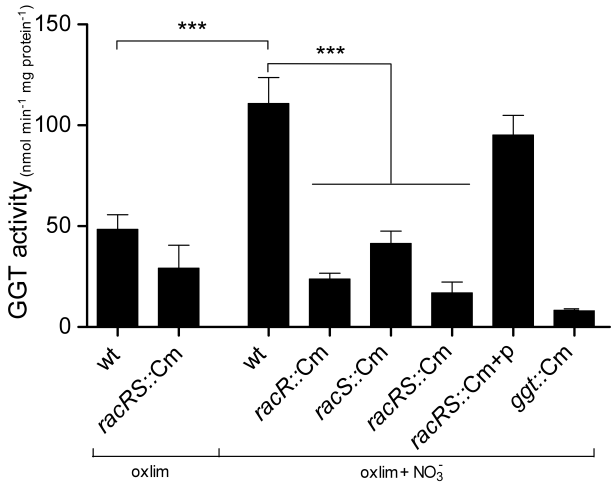

Figure 2: RacR influences the GGT activity. GGT activity was measured in cell lysate from stationary phase (20 h) cultures of wildtype 81116, the *racR*, *racS* and *racRS* mutant strains, as well as the complemented *racRS* mutant, grown in HI medium at oxygen limiting conditions with or without the addition of nitrate. Mean and standard errors of at least three independent experiments are shown. ***, p<0.001.

3

RacR protein binds to the promoter region of the *ggt* gene.

To investigate whether RacR activates the *ggt* gene directly by binding to the *ggt* promoter region, electrophoretic mobility shift assays (EMSA) were performed. Hereto the RacR response regulator and the cytoplasmic region of RacS were isolated as His-tagged recombinant proteins and together with ATP incubated with DIG-labeled DNA fragments containing the promoter region of the *ggt* gene or, as a negative control, the promoter regions of the *phoX* and *Cj0200c* genes. Incubation of RacR and the cytosolic region of RacS in the presence of ATP led to rapid phosphorylation of RacR (van der Stel et al., 2015). Unphosphorylated RacR bound to the *ggt* promoter but less RacR was needed when it was phosphorylated by the cytoplasmic part of RacS (Fig. 3A). Phosphorylated RacR did not bind to the *phoX* and *Cj0200c* promoter fragments as no band shifts were observed, while a clear bandshift was seen for the *ggt* promoter fragment (Fig. 3B). These results indicate that RacR specifically binds to the *ggt* promoter and that phosphorylation of RacR enhances the binding affinity.

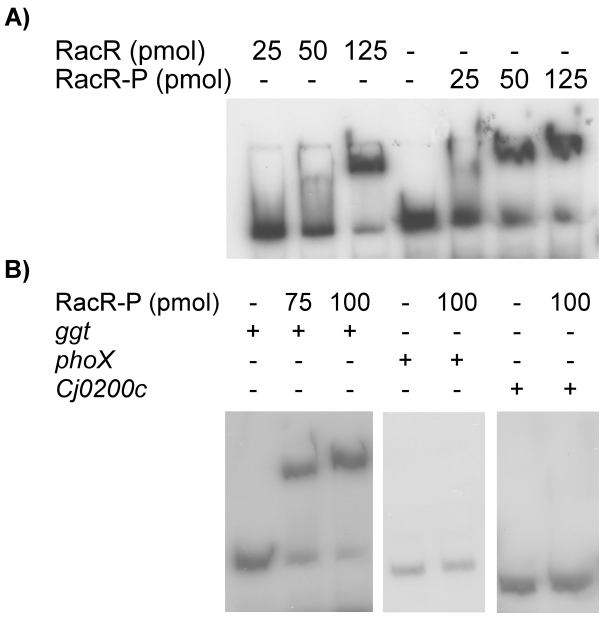

Figure 3: RacR binds to the ggt promoter region as shown by *electrophoretic* mobility shift assays. DIG-labeled PCR fragments (~50 fmol) containing the *ggt*, *phoX* or *Cj0200c* promoter regions were incubated with RacR as indicated. **A.** Influence of the phosphorylation of RacR on the binding to the *ggt* promoter. RacR was phosphorylated by RacScyto in the presence of ATP. **B**. E*lectrophoretic* mobility shift assays of the *ggt*, *phoX* and *Cj0200c* promoter regions with phosphorylated RacR protein. The *phoX* and *Cj0200c* promoter regions were used as negative controls. RacR-P: phosphorylated RacR.

3

RacR binds to a RacR binding consensus sequence in front of the *ggt* promoter.

To investigate where RacR binds on the *ggt* promoter, an *in-silico* analysis of the promoter region was performed (Fig. 4A). Based on this analysis, primers were designed to amplify DNA fragments in order to study the different elements on the *ggt* promoter region. Besides the full intergenic region between the *ggt* and *c8j_0034* gene (-204 nt fragment) three truncated *ggt* promoter elements were generated; 1) a -104 nt fragment containing a putative RacR binding consensus sequence, a palindromic sequence and the putative -35, -16 and -10 region; 2) a -69 nt fragment lacking the putative RacR binding consensus sequence and 3) a -35 nt fragment only containing the putative -35, -16 and -10 regions. These promoter elements were cloned in front of the luciferase reporter gene, replacing the *metK* promoter located on plasmid pMA5-metK-luc (Bouwman et al., 2013). Luciferase activity was measured in the wt and *racRS* mutant strain under RacR inducing conditions. Because of poor stability of the luciferase enzyme at 42°C (data not shown), all luciferase reporter assay experiments were performed at 37°C. In wt bacteria high luciferase activity was measured only from the promoter elements -204 and -104, both containing the predicted RacR nucleotide binding site (Fig. 4B). All promoter fragments resulted in a low luciferase activity in the *racRS* mutant, which was however still higher than the luciferase activity of the strain carrying a promoterless luciferase plasmid. These results indicate that the region upstream of the *ggt* gene containing the predicted RacR consensus sequence is important for enhancing of the *ggt* transcription in a RacRS dependent manner.

In order to verify the luciferase reporter results, the different *ggt* promoter elements were subjected to EMSA experiments (Fig. 4C). The -204 and -104 *ggt* promoter elements,

harbouring the predicted RacR binding site showed a distinct bandshift when phosphorylated RacR was present. In accordance with the luciferase assay results RacR did not bind to the two shorter fragments. These results prove that phosphorylated RacR binds to the upstream region of the *ggt* promoter region containing the predicted RacR binding consensus nucleotide site.

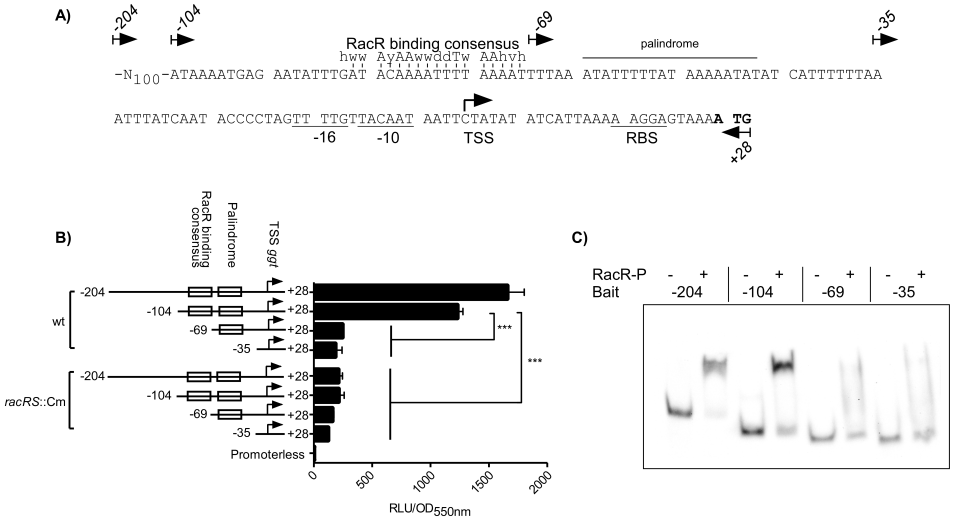

Figure 4: RacR binds to a specific region on the *ggt* promoter. **A**. Nucleotide sequence and features of the *ggt* promoter. The start codon ATG is indicated in bold face, the putative -10 and -16 regions and ribosomal binding site (RBS) are underlined. A palindromic sequence is indicated with a horizontal bar. The previously identified RacR binding consensus sequence (van der Stel et al., 2015) is indicated above the predicted RacR binding site, vertical lines indicate matching nucleotides. Arrows indicate the 5' termini and direction of the primers used to generate the *ggt* promoter elements for the luciferase reporter plasmids and EMSA bait DNA. The transcriptional start site of the *ggt* gene identified by (Dugar et al., 2013) is indicated with a hooked arrow. **B**. Luciferase activities using different lengths of the region upstream of the *ggt* gene are determined in wt and *racRS*::Cm mutant bacteria. Cultures were grown until late-log phase at oxygen limiting conditions with the addition of nitrate. Data represents the mean and standard error of three independent experiments. **C**. EMSA experiments using the different *ggt* promoter elements. DIG-labeled PCR fragments (~50 fmol) were mixed with or without 50 pmol RacR and 25 pmol RacScyto in the presence of ATP. RacR-P: phosphorylated RacR.

RacR is important for *C. jejuni* to generate more biomass out of glutamine, under RacRS inducing conditions.

To investigate whether the GGT activity contributes to an increased bacterial fitness, growth curves in DMEM medium with or without 10 mM glutamate or 10 mM glutamine were recorded for the wt, the *racR* and *ggt* mutant strains under RacR inducing conditions. To facilitate growth TMAO was used as electron acceptor, because nitrate proved to be detrimental for growth at these nutrient poor conditions. The growth rates of the wt grown with either glutamate or glutamine as sole carbon source were very similar, although the maximum OD_{600nm} was higher when glutamine was present (Fig. 5A). The *racR* mutant strain on the other hand showed a reduced growth rate when grown on glutamine compared to glutamate and consistently reached a slightly lower OD_{600nm} (Fig.

5B). Furthermore, compared to the wt, the *racR* mutant shows a reduced growth rate and a lower maximum growth yield. The *ggt* mutant strain grew comparable to the wt on glutamate, but hardly grew on glutamine (Fig. 5B), verifying that GGT is needed to utilize glutamine as sole carbon/energy source (Hofreuter et al., 2006). These results prove that under low oxygen conditions in the presence of alternative electron acceptors the RacRS system is important for the conversion of glutamine to glutamate.

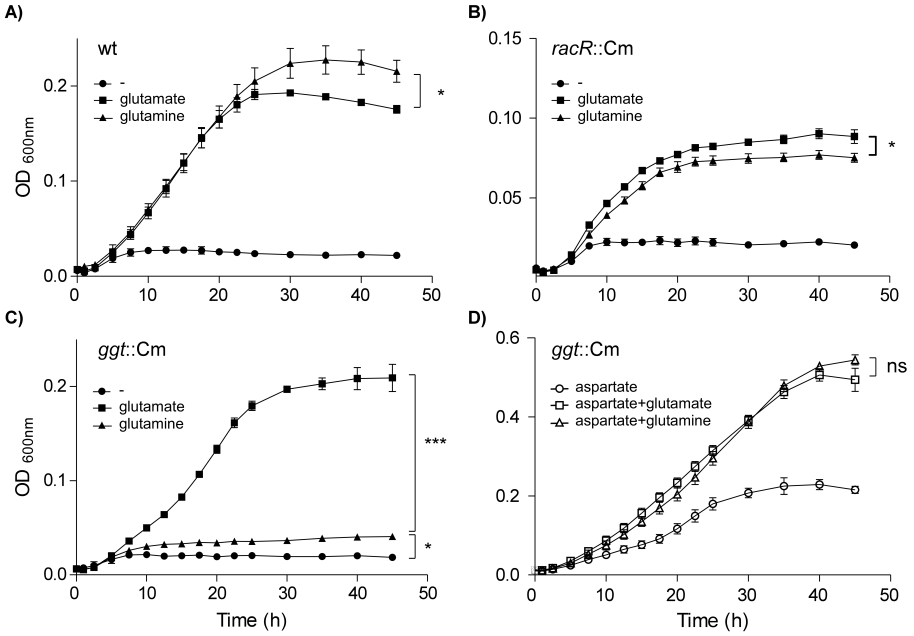

Figure 5: The RacRS system is important for the generation of glutamate out of glutamine. Growth curves were generated of the wt 81116 (**A**), the *racR* (**B**) and *ggt* (**C**) mutants grown in DMEM (circles) or with the addition of 10 mM glutamate (squares) or 10 mM glutamine (triangles) at 1% O_2 with the addition of 10 mM TMAO as electron acceptor. (**D**) The same as C but with the extra addition of 10 mM aspartate to the medium. Data represent the mean and standard error of three independent experiments. Significance was calculated using the maximally obtained OD_{600} values. ns, not significant; *, p<0.05; ***, p<0.001.

C. jejuni is capable of utilizing glutamine in a GGT independent manner.

Although GGT is required for growth with glutamine as sole carbon source, we observed that the *ggt* mutant reached a higher OD_{600nm} when grown on DMEM plus glutamine compared to DMEM alone (Fig. 5B). *C. jejuni* possess a glutamine transporter PaqPQ that has been shown to be functional under nutrient rich conditions (Lin et al., 2009). To investigate whether the *ggt* mutant is able to grow on glutamine under nutrient rich conditions, aspartate was added to the DMEM medium. Both the growth rate and bacterial yield of the *ggt* mutant increased with glutamate and glutamine compared to aspartate alone (Fig. 5D). Similar results were obtained when serine was used instead of aspartate (data not shown). This GGT independent glutamine utilization phenotype was also seen for the *C. jejuni* 81-176 *ggt* mutant strain and the *C. jejuni* 11168 strain, which

naturally lacks the *ggt* gene (data not shown). These results indicate that GGT is important when other energy sources than glutamine are not available.

DISCUSSION

The highly conserved enzyme γ-glutamyltranspeptidase (GGT) in prokaryotes plays a key role in the glutamine and glutathione metabolism. However, the regulation of the transcription of this gene is poorly understood. Here we identified the response regulator RacR as the first prokaryotic transcription factor that directly regulates *ggt* gene transcription. The *C. jejuni* RacR activates *ggt* gene transcription under low oxygen conditions in the presence of alternative electron acceptors. The RacRS system therefore not only activates the cytoplasmic production of glutamate by upregulating the GOGAT system (van der Stel et al., 2015), but also influences the periplasmic production of glutamate by upregulating the GGT enzyme, and thus ensuring the use of extracellular glutamine as energy source under RacR inducing conditions.

Previously, the *ggt* gene was not identified as part of the RacR regulon as the micro-arrays used in that study were based on a *C. jejuni* strain that lacks the *ggt* gene (van der Stel et al., 2015). Only 31% of *C. jejuni* strains contain a *ggt* gene (de Haan et al., 2012). Because both RacR and GGT have been shown to be important for host colonization (Brás et al., 1999; Barnes et al., 2007; Hofreuter et al., 2008), we assumed that the RacRS system might also regulate the *ggt* gene. Real-time RT-PCR and GGT enzyme activity assays clearly showed that RacR activates the transcription of the *ggt* gene and GGT enzyme activity under limited oxygen conditions in the presence of alternative electron acceptors (Fig. 1 & 2), the same result was observed in a *C. jejuni* 81-176 *racR* mutant (data not shown). Similar results were obtained in the *racRS* and the *racS* mutants, showing that both RacR and RacS are needed to activate *ggt* transcription.

Unequivocal evidence that *ggt* is a member of the RacRS regulon was obtained from EMSA and luciferase reporter assays (Fig 3 & 4). EMSA results showed that phosphorylated RacR strongly interacts with the *ggt* promoter, indicating direct regulation of *ggt* by RacR upon phosphorylation. The *C. jejuni* 81116 *ggt* promoter contains a conserved -10 and -16 region but no -35 region (Petersen et al., 2003; Dugar et al., 2013). Apparently a strongly conserved -10 and -16 region are not sufficient to activate the *ggt* transcription without additional transcription factors, as seen by real-time RT-PCR and luciferase assay (Fig. 1&4). These results suggest that although a conserved consensus sequence for the -35 region of sigma 70 regulated promoters in *C. jejuni* is lacking the region upstream of the -16 region is essential to activate sigma 70 regulated promoters as has been observed already (Salamasznska-Guz et al., 2013; Dugar et al., 2013). Using the previously obtained RacR binding consensus (van der Stel et al., 2015), a potential RacR binding site was found ~80-bp upstream of the transcriptional start site (TSS) (Dugar et al., 2013), besides that, a palindromic sequence was found ~60-bp upstream of the TSS, which could be a potential regulatory element. Different lengths of the *ggt* promoter cloned in front of a promoterless luciferase reporter gene and EMSA experiments showed that only

the fragments containing the predicted RacR binding nucleotide sequence were activated in the luciferase assay and bandshifted in the EMSA experiment. This result clearly shows that the *ggt* gene belongs to the RacRS regulon.

Although present in many bacterial species, knowledge regarding the regulation of the γ-glutamyltranspeptidase is limited. In *E. coli* and *B. subtilis* GGT activity is maximal in stationary growth phase (Tabor and Richardson, 1985; Xu and Strauch, 1996), while in *H. pylori,* an organism closely related to *C. jejuni*, *ggt* transcription is growth phase independent (Wachino et al., 2010). In *C. jejuni* the highest GGT activity measured at microaerophilic conditions is seen on plates in logarithmic growth phase (Barnes et al., 2007), however when we used HI liquid medium the highest GGT activity was observed in stationary growth phase, independent of the oxygen concentration (data not shown). Nutrient availability might explain this differences as nitrogen-limiting conditions has been shown to activate the *B. subtilis* GGT (Kimura et al., 2004). So far, only in *B. subtilis* a transcription factor ComA has been identified to indirectly regulate the *ggt* gene. ComA of the quorum sensing two-component system ComP/ComA activates the *ggt* transcription, but has no influence on the *gltBD* genes (Ogura et al., 2001). The *C. jejuni* RacRS system is therefore unique that it directly regulates the periplasmic as well as cytoplasmic glutamate production in response to the available electron acceptors.

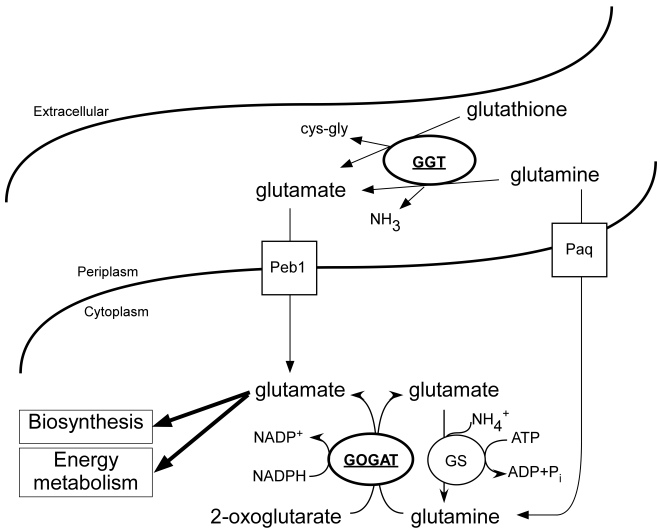

Figure 6: Overview of the *C. jejuni* glutamate generating enzymes and involved transport systems. Under low oxygen conditions in the presence of additional electron acceptors, RacR is activated and enhances the production of GGT and GOGAT (bold and underlined). Glutamine and glutathione are converted to glutamate in the periplasm by GGT; glutamate is consequently imported and used as carbon/energy source and anabolic precursor. Independent of GGT, glutamine can be imported and converted to glutamate by GOGAT, however this process is dependent on the presence of additional carbon/energy sources.

The GOGAT activity in other bacteria is strictly regulated, based on carbon, nitrogen and energy status of the cell (Ninfa and Jiang, 2005), also because it requires the high energetic cofactor NADPH to generate glutamate. GGT does however not require high energetic co-factors, this could be a reason why it is expressed by *C. jejuni* in late log/stationary phase to scavenge for nutrients when energy levels are low.
The role of GGT varies among organisms, in animals the enzyme is used to recycle glutathione, while in yeast cells GGT is used as metabolic enzyme to utilize nitrogen

sources (Mehdi and Penninckx, 1997). In *H. pylori* it was shown that an ammonia generating cycle consisting of periplasmic GGT and AnsA is present that aids in acidic resistance (Miller and Maier, 2014) and is essential for colonization and pathogenicity. The role of GGT in *C. jejuni* has been proposed to be metabolic, necessary for the acquisition of energy, carbon and nitrogen, by deamination of glutamine, or acquisition of additional sulphur by metabolizing glutathione (Hofreuter, 2014; Vorwerk et al., 2014). Furthermore, GGT has been shown to be important for the pathogenesis of *C. jejuni*, by inhibiting cell proliferation and causing apoptosis (Barnes et al., 2007; Floch et al., 2014). Here we show that *ggt* is co-regulated with other metabolic genes in *C. jejuni* by RacR (Fig. 1). In the periplasm GGT converts glutamine and glutathione to glutamate (Fig. 6), which is subsequently transported to the cytoplasm via glutamate transporter PEB1 (Del Rocio Leon-Kempis et al., 2006). Upregulation of *ggt* yields a growth advantage when grown on glutamine, confirming a metabolic role for GGT. This growth advantage is, however, only observed when other more favourable nutrients are less available (Fig. 5D). When other carbon sources are present, the transporter PaqPQ (Lin et al., 2009) probably imports glutamine, which is converted to glutamate by the GOGAT system. Recently it has been reported that most Campylobacter cow isolates lack the *ggt* gene, while *ggt* is common in poultry and human isolates (de Haan et al., 2012). *C. jejuni* clades that lack *ggt* often harbour the fucose utilisation gene cluster, while co-occurrence is rarely observed (de Haan et al., 2012), which could explain why strains lacking *ggt* are able to colonize chickens while a constructed *ggt* mutant cannot. As humans and poultry are both omnivores, they consume a diet richer in protein than cattle. This results in a higher glutamine concentration in the gut (Stella et al., 2006), which is in favour of GGT possessing *C. jejuni* strains.

Overall, we show that the *C. jejuni* RacRS two-component system directly regulates the *ggt* gene transcription under limited oxygen conditions when alternative electron acceptors are present. The RacRS system is the first identified system that directly regulates both the periplasmic glutamate production (GGT) as well as the cytoplasmic glutamate production (GOGAT) and plays an important role in the metabolism of *C. jejuni.*

MATERIAL AND METHODS

Bacterial strains, media and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *C. jejuni* strains were cultured at 37°C or 42°C on Blood Agar Base No. 2 (BA) medium containing 5% horse blood or in Heart Infusion broth (HI) (Oxoid), under microaerobic conditions (5% O_2, 7.5% CO_2, 7.5% H_2, 80%N_2) or under oxygen limited conditions (0.3% O_2, 10% CO_2, 10% H_2, 80% N_2). Kanamycin (25 μg ml^{-1}) and/or chloramphenicol (15 μg ml^{-1}) were added when appropriate. *E. coli* strains were routinely grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates supplemented with ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}) or chloramphenicol (30 μg ml^{-1}).

Construction of C. jejuni *ggt*, *racS* and *racRS* mutants.

To inactivate the *ggt* gene, the *ggt* gene was amplified by PCR using the oligonucleotides Cjj67Sac and Cjj67Xba (Table 2). The obtained PCR product was digested with *Cla*I and *Pst*I resulting in an 1.2-kb DNA fragment, which was ligated into pBluescript II KS to give plasmid pUWM799. Plasmid pUWM799 was digested with *Bgl*II to remove a 0.1-kb internal *ggt* fragment and was ligated to a 0.8-kb *Bam*HI fragment containing the *cat* cassette (0.8 kb) of pRY109. The resulting *ggt* knockout construct pUWM804 contained the *cat* cassette in the same orientation as the *ggt* gene.

To construct the *racS* and *racRS* knockout constructs plasmid pGEM1261 (van der Stel et al., 2015), containing the genes Cj1261-Cj1263 was amplified by PCR using the primers RacSendBamHI/RacSstart2BamHI or Cj1261FBamHI/RacSendBamHI, respectively. The resulting PCR products were digested with *Bam*HI and ligated to a 0.8-kb *Bam*HI *cat* cassette from pAV35 (Vliet et al., 1998), resulting in plasmids pGEM1262::Cm and pGEM1261-1262::Cm, respectively.

The genes *ggt, racS* and the *racRS* were disrupted in *C. jejuni* strain 81116 or 81-176 by natural transformation using the knock-out plasmids pUWM804, pJET1262::Cm and pGEM1261-1262::Cm, respectively. Double cross-over recombination events were confirmed by PCR.

Construction of *racRS* complementation strain.

To complement the *racRS* mutant, the complementation plasmid pMA1-1261-1263 (van der Stel et al., 2015) was first transformed into *E. coli* S17 and then conjugated (Labigne-Roussel et al., 1987) to the *racRS*::Cm mutant strain.

Purification of recombinant RacR and cytoplasmic RacS.

RacR(N-His) and RacScyto(N-His) were expressed and purified as described before (Wösten et al., 2004) using plasmids pT7.7-RacR(N-his) and pT7.7-RacScyto(N-his). Protein concentrations were determined using the BCA protein assay kit (Pierce).

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Relevant characteristics	Origin or reference
Strains		
E. coli TG1	supE hsdΔ5 thi Δ(lac-proAB) F′ (traD36 proAB$^+$ lacI^q lacZΔM15); used for general cloning	(Green and Sambrook, 2012)
E. coli BL21(DE3)	F$^-$ *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3); used for protein overexpression	Novagen
E. coli S17	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* (Tmp^r Str^r); used for conjugation	(Parke, 1990)
C. jejuni 81116	Wildtype	(Palmer et al., 1983)
C. jejuni ggt	81116 derivative *ggt*::Cm	This study
C. jejuni racR	81116 derivative *racR*::Cm	(van der Stel et al., 2015)
C. jejuni racS	81116 derivative *racS*::Cm	This study
C. jejuni racRS	81116 derivative *racRS*::Cm	This study
C. jejuni racR+p	81116 derivative *racR*::Cm + pMA1-1261-1263	This study
	81116 derivative *racRS*::Cm + pMA1-1261-1263	
C. jejuni racRS+p	Wildtype	This study
	81-176 derivative *ggt*::Cm	
C. jejuni 11168		(Palmer et al., 1983)
C. jejuni 81-176 ggt		This study
Plasmids		
pBluescript II KS	Ap^R; 3.0 kb; LacZα	Stratagene
pGEM-T Easy	Ap^R; 3.0 kb; LacZα; TA cloning vector	Promega
pT7.7	Ap^R; 2.5 kb; expression vector	(Tabor and Richardson, 1985)
pMA1	Km^R; 10 kb; *E.coli/C. jejuni* shuttle vector	(van Mourik et al., 2008)
Plasmids constructed for mutagenesis		
pUWM799	Ap^R; 4.2 kb; pBluescript II KS/internal fragment of *C. jejuni ggt*	This study
pUWM804	Ap^R Cm^R; 4.9 kb; pBluescript II KS/*ggt::*Cm	This study
pGEM-1261-1263	Ap^R; 5.5 kb; pGEM-T Easy/*C. jejuni 1261-1263*	This study
pGEM1261::Cm	Ap^R Cm^R; 6.3 kb; pGEM-T Easy/*racR::*Cm	This study
pGEM1262::Cm	Ap^R Cm^R; 6.3 kb; pGEM-T Easy/*racS::*Cm	This study
pGEM1261-62::Cm	Ap^R Cm^R; 5.3 kb; pGEM-T Easy/*racRS::*Cm	This study
Plasmids constructed for complementation		
pMA1-1261-1263	Km^R ;12.5 kb; pMA1/*Cj1261-1263*	(van der Stel et al., 2015)

Real-time RT-PCR.

Total RNA was extracted from late logarithmic phase *C. jejuni* cultures grown in HI medium at 42°C under oxygen limiting conditions with the addition of 50 mM nitrate, using RNA-Bee^{TM} kit (Tel-Test, Inc) according to the manufacturer's specifications. Real-time RT-PCR analysis was performed as previously described (van der Stel et al., 2015). Primers used in this assay are listed in Table 2. Experiments were repeated with three independently grown cultures. Fold increase was calculated with the $2^{\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) using *rpoD* as reference gene.

Table 2. Oligonucleotides used in this study

Name	Sequence (5'-3')[a]
Cjj67Sac	CGCGAGCTCGCTTTTTGCGGTGGTAGG
Cjj67Xba	AGTTCTAGAGGAGATCCTGTGCCTGTG
GGT204	AAAGCATGCATTGCACTTTCAATAAATTTTAAATATTTTAGC
GGT104	AAAGCATGCATAAAATGAGAATATTTGATAC
GGT69	AAAGCATGCTTTAAATATTTTTATAAAAATATATC
GGT35	AAAGCATGCAATTTATCAATACCCCTAGTTTTG
GGT28	AAAGAGCTCCATTTTACTCCTTTTTAATGATATATAG
Primers for gel mobility shift assay	
GGTprR	GCTTCAAATTTCATATTGCACTT
GGTprFDIG	TTGAAATCGCAAATATAGCT
Cj200RDIG	GTTTTAGACTATCTGCAAAA
Cj201F	TTTCATCTTCAATATACTCTAA
CJ0145RDIG	TTAAAAACAATCTTCTTTCCAT
CJ0145F	TTTCTAGTACAGTAAGTGATATAGC
Primers used for real-time RT-PCR	
ggtftaq	TGCGAGTTATGGTTCAGGTG
ggtrtaq	TTAGCTTCTCCGCCTACAAG
gltBftaq2	acacgatgcctgtggtatcg
gltBrtaq2	tcggtgttcaagattcatcaaaat
aspAftaq	TATGGGATAAGCATAGTGAAGTTCAAG
aspArtaq	CGCTTTAATAATCGCATCTTGGA
rpoDftaq	GAACGAATTTGATTTAGCCAATGA
rpoDrtaq	TGTCCCATTTCTCTTAAATACATACGA

[a] Restriction sites introduced for cloning purposes are underlined.

Construction of *ggt* promoter luciferase constructs.

To localize where RacR binds on the *ggt* promoter, different lengths of the upstream region of the *ggt* gene were amplified by PCR. The PCRs were generated by fusion polymerase (Thermo) with *Cj*81116 genomic DNA as template and one of the primer pairs GGT28/GGT204, GGT28/GGT104, GGT28/GGT69 or GGT28/GGT35. The PCR products were digested with *Sac*I and *Sph*I (Thermo) and cloned into *Sac*I, *Sph*I digested plasmid pMA5-metK-luc (Bouwman et al., 2013) to replace the *metK* promoter. To obtain a promotorless luciferase vector, pMA5-metK-luc was digested with *Sac*I and *Sph*I, blunted with the blunting enzyme from the CloneJET PCR Cloning Kit (Thermo) and finally self-ligated. The plasmids were verified by sequencing (Macrogen). The obtained plasmids were transformed to *E. coli* S17 and subsequently conjugated to *C. jejuni* 81116 and the *racRS* mutant.

GGT activity assay.

To assay the GGT activity the production of 3-carboxy-4-nitroaniline was followed by measuring the absorbance at 405 nm according to a modified procedure described by (Chevalier et al., 1999). Briefly, 1 mL of bacterial culture was pelleted and stored at -80°C for at least 1 hour. The pellet was resuspended in 250 µL buffer A (50mM Tris/HCl (pH 7.6), 1 µg/mL lysozyme), and incubated for 30 min on ice. Next, the bacteria were disrupted by sonication followed by centrifugation (10 min at 12000 x *g* at 4°C). From the cell free bacterial lysate 20 µl was mixed together with 180 µl of a reagent containing

2.9 mM L-γ-glutamyl-3-carboxy-4-nitroanilide, 100 mM glycylglycine and 100 mM Tris-HCl (pH 8.2). Samples were measured every 60 s during an incubation period of 30 min at 37°C. From these graphs the slope of all values in a linear range was calculated. Protein concentration was determined using the BCA method (Pierce). GGT activity is expressed as nmol min^{-1} mg $protein^{-1}$. The data shown represents at least three independent experiments.

Luciferase assay.

Expression of the luciferase in *C. jejuni* 81116 and *racRS* mutant strain harbouring the pMA5-ggtprom-luc plasmids was measured as previously described (Bouwman et al., 2013). Briefly, overnight cultures were diluted to an OD_{550nm} of 0.05 and grown for 7.5 h in HI with 50 mM KNO_3 in an oxygen limiting atmosphere at 37°C. One mL of each culture was pelleted (8000 x *g*, 5 min, 4°C) and suspended in 100 µL RLB buffer (Promega) supplemented with 0.5% Triton-X100. Suspensions were stored at -80°C for at least 30 min to disrupt the bacteria. Bacterial lysate (20 µL) was mixed with 50 µL of luciferase reagent (Promega) and RLU's were measured immediately on a luminometer (TD20/20, Turner Designs). The data shown represents at least three independent experiments.

Gel mobility shift assay.

The promoter regions upstream of *ggt, phoX* (Cj0145) and Cj0200c were amplified by PCR using Dreamtaq polymerase (Thermo) and one of the primers sets GGTpromDIG/GGT204, GGTpromDIG/GGT104, GGTpromDIG/GGT69, GGTpromDIG/GGT35, Cj145F/CJ0145RDIG and Cj200F/Cj200RDIG, respectively (Table 2) and *Cj*81116 chromosomal DNA as template. Primers GGTpromDIG, Cj0145RDIG and Cj200RDIG were ordered with a digoxigenin (DIG) label (Eurofins genomics). Approximately, 50 fmol DIG-labeled PCR fragments was incubated with His-tagged RacR, RacScyto and 2 mM ATP for 30 min on ice. RacR, RacScyto and ATP were preincubated for 5 min at 37°C to allow phosphorylation. The binding buffer used for protein-DNA incubations was 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 100 µg/ml bovine serum albumin, 1 µg/ml poly-(dI-dC) and 10% glycerol. Samples (10 µl) were run on a 4% non-denaturing Tris glycine polyacrylamide gel at 4°C. After electrophoresis, the DNA was blotted on a hybond-N+ membrane (Amersham) and PCR fragments were visualized using α-DIG-AP, Fab fragments and CSPD substrate (Roche).

Growth curves.

C. jejuni precultures were grown for 7 h in HI medium with the addition of 50 mM nitrate at oxygen limiting conditions at 42°C. The precultures were diluted 50 times in 300 µL DMEM (without glucose, glutamine, pyruvate, bicarbonate and phenol red, D5030, Sigma) with the addition of 10 mM TMAO and 10 mM glutamate, glutamine or aspartate and growth curves were generated at 42°C in a 100 well honeycomb plate which was continuously shaking in a Bioscreen C MRB (Oy Growth Curves Ab) computer-controlled incubator. The incubator was placed inside an anaerobic chamber (Coy Labs, MI, USA), due to suboptimal gas exchange in the honeycomb plate the oxygen concentration was set to 1%, which yielded comparable growth as when bacteria were grown in rectangular flasks inside an anaerobic jar containing 0.3% O_2. The OD_{600nm} of cultures was recorded

every 15 min during 45 hours. For clarity reasons only point at 2.5 h, or 5 h intervals are shown. Experiments were repeated three times in duplicate.

Statistical analysis

Prism software (GraphPad, San Diego, CA) was used for statistical analysis. Results are shown as mean ± SEM. Data was analyzed by one-way ANOVA, followed with Bonferroni post-hoc tests; $P < 0.05$ was considered statistically significant.

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AUTHOR CONTRIBUTION

AXS, AvM, EJK, JvP and MW contributed to the conception of the experiments. AXS, AvM and PL performed the experiments. Cloning, GGT assays, RT-qPCR, luciferase assasys, EMSA and growth curves were performed by AXS. EMSA experiments were performed by AvM. The *ggt* knockout construct was created by PL. AXS, AvM, and MW performed analysis of the data. AXS, AvM, JvP and MW wrote the manuscript.

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Chapter 4

Generation of the membrane potential and its impact on the motility, ATP production and growth in *Campylobacter jejuni*

Anne-Xander van der Stel[1], Fred C. Boogerd[2], Steven Huynh[c], Craig T. Parker[3], Linda van Dijk[1], Jos P. M. van Putten[1], Marc M.S.M. Wösten[1]

[1]Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands.
[2]Department of Molecular Cell Biology, VU University Amsterdam, Amsterdam, The Netherlands
[3]Produce Safety and Microbiology Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Albany, California, USA

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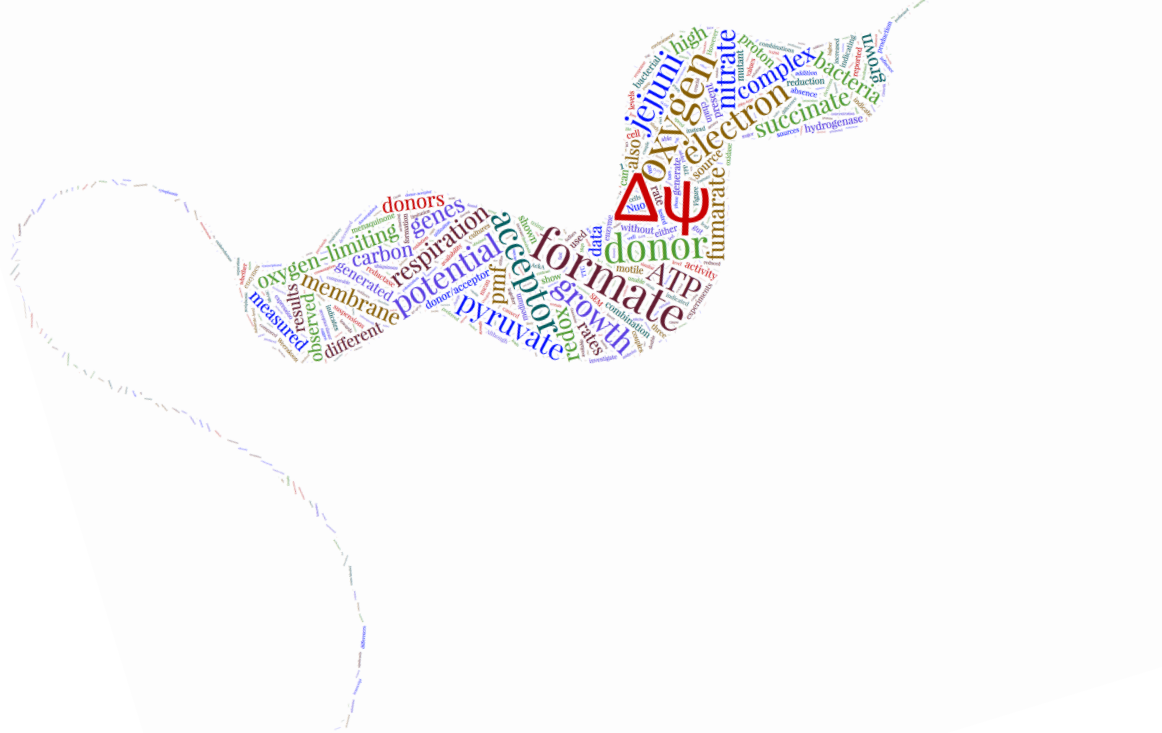

SUMMARY

The generation of a membrane potential (Δψ), the major constituent of the proton motive force (pmf), is crucial for ATP synthesis, transport of nutrients and flagellar rotation. *Campylobacter jejuni* harbors a branched electron transport chain, enabling respiration with different electron donors and acceptors. Here, we demonstrate that a relatively high Δψ is only generated in the presence of either formate as electron donor or oxygen as electron acceptor, in combination with an acceptor/donor, respectively. We show the necessity of the pmf for motility and growth of *C. jejuni*. ATP generation is not only accomplished by oxidative phosphorylation via the pmf, but also by substrate level phosphorylation via the enzyme AckA. In response to a low oxygen tension, *C. jejuni* increases the transcription and activity of the donor complexes formate dehydrogenase (FdhABC) and hydrogenase (HydABCD) as well as the transcription of the alternative respiratory acceptor complexes. Our findings suggest that in the gut of warm-blooded animals, *C. jejuni* depends on at least formate or hydrogen as donor (in the anaerobic lumen) or oxygen as acceptor (near the epithelial cells) to generate a pmf that sustains efficient motility and growth for colonization and pathogenesis.

INTRODUCTION

Respiration in prokaryotic organisms leads to the generation of the electrical transmembrane potential (Δψ), which together with the proton gradient across the cytoplasmic membrane (ΔpH) forms the proton motive force (pmf). The pmf is a major store of free energy in the bacterial cell that drives the transport of nutrients, generation of ATP and rotation of the flagella. The pmf is generated either by proton pumping complexes or by (half) redox loops constituted by certain arrangements of electron donor and electron acceptor complexes (Boogerd et al., 1981; Mitchell, 1961; Simon et al., 2008). Electron and proton transport between various electron donor and acceptor complexes is facilitated by quinones, which serve as membrane-associated redox carriers.

Campylobacter jejuni is the leading cause of bacterial food-borne diarrhea in humans causing approximately 400 million cases each year worldwide. This microaerophilic organism is often present as a commensal in the oxygen-limiting environment of the gastro-intestinal tract of birds. *C. jejuni* utilizes amino acids and TCA-cycle intermediates as main carbon sources as it is unable to metabolize glucose due to the lack of the glycolytic enzymes glucokinase and 6-phosphofructokinase. *C. jejuni* possesses a branched electron transport chain with multiple redox-enzymes that utilize an array of molecules as electron donor or acceptor (Sellars et al., 2002). Electrons of the carbon sources, pyruvate, succinate and malate are donated to the menaquinone (MK) pool, as this microaerophilic organism lacks ubiquinone, as is present in *Escherichia coli*. Pyruvate is oxidized to acetyl-CoA by pyruvate oxidoreductase (POR; Cj1476), the electrons derived from pyruvate are subsequently donated to the NADH ubiquinone oxidoreductase (Nuo) complex, which in *C. jejuni* uses flavodoxin (FldA) and menaquinone instead of NADH and ubiquinone (Fig. 1A) (Hughes et al., 1998; Maurice et al., 2007; Weerakoon and Olson,

2008). The Nuo complex is predicted to be a proton pump (Smith et al., 2000). Malate, via malate:quinone oxidoreductase (Mqo; Cj0393), and succinate, via Fumarate reductase (FrdABC), donate their electrons directly to the menaquinone pool (Fig. 1A). Besides these carbon sources, C. jejuni can use formate and hydrogen as electron donor. Formate and hydrogen are oxidized by the periplasmic formate dehydrogenase (*fdhABC*) and hydrogenase (*hydABC*), respectively (Weerakoon et al., 2009).

Despite its sensitivity to atmospheric oxygen levels (Kendall et al., 2014), oxygen is the preferred electron acceptor of *C. jejuni* (Sellars et al., 2002). Two cytoplasmically oriented oxidases are present; a cyanide insensitive-oxidase (*cioAB*; previously *cydAB*), which oxidizes menaquinones, and a cytochrome cbb_3-type oxidase (*ccoNOPQ*) (Jackson et al., 2007), which accepts electrons from Cyt *c* (Cj0037), which in turn is reduced by the cytochrome bc_1 complex (*petABC*) (Fig. 1A). Both the cytochrome bc_1 complex, as well as the cbb_3-type oxidase are predicted to have proton translocating capabilities (Smith et al., 2000), as reported for the closely related bacterium *Helicobacter pylori* (Tanigawa et al., 2010). *C. jejuni* is unable to grow under strictly anaerobic conditions, probably due to the need of molecular oxygen for anabolic purposes (Sellars et al., 2002). However, when oxygen is limiting, respiration with alternative electron acceptors, like nitrate and fumarate can support growth (Sellars et al., 2002). The periplasmic Nap complex reduces nitrate to nitrite (Pittman and Kelly, 2005). Fumarate is reduced either by the cytoplasmic fumarate reductase (*frdABC*), which also acts as succinate dehydrogenase, or by the periplasmic methylmenaquinone:fumarate reductase (*mfrABC*; previously *sdhABC*) (Fig. 1A) (Guccione et al., 2010). The acceptor complexes Mfr and Nap do not function as proton pump. On top of that, both the menaquinol- and substrate redox sites of these complexes are located at the periplasmic side of the membrane. This means that no charge separation takes place and therefore, these complexes do not contribute to the membrane potential, but function solely as an electron sink (Simon et al., 2008). In order to generate a $\Delta\psi$ under these conditions, a donor complex must accomplish charge separation, either as a proton pump or via a differential substrate- and menaquinone redox site. When both donor and acceptor complex are electroneutral, respiration does not lead to a $\Delta\psi$ (Biel et al., 2002; Simon et al., 2008).

Although the majority of the electron transport chain enzymes of C. jejuni have been characterized (Guccione et al., 2010; Jackson et al., 2007; Liu et al., 2013; Pittman and Kelly, 2005; Sellars et al., 2002; Weerakoon and Olson, 2008), it remains unclear which electron transport routes are able to generate a membrane potential in C. jejuni. Furthermore, knowledge on which electron donors are preferred by *C. jejuni* and whether this varies between growth conditions is scarce. In this study, we measured the $\Delta\psi$ of *C. jejuni*, generated with different electron transport routes and what effect the use of the different electron donor/acceptor couples has on bacterial ATP production, motility and growth. Moreover, we studied the transcriptional regulation of the electron transport chain genes.

RESULTS

Generation of Δψ in *C. jejuni*

To investigate which electron donor-acceptor pairs generate an electrical gradient in *C. jejuni* we measured the distribution of the lipophilic cation tetraphenyl phosphonium (TPP^+) in bacterial suspensions after addition of different electron donor and acceptor combinations (Fig. S1). TPP^+ freely diffuses across the inner membrane and accumulates in the cytoplasm, as a result of the existing Δψ. *C. jejuni* was grown in complex medium under microaerobic conditions until early stationary phase. The cultures were washed and resuspended in buffer containing EDTA, to permeabilize the outer membrane (Bakker, 1982). The experiments were performed at pH 8.0 to minimize the pH difference between both sides of the membrane. When the ΔpH is negligible, the pmf is entirely dependent on the Δψ (Krulwich et al., 2011).

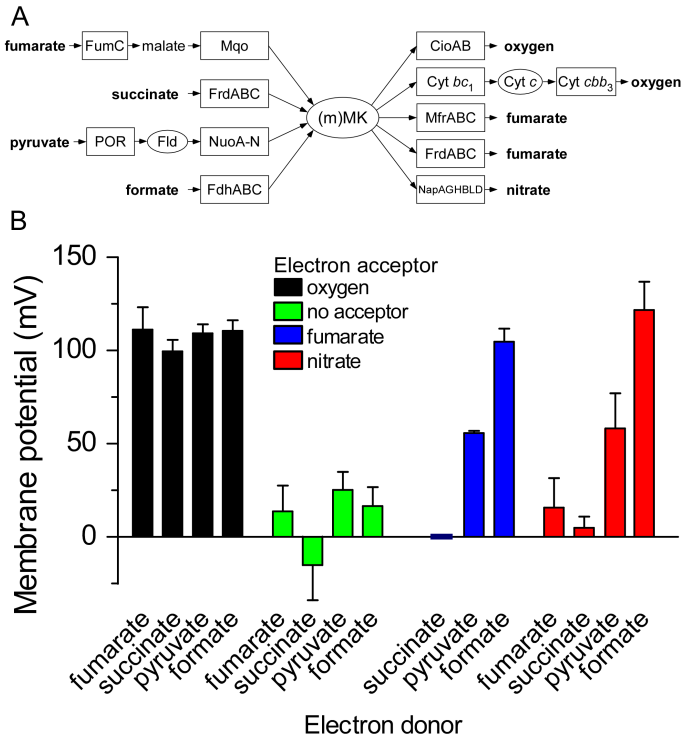

Figure 1: Membrane potential (Δψ) generated by *C. jejuni*. **A**: Schematic model of the electron transport chain of *C. jejuni*, showing the possible combinations of the electron donor and –acceptor complexes of the here studied compounds. **B**: Δψ values measured in microaerobic grown *C. jejuni* cell suspension in the presence of different electron donors and acceptors. The distribution of TPP^+ across the inner membrane was used to calculate the Δψ, in response to respiration with the indicated electron donor/acceptor couples. Data are shown as the mean of three biologically independent experiments with SEM as error bar.

A Δψ of 100-110 mV was measured when the electron donors fumarate, succinate, pyruvate or formate were tested in combination with oxygen as electron acceptor. With formate as electron donor a Δψ of 105-120 mV was measured independent of the electron acceptor (oxygen, fumarate or nitrate) (Fig. 1B). As expected, none of the electron donors was capable of generating a Δψ in the absence of an electron acceptor. In the presence of fumarate or nitrate as electron acceptor, *C. jejuni* generated a Δψ of only ~55 mV with pyruvate as electron donor. With succinate or fumarate as electron donors, no Δψ was detected. The membrane potential dissipated when the uncoupler agent carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was added (data not shown). FCCP disrupts the Δψ by allowing the transportation of protons through the cytoplasmic membrane resulting in the release of TPP^+ from the cells. The results (Fig. 1B) imply that all dehydrogenases, oxidases and reductases necessary for electron transfer between the tested electron donors and acceptors were present under the conditions employed and thus could in principle be operative in any donor-acceptor combination. This was substantiated when the experiments were repeated with bacteria grown under different conditions, which might lead to expression of other electron transport chain enzymes. When *C. jejuni* was grown under oxygen-limiting conditions in the presence of nitrate, nearly identical results as with microaerobic grown bacteria were obtained (data not shown). Overall, the experiments indicate that in the presence of oxygen as electron acceptor or formate as electron donor, *C. jejuni* generates a relatively high Δψ. However, in combination with electron acceptors other than oxygen, the donor pyruvate leads to a lower Δψ and fumarate and succinate do not yield a membrane potential at all.

Association between respiration and Δψ generation

To investigate whether the differences in Δψ, observed for the tested electron donor-acceptor combinations were caused by a difference in bacterial respiration, we measured the *C. jejuni* respiration rates on the basis of the consumption of O_2 and the production of nitrite or succinate (μmol min^{-1} mg $protein^{-1}$). Although the Δψ was similar for all electron donors when oxygen was present as electron acceptor (Fig. 1B), the rate of oxygen consumption was 10-fold higher when formate was used instead of pyruvate, fumarate, or succinate (Fig. 2A). This suggests that the magnitude of the respiration rate is not per se a good predictor of the generation of Δψ. On the other hand, nitrate respiration rates showed no major differences between the tested electron donors (Fig. 2C). Since nitrite can also be used by *C. jejuni* as electron acceptor and is converted to ammonium by NrfA, we also measured the ammonium production. No ammonium was detected (data not shown), indicating that the measured nitrite production is representative for the nitrate respiration in this assay. The formation of succinate was dependent on the presence of fumarate as electron acceptor (Fig. 2B). In contrast to nitrate respiration, fumarate respiration was also observed in the absence of an added electron donor, consistent with fumarate being used as electron donor (via malate and Mqo) and acceptor simultaneously (van der Stel et al., 2015). The fumarate respiration rates with formate or pyruvate as electron donors were comparable, but succinate seemed to cause product inhibition. In some cases (e.g. succinate/fumarate or fumarate/nitrate), respiration did occur, but was not accompanied with the formation of a Δψ (Fig 1B and 2). Altogether, the data indicate that although respiration is necessary for Δψ formation, respiration and the respiration rate are not obligatory correlated to the formation and amplitude of the Δψ, respectively.

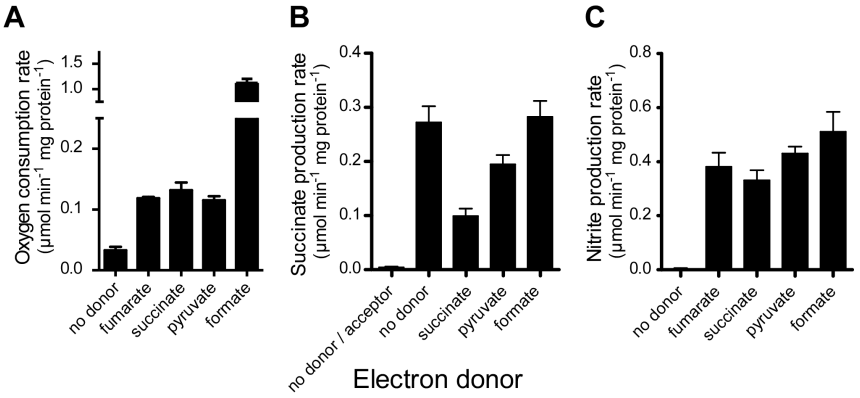

Figure 2: Respiration rates *C. jejuni* in cell suspensions. Microaerobic grown bacteria were used to obtaine respiration rates with different electron donors. **A**: Oxygen respiration rate as determined with a Clark-type oxygen electrode. Oxygen consumption was measured in parallel with the Δψ measurements. **B**: Fumarate respiration rates. Succinate accumulation was measured using LC/MS/MS. **C**: Nitrate respiration rates. Nitrite accumulation was determined using the Griess-assay. Bars show the mean of three biologically independent experiments ± SEM.

Motility is dependent on oxygen or formate

As rotation of bacterial flagella is driven by the pmf, bacterial motility can be used as an indirect indicator of variation of the pmf under different conditions. To investigate the influence of the different electron donors /acceptors on the pmf of *C. jejuni*, we measured the number of motile bacteria and their swimming speed by video tracking in cell suspensions. Addition of all electron donors, especially formate, led to more motile bacteria in the presence of oxygen (Fig. 3A). Also, more bacteria were motile when instead of oxygen, nitrate or fumarate was present together with formate. Succinate together with fumarate or nitrate did not increase the number of motile bacteria, indicating that no pmf sufficient to drive flagellar rotation is generated under these conditions. Also, addition of pyruvate did not lead to more motile bacteria, which in contrast to succinate was able to generate a small Δψ of 55 mV (Fig. 1B).

The motile bacteria reached a swimming speed of more than 15 µm/s, when oxygen and an electron donor were present (Fig. 3B). Swimming speeds of 20 µm/s were measured with the electron donor formate, no matter which electron acceptor was available. This high swimming speed was also reached when fumarate was present as electron acceptor in combination with pyruvate but not with succinate as electron donor. Based on these results we concluded that there is a good correlation between the percentage of motile bacteria and the swimming speed (r^2=0.79) (Fig. S3), as has been reported before (Wright et al., 2009). These results further indicate that *C. jejuni* is unable to generate a substantial pmf in the absence of either oxygen as electron acceptor in combination with any donor or formate as electron donor with any acceptor, which corresponds to the Δψ measurements.

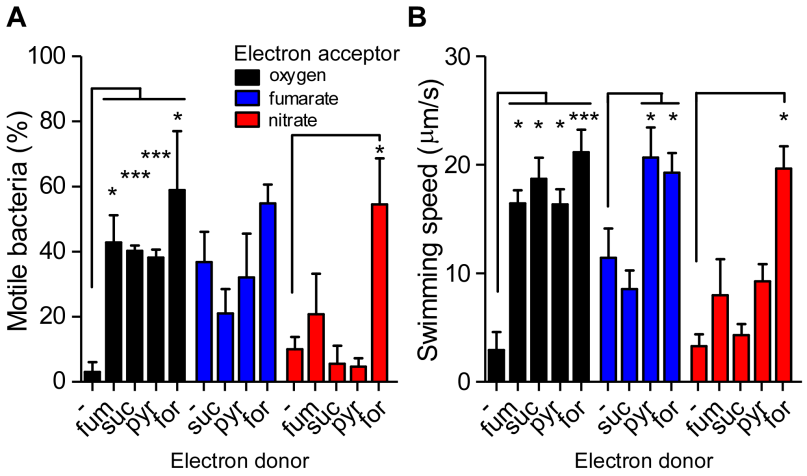

Figure 3: The influence of electron acceptor/donor couple on the motility of *C. jejuni* wt bacteria. *C. jejuni* was grown in HI-medium under microaerobic conditions and subsequently diluted into HEPES buffer with or without an electron donor and acceptor and incubated for 30 min at 42°C before the motility was monitored by video analysis. **A**: The percentage of motile vs immotile bacteria (n=~100) in the presence of the indicated electron acceptor/donor couple. **B**: Swimming speed of the bacteria that are motile under the indicated electron acceptor/donor couple (n=9). The data represents three independent experiments and shows the mean with SEM as error bars.

ATP is generated by the pmf and by substrate-level phosphorylation

ATP is the most important energy carrier in organisms and drives a multitude of reactions inside the cell. In general, ATP can be generated either by oxidative phosphorylation where the pmf functions as the driving force or by substrate-level phosphorylation coupled to certain enzymatic reactions. Although *C. jejuni* harbors the enzyme AckA, which converts acetyl-P and ADP to acetate and ATP, so far no ATP generation independent of respiration has been described for *C. jejuni.* To study the influence of the respiration on ATP synthesis, the ATP levels were determined in bacterial cell suspensions incubated with different electron donor/acceptor combinations. Without an electron acceptor, ATP levels were low, except when pyruvate was present (Fig. 4). This indicates that ATP can be generated independent of the pmf. To investigate whether this was caused by the AckA enzyme we measured the ATP levels in an *ackA* mutant. ATP levels did not increase when this mutant was incubated with pyruvate in the absence of an electron acceptor, indicating that ATP in the wild type is produced via AckA (Fig. S3A). To ensure that the ATP generation was not caused by traces of oxygen, a *ccoN/cioA* double mutant was constructed, which is defective in both oxidases and therefore unable to utilize oxygen as electron acceptor. This double mutant strain produced small colonies and did not reach high densities in liquid cultures (HI-medium with formate and pyruvate and nitrate or fumarate under oxygen limitation). Also, no growth was observed under microaerobic conditions, similar as has been described previously (Weingarten et al., 2008). The ATP levels of this double mutant strain gave identical results as the parent strain (Fig. S3), confirming that *C. jejuni* is able to generate ATP by substrate-level phosphorylation. These data (Fig. 3 & Fig. S3) show that *C. jejuni* is able to generate ATP by both oxidative phosphorylation using the pmf and by substrate-level phosphorylation via AckA.

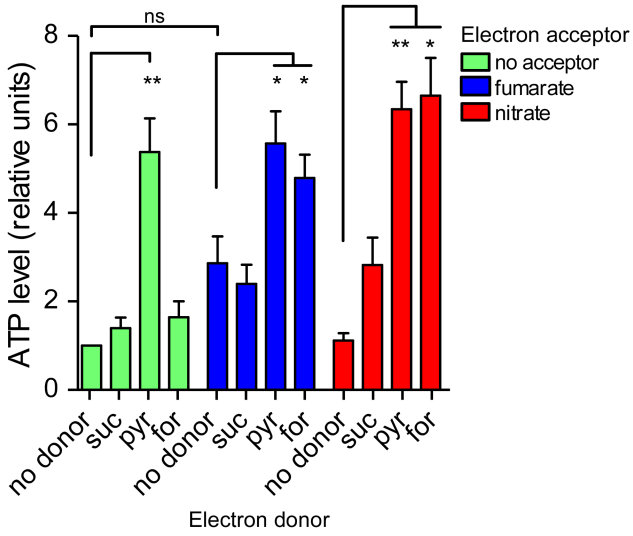

Figure 4: ATP level of *C. jejuni* cell suspensions, measured in the presence of different electron acceptor/donor couples. *C. jejuni* was grown in HI-medium under microaerobic conditions, whereafter the cells were harvested and resuspended in HEPES buffer. After incubation under oxygen- limiting conditions, with the indicated electron donor/acceptor couple, the ATP level of the total bacterial suspension was determined using a luciferase-coupled ATP assay. The relative ATP concentrations are presented, in which the condition with no electron donor/acceptor was set to 1. The experiment was performed three times independently and results are presented as mean ± SEM.

Growth rate correlates to the Δψ

To determine the effect of the presence or absence of the membrane potential on the growth characteristics of *C. jejuni,* growth curves were recorded in the presence of different electron donor/acceptor combinations in an atmosphere of 10% CO_2 with either 10% or 0.5% oxygen. Hereto, bacteria were grown in a defined medium supplemented with 2 mM aspartate as nitrogen source and 1 mM pyruvate to ensure reproducible growth. In the presence of 10% oxygen, poor growth of *C. jejuni* was observed in this medium (Table 1). Addition of 25 mM of fumarate, succinate or pyruvate as electron donor/carbon source increased the specific growth rate and the production of biomass (Table 1, Table S1). Addition of the electron donor formate led to an increased growth rate with fumarate and pyruvate as carbon source. Formate also strongly reduced the lag phase as observed with all carbon sources, but slightly reduced the biomass production. These results indicate that formate has a strong influence on the growth performance of *C. jejuni*.

At an atmospheric oxygen concentration of 0.5%, without an added electron acceptor, the addition of the carbon sources fumarate or pyruvate caused a slow growth rate, while no growth was observed with succinate. This indicates that the electron acceptor oxygen was not sufficiently available to support growth at this concentration. In the case of fumarate, but not with pyruvate, growth was enhanced by adding the electron donor formate. This difference can be explained by the fact that fumarate is used as carbon source, but also as electron acceptor. This was confirmed with a constructed *frdA/mfrA* double mutant which can still use fumarate as carbon source/electron donor, but is unable to utilize fumarate as electron acceptor (Guccione et al., 2010). The double mutant grew poorly with formate and fumarate as carbon source (μ=0.05 h^{-1}), comparable to wt bacteria without an added electron acceptor. This indicates that without an electron acceptor *C. jejuni* is not able to grow efficiently.

Table 1: Growth rates of *C. jejuni* in defined medium with different electron donors/acceptors.

		Growth rate (h^{-1}) (±SEM)	
electron acceptor	carbon source	without formate	with formate
oxygen	no carbon source	0.07 (0.15)	0.08 (0.01)
	fumarate	0.29 (0.02)	0.38 (0.05)
	succinate	0.18 (0.02)	0.17 (0.01)
	pyruvate	0.31 (0.04)	0.38 (0.02)
no acceptor	no carbon source	nc	nc
	fumarate	0.14 (0.03)	0.25 (0.04)
	succinate	0.06 (0.02)	nc
	pyruvate	0.12 (0.03)	0.09 (0.03)
fumarate	no carbon source	0.07 (0.1)	0.12 (0.05)
	fumarate	0.18 (0.05)	0.37 (0.07)
	succinate	0.11 (0.04)	0.28 (0.09)
	pyruvate	0.18 (0.03)	0.37 (0.11)
nitrate	no carbon source	nc	0.05 (0.03)
	fumarate	0.03 (0.01)	0.31 (0.05)
	succinate	nc	0.09 (0.06)
	pyruvate	0.10 (0.02)	0.32 (0.06)

Growth curves were recorded in a minimal medium with 25 mM carbon source and 10 mM electron donor and/or acceptor, or in the case of oxygen 10% in the gas phase. nc: growth curve did not converge to the Gompertz model, because of lack of growth. The data is presented as the mean and SEM of three independent experiments.

The addition of the electron acceptor fumarate under oxygen-limiting conditions led to a slight increase in growth rates and biomass. However, addition of the electron donor formate strongly increased the growth rates to >0.30 h^{-1} with the carbon sources fumarate and pyruvate. These high growth rates obtained with formate/fumarate as electron donor/acceptor are comparable to the growth rates with the couple formate/oxygen. For both donor-acceptor couples also high Δψ values were obtained (Fig. 1B), indicating that a substantial Δψ is needed for growth rates >0.30 h-1.

When *C. jejuni* was using nitrate as electron acceptor, low growth rates were measured with all carbon sources. However, growth rates increased when formate was also added to the culture in combination with fumarate or pyruvate as carbon source. This observation fits with the high Δψ generated with formate and nitrate (Fig. 1B). Consistent with this finding is the fact that a high growth rate was not reached in a *napA* mutant (Table S1), which cannot utilize nitrate as electron acceptor. These results further prove that a high Δψ is indispensable to obtain high growth rates, since fast growth was only observed in conditions where a high Δψ was generated.

The transcription of electron transport chain genes is dependent on the oxygen concentration

As only certain electron donor/acceptor couples generated a Δψ, we wondered whether the available electron acceptor influenced the transcription of genes involved in these processes. Hereto we compared the transcriptomes of exponentially growing C. jejuni cells grown in HI medium under three different conditions; microaerobic, oxygen-limiting and oxygen-limiting in the presence of the alternative electron acceptor nitrate. RNA-seq analysis revealed that under oxygen-limiting conditions, the oxygen electron acceptor genes *cioAB*, *ccoNOPQ*, as well as *petABC* were downregulated (Fig. 5A). On the other hand, all alternative electron acceptor reductase genes (*napAGHBLD*, *torA*/*cj0265c*, *mfrABC* and *nrfAH*) were upregulated, independent of the availability of their corresponding substrates. Similar results were seen in the presence of the alternative electron acceptor nitrate, however for the alternative electron acceptor reductase genes less transcript differences were observed. Apart from electron acceptor complexes, transcription of genes encoding components of electron donor complexes were also affected by the availability of oxygen. Under oxygen-limiting conditions, the formate dehydrogenase encoding genes (*fdhABC*) and the hydrogenase operon (*hydABCD*) were upregulated, while the gluconate dehydrogenase (*gdhAB*), 2-oxoglutarate reductase (*oorABCD*), NADH ubiquinone oxidoreductase (*nuoA*-*N*), as well as flavodoxin (*fldA*) were downregulated. The presence of nitrate increased the expression of the *fdhABC* operon even further, but in contrast to the oxygen-limiting conditions transcription of *gdhAB* genes was upregulated. These transcriptomic data suggest that dependent on the availability of oxygen as electron acceptor, the gene transcription of electron donor and acceptor complexes that allow for the generation of a high Δψ are upregulated.

To confirm the transcriptomic results and to investigate the transcriptional response when fumarate is used as electron acceptor instead of nitrate, an RT-qPCR experiment was performed on the electron transport genes. The RT-qPCR results confirmed the outcome of the transcriptomic data as even larger differences in transcript abundance were observed for the investigated genes (Fig. 5B). The genes necessary for oxygen utilization, *petC*, *ccoN* and *cioA*, were downregulated under oxygen-limiting conditions and the alternative electron acceptor reductase genes mfrA, napH and nrfH were upregulated, just like the electron donor complexes *fdhA* and *hydD*. In contrast to the RNA-seq data *cj0037* (putative Cyt *c*) was found to be slightly downregulated instead of upregulated. No obvious difference was observed between cultures grown under oxygen-limitation with or without fumarate or nitrate, except for the *cioA* gene, which was upregulated in the presence of nitrate. These results indicate that the gene regulation of the electron acceptor and -donor genes are almost exclusively dependent on the availability of oxygen.

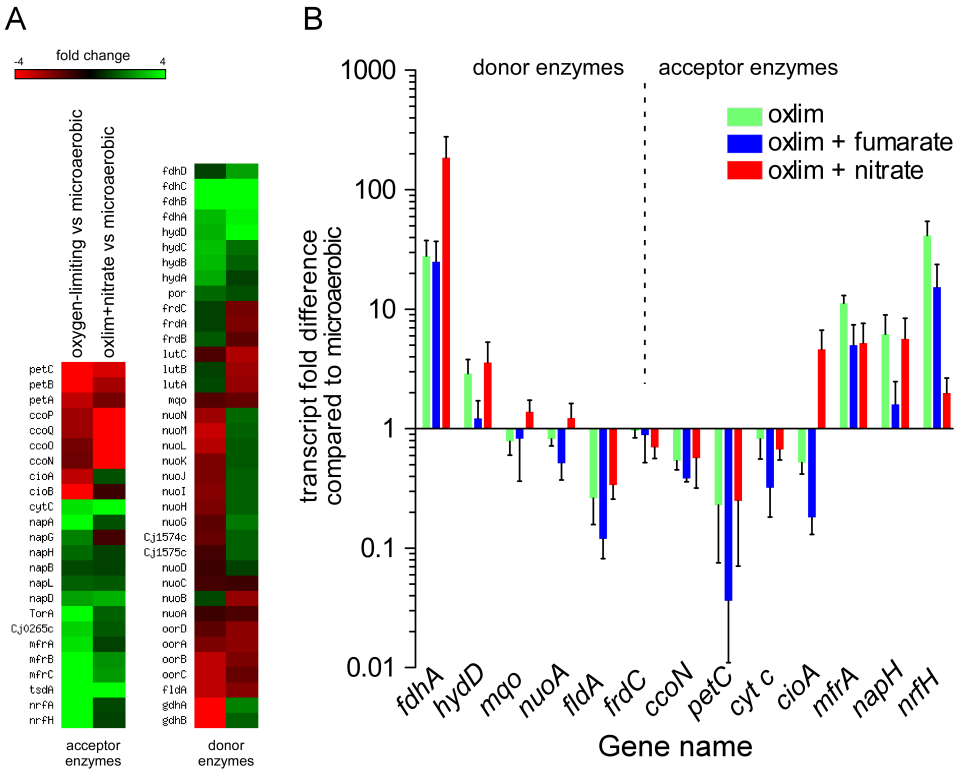

Figure 5: Transcriptional adaptation towards low oxygen. **A**: Heat map of RNA-seq data of *C. jejuni* grown under oxygen limitation with or without 10 mM nitrate compared to the microaerobic grown cultures. Genes are clustered per regulatory operon unit and subsequently ordered on the basis of expression under oxygen limitation; *frdABC* is listed as donor complex. Heat map was created using matrix2png (Pavlidis and Noble, 2003) **B**: RT-qPCR experiment showing the transcript fold differences of the respiratory genes under oxygen-limiting conditions, with or without the addition of 10 mM fumarate or nitrate as electron acceptor, compared to microaerobic conditions. Total RNA from logarithmic phase cultures in HI medium was analyzed. The data is shown as mean ± SEM of three independent RNA isolations as error bars.

The formate dehydrogenase and hydrogenase activity are strongly upregulated under oxygen-limiting conditions.

Under oxygen-limiting conditions, the electron donors formate and hydrogen are crucial for the generation of a membrane potential (Fig. 1B). The transcription of especially the formate dehydrogenase genes, but also of the hydrogenase genes, is strongly upregulated under these conditions (Fig. 5). To investigate whether this upregulation also led to higher formate dehydrogenase and hydrogenase activity in bacteria grown under oxygen-limiting conditions and whether this is conserved among *C. jejuni* strains, a 2,3,5- Triphenyl-tetrazolium chloride (TTC) reduction assay was performed. Therefore, six wild type strains were grown under microaerobic or oxygen-limiting conditions. Next, TTC reduction, dependent on substrate oxidation, was measured in cell suspensions in the presence of the electron donors, formate, hydrogen or pyruvate. All strains showed significantly more formate dependent TTC reduction (3-25 fold) in oxygen-limiting compared to microaerobic grown cultures, indicating increased formate dehydrogenase activity (Fig.

6A). Similar but less pronounced results were observed for the hydrogenase activity (1.2-2.9 fold) (Fig. 6B). Pyruvate dependent TTC reduction was independent of the growth condition (Fig. 6C) which is in agreement with the unaltered transcript levels of the *por, nuo, and fldA* genes that are needed to use pyruvate as electron donor. These data suggest a strong preference for formate and hydrogen utilization when oxygen cannot be used as electron acceptor.

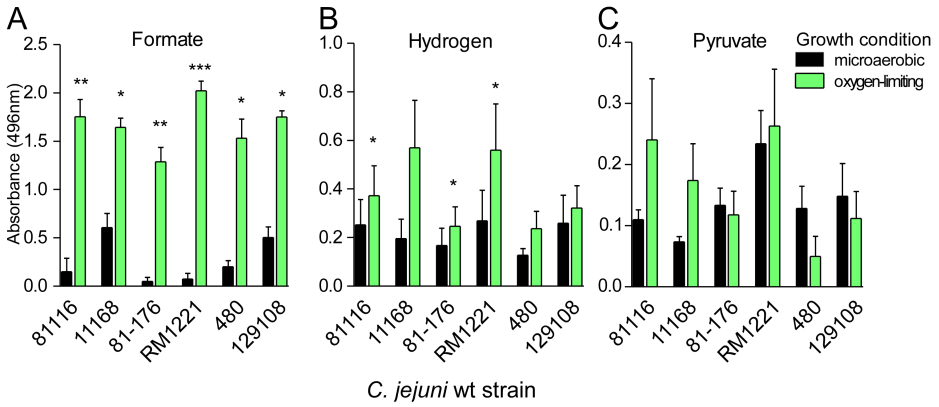

Figure 6: Formate, hydrogen and pyruvate dependent TTC reduction showing the formate dehydrogenase (**A**), hydrogenase (**B**) and pyruvate oxidation (**C**) activity of six *C. jejuni* wt strains. TTC reduction was measured at the absorbance of 496 nm in bacterial suspensions from cultures grown in HI medium under either microaerobic (black bars) or oxygen-limiting (green bars) conditions until logarithmic phase. Data is shown as mean ± SEM as error bars of three independent experiments.

4

DISCUSSION

Here we show which electron donor/acceptor couples allow the formation of an electric membrane potential in *C. jejuni*. The generated Δψ, the major contributor of the pmf, is crucial for efficient *C. jejuni* motility, growth and ATP generation, however the latter is also driven by substrate level phosphorylation. Transcriptomics revealed that the oxygen availability is the key regulator of electron transport chain genes in *C. jejuni.* When oxygen is not available as electron acceptor, the formate dehydrogenase and hydrogenase are upregulated, ensuring Δψ formation via alternative electron acceptors.

The Δψ formed with any electron donors in combination with oxygen is equivalent to the Δψ formed with formate and alternative electron acceptors (Fig. 1B). When grown with nitrate as electron acceptor, instead of oxygen, the the membrane potential did not significantly change, indicating that all respiratory proteins are already expressed under microaerobic conditions, although they are further induced when oxygen is limiting (Fig. 5). Previous studies also found that *C. jejuni* has fumarate and nitrate reductase activity under microaerobic growth conditions, which is enhanced under oxygen-limiting growth (Guccione et al., 2010; Weingarten et al., 2008). Similar results are reported for *E. coli,* where the Δψ remains stable under growth conditions with different electron acceptors (Tran and Unden, 1998).

The Δψ of *C. jejuni* is lower than reported for other proteobacteria, but is within the range that has been reported for bacteria in general (Geisler et al., 1994; Rao et al., 2008; Schnorpfeil et al., 2001; Tran and Unden, 1998). During the Δψ measurements a large amount of TPP^+ is bound to cellular components and is excluded when calculating the free $[TPP^+]_{in}$. Although this is observed for many bacteria, these corrections could underestimate the real Δψ values of *C. jejuni*. Some bacteria code for efflux pumps that can actively excrete cations like TPP^+ from the cytoplasm (Poole, 2004). This has not been tested for *C. jejuni,* but could potentially also lower the observed Δψ. Lower Δψ values are reported in another *C. jejuni* study (Tholozan et al., 1999), where a higher experimental $[^3H]TPP^+$ concentration (10 mM) was used, which likely led to Δψ dissipation (Bakker, 1982). Despite the possible underestimation of the actual Δψ values, we were able to show which electron donor/acceptor couples are capable of generating a pmf in *C. jejuni*.

Oxygen is the preferred electron acceptor for *C. jejuni* (Table 1 & S1). The bc_1 complex and the cbb_3-type oxidase are both proton translocating enzymes and this route is thus electrogenic (Fig. 7 & Table 2) (Hoffman and Goodman, 1982; Rauhamäki et al., 2012). With a mid-point redox potential of +820 mV, oxygen is the strongest electron acceptor *C. jejuni* can use. Because oxygen is such a potent electron acceptor, all electron donors yield comparable membrane potentials, growth yields and motility performance (Fig. 1, 3 and Table 1). The growth yields on all carbon sources/electron donors are significantly higher with oxygen than with other electron acceptors (Table S1). A *cioA* mutant had similar growth characteristics as the wt (data not shown) and it was previously reported that the CioAB complex is not involved in oxygen respiration under microaerobic conditions, despite its relative high affinity towards oxygen (Jackson et al., 2007). This suggests that the pathway through the bc_1 complex and cbb_3-type oxidase is solely responsible for pmf generation with oxygen respiration. CioAB homologues of other bacteria have repeatedly been implicated in ROS and RNS resistance (Borisov et al., 2015). Our observation that the transcription of *cioA* is upregulated in the presence of nitrate (Fig. 5) supports the notion that the *C. jejuni* CioAB might have a similar role in nitrosative stress resistance.

When oxygen is not available as electron acceptor, the transcription of all genes coding for alternative electron acceptor complexes are upregulated independent of the presence of their corresponding substrates (Fig. 5B). Besides these genes, also the genes coding for the electron donor complexes, formate dehydrogenase and hydrogenase are upregulated, indicating that these electron donor/acceptor combinations are preferred under these conditions. Indeed, formate (hydrogen was not tested due to safety duties) together with the alternative electron acceptors generates a high Δψ, while with pyruvate a moderate and with succinate no Δψ is generated (Fig. 1B), even though respiration is observed in all cases. Although the different expression of respiratory complexes does not lead to altered Δψ values (data not shown), it does influence substrate kinetics (Fig. 6) and this will likely give an advantage to the bacterium *in vivo* where substrate might be scarce. Other donor complexes, which do not contribute to the pmf, were not upregulated, such as Mqo and FrdABC. Malate, produced from fumarate by the enzyme FumC, donates its electrons to the menaquinone pool via the electroneutral enzyme Mqo (Fig. 7 & Table 2). Despite not being coupled to proton translocation, this reaction still has a role in carbon assimilation

in the TCA cycle. Succinate donates electrons to the MK-pool via the FrdABC complex (Fig. 7). However, the mid-point redox potential of succinate (+35 mV) is more positive than that of both types of menaquinones of *C. jejuni* —menaquinone (-74 mV) and methylmenaquinone (-124 mV) — consequently, this reaction is endergonic and consumes pmf to use succinate as electron donor (Fig. 7 & Table 2) (Dietrich and Klimmek, 2002; Juhnke et al., 2009). This likely explains the poor growth observed with succinate as carbon source in the absence of oxygen as electron acceptor, even in the presence of the electron donor formate (Table 1).

A small Δψ was observed when pyruvate was present with nitrate or fumarate as electron acceptor. The Nuo complex is linked to menaquinone reduction and acts as proton pump (Fig. 7 & Table 2). The Nuo complex is the major electron donor complex in many organisms (Brandt, 2006), however we measured lower Δψ values with pyruvate, compared to formate with alternative electron acceptors (Fig. 1B), even though the substrate of the Nuo complex, Flavodoxin, has a midpoint redox potential of around -400 mV (Freigang et al., 2002). Furthermore, the *nuo* genes are not upregulated, like *fdhABC* and *hydABCD*, suggesting no preference for the Nuo complex in the absence of oxygen. The ATP levels indicate that *C. jejuni* is capable of ATP synthesis with pyruvate as substrate, yet this occurs independently of an electron acceptor, indicating that the Nuo complex does not play a role in this event. It turned out that the enzyme AckA is responsible for the generation of ATP independent of the pmf via substrate level phosphorylation (Fig. S3A & 7). However, the low Δψ and the AckA-generated ATP combined are insufficient to sustain high motility and fast growth rates (Fig. 3 & Table 1). Together, our data suggest that the Nuo complex of *C. jejuni* functions optimally in combination with oxygen, but not with alternative electron acceptors. Compared to other bacteria this function dissimilarity could be caused by the substrate of the *C. jejuni* Nuo complex, which is Flavodoxin rather than NADH or the absence of ubiquinone and demethylmenaquinone (Carlone and Anet, 1983; Weerakoon and Olson, 2008).

A high Δψ is formed with formate together with any electron acceptor. This is accompanied by fast growth rates (Table 1), more motile bacteria and ATP generation (Fig. 3, 4). Strong correlations were found between the Δψ and the growth rate, and the Δψ and the motility, with r^2=0.81 and r^2=0.69 respectively (Fig. S2). This substantiates that the Δψ is required for growth and motility. The transcription and activity of the formate dehydrogenase (FdhABC) and hydrogenase (HydABCD) enzymes were the only donor complexes upregulated under oxygen-limiting conditions (Fig. 5 & 6), suggesting that these two complexes are involved in the generation of a high Δψ under these conditions. The substrate binding site for formate of the integral membrane complex formate dehydrogenase is located in the periplasm, but menaquinone reduction takes place at the cytoplasmic side of the membrane (Jormakka et al., 2002). Therefore, formate oxidation leads to charge separation via a redox half loop (Fig. 7). Of all electron donors used by *C. jejuni*, formate has the most negative standard midpoint redox potential (-430 mV) followed by hydrogen (-420 mV). Although not studied here, hydrogen is expected to have a similar effect on the membrane potential as formate, based on the enzyme subunit layout, the comparable redox potential, as well as previous reports (Biel et al., 2002; Laanbroek et al., 1978; Weerakoon et al., 2009). Overall, the data show that formate and

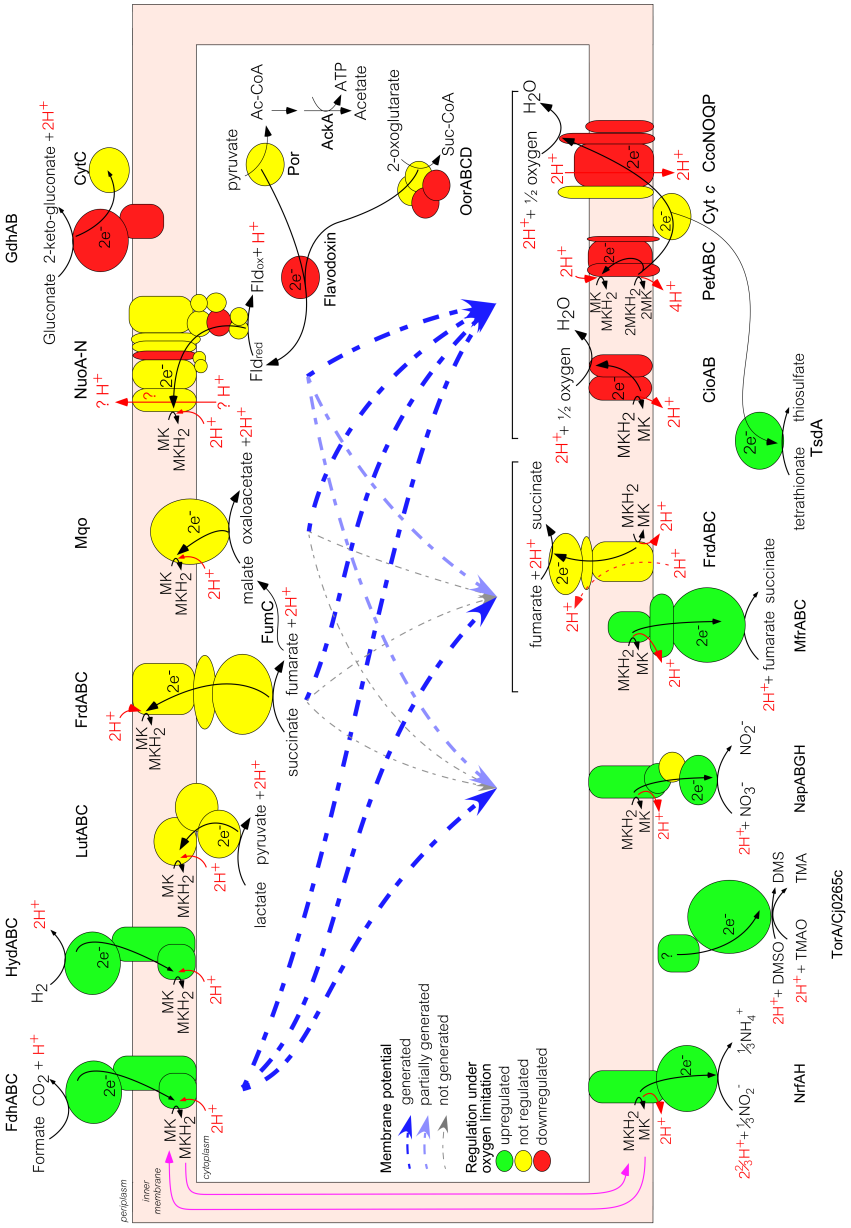

Figure 7: Model of electron/proton flow in the *C. jejuni* electron transport chain. Respiratory donor (top) and acceptor (bottom) complexes are shown, including electron movements (black arrows) between donor, carrier — MK(H_2), cyt *c*, flavodoxin — and acceptor molecules. Red arrows show proton translocations. A membrane potential (Δψ) is generated either by proton pumping enzymes or through translocations of protons by menaquinones via a redox loop, as depicted by the pink arrows. The generation of a Δψ in *C. jejuni* with different substrates (as shown in Fig. 1B), is indicated with blue dot dashed arrows (darkblue: high Δψ; light blue: partial Δψ; grey: no Δψ). The transcriptional response towards oxygen limitation of the genes coding for the electron transport proteins (as shown in Fig. 5) is indicated as follows: green fill indicates upregulation upon oxygen limitation, red indicates downregulation and yellow indicates equal expression.

hydrogen are the preferred electron donors of *C. jejuni,* and the only donors that generate a high Δψ, when respiring with electron acceptors other than oxygen.

The main reservoir of *C. jejuni* is the oxygen-limiting intestine of warm-blooded animals. The gut is oxygenated to a certain extent from the epithelial cells (Espey, 2013), and *C. jejuni* uses energy taxis to swim towards higher concentrations of oxygen (Reuter and Vliet, 2013) and is frequently found penetrating the mucus layer. Oxygen in the gut of chicken is essential for *C. jejuni* as a *ccoN* mutant strain is unable to colonize this environment (Weingarten et al., 2008). These features indicate that *C. jejuni* actively searches for oxygen and has access to oxygen near the epithelial cell layer *in vivo*.

Table 2: Comparison between midpoint redox potential difference ($\Delta E_0'$), the theoretical charge separation per electron (based on electron and proton movements; Fig 7) and the experimentally observed membrane potential (Δψ; Fig. 1).

Acceptor	Donor	$\Delta E_0'$ (mV)	theoretical q^+/e^-	experimental Δψ (mV)
oxygen	formate	1250	3	110
	pyruvate	~1220[a]	~4[b]	109
	fumarate[c]	986	2	111
	succinate	790	1	100
fumarate	formate	460	1	105
	pyruvate	~430[a]	~2[b]	56
	fumarate[c]	196	0	n.d.
	succinate	0	-1	0
nitrate	formate	850	1	122
	pyruvate	~820[a]	~2[b]	58
	fumarate[c]	586	0	15
	succinate	390	-1	5

[a]The midpoint redox potential of flavodoxin, the substrate for the Nuo complex is not exactly known (Freigang et al., 2002).
[b]The number of protons translocated over the membrane by the Nuo complex is not known.
[c]Fumarate is converted to malate in C. jejuni, which consequently acts as electron donor via the Mqo enzyme.
n.d.: not determined

Our results show that formate plays a significant role in the motility, growth rate and ATP production of *C. jejuni* in an oxygen-limiting environment. Therefore, *C. jejuni* would have difficulties colonizing chickens or cause disease in humans without the electron donor formate (or hydrogen). A chemotactic response towards formate has been reported, through the transducer-like protein Tlp9 (Tareen et al., 2010), emphasizing the preference of *C. jejuni* for formate as electron donor. Mixed acid fermentation by the gut microbiota leads to the formation of formate and hydrogen in the intestine, as can be assumed from the expression of formate- and hydrogen-generating enzymes by the gut microbiota (Polansky et al., 2016; Sergeant et al., 2014). Induction of formate dehydrogenase is observed when *C. jejuni* is grown in a rabbit ileal-loop *ex vivo* (Stintzi et al., 2005), but not during chicken colonization (Taveirne et al., 2013; Woodall et al., 2005). Moreover, formate utilization is crucial for piglet colonization (Vries et al., 2017), efficient colonization of chickens (Weerakoon et al., 2009) and pathology in mouse models (Bereswill et al., 2011; Kassem et al., 2012). Formate also acts as invasion signal for the pathogenic bacterium *Salmonella enterica* serovar Enteritidis (Huang et al., 2008; Van

Immerseel et al., 2003). This all indicates that without formate metabolism, *C. jejuni* loses its potential to colonize the gut and cause pathogenicity.

Very recently a study was published that confirms our results that formate utilization stimulates *C. jejuni* growth under oxygen-limiting conditions (Kassem et al., 2017). However, in that study it is suggested that formate reduces oxidase activity, since TMPD oxidation, employed to read out the oxidase activity, is reduced when bacteria are incubated in the presence of formate. Our results on the contrary, show a vast increase in oxygen consumption when formate is present, suggesting an enhanced oxidase activity (Fig. 2A). This discrepancy could be caused by: 1) competition between formate and the TMPD dye as electron donor for the oxidases in *C. jejuni,* or by 2) fast depletion of oxygen by the cells, whereafter dye oxidation would be diminished.

In this report, we demonstrate that *C. jejuni* is dependent on oxygen (plus an electron donor) or, under anoxic conditions, on formate/hydrogen (plus any alternative electron acceptor) to generate a high $\Delta\psi$, which is crucial for fast growth, motility and ATP generation of *C. jejuni*. Theoretically, *C. jejuni* must therefore have access to a suitable combination of these compounds in order to efficiently colonize and persist in the oxygen-limiting environment of the gut of warm-blooded animals. Reducing the availability or utilization of these compounds in the gut may finally lead to reduction of *C. jejuni* in its natural environment.

MATERIAL AND METHODS

General growth conditions

C. jejuni strain 81116 (Table S2) was routinely grown on saponin plates (Biotrading, Mijdrecht, The Netherlands), supplemented with chloramphenicol (30 µg/mL) and/or kanamycin (50 µg/mL) when appropriate. Liquid cultures were grown in hearth infusion (HI) broth (Biotrading) without antibiotics under microaerobic (5% O_2, 8% CO_2, 8% H_2) or oxygen-limiting (0.3% O_2, 10% CO_2, 10% H_2) conditions in anaerobic jars (MART, Drachten, The Netherlands) at 42^{0}C, unless specified otherwise. *E. coli* DH5α was grown in Luria–Bertani (LB) broth or on LB agar plates (Biotrading) at 37^{0}C, supplemented with ampicillin (50 µg/ml), chloramphenicol (30 µg/mL) and/or kanamycin (50 µg/mL).

Mutagenesis of the *cioA, ccoN, mfrA, frdA* and *ackA* genes

The genes including the flanking regions were amplified using primers cioAF/cioAR, cconF/cconR, mfrAF/mfrAR, frdAF/frdAR or ackAF/ackAR (Table S3) respectively, and subsequently cloned into plasmid pJET (Thermo, Waltham, USA) or for *mfrA* into plasmid pGEM (Promega, Madison, USA). The obtained plasmids were used as template to partially delete the targeted genes by PCR using the primers CioAstart/CioAend, CcoNstart/CcoNend, mfrAstart/mfrAend, frdAstart/frdAend or ackAstart/ackAend. These PCR products were digested with BamHI (Thermo) or BglII for the Δ*mfrA* PCR product, and were subsequently ligated to a BamHI digested chloramphenicol or kanamycin resistance gene, from pAV35 or pMW2, respectively. *C. jejuni* 81116 was naturally transformed using the knockout plasmids as described before (van der Stel et al., 2015). Colonies with the *ccoN* mutation were recovered on fresh saponin plates supplemented with chloramphenicol (17 µg/mL) and 10 mM formate, pyruvate, fumarate and nitrate under oxygen-limiting conditions (0.3% O_2, 10% CO_2, 10% H_2). Gene knockouts were verified with PCR.

Membrane potential measurements

Campylobacter cultures, grown in BHI (oxoid) medium in a microaerobic atmosphere, were harvested, washed, and resuspended in buffer A (100 mM TRIS/HCl pH 8.0, 100 µM EDTA) to an OD_{550} of 60-80. Of the bacterial suspension 25 µL was added to 1.5 mL buffer A, supplemented with 10 µL electron donor (1 M stock) after calibration with 5 pulses (6 µL, 25 µM stock solution) of the lipophilic cation tetraphenylphosphonium-bromide (TPP^+) (Sigma). The extracellular concentration of TPP^+ was measured in a stirred, heated vessel (30°C) using a selective TPP^+ electrode (Kamo et al., 1979). After the TTP^+ stabilization 5 µL electron acceptor (1 M stock solution) was added to determine the Δψ under anoxic conditions. Δψ was calculated using a modified Nernst-equation, as described by Lolkema *et. al.* (Lolkema et al., 1982) (Eq. 1), where C_0 and C_e are the initial and experimental concentration of TPP^+, respectively, x is the fractional internal volume and $f_{cm}K_{cm}$ defines the binding of the probe to cellular components, by combination of f_{cm} (ratio of the fractional cytoplasmic membrane and intracellular volume) and K_{cm} (cytoplasmic membrane partition coefficient).

$$\Delta\psi = \mathrm{Z}\log\frac{\frac{C_0}{C_e}-1+x\left(1-\frac{1}{2}f_{cm}K_{cm}\right)}{x\left(1+\frac{1}{2}f_{cm}K_{cm}\right)}$$ Equation 1

The intracellular volume of *C. jejuni* was taken from Tholozan *et. al*. (Tholozan et al., 1999); a value of 1.8 µL/mg protein was used for the calculations. The correction factor $f_{cm}K_{cm}$ was determined by measuring the aspecific binding between permeabilized cells (1% toluene, 1 h at 42°C) and TPP^+ under identical experimental conditions (Beilen et al., 2014; Lolkema et al., 1982). The $f_{cm}K_{cm}$ was found to have a value of 126 with a SEM of 9 using four independent experiments.

Respiration rates

Oxygen consumption rates were measured simultaneously during the Δψ measurements, using a Clark-type electrode. The rates of fumarate and nitrate reduction were determined by analyzing the supernatant for the presence of nitrite and succinate, products obtained after reduction of nitrate and fumarate, respectively. To 1.5 mL buffer A with or without 10 µL electron donor (1 M stock), 20 µL of cell suspension (OD_{550nm} ~60-80) was added and incubated in an Eppendorf mixer 5432 (Eppendorf, Hamburg) for 10 min. Then, 5 µL of electron acceptor (1 M stock) was added and five samples of 60 µL were harvested in a time course of 15 min (nitrate) or 30 min (fumarate). Samples were immediately centrifuged and supernatants were stored at -20°C. Nitrite accumulation was analyzed using the Griess assay (Promega). Succinate accumulation was determined using LC/MS/MS as described previously (van der Stel et al., 2015). Respiration rates were calculated and reported as µmol min^{-1} mg $protein^{-1}$.

Motility

The motility of *C. jejuni* in response to the available electron acceptors/donors was measured by video tracking. *C. jejuni* was grown under microaerobic conditions until late exponential phase and diluted 50x in HEPES buffer (10 mM HEPES pH 8.0, 100 mM NaCl) supplemented with 5% glycerol. The cell suspensions were subsequently incubated for 30-60 min in an atmosphere of 10% CO_2 and 0.3% oxygen at 42°C, with or without the addition of 10 mM electron donor and 5 mM electron acceptor. In case of anaerobic respiration conditions, 10% (v/v) of a sulfite solution with cobalt as catalyst (0.1 g/mL Na_2SO_3; 50 µL/mL $Co(NO_3)_2$ of a stock of 1 g/L in 0.5 M HCl) was added to scavenge oxygen. Short videos of 10 s were made of each condition tested with a CMEX5000 camera (Euromex, Arnhem, The Netherlands) placed on a microscope and the speed (µm/s) of the bacteria was determined using software Tracker (http://www.opensourcephysics.org/).

Measuring the ATP level

Campylobacter grown until mid-exponential phase in HI medium were harvested and resuspended in HEPES buffer (10 mM HEPES pH 8.0, 100 mM NaCl) to an OD_{550} of 1.0. Handling was performed inside a hypoxic glovebox with an atmosphere set to 0.5% O_2, 10% CO_2. The bacterial suspension was incubated for 30 min on ice and subsequently aliquots (180 µL) were incubated with or without 10 mM electron donor for 30 min at 30°C. Next, if appropriate, 5 mM electron acceptor was added, and the suspension was further incubated for 30 min. Of each reaction, 20 µL was lysed with 10 µL trichloroacetic

acid (final concentration 1%) for 10 min and subsequently neutralized with NaOH. Finally, the lysed samples were diluted ten-fold in TRIS buffer (100 mM TRIS/HCl, pH 7.5). ATP concentration was determined by mixing equal amounts of sample and Bac-titer-GLO (Promega, Madison, USA) and measuring relative light units (RLU) on a Tristar^2 multimode reader (Berthold Technologies, Wildbad, Germany). The RLU of the sample without electron donor and electron acceptor was set to 1.

Growth curves

Campylobacter precultures were diluted to an OD_{550} of ~0.01 in modified defined media (pH 7.4) (Leach et al., 1997; van der Stel et al., 2015) supplemented with 10 nM selenite, 2 mM aspartate as nitrogen source, and 1 mM pyruvate. Carbon sources were added to a final concentration of 25 mM, as indicated. Formate, fumarate and nitrate were added to a final concentration of 10 mM as indicated. Growth curves (175 µL/well) were generated in a 96-wells plate (Costar) using a Synergy HTX multi-mode reader (Biotek, Winooski, USA), placed inside a hypoxic chamber (Coy labs, Grass Lake, USA) set to 10% CO_2 and 10% or 0.5% O_2, balanced with N_2. Because the cultures in the microplate are less aerated than the larger cultures in culture flasks, the oxygen tension was increased to empirically determined values of 10% or 0.5% (oxygen-limiting). The outer wells were filled with water to prevent evaporation. The microplate was shaken continuously (567 cpm) and the OD_{550} was monitored every 10 min for 30 h. The lag phase and growth rate were calculated using the Gompertz model (Zwietering et al., 1990). The biomass production was calculated by subtracting the starting OD_{550} from the maximally obtained OD_{550}. Growth experiments were conducted three times independently.

RNA-isolation

Campylobacter precultures were diluted to an OD_{550} of 0.05 in HI medium and grown either microaerobic or under oxygen-limiting conditions, with or without addition of 10 mM nitrate or fumarate. RNA was isolated from mid-log phase cultures using the RNA-Bee kit (Tel-Test) according to the instructions of the manufacturer. Subsequently, RNA was treated with RNAse-free DNAseI (Thermo) according to the instructions of the manufacturer.

RNA sequence and transcriptomic analysis

RNAseq was performed using an Illumina MiSeq sequencer (Illumina, San Diego, CA). Initially, total RNA samples were rRNA depleted using the Ribozero Magnetic Kit for-Gram negative bacteria (Illumina) following manufacturer's instructions (option 1). Illumina libraries were prepared using the KAPA stranded RNA-seq kit (Kapa Biosystems, Wilmington, MA), following manufacturer's instructions except for the following changes: 159-400 ng RNA was sheared for 6 minutes at 85^{o}C. Standard desalting TruSeq LT primers were ordered from Integrated DNA Technologies (Coralville, IA) and used at 50-100 nM final concentration based on starting RNA amount. The PCR step was reduced to 6 cycles. Libraries were quantified using the KAPA Library Quantification Kit (Kapa), except with 10 µl volume and 90 sec annealing/extension PCR. Libraries were pooled and normalized to 4 nM. Pooled libraries were re-quantified by ddPCR on a QX200 system (Bio-Rad), using the Illumina TruSeq ddPCR Library Quantification Kit and following manufacturer's protocols.

The libraries were sequenced in two 2x76 bp paired end v3 runs on a MiSeq instrument (Illumina) at 13.5 pM, following manufacturer's protocols.
Fastq files were generated for each sample by the MiSeq Instrument Software. Subsequent processes were performed with Geneious 9.1. The fastq sequences were trimmed, mapped to remove poor quality bases and then assembled to reference genome CP000814 using Bowtie within Geneious. Geneious software was used to calculate the normalized transcripts per million (TPM) and to compare expression levels between the control growth condition (microaerobic) and experimental conditions (oxygen-limiting+nitrate and oxygen-limiting).

RT-qPCR

Reverse transcriptase quantative PCR analysis was performed using DNAse treated RNA (10 ng) and the Takyon No Rox qPCR-kit with Euroscript II reverse transcriptase (Eurogentec, Seraing, Belgium), according to the manufacture's protocol in a Lightcycler 480 machine (Roche, Penzberg, Germany). Differential gene expression was calculated using the 2ΔΔCt method using rpoD as reference gene (Schmittgen and Livak, 2008).

Formate, hydrogen and pyruvate utilization assays

Campylobacter cultures in HI medium were harvested and resuspended in HEPES buffer (100 mM HEPES, pH 8.0, 100 mM NaCl) to an OD_{550} of 0.1 (formate/H_2) or 0.4 (pyruvate). Bacterial suspensions (120 µL) were mixed with 15 µL formate or pyruvate (100 mM stock), and 15 µL 2,3,5- Triphenyl-tetrazolium chloride (TTC) (1% w/v stock solution) in a microtiter plate. The bacterial suspensions were incubated under an atmosphere of 5% O_2 and 10% CO_2, or in the case of hydrogen 5% O_2, 8% CO_2 and 8% H_2. The purple color formation was measured at 496 nm after 1 hour using the Synergy HTX multi-mode reader.

Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad, San Diego, CA). Data is expressed as mean with SEM. Results were analyzed using paired t-tests and a p-value of <0.05 was considered statistically significant. (*:$p<0.05$, **:$p<0.01$, ***:$p<0.001$)

Accession numbers

Gene expression ratios from the RNA-seq experiments are deposited in the GEO database under accession number GSE92644.

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AUTHOR CONTRIBUTIONS

AXS, FB and MW performed the conception and design of the study; AXS, SH, CP and LD performed the experiments. The membrane potential measurements, respiration rates, motility measurements, ATP assays, cloning, RNA isolation, RT-qPCRs, growth experiments and TTC assays were performed by AXS. RNA-seq was performed by SH and CP. The construction of the *frdA* mutant was performed by LD. AXS, FB, SH, CP, JP and MW performed analysis or interpretation of the data; AXS, FB and MW wrote the manuscript.

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SUPPLEMENTAL MATERIAL

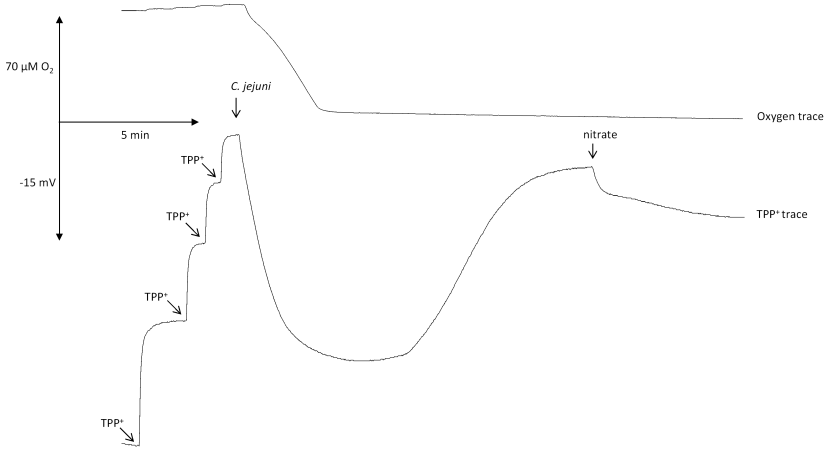


Figure S1: Typical traces to determine respiration and membrane potential. Traces of oxygen concentration (upper) and TPP^+ signal (lower) were recorded using pyruvate as electron donor and oxygen and nitrate as electron acceptor, sequentially. Arrows with text indicate additions to the reaction vessel. The drop in the external TPP^+ concentration is due to TPP^+ ions migrating towards the negatively charged inner aspect of the cytoplasmic membrane of the bacteria, caused by the membrane potential. First, oxygen was used as electron acceptor and once all oxygen was consumed, the TPP^+ signal went up again, indicating that the membrane potential was gradually lost. The subsequent addition of the alternative electron acceptor nitrate yielded a second drop in TPP^+ concentration, indicating the generation of a membrane potential independent of oxygen via anaerobic nitrate respiration.

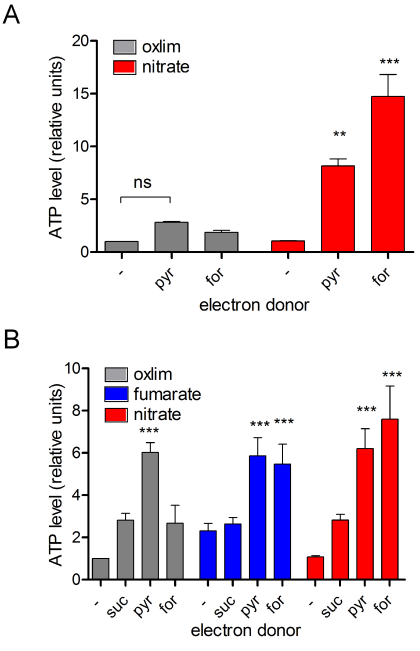
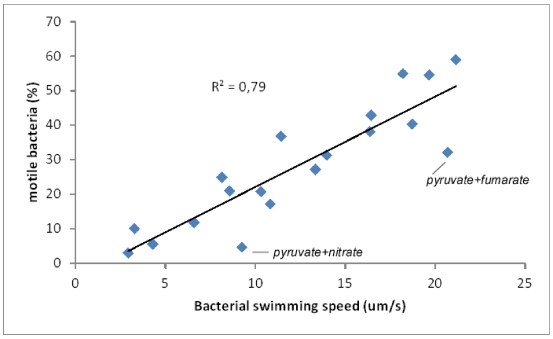


Figure S2: ATP levels in *C. jejuni* mutant strains. The influence of the available electron acceptor/donor couple on the ATP level of C. jejuni *ackA*::Km (**A**) and *cioA*::Km/*ccoN*::Cm (**B**) mutant strains. Bacteria were harvested from plates, washed, and resuspended in HEPES buffer. After incubation under oxygen-limiting conditions, with the indicated electron donor/acceptor couple, the ATP level of the total bacterial suspension was determined using a luciferase-coupled ATP assay. The relative ATP concentrations are presented, in which the condition with no electron donor/acceptor was set to 1. The experiment was performed three times independently and results are presented as mean ± SEM.

A



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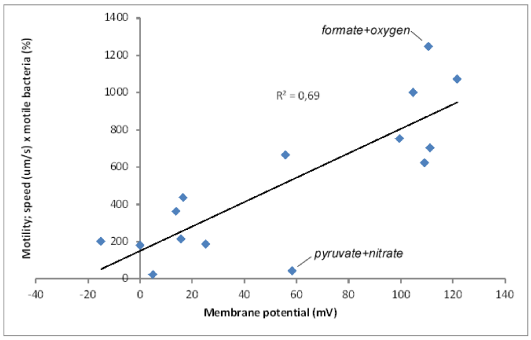


C

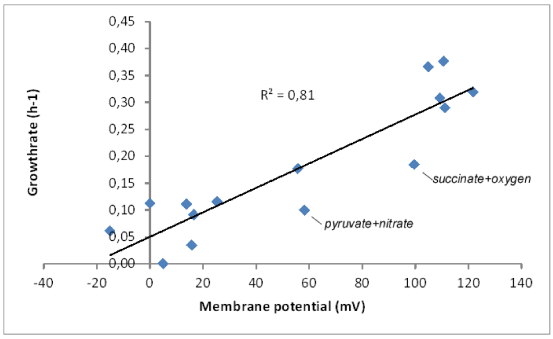


Figure S3: Correlations between different experiments. **A**: Correlation between percentage of motile bacteria and swimming speed (Fig. 3). **B**: Correlation between membrane potential (Fig. 1) and motility (product of swimming speed and percentage of motile bacteria) (Fig. 3). **C**: Correlation between membrane potential (Fig. 1) and specific growth rate of *C. jejuni* (Table 1). Obvious outliers are annotated in the graphs.

Table S1: Growth characteristics of *C. jejuni* in defined medium with different electron donors/acceptors. Growth curves were recorded in a minimal medium with 25 mM carbon source and 10 mM electron donor and/or acceptor, or in the case of oxygen 10% in the gas phase. *nc*: growth curve did not converge to the Gompertz model, because of lack of growth. The data is presented as the mean and SEM of three independent experiments.

		Wildtype *C. jejuni*				Mutants of *C. jejuni*		
electron	carbon	Growth h^{-1}	Biomass	Lag	*mutant*	Growth h^{-1}	Biomass	Lag
oxygen	no	0.07	0.030	22.5				
	fum	0.29	0.262	9.1 (0.3)				
	succ	0.18	0.257	9.8 (0.8)				
	pyr	0.31	0.207	4.1 (0.6)				
	for	0.08	0.034	1.4 (1.0)				
	for+fu	0.38	0.172	2.0 (0.5)				
	for+suc	0.17	0.230	0.3 (0.6)				
	for+pyr	0.38	0.201	1.3 (0.3)				
no acceptor	no	*nc*	0.003	*nc*				
	fum	0.14	0.049	6.7 (1.3)				
	succ	0.06	0.025	12.1				
	pyr	0.12	0.032	2.7 (1.3)				
	for	*nc*	-	*nc*				
	for+fu	0.25	0.042	2.9 (0.6)	*frdA/mfr*	0.05	0.028	0
	for+suc	*nc*	0.003	*nc*				
	for+pyr	0.09	0.023	0 (2.0)				
fumarate	no	0.07	0.013	17 (7.1)				
	fum	0.18	0.059	3.8 (1.6)				
	succ	0.11	0.033	2.5 (2.0)				
	pyr	0.18	0.051	0.6 (0.8)				
	for	0.12	0.017	0.9 (2.1)				
	for+fu	0.37	0.057	1.4 (0.6)	*frdA/mfr*	0.12	0.031	0
	for+suc	0.28	0.024	1.4 (0.9)				
	for+pyr	0.37	0.042	1.3 (0.8)	*frdA/mfr*	0.16	0.032	0
nitrate	no	*nc*	0.006	*nc*				
	fum	0.03	0.016	0.8 (5.5)				
	succ	*nc*	0.010	*nc*				
	pyr	0.10	0.030	0.7 (1.5)				
	for	0.05	0.008	0.9 (3.1)				
	for+fu	0.31	0.030	0.9 (1.1)				
	for+suc	0.09	0.011	1.4 (3.0)				
	for+pyr	0.32	0.043	1.1 (0.6)	*napA*	0.04	0.021	0

Table S2: Strains and plasmids used in this study

Strain/plasmid	Characteristics	Reference
C. jejuni		
81116	Human isolate	Manning *et. al.*, 2001
11168	Human isolate	Parkhill *et. al.*, 2000
81-176	Human isolate	Black *et. al.*, 1988
RM1221	Chicken isolate	Fouts *et. al.*, 2005
480		King *et. al.*, 1991
129108	Human isolate	Endtz *et. al.*, 1993
81116 *napA*::Cm	*napA* gene disruption	Vaezirad *et.al.* in preparation
81116 *frdA*::Km	*frdA* gene disruption	This study
81116 *mfrA*::Cm	*mfrA* gene disruption	This study
81116 *frdA*::Km *mfr*::Cm	*frdA* and *mfrA* gene disruption	This study
81116 *cydA*::Km *ccoN*::Cm	*cydA* and *ccoN* gene disruption	This study
81116 *ackA*::Km	*ackA* gene disruption	This study
E. coli		
DH5a	General cloning strain	Labstock
Plasmids		
pMW2	Plasmid containing kanamycine resistance gene	Wösten *et. al.*, 2010
pAV35	Plasmid containing chloramphenicol resistance gene	Vliet *et. al.*, 1998
pJET cydA Int	pJet with *cydA* with flanking regions	This study
pJET ccoN Int	pJet with *ccoN* with flanking regions	This study
pJet cydA::Km	pJet with *cydA* gene disruption	This study
pJET ccoN::Cm	pJet with ccoN gene disruption	This study
pJET frdA::Km	pJet with *frdA* gene disruption	This study
pGEM mfrA:: Cm	pGEM with *mfrA* gene disruption	This study
pJET ackA::Km	pJet with *ackA* gene disruption	This study

Table S3: Primers used in this study. Nucleotides displayed in lowercase indicate restriction sites for cloning purposes.

Primer name	Sequence 5' to 3'
RT-qPCR	
Cj1511ftaq	CCATTGTCCGCTACGATTTGT
Cj1511rtaq	ATTTATCTTTTACCTGCAGCAACACA
hydDftaq	TGCTTCAAACCTTACAATACATGGA
hydDrtaq	TCAATACGCTTTGGCACACAA
malateFtaq	AGGTTGTTTTAGGAGAAGATTGCAA
malateRtaq	AAAACCTCGCCGCTTTCA
nuoAFtaq	CTTTAAAAATCAAAGCCCAAGGAAA
nuoARtaq	CCTATTCCTGTTAAACAAGCTAACAAGA
fldAftaq	GGGAAGTGGTGATTTACAAGATGA
fldArtaq	AAATACAGCTACAGTTTTTCCACCAA
FRDCFTAQ	TACTGACGGTAATTAATAGGAAATTTTCTC
FRDCRTAQ	TTCATTTTTAGCCGCCTGTGT
cconftaq	CCCAACCTAATGTTCCATCATGA
cconrtaq	GGCCAATTCTTTCAATTAAATCTGTT
cj1184Frtaq	GCTATGCCAAGAGTAGGTTTAACAGA
cj1184rtaq	TCTTTTTTGCTATCACCCACTTGA
cj0037Ftaq	TGTCCGCTACCTTGTTCGTAATTA
Cj0037Rtaq	TGAAGAAAATTGCGTTGCTTGT
cioAFtaq	TGTTATCCATTCCATTGGGTTATATTG
cioARtaq	GCCCAAGGTTGTCTGCCTACT
SDHAFTAQ	TGCAAATGGTGGAACTCTTATTACA
SDHARTAQ	GCTCACCACGATTATTTAAAAGATAT
napHftaq	CACTAAATGTTATGAATGTATCCGTATTTG
napHrtaq	TGCACACCTTATACACTCTCCTGATT
Cj1358ftaq	GGCAAATGACAATCCACACAAG
Cj1358rtaq	GGCAGGACCACACTCTCAAAA
rpoDFtaq	GAACGAATTTGATTTAGCCAATGA
rpoDrtaq	TGTCCCATTTCTCTTAAATACATACGA
Mutagenesis	
cioAF	GTATGCATTTTCCATTTCCTA
cioAR	CTTAGAGTATAATAGCTTGAACT
cioAstart	AggatccAACGCTACTAAGTTCGTTCA
cioAend	AggatccACATACTCCACTTATGGGCA
cconF	TAGCTCCAATTAAAGATAAGAGA
cconR	GCTGGTGGGATTGATAAA
ccoNstart	AAagatctTCGCCTATGGCAGCATAA
ccoNend	AAagatctTTAATACATTACCTGGATGCA
sdhAF	AAATCTTTTGCTAAAGAAGGAGCA
sdhAR	ACACAACCTGGAAAAAAA
sdhAstart	GAagatctTCTAGAAAATTCACCCACAT
sdhAeind	GAagatctGTCGTGGTAACTG
frdAF	CTTATTCATTAGCAAGATTTATAT
frdAR	AATAAAACCTGATTTAAAAAGATTT
frdAstart	AggatccATCACTATATTGTATATTCATAATTT
frdAeind	AggatccTATAAAGCACCAAACCAAAGAA
ackAF	GAAGAGTATGATTTTATAACTCCACATCG
ackAR	GATACAAAATTCTCAAACTATAGAAAAACG
ackAstart	AAAggatccCAAATTCTAAAGTTAAAGTGCTTGTTATTCC
ackAend	AAAggatccTTTCTCTACTAAACCACTCGCTTTAACG

4

Chapter 5

Catabolite repression by intracellular succinate in *Campylobacter jejuni*

Anne-Xander van der Stel[1], Chris H.A. van de Lest[2], Steven Huynh[3], Craig Parker[3], Jos P. van Putten[1], Marc M.S.M. Wösten[1]

Affiliations:
[1]Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands
[2]Department of Biochemistry and Cell Biology, Utrecht University, Utrecht, The Netherlands
[3]Produce Safety and Microbiology Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Albany, California, USA

Submitted

SUMMARY

Bacteria have evolved different mechanisms to catabolize carbon sources from a mixture of nutrients. They first consume their preferred carbon source, before others are used. Regulatory mechanisms adapt the metabolism accordingly to maximize growth and to outcompete other organisms.

The human pathogen *Campylobacter jejuni* is an asaccharolytic Gram-negative bacterium that catabolizes amino acids and organic acids for growth. It prefers serine and aspartate as carbon sources, however it lacks all regulators known to be involved in regulating carbon source utilization in other organisms. In which manner *C. jejuni* adapts its metabolism towards the presence or absence of preferred carbon sources is unknown.

In this study, we show with transcriptomic analysis and enzyme assays how *C. jejuni* adapts its metabolism in response to its preferred carbon source serine. In the presence of serine as well as lactate and pyruvate *C. jejuni* represses the utilization of other carbon sources, by repressing the expression of a number of central metabolic enzymes. The regulatory proteins RacR, Cj1000 and CsrA play a role in the regulation of these metabolic enzymes. This metabolism dependent transcriptional repression correlates with an accumulation of intracellular succinate. Hence, we propose a demand-based catabolite repression mechanism in *C. jejuni,* which depends on the intracellular succinate level.

INTRODUCTION

An important factor in bacterial adaptation to changing environmental conditions is the plasticity in bacterial metabolism. Different bacterial species have evolved all kinds of mechanisms to balance their carbon, nitrogen and energy supplies (Chubukov et al., 2014). Carbon sources are used for anabolic purposes as well as to generate energy. Adaptations in bacterial carbon metabolism are generally driven by the availability of carbon sources in the environment and the bacterial need for anabolic precursors and energy to maximize growth rates (Ramkrishna et al., 1987). When multiple carbon sources are available, bacteria often use them sequentially in a hierarchical fashion. They first deplete the preferred carbon source and then switch their metabolism to utilize less-preferred carbon source(s). This process is often referred to as catabolite repression control (Brückner and Titgemeyer, 2002; Görke and Stülke, 2008; Hueck and Hillen, 1995; Rojo, 2010). To maximize growth rates, bacteria can employ energy inefficient pathways (Peebo et al., 2015; Valgepea et al., 2010). This counter-intuitive strategy is rationalized when the protein synthesis 'costs' are taken into account (Basan et al., 2015). At high growth rates*, Escherichia coli* favors fermentation rather than respiration, even in the presence of oxygen. Although respiration leads to the production of more ATP, expressing all necessary proteins for oxidative phosphorylation is a higher burden (Basan et al., 2015).

Bacteria can detect changes in the environment via transmembrane sensor proteins (Golby et al., 1999; Nishijyo et al., 2001; Sevvana et al., 2008) as well as via intracellular sensors. Typical cytosolic sensors are transcription factors that sense changing concentrations of metabolites or fluxes inside the cell and then provide a regulatory output to balance catabolic and anabolic fluxes (Kochanowski et al., 2013; Nam et al., 2005; Sauer and Eikmanns, 2005). In this way, the accumulation or deprivation of a metabolic intermediate signals nutrient availability and this information is transferred via a transcription factor to adjust the magnitude of the uptake flux. This process is called the demand-based regulation (Chubukov et al., 2014; Jiang and Ninfa, 2009; Millard et al., 2017; You et al., 2013).

Besides metabolic pathway-specific regulation, bacteria display global regulatory mechanisms (Peng and Shimizu, 2003) that often sense key-metabolites that indicate the general metabolic status of the cell (Kochanowski et al., 2017). With these systems, a plethora of genes is differently regulated and the metabolism is coordinately shifted up or down.

Campylobacter jejuni is the most common bacterial foodborne pathogen associated with human gastroenteritis worldwide. Unlike most other bacteria, *C. jejuni* lacks the ability to metabolize glucose and other hexose sugars. Because of this feature, *C. jejuni* metabolism is dependent on amino acids and organic acids as carbon sources (Guccione et al., 2008; Leach et al., 1997). *C. jejuni* encodes a complete TCA cycle (Kelly, 2001). The carbon sources serine and lactate are both metabolized to pyruvate, which enters the TCA cycle through acetyl-CoA (Thomas et al., 2011; Velayudhan et al., 2004). Pyruvate acts as a central precursor for anabolism of certain amino acids and is a starting point for the gluconeogenesis and the production of fatty acids via acetyl-CoA. This intermediate adds to the conservation of energy through its oxidation in the TCA cycle or the generation of ATP via substrate level phosphorylation during its conversion into acetate. The catabolism of serine to pyruvate, as well as other enzymes in any of these pathways are indispensable for efficient *C. jejuni* growth *in vitro* and *in vivo* (Gao et al., 2017; van der Stel et al., 2017). Other amino acids used by *C. jejuni* are all catabolized to aspartate. Aspartate enters the TCA cycle as fumarate after deamination by AspA (Guccione et al., 2008).

Compared to the more than 300 transcriptional regulators in *E. coli,* the *C. jejuni* genome encodes a small number of regulators; ten response regulators and less than 20 additional transcriptional regulators from different protein families (Pérez-Rueda and Collado-Vides, 2000) (Parkhill et al., 2000). Because of its aberrant metabolism, *C. jejuni* also regulates its metabolism differently than glucose metabolizing bacteria. This is reflected in the absence of conserved global metabolic regulators known from the well-studied organisms *E. coli* or *B. subtilis* (e.g. CcpA, Crp, Lrp, GlnB or NtrC) (Chubukov et al., 2014). *C. jejuni* also lacks the stationary phase stress sigma-factor, RpoS, and the respiration sensitive regulators ArcAB and Fnr (Parkhill et al., 2000). Despite its small number of regulators and the absence of well-studied metabolic regulators, *C. jejuni* does seem to regulate its carbon source uptake and metabolism (Line et al., 2010; Wright et al., 2009). Serine and aspartate are described as preferred carbon sources. During the stationary phase, a distinct acetate switch is observed, where previously excreted acetate is reabsorbed and metabolized

(Wright et al., 2009). In the gut, *C. jejuni* uses the presence of microbiota derived short-chain fatty acids as a cue to adjust its carbon metabolism until it reaches its preferred niche (Luethy et al., 2017).

To date, knowledge about the mechanisms how *C. jejuni* adapts its metabolism towards different carbon source availability is scarce. The metabolites that are being sensed and the transcription factors involved in this process are largely unknown. In this study, we elucidated the metabolic response of *C. jejuni* towards the preferred carbon sources serine and aspartate.

RESULTS

Carbon source utilization

To investigate how *C. jejuni* adapts its metabolism towards the use of preferred carbon sources, a triphenyl-tetrazolium chloride (TTC)-coupled substrate oxidation assay was employed. Hereto cultures were grown in Hearth Infusion broth (HI) and in HI broth enriched with 25 mM serine or aspartate. The addition of serine led to a slight increase of the bacterial growth rate compared to growth in HI or HI with aspartate. However, both the addition of serine and aspartate to HI led to a higher final optical density (Fig. 1A). At different time points, the bacterial cell suspensions were harvested and the TTC reduction, quantified by the colorimetric change, was measured by adding different carbon sources. TTC reduction for most carbon sources was dependent on the growth phase and was highest at the transition from exponential to stationary phase (6 and 9 hours of growth). Addition of serine led to a repression of the utilization of most carbon sources (Fig. 1 & S1) at exponential growth phase compared to the other growth conditions (HI and HI+asp). However, at the 15-hour time-point an increase was measured in the utilization capacity of the carbon sources aspartate, glutamine, fumarate, asparagine, glutamate, and proline (Fig. 1E, F & data not shown), indicating a de-repression of these metabolic pathways. These data indicate that *C. jejuni* controls its metabolism at different growth phases in batch cultures and that the addition of serine leads to a metabolic downshift during the exponential growth phase, but this is not at the expense of a lower growth rate.

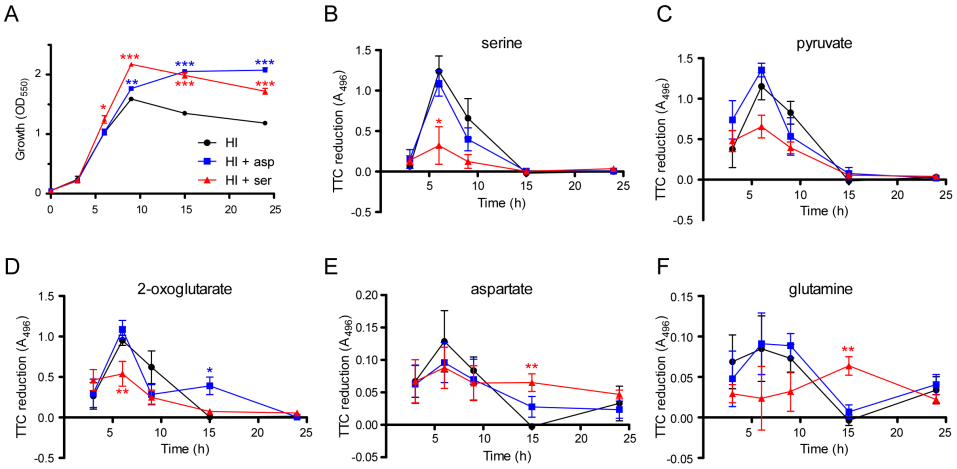


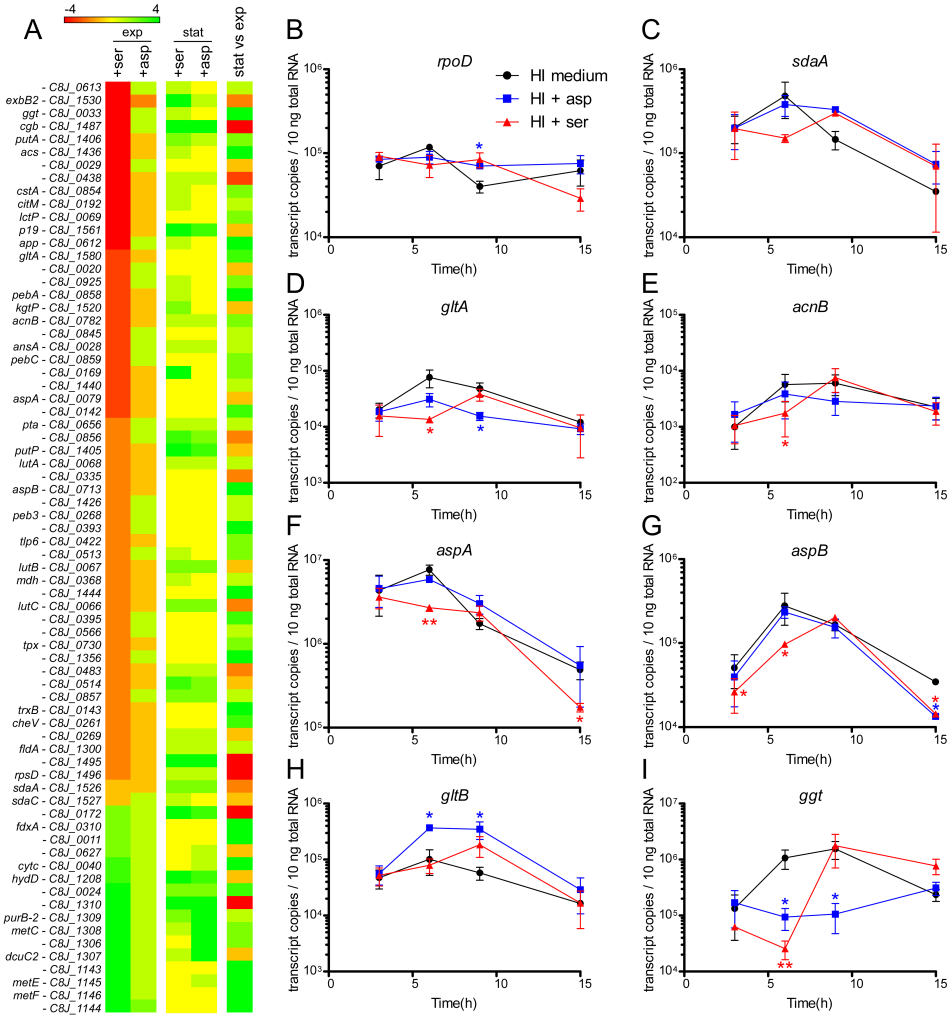
Figure 1: Carbon source utilization of *C. jejuni*. TTC reduction was measured during microaerophilic batch cultures in HI-medium (black circles) or with the addition of 25 mM serine (red triangles) or aspartate (blue squares). **A**: Growth of the batch cultures during a 24-hour period. **B-F**: Carbon source utilization based on TTC reduction-coupled oxidation of serine, pyruvate, 2-oxoglutarate, aspartate and glutamine during the batch cultures. At the indicated time-points, cultures were harvested and optical density was determined. Carbon source utilization was probed using a TTC-reduction assay with the indicated compound. Results shown represent the mean ± SEM of three independent experiments. Statistical significance was calculated with a student *t*-test. (*:p<0.05, **:p<0.01, ***:p<0.001)

Transcriptomic analysis

To determine if the differential carbon source utilization by *C. jejuni* in the presence of serine is regulated at the transcriptional level, RNA-seq analysis was performed on RNA isolated from *C. jejuni* cultures in their exponential (6 hr) or early stationary (12 hr) growth phase in HI without or with additional 25 mM of serine or aspartate. Addition of aspartate to the growth medium yielded largely similar gene expression profiles in the exponential and in the early stationary growth phase compared to *C. jejuni* cultures grown in HI alone (Figure 2A). Addition of serine however, resulted in a reduced expression of many metabolic genes in the exponential growth phase. This was found for genes encoding components of the TCA-cycle (e.g. *gltA, acnB,* and *mqo)*, as well as for genes encoding proteins needed to catabolize (e.g. *ansA*, *ggt*, *putA*, *sdaA*, *cstA*, *aspA*, *aspB* and *acs)* and transport (e.g. *kgtP*, *lcpT*, *dcuC*, *peb1*, *cstA*, *cj0203* and *c8j_0613)* specific carbon sources. No genes encoding regulatory proteins were differentially regulated.

Apart from many downregulated genes, three operons (*c8j_1306 - c8j1310, c8j_1143 – c8j_1146* and *c8j_0024*) were highly upregulated. The first two operons harbor genes required for methionine biosynthesis. The first operon encodes MetC, which converts cystathionine into homocysteine. The second operon harbors the genes encoding MetE, which produces methionine from homocysteine, and MetF, which synthesizes 5-methyltetrahydrofolate, crucial for the MetE reaction. The gene c8j_0024, encodes for a sodium:dicarboxylate family transmembrane symporter that has homology to the TcyP protein family, which includes L-cystine importers.

Like the addition of aspartate to the growth medium, addition of serine did not cause a large number of alterations in gene expression in the early stationary growth phase when compared to *C. jejuni* cultures grown in HI alone. However, several genes encoding proteins necessary to import and metabolize non-serine substrates were more strongly expressed in early stationary phase compared to exponential phase. These genes include *acs*, *ggt*, *putA*, *cstA*, *gltA*, *pebA*, *acnB*, *ansA*, *pebC* and *aspB*. Addition of serine to the growth medium made these effects more pronounced than in cultures grown in HI-medium alone or HI with additional aspartate. Together the RNA-seq results indicate strong metabolic adaptation at the transcriptional level and a differential regulation of genes necessary for the utilization of different growth substrates. This regulation is not only dependent on the growth phase, but also on the available carbon sources in the medium.



←**Figure 2: Transcriptional regulation of *C. jejuni* by carbon sources**. RNA seq analysis was performed on bacteria grown in microaerophilic batch-culture in HI-medium with or without the addition of 25 mM serine or aspartate. **A**: RNA-seq analysis of exponential and stationary grown bacteria in HI medium or with the addition of carbon source. Fold difference was calculated between the conditions with added carbon source compared to HI broth alone in both exponential- and stationary growth phase, and between stationary- and exponential growth phase without carbon source added (stat vs exp). The displayed genes were selected by filtering for >2-fold difference and statistically significant differentially regulated genes of exponentially growing cells with serine compared to HI broth alone. **B**: RT-qPCR analysis of metabolic genes on RNA isolated from *C. jejuni* during microaerophilic batch cultures in HI-medium (black circles) or with the addition of 25 mM serine (red triangles) or aspartate (blue squares), harvested at different time points. The transcript copies were calculated based on a standard range with PCR-amplified template DNA per reaction containing 10 ng of total RNA. The results shown represent the mean of three independent experiments. The error bars represent SEMs. Statistical significance was calculated with a student t-test. (*:p<0.05, **:p<0.01, ***:p<0.001)

To verify the RNA-seq data and to study the effect of carbon source addition in relation to the growth phase on the expression of metabolic genes in more detail, RT-qPCR was performed on the genes *sdaA*, *Cj0075c, sucD, putP, aspA, glnA, gltB, metF, dcuD*, *acs*, *ggt*, *putA*, *cstA*, *gltA*, *pebA*, *acnB*, *ansA*, *pebC* and *aspB*. Hereto RNA was harvested from *C. jejuni* cultures grown for 3, 6, 9, and 15 hours in HI broth or HI broth with additional 25 mM serine or aspartate. The expression analysis showed that most metabolic genes have highest expression at the end of the exponential phase and the beginning of stationary phase (Fig. 2B-I and Fig. S2). This peak in expression was not observed for the household gene *rpoD* (encoding the main sigma-factor of RNA-polymerase, σ^{70}) (Fig. 2B). In line with the RNA-seq data, addition of serine reduced the transcription of most of tested metabolic genes at the (late) exponential growth phase (6 hr). The central metabolic genes *gltA* (6-fold), *acnB* (3-fold) and *acs* (12-fold) were downregulated as well as the amino acid catabolism genes *aspA* (3-fold), *putP* (10-fold), *ggt* (42-fold), *aspB* (3-fold) and *sdaA* (3-fold). At the early stationary growth phase (9 hr), expression of most tested genes had increased in the cultures with additional serine, which was also in agreement with the RNA-seq data. The expression of the genes *ggt* (15 h; 3-fold), *putP* (9 h; 5-fold) and *sucD* (9, 15 h; 4-fold) even increased above the expression in the control cultures. The expression of some genes was also affected by aspartate. For instance, the expression of *acs* and *ggt* genes was repressed, while *glnA, gltB* and *dcuD* transcripts were more abundant in *C. jejuni* cultures grown in medium with additional aspartate (Fig. 2C-I & S2). The transcriptional data confirm that metabolic regulation, as observed in the phenotypic TTC assays, takes place at the transcriptional level and is dependent on the available carbon source.

Transcription regulation correlates with the enzyme activity

To ascertain that the observed regulation of transcription can be extrapolated to the protein level, we measured the enzyme activity of GGT and GOGAT. These enzyme activities were investigated as the transcription of the *ggt* gene was greatly affected by different carbon source additions, while the expression of *glnA* and *gltB*, encoding for the GS/GOGAT system enzymes, was uniquely upregulated by addition of aspartate (Fig. 2 & S2). The enzyme activities were measured in cell-free lysates obtained from batch cultures grown in HI with or without additional 25 mM of serine or aspartate for 3, 6, 9, or 15 hr. The GGT activity of the bacteria grown in HI broth steadily increased during growth and peaked at stationary phase (Fig. 3A). The addition of aspartate to the medium did not influence the activity in the exponential growth phase, but did not lead to an increase of

the activity in the stationary phase. Addition of serine to the medium had exactly opposite effects, GGT activity was highly repressed during logarithmic phase, but was high at stationary phase (Fig. 3A). The GOGAT activity, on the other hand, was found to peak in the exponential growth phase. The addition of aspartate to the medium led to higher GOGAT activity, while serine led to lower activity at all time-points. The GOGAT and GGT enzyme activity results correlate well with the expression of the corresponding genes, indicating that transcriptional regulation is the major determinant of the activity of these enzymes.

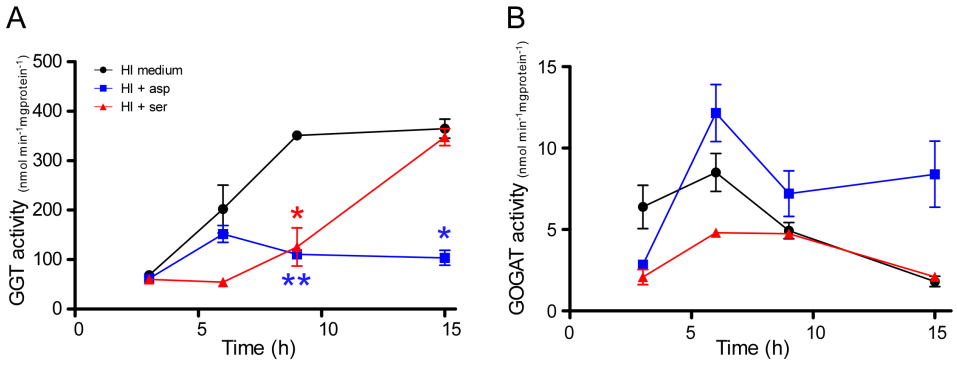


Figure 3: Enzyme activities of GGT and GOGAT in *C. jejuni* in batch cultures. **A**: GGT activity of *C. jejuni* grown in microaerophilic batch cultures in HI-medium (black circles) or with the addition of 25 mM serine (red triangles) or aspartate (blue squares), harvested at different time points. **B**: GOGAT activity under ditto conditions as in A.

Identification of transcription regulators involved in the differential carbon source utilization

The finding that the regulation of the carbon utilization is regulated at the transcription level led us to search for transcription factors involved in this process. To identify these factors the GGT and GOGAT enzyme activities were measured in 21 different *C. jejuni* regulatory protein mutants grown in HI or HI medium enriched with aspartate or serine. Two mutant strains *Cj1000* and *racR* showed altered GGT activity (Fig. 4A). The *cj1000* mutant strain showed higher GGT activity under all conditions, but most strongly in the exponential growth phase (6 hr). The *racR* mutant strain had lower GGT activity than the wt under all conditions (Fig. 4A-B).

GOGAT activity was similar for all strains tested, except for the *csrA* mutant strain. The *csrA* gene codes for a post-transcriptional regulator and has previously been implicated in metabolic regulation (Fields et al., 2016). The GOGAT activity was more than twice as high in the *csrA* mutant strain, compared to the wt and the other strains (Fig. 4C). However, no GGT expression phenotype was observed for the *csrA* mutant strain (Fig. 4A-B). Based on these results the transcription factors Cj1000, RacR and CrsA might have a role in the regulation of the carbon utilization in *C. jejuni*.

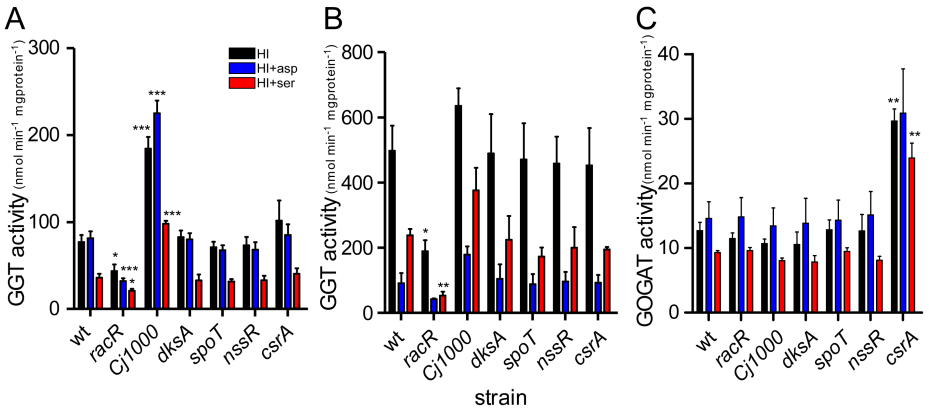


Figure 4: Metabolic enzyme activity of *C. jejuni* wt and metabolic regulator mutant strains. *C. jejuni* was grown in microaerophilic HI medium (black bars) or with the addition of 25 mM aspartate (blue bars) or serine (red bars). **A**-**B**: GGT activity of *C. jejuni* strains in exponentially (6 hr)(A) or stationary phase (15 hr) (B) growing bacteria. **C**: GOGAT activity of *C. jejuni* strains in exponential phase (6 hr) growing bacteria. Results represent the mean ± SEM of three independent experiments. Statistical significance was calculated with a student *t*-test. (*:p<0.05, **:p<0.01, ***:p<0.001)

Metabolic gene expression is regulated by intracellular metabolites

Bacteria can sense and change their metabolism in response to the available carbon sources in- or outside the cell. To determine if *C. jejuni* senses specific metabolites outside the cell, or uses intracellular metabolite pools to alter its metabolic profile, the effect of various carbon sources on the expression of GGT, GOGAT and TTC-coupled serine oxidation in exponential growth phase was determined and compared to the phenotypes already known for serine and aspartate addition. In the exponential growth phase pyruvate and lactate exerted similar effects on the GGT and GOGAT activity as serine (Fig. 5A-B). The addition of pyruvate but not that of lactate, showed also the same phenotype as serine in the TTC-coupled serine oxidation assay (Fig. 5C). The GGT and GOGAT activity and TTC-coupled serine oxidation of the bacteria did not change when grown with additional fumarate, succinate, 2-oxoglutarate or aspartate. During stationary phase, the additional presence of fumarate, succinate and 2-oxoglutarate inhibited the induction of high GGT activity, (Fig. 5D), as was observed after the addition of aspartate. These results suggest that under the conditions employed, *C. jejuni* senses intracellular and not extracellular metabolites to alter its metabolism, since carbon sources that share metabolic pathways exerted the same metabolic effects.

To further investigate this and to address which intracellular metabolite(s) might influence the metabolic response, the GGT activity was measured in strains with isogenic mutations in the metabolic genes *frdA* and *aspA*. The strains were grown in HI medium alone or with addition of aspartate, serine, fumarate or succinate. GGT activity was measured in stationary growth phase. Mutation in *aspA* leads to accumulation of aspartate and glutamate, due to the inability to metabolize these compounds to fumarate (Guccione et al., 2008; Howlett et al., 2014) (Fig. 5D). The *aspA* mutant strain showed comparable GGT

activity when grown with addition of fumarate or serine, however addition of aspartate led to high GGT activity compared to the wt (Fig. 5E&F). This indicates that the deamination step from aspartate to fumarate, catalyzed by AspA in wt bacteria, is crucial for the repression of the GGT activity. Therefore, a potential metabolite that influences the expression of GGT must be located downstream of the AspA enzyme. This ruled out aspartate and glutamate as regulatory metabolites that are sensed by *C. jejuni* to determine the metabolic status of the cell. The *frdA* mutant is unable to metabolize the carbon sources succinate, 2-oxoglutarate, (iso)citrate (Weingarten et al., 2009), and these likely accumulate intracellularly. The *frdA* mutant strain showed low GGT expression with all carbon sources tested, even with serine (Fig. 5G). This is likely caused by the accumulation of a metabolite with a regulatory role that is formed upstream of the FrdABC enzyme. To ensure that an intracellular metabolite rather than excreted metabolites is sensed as a cue, GGT activity was measured in a *dctA* mutant strain, which cannot import aspartate and succinate (Wösten et al., 2017). The addition of aspartate or succinate to the medium repressed the GGT activity level in the wt bacteria but not in the *dctA* mutant strain (Figure S4). This indicates that not extracellular but intracellular succinate is likely responsible for the observed phenotype. These results show that *C. jejuni* alters its carbon metabolism in response to an intercellular metabolite upstream of the FrdABC enzyme.

Identification of the intracellular metabolite that regulates *C. jejuni* carbon metabolism.

To identify the intracellular metabolite(s) to which *C. jejuni* adapts its carbon metabolism, we measured their concentrations in 6 hour old cultures of the wt and the *aspA* and *frdA* mutant strains. The concentration of several intracellular metabolites in the wt appeared to be dependent on the available carbon source. The addition of serine, aspartate and fumarate to the culture medium led to an elevated concentration of citrate when compared to the levels measured in bacteria grown in HI (Fig. S5). Addition of serine to the medium, which had no influence on the GGT activity, increased intercellular serine and pyruvate levels. The intracellular concentration of aspartate and fumarate increased when aspartate was added to the culture medium. Moreover, both the addition of aspartate and fumarate, which reduce the GGT activity (Fig. 5D), caused an increase in the intracellular succinate concentration (Fig. 5H). The *aspA* mutant showed accumulation of aspartate and glutamate, as previously noted (Fig. S5)(Howlett et al., 2014). Only when fumarate was added to the medium a high intercellular succinate concentration was measured in the *aspA* mutant corresponding with a low GGT activity (Fig. 5F and I). In the *frdA* mutant, high levels of glutamate were measured which was partially reversed upon the addition of aspartate and fumarate, which can be used as growth substrate by the *frdA* mutant (Fig. 5J). Consequently, these growth conditions led to increased accumulation of 2-oxoglutarate. In all growth conditions, the intracellular succinate concentration of the *frdA* mutant was high and the the GGT activity low (Fig. 5G&J). Together, these results indicate that the intracellular succinate concentration is inversely correlated with the GGT activity levels (Fig. 5K). This suggests that the intracellular concentration of the succinate pool likely influences gene expression of metabolic genes, by using a demand-based mechanism.

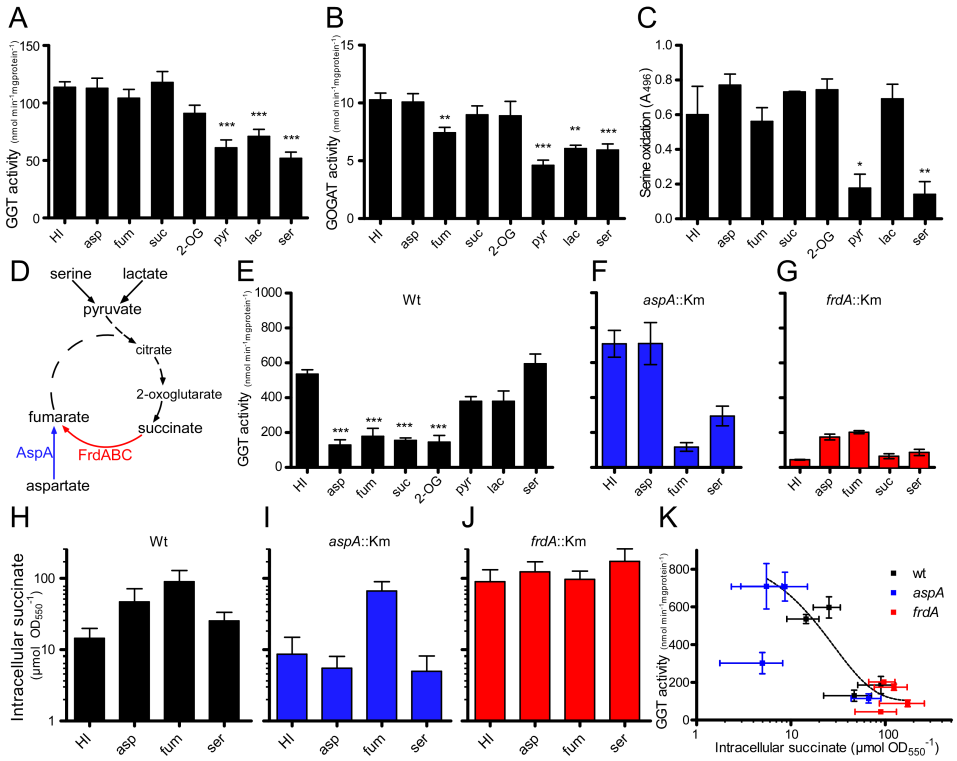


Figure 5: Metabolic enzyme activity is regulated by intracellular metabolites A-C: GGT activity (A), GOGAT activity (B), and serine oxidation (determined by TTC-coupled reduction) (C), in exponentially growing *C. jejuni* (6 h batch culture) grown in HI medium or with the addition of 25 mM carbon source. **D**: Metabolic map of the central metabolism in *C. jejuni*, which indicates the location of the carbon sources tested and the enzymes that are absent in the mutant strains. Dotted arrows indicate multiple enzymatic reactions. **E-G**: GGT activity of *C. jejuni* wildtype (D), the *frdA*::Km (E), and the *aspA*::Km (F) strains in the stationary phase (15 h batch culture) grown in HI medium alone or with the addition of 25 mM carbon source. **H-J**: Semi-quantitative intracellular succinate concentration in exponentially growing *C. jejuni* in HI medium with or without the addition of 25 mM carbon source. **K**: Correlation between the intracellular succinate concentrations and GGT activity levels. The black line represent the least-square fit of an exponential-decay fit of all data points (except the single outlier; *aspA*::Km with serine) and has an R^2 of 0.82. Results are the mean ± SEM of three independent experiments. Statistical significance was calculated with a student *t*-test. (*:p<0.05, **:p<0.01, ***:p<0.001)

DISCUSSION

In this study, we provide evidence that *C. jejuni* regulates its central metabolism at the transcriptional level in response to available carbon sources (Fig. 6). Carbon sources that feed the TCA cycle through acetyl-CoA induce a different metabolic adaptation profile than carbon sources that feed the TCA cycle through conversion to fumarate via the key enzyme AspA. The regulatory proteins Cj1000, RacR and the CsrA play a role in the regulation of the carbon utilization in *C. jejuni*. Intracellular succinate appears to be a key

metabolite that influences the gene expression of the metabolic genes by using a demand-based mechanism. The results indicate that *C. jejuni* which lacks all classical conserved metabolic transcription factors, has evolved alternative strategies to regulate its metabolism at the transcriptional level.

A first indication of regulation of metabolism in *C. jejuni* was the increase of the growth rate when grown in media with addition of serine but not addition of aspartate. (Fig. 1A). The efficient use of serine was accompanied by lower metabolic rates of catabolic substrate usage, as determined by the TTC-coupled oxidation assays (Fig. 1&S1) and by a lower expression of metabolic genes (Fig. 2&S2). This paradoxical mechanism, whereby less enzymes are needed to yield higher growth rates can be best explained when the substrate used for growth is more efficiently catabolized and yields sufficient anabolic precursors and energy to meet the demands of the organism. These nutrients will be the preferred substrate of the cell.

Interestingly, the catabolism of several carbon sources increased during the transition from the exponential to the (early) stationary phase. This increase was most evident when serine was present as original carbon source (Fig 1 & S1). This indicates that *C. jejuni* has a preferential order of catabolizing substrates. When at the end of the exponential growth phase all or most of the serine is depleted, the pathways used to metabolize amino acids that are catabolized via fumarate (i.e. aspartate, asparagine, glutamate, proline and glutamine) are upregulated (Fig. S1). These substrates are less preferred and these catabolic pathways are only expressed when a preferred substrate (serine, lactate or pyruvate) is not available. Our results indicate that serine, lactate and pyruvate, which enter the TCA cycle via acetyl-CoA, are the preferred growth substrates for *C. jejuni* (Fig. 1&5). In *E. coli,* metabolic gene expression is inversely correlated with the quality of the carbon source; carbon sources that sustain low growth rates lead to upregulation of more catabolic pathways (Liu et al., 2005). It is still unknown how this foraging mechanism is working in detail, but the similarities with our results are evident and could indicate a conserved mechanism to adapt to different carbon sources in proteobacteria.

Apart from many downregulated genes, two operons (*c8j_1306* - *c8j1310* and *c8j_1143* – *c8j_1146*) were found to be highly upregulated in the presence of serine (Fig. 2A). Both these operons contain genes involved in methionine synthesis. Therefor we checked whether methionine might be a limiting factor under these growth conditions, but no difference was found when these cultures were also supplemented with methionine (data not shown). Further research needs to be done to explain why these operons are upregulated in the presence of serine.

The expression of metabolic genes in *C. jejuni* is under control of σ^{70} (Wösten et al., 2008; Wright et al., 2009). However, neither the growth phase, nor the addition of a carbon source influenced the expression of *rpoD*, encoding σ^{70} (Fig. 2B). This hinted to the existence of other transcription factors or regulatory mechanisms. To investigate this, we inactivated most of the *C. jejuni* transcription factors to decipher their potential role in the carbon metabolism. GGT and GOGAT enzyme assays were used as read system as the activity of these enzymes showed to be highly dependent on the available carbon source

and the growth phase of the culture (Fig. 3). This approach showed that the DskA and SpoT proteins, which regulate the stringent response during amino acid starvation (Gaynor et al., 2005; Yun et al., 2008) and the NssR protein (a family member of the CRP/FNR-type regulators, which are often stimulated by the secondary messenger molecule cAMP (Tutar, 2008)), did not influence the GGT and GOGAT activities (Fig. 4). A *C. jejuni csrA* mutant strain however, showed elevated GOGAT activity and slightly increased GGT activity in the exponential growth phase compared to the wt. CsrA is a post-transcriptional regulator that is involved in regulation of central metabolic pathways, motility, biofilm, cell invasion and flagella synthesis (Fields et al., 2016; Radomska et al., 2016). Since both the *gltB* and the *ggt* 5'UTR mRNA do not form a required stem loop for CsrA binding (data not shown) (Radomska et al., 2016), these effects are likely to be indirect. A previous CsrA proteomics study found higher GGT protein levels, but not GOGAT (Fields et al., 2016). It is unclear why our results are different, but this could be due to the use of other culture media and growth conditions.

Inactivation of the two-component regulator *racR* resulted in lower GGT activity. This phenotype was not observed for its cognate histidine kinase RacS mutant (Fig. 4A & data not shown). The two-component system RacRS has been shown to be a positive regulator of both *gltB* and *ggt* under oxygen-limiting conditions, especially when nitrate is available (van der Stel et al., 2015b). Because GGT activity is only dependent on RacR and not RacS, RacR might be cross-phosphorylated by other histidine kinases, or activated through high-energy phosphodonors like acetylphosphate under microaerobic conditions. However, we were unable to show that acetylphosphate can phosphorylate RacR in *in vitro* experiments, nor did we observe contrasting phenotypes in *pta* and *ackA* mutant strains, which produce low or high levels of acetylphosphate, respectively (data not shown). Another possibility is that unphosphorylated RacR has residual transcription inducing activity, as it still binds to its target promotors in the unphosphorylated state *in vitro* (van der Stel et al., 2015a). The *cj1000* mutant strain showed an increase in GGT activity in the exponential growth phase but no change in GOGAT activity (Fig. 4) or growth (data not shown). This mutant has been reported to affect *C. jejuni* respiratory activity and the expression of a large number of genes, including genes that encode central metabolic proteins (Dufour et al., 2013). However, it is unclear what role Cj1000 plays in the carbon utilization in *C. jejuni*.

Our results make it plausible that the intracellular concentration of succinate influences the expression of central metabolic genes (Fig. 5&5S). The concentration of intracellular succinate highly correlates with the expression of the metabolic enzyme GGT in both the wt strain and in strains with mutations in the *aspA* and *frdA* genes. Moreover, expression of GGT was not influenced in a *dctA* mutant, which is severely impaired in succinate uptake. This indicates that intracellular and not extracellular succinate is needed for metabolic regulation in *C. jejuni*. On the basis of our results a scenario is enfolding in which when sufficient intracellular succinate is present, the TCA-cycle as well as genes encoding proteins needed to catabolize and transport specific carbon sources are downregulated. Under conditions that carbon (i.e. succinate) is becoming scarce, alternative catabolic pathways are upregulated to scavenge more carbon from the environment (Fig. 1, 2 and 5). This regulatory mechanism enables for global regulation

towards a multitude of carbon sources, likely encountered in the natural environments of *C. jejuni*. Although fine-tuning may not be possible with such a system, the strategy does not require an extensive arsenal of regulators. This might be evolutionary driven by the host-associated lifestyle of *C. jejuni*, which secures a rich environment, where a more specific regulation is not necessary (Cases et al., 2003). In the alphaproteobacterium *Sinorhizobium meliloti* extracellular succinate is sensed and leads to selective succinate catabolism (Garcia et al., 2010). In *Pseudomonas* species, carbon catabolite repression leads to repression of the catabolic pathways of lesser preferred substrates in response to intracellular succinate. The presence of succinate also leads to high transcription of a small RNA *crcZ*, which binds the translational repressor Crc (Müller et al., 1996; Valentini and Lapouge, 2013). *C. jejuni* does not contain the *crcZ* sRNA, nor the CRC protein, but does transcribe a high number of non-coding RNA species, which might be involved in gene regulation (Dugar et al., 2013; Porcelli et al., 2013)

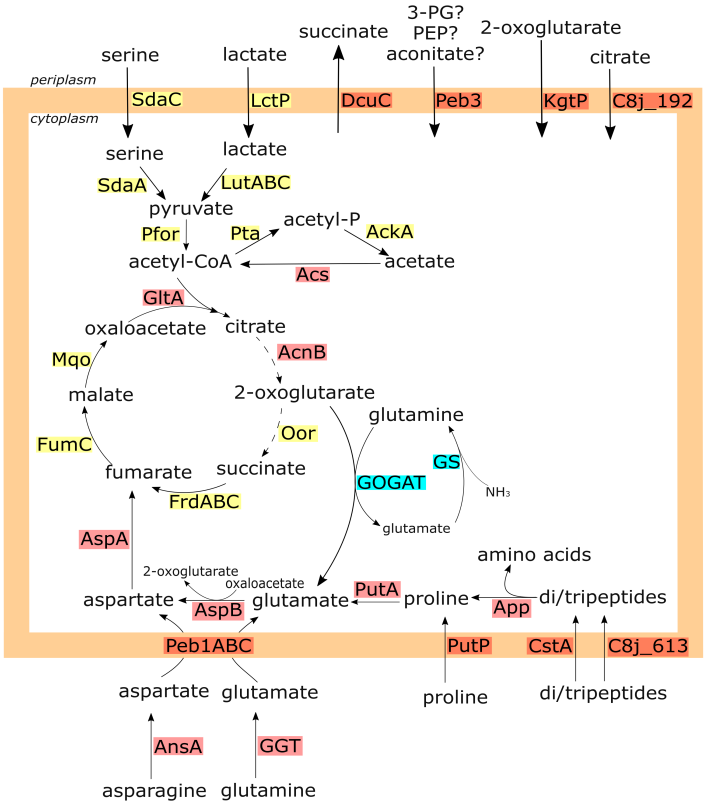


Figure 6: Model of central metabolic regulation in *C. jejuni*. Upon the presence of serine, lactate or pyruvate, a metabolic down-shift takes place; enzymes and transporters shaded in red are down-regulated, while yellow shaded proteins are not differential regulated. Blue shaded indicate enzymes, which are upregulated by the presence of aspartate.

Recently a study was published, which identified a set of genes that overlaps with the genes found in this study. This set of genes is upregulated by the presence of extracellular acetate, butyrate and propionate, and could be used by *C. jejuni* to determine its spatial localization in the host (Luethy et al., 2017). Our results indicate a mechanism via which the identified genes are regulated by the intracellular carbon status of the cell. Together, the results suggest that *C. jejuni* utilizes multiple regulatory mechanisms to regulate its central metabolism. Unfortunately, the involved transcriptional regulatory molecules have not been identified yet. Therefore, the exact mechanism of metabolic awareness of *C. jejuni* is not clarified. Multiple signals could potentially lead to the activation or repression of the same transcription factor. Besides, our results indicate that multiple regulators influence the expression of metabolic genes, so the expression of these central metabolic enzymes is likely controlled by a network of regulatory elements.

In this study, we describe for the first time metabolic regulation in ε-proteobacteria towards different carbon sources. We show that preferred carbon sources of *C. jejuni* that feed the TCA cycle through acetyl-CoA represses the central metabolism at the transcriptional level. This metabolic adaptation is likely regulated by the intracellular level of succinate, which is depending on the available carbon source and growth phase.

MATERIAL AND METHODS

General growth conditions

Strains and plasmids used in this study are listed in Table S2. *Campylobacter jejuni* strain 81116 and derivatives were grown on saponin plates (Biotrading, Mijdrecht, The Netherlands) supplemented (when appropriate) with the antibiotics chloramphenicol (20 µg/ml) or kanamycin (50 µg/ml), or in Hearth Infusion medium (HI-medium, Biotrading, Mijdrecht, The Netherlands) without antibiotics) at 42°C in a microaerophilic atmosphere (5% O_2, 8%CO_2, 8%H_2, 79%N_2). *E. coli* strains were grown on Luria-Bertani (LB) agar plates or in LB broth (Biotrading Mijdrecht, The Netherlands) at 37^{o}C.

Mutagenesis

To disrupt the genes encoding regulatory or metabolic proteins (Table S1), a PCR was performed to amplify the target gene and ~1000 bp flanking regions at both sides using the appropriate fw and rev primers (Table S2). The PCR product was subsequently cloned into the vector pJET1.2 (Thermo). Next, an inverse PCR was performed on the obtained construct with BamHI containing primers to disrupt the gene of interest. The inverse PCR product was digested with BamHI and ligated to a BamHI digested DNA fragment harbouring either a chloramphenicol- or kanamycin antibiotic resistance gene from the vector pAV35 or pMW2, respectively (Table S1). The resulting knock-out construct was checked with sequencing and subsequently used to mutate *C. jejuni* 81116 wt, using natural transformation. Homologous recombination resulting in double cross-over events were verified by PCR.

TTC assays

Precultures of *C. jejuni* were diluted to an OD_{550} of 0.05 in 5 ml of HI medium or HI enriched with 25 mM of aspartate or serine. Cultures were grown for 3, 6, 9, 15 or 24 hours under microaerobic conditions at 42^{o}C, 160rpm. Bacteria were harvested by centrifugation, washed and resuspended to an OD_{550} of 0.4 in HEPES buffer (10 mM HEPES, pH 8.0, 100 mM NaCl). The cell suspension (135 uL) was mixed with 15 uL of 1% TTC and 15 uL of 100 mM carbon source. As blank value, reactions with water were used instead of carbon source. Absorbance was measured after 1 h incubation under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C, at 496 nm on a Synergy HTX multi-mode reader (Biotek, Winooski, USA).

RNA-seq

To identify genes that are differentially expressed upon addition of carbon source, *C. jejuni* pre-cultures were diluted to an OD_{550} of 0.05 in HI broth or medium with 25 mM aspartate or serine, and grown for either 6 h (exponential) or 12 h (early stationary). RNA was immediately isolated using the RNA-Bee kit (Tel-Test) according to the instructions of the manufacturer. RNA aliquots were treated with RNAse-free DNAse (Thermo), following the manufacturer's instructions. RNA-seq was performed as described before (van der Stel et al., 2017).

RT-qPCR

Precultures were diluted to an OD_{550} of 0.05 and grown in HI medium or HI enriched with 25 mM of aspartate or 25 mM serine. After 3, 6, 9 or 15 h cultures were harvested and RNA was isolated immediately using the RNA-Bee kit. RT-qPCR was performed as before (van der Stel et al., 2015a). In brief, DNAse treated RNA (10 ng) was used to determine the amount of specific transcripts using the Takyon No Rox qPCR-kit with Euroscript II reverse transcriptase (Eurogentec, Seraing, Belgium), according to the manufacture's protocol in a Lightcycler 480 machine (Roche, Penzberg, Germany) using custom DNA-oligo's (Table S2). Transcript abundance was determined semi-quantitative by comparison to a standard range. The standard range was made by performing qPCR reactions on known quantities of target DNA for each gene separately. Target DNA was made with RT-qPCR primers on genomic DNA and subsequent purification and diluted to 10^4-10^8 molecules per reaction.

Enzyme activity assays

GGT activity was determined as described before (van der Stel et al., 2015b). GOGAT activity was measured by following the consumption of NADPH (Meers et al., 1970). Briefly, culture aliquots (1 mL) were pelleted and stored at -80°C for at least 1 hour. The pellet was resuspended in 250 µL of 50 mM Tris/HCl (pH 7.6) and 1 µg/mL of lysozyme, and incubated for 30 min on ice. The bacteria were disrupted by sonication. After centrifugation (10 min at 12000 x *g* at 4°C), the cleared lysate (20 µL) was mixed with assay buffer (50 mM TRIS/HCl, pH7.6, 5 mM 2-oxoglutarate, 0.25 mM NADPH) with or without substrate (5 mM glutamine). The rate of NADPH consumption was measured by following the absorbance at 340 nm during 30 min incubation at 37°C in a UV-transparent 96-wells plate (Falcon). The reaction rate with glutamine was corrected by subtracting the auto-oxidation rate (from the reaction without glutamine). Protein content was measured in parallel using a BCA kit (Thermo), according to manufacturer's specifications. The activities were quantified using an extinction coefficient of 6220 $M^{-1}cm^{-1}$ for NADPH.

Metabolomics

Bacteria were grown in 5 mL HI broth (or with the addition of 25 mM carbon source as indicated) under microaerobic conditions for 6 h. The bacteria were harvested by centrifugation for 10 min, 5,000 x g at -9°C and washed in 1 mL of ice-cold saline (0,9% NaCl). After centrifugation for 1 min, 12,000 x g at -9°C, the pellets were resuspended in a 1.6 mL of methanol and chloroform 1:1 mixture (-20°C) and stored at -80°C. After the addition of 320 µL ice-cold Milli-Q-water, the cells were lysed 4 times for 5 min in an iced water Sonorex super sonicator bath (Brandelin). After centrifugation (14,000 g, 5 min, 4°C), the water phase was collected and concentrated using a centrivac at room-temperature for 20 min.

Prior to MS analysis metabolites were separated on a ZIC®-pHILIC column (5 µm, 2.1 mm × 100 mm) (Merck KGaA, Darmstadt, Germany). The injection volume was 5 µl. Mobile phase A consisted of acetonitrile, while mobile phase B consisted of 10 mM ammonium carbonate (adjusted to pH 9.4 with ammonia). The gradient started with 80% A and increased linearly to 20% A in 14 min. The mobile phase was kept at isocratic conditions (20% A) for 4 min before the gradient was allowed to reach 80% A in 30 s and then equilibrated for 1.5 min. The total analysis time was 20 min and the flow rate was 200

µl/min. Sample tray temperature was controlled at 4 ◦C during the measurement. The effluent of the column was introduced to a heated electrospray ionization source, coupled with an Orbitrap Fusion mass spectrometer (Thermo Scientific, Waltham, MA, USA). The mass scanning range was m/z 50-500, at an orbitrap resolution of 120k, the needle voltage was −2.5 kV (negative mode), capillary temperature was 275 ◦C and the sheath and auxiliary gas flow rates were 70 and 5 arbitrary units, respectively. During orbitrap scanning five ms2 spectra were recorded of the 5 most intense precursor ions of the previous orbitrap scan, using the linear ion trap at 'rapid scan rate' and a collision energy of 30%.

Raw-data were converted to mzXML format and analyzed using XCMS v1.50.1 running under R v3.3.2 (Smith et al., 2006; Tautenhahn et al., 2008). Featured peaks were annotated based on their m/z value, retention time and, if available, MS^2 spectrum. Metabolites were quantified based on reference curves of standard metabolites.

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AUTHOR CONTRIBUTION

AXS, JvP and MW contributed to the conception of the experiments. AXS, CP, LH and CL performed the experiments. Growth curves, TTC assays, RNA isolation, RT-qPCR and enzyme assays were performed by AXS. RNA-seq analysis was performed by SH and CP. HPLC/MS/MS analysis experiments were performed by CL. Mutagenesis of *C. jejuni* 81116 was performed by AXS, AA and CA. AXS, CP and MW performed analysis of the data. AXS, JvP and MW wrote the manuscript.

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SUPPLEMENTARY MATERIAL

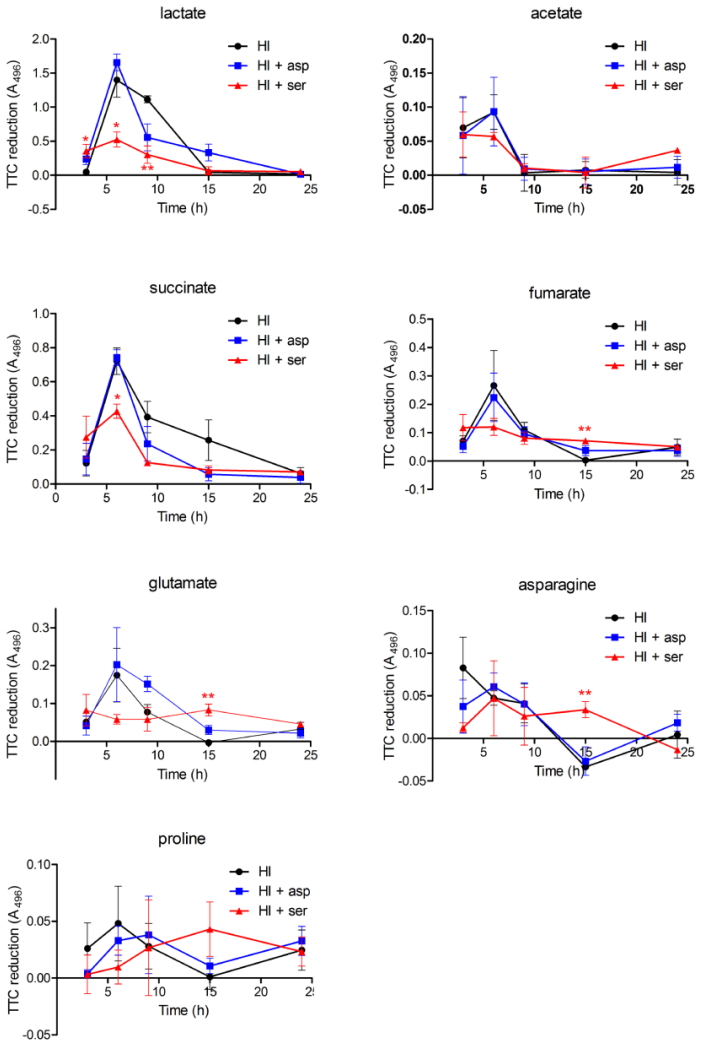


Figure S1: Carbon source utilization of *C. jejuni*. TTC reduction was measured in bacteria during microaerophilic batch cultures in HI-medium (black circles) or with the addition of 25 mM serine (red triangles) or 25 mM aspartate (blue squares). Carbon source utilization based on TTC reduction-coupled oxidation of electron donors during the batch cultures. After indicated time-points, cultures were harvested and optical density and carbon source utilization was probed using a TTC-reduction assay with single compounds. The results shown represent the mean of three independent experiments. The error bars represent SEMs. Statistical significance was calculated with a student t-test. (*:p<0.05, **:p<0.01, ***:p<0.001).

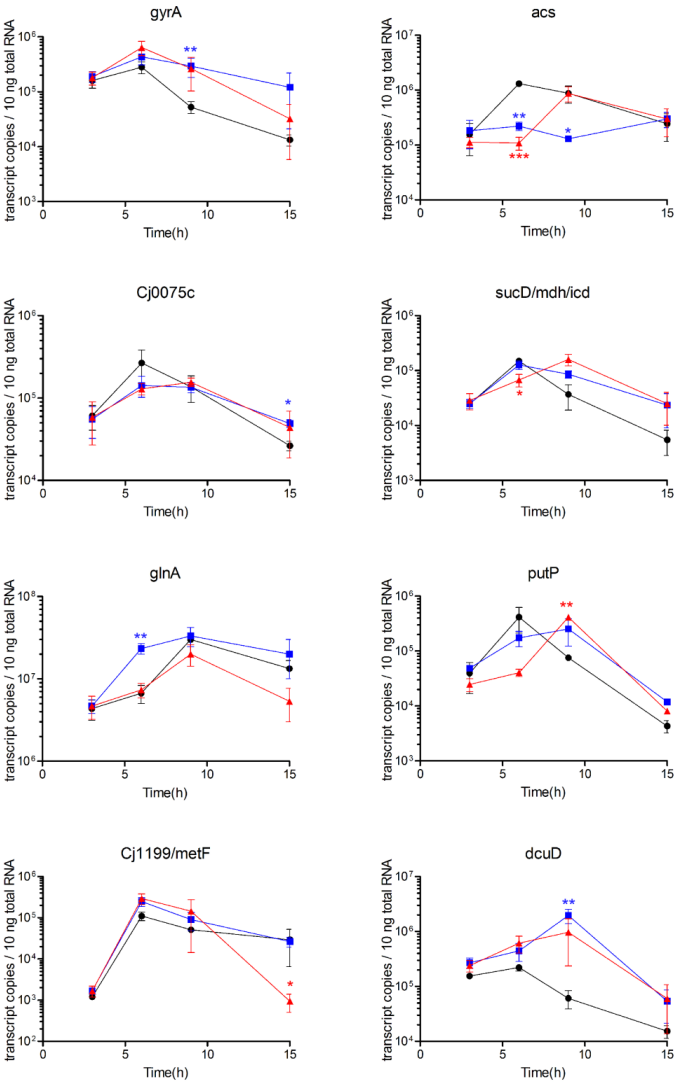


Figure S2: RT-qPCR analysis of metabolic genes from *C. jejuni*. RNA was isolated during microaerophilic batch cultures in HI-medium (black circles) or with the addition of 25 mM serine (red triangles) or aspartate (blue squares), harvested at different time points. The transcript copies were calculated based on a standard range with PCR-amplified template DNA per reaction containing 10 ng of total RNA. The results shown represent the mean of three independent experiments. The error bars represent SEMs. Statistical significance was calculated with a student t-test. (*:p<0.05, **:p<0.01, ***:p<0.001).

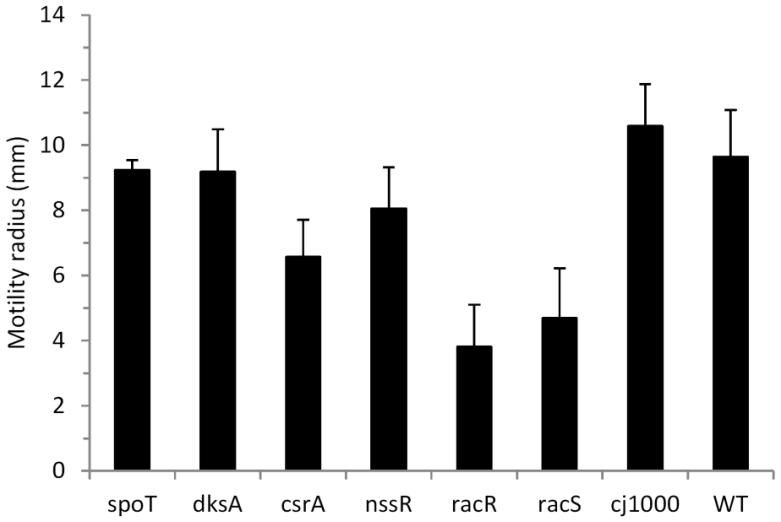


Figure S3: Motility zones of *C. jejuni* wt and regulatory mutant strains in soft agar plates. *C. jejuni* strains were harvested from plate using a pipette tip and stabbed in 0.4% Mueller-Hinton agar plates and incubated for 16 h under microaerophilic conditions. The results shown represent the mean of three independent experiments. The error bars represent SEMs. Statistical significance was calculated with a student t-test. (*:p<0.05, **:p<0.01, ***:p<0.001).

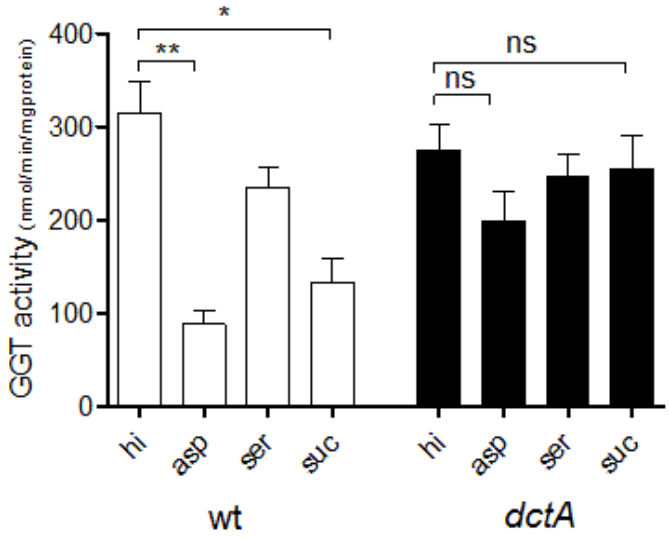


Figure S4: GGT activity in the *dctA* mutant strain is not susceptible to aspartate and succinate addition. Bacteria were grown for 15 hours under microaerobic conditions in HI medium (with the addition of 25 mM carbon source). The results shown represent the mean of three independent experiments. The error bars represent SEMs. Statistical significance was calculated with a student t-test. (*:p<0.05, **:p<0.01).

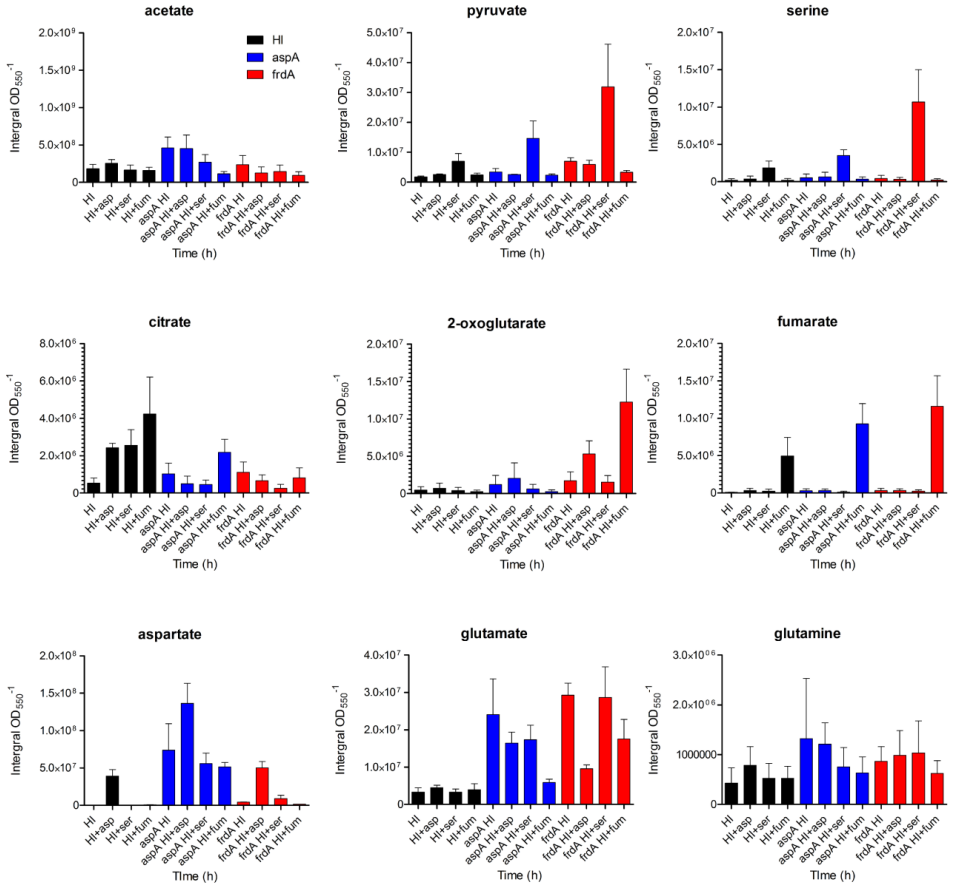


Figure S5: Intracellular metabolite levels in *C. jejuni*. Bacteria were grown for six hours in HI-medium under microaerobic conditions with or without the addition of 25 mM carbon source, as indicated. Polar metabolites were extracted and the amount in each sample was determined by HPLC. The integrals of each central metabolite was determined and normalized with OD_{550} values. The results shown represent the mean of three independent experiments. The error bars represent SEMs.

Table S1: Primers used in this study.

Primer	Sequence (5'-3')
Mutagenesis	
cj1000 fw	GAAATAATATCACTCGTAGCAATACC
cj1000 rev	TCTTTTAAAATATCTGCATGGCG
cj1000 ow fw	AAAGGATCCGAAAAAATTATAATTTTAACAGAGC
cj1000 ow rev	AAAGGATCCGTTTAAAAGATCTAAAAATATTTCC
dksa fw	GCTAAAATCTATCATTGTATGAGTTGG
dksa rev	GGTAGAAAAAGTTCTGTCATCAGCGG
dksa ow fw	AAAGGATCCATTGCTTTGCAAATTTTCTAAGATGGC
dksa ow rev	AAAGGATCCCTCAAAGACTAAAAGTCAAACCTCACGC
spot fw	CTCAGCCATATCACTTGGTAAAGC
spot rev	CTCATTAGCTTAAATTAGCTAAATTTAATGG
spot ow fw	AAAGGATCCTTTCGAAAAGCAAAGCCTTAGCAGC
spot ow rev	AAAGGATCCGTGTCAAGGATAGGTTAAAATCTCG
nssr fw	ATCATCAAATTATCATTATTTATCCC
nssr rev	CAAATGCTAAAAACAATAATAGCTGC
nssr ow fw	AAAGGATCCCAGAACTTATTGATACAAAAAAAGG
nssr ow rev	AAAGGATCCAAAATTTTTTAGCCTCTTCGCCCTC
1042 fw	ATCAATAATAGGCCCCACTGCAGTAATTGGGG
1042 rev	TGATTTTAGAAAAGACGCCCTTGCTACGGC
1042 ow fw	AAAGGATCCAGTATCCGTTTGTGTATATTTAGCAAAAGTGC
1042 ow rev	AAAGGATCCGGTTTATTGAAAGATTTAAAGAGAAATATGAGC
1387 fw	TGAACAAGCTGCCTTTAGTCCAAGCAATATCG
1387 rev	ATATATTGACAAATAGCAATCACACTTAAGGC
1387 ow fw	AAAGGATCCCTCATATTGCTCACCTAAAACTTCTCC
1387 ow rev	AAAGGATCCAATATAAAAGGAGCGGTTCCTATCGTAGC
571 fw	ATGAACCACCTTTTAATCCTTTATAATCCC
571 rev	ATCTGAAGTTAAAAGCTCAAAATCACTACC
571 ow fw	AAAGGATCCAGATGAAATTTTGCATTTGGTTAAGCAGTGG
571 ow rev	AAAGGATCCAACTCTTGTGCAAGCTCTTGGGCGC
883 fw	GAGATGCAGCTGCAGCACTTGGTTTGC
883 rev	TGGAATTCACTTGATTGTAGCCACACAACGGCC
883 ow fw	AAAGGATCCTAGCTAAAAAACTTTTAGGAATATCAAGC
883 ow rev	AAAGGATCCAAGAAGAAAATTGCACCTTAATGCC
1491 fw	TAAGGCTAAATCAGTCTTAT
1491 rev	TGAATTTGATGTGAGTATAGA
1491 ow fw	CATGGATCCTAGGCATAGCAATATCTGTG
1491 ow rev	TATGGATCCACTGGATATGGCAAGAGAGA
1546 fw	GGAATCATAGTTGTAACCAAAACACCG
1546 rev	CATAAGGTTGCGCCTTTGCTTTGTGCGGG
1546 ow fw	AAAGGATCCTTAATTCCTATTTTACAAGCTATGTGC
1546 ow rev	AAAGGATCCATTAATTAATGCTAGAGTATAGTTAAATCCG
pta fw	TAAATGCTTTAGGTGAAGCTAAGGGCGC
pta rev	GCCACCTGTAAAAATAATAGCATCGG
pta ow fw	AAAGGATCCGTTAATGATTTAAGTCGTGGATGTTTGG
pta ow rev	AAAGGATCCATTTTTGTAAATTTTGCTATAATTTTTAAGC
acka fw	GAAGAGTATGATTTTATAACTCCACATCG
acka rev	GATACAAAATTCTCAAACTATAGAAAAACG
acka ow fw	AAAGGATCCCAAATTCTAAAGTTAAAGTGCTTGTTATTCC
acka ow rev	AAAGGATCCTTTCTCTACTAAACCACTCGCTTTAACG
RT-qPCR	
rpoD fw taq	GAACGAATTTGATTTAGCCAATGA
rpoD rev taq	TGTCCCATTTCTCTTAAATACATACGA
gyrA fw taq	ACGACTTACACGACCGATTTCA

gyrA rev taq	ATGCTCTTTGCAGTAACCAAAAAA
sdaA fw taq	GGAGCAGAAGCAGGTTGTCAA
sdaA rev taq	GCCCCTAAAACTGTTGCCATAG
cj0075 fw taq	TTTTACGCCACATGCTTAGGAA
cj0076 rev taq	CACGCCTTAAAAGTTTTATTGCATT
gltA fw taq	CGTCTTGAAGCATTTCGTTATGA
gltA rev taq	AGCATTATCAGGGAAAGAATCAAAA
sucD fw taq	ACCGTTGCAGATGCGGTTA
sucD rev taq	CACCTACAGCAAAAGCAGGTACA
acnB fw taq	GGAAACAAATACCGATGATCTAAGC
acnB rev taq	CTTACCTTTAGCATGGCATTTGC
acs fw taq	CTGAAATTGAAAGCGCTATTGCT
acs rev taq	CGCCCTTAATCGCATCCA
putP fw taq	TGCTACTGTGGTACTTTGGAAGAATT
putP rev taq	TCACCACCGAAGCTGCTAAA
ggt fw taq	TGCGAGTTATGGTTCAGGTG
ggt rev taq	TTAGCTTCTCCGCCTACAAG
aspB fw taq	GCCGTTGTTCCAGGTGTAGGT
aspB rev taq	AGCTCATCGCTTGTGGCATA
aspA fw taq	TTTGTTAGAGCTTTGGCTAGAGTAAAAA
aspA rev taq	CGCTTTAATAATCGCATCTTGGA
glnA fw taq	CAATACTGGACACAGGCCAAGA
glnA rev taq	CCATTTCAGAACGAATATCTACTAAAGAAT
gltB fw taq	acacgatgcctgtggtatcg
gltB rev taq	tcggtgttcaagattcatcaaaat
cj1199 fw taq	TGGCCTAGTGAAATTCCAGAACTT
cj1199 rev taq	GAGCTTGAGCGAAGGCTTTTAA
dcuD fw taq	TGCTTGCAGTTTGTGCTTTCTT
dcuD rev taq	TTGGGATAAGTGGAGCAAAGCT

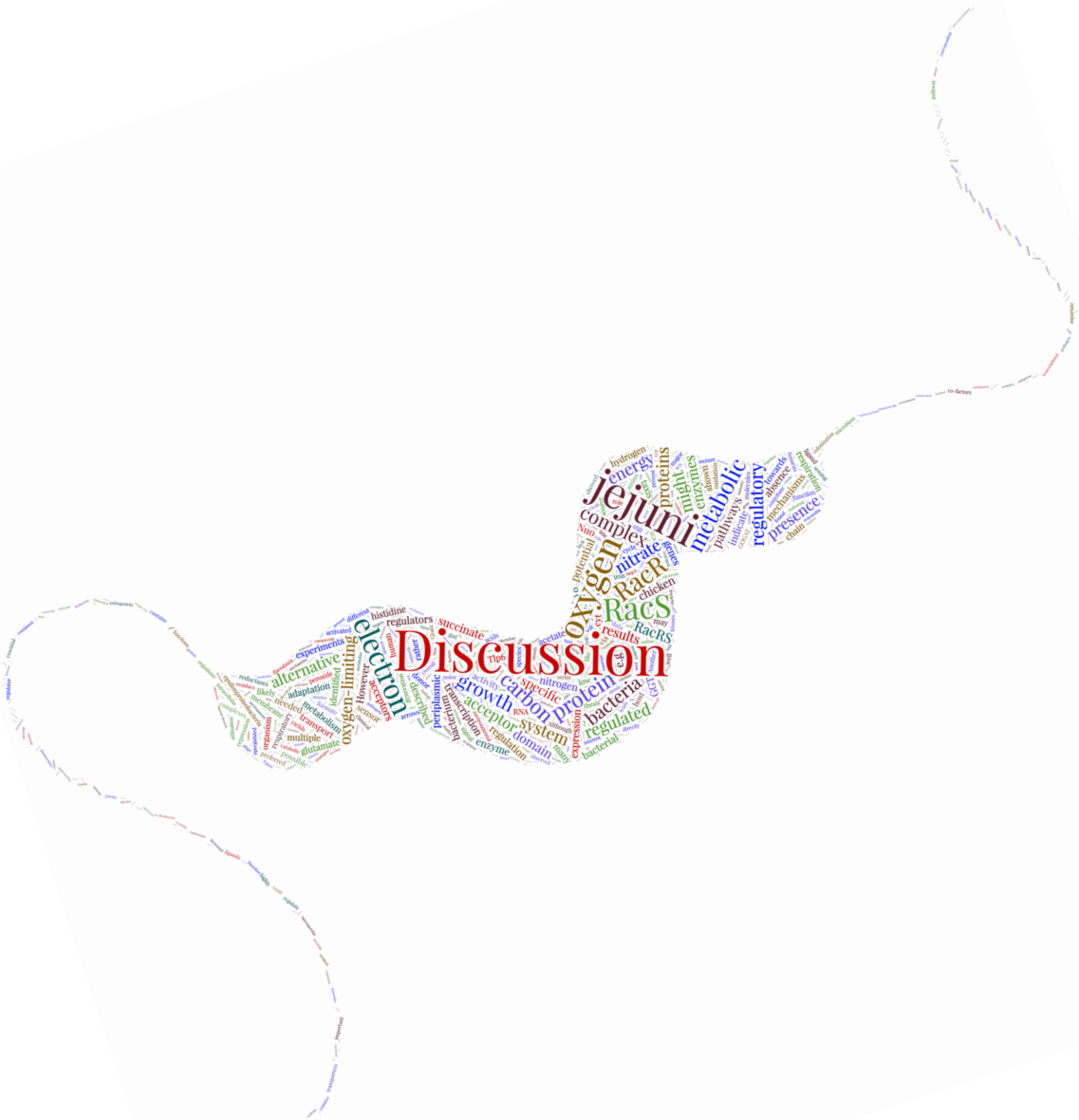
Table S2: Strains and plasmids used in this study

Strain/plasmid	Characteristic	Reference
C. jejuni		
81116 wt	Human isolate	(Manning et al., 2001)
81116 *aspA*::Km	*aspA* gene disruption	This study
81116 *frdA*::Cm	*frdA* gene disruption	(van der Stel et al.,
81116 *racR*::Cm	*racR* gene disruption	(van der Stel et al.,
81116 *cj1000*::Cm	*cj1000* gene disruption	This study
81116 *csrA*::Cm	*csrA* gene disruption	(Radomska et al.,
81116 *dksA*::Cm	*dksA* gene disruption	This study
81116 *spoT*::Cm	*spoT* gene disruption	This study
81116 *nssR*::Cm	*nssR* gene disruption	This study
81116 *cj1042*::Cm	*cj1042* gene disruption	This study
81116 *cj1387*::Cm	*cj1387* gene disruption	This study
81116 *fur*::Cm	*fur* gene disruption	(van Vliet et al., 1998)
81116 *cj0571*::Cm	*cj0571* gene disruption	This study
81116 *dccR*::Cm	*dccR* gene disruption	(Wösten et al., 2010)
81116 *cj0883*::Cm	*cj0883* gene disruption	This study
81116 *flgR*::Cm	*flgR* gene disruption	(Wösten et al., 2004)
81116 *cj1491*::Cm	*cj1491* gene disruption	This study
81116 *cj1546*::Cm	*cj1546* gene disruption	This study
81116 *cj0440*::Cm	*Cj0440* gene disruption	This study
81116 *cj1505*::Cm	*cj1505* gene disruption	This study
81116 *cj1507*::Cm	*cj1507* gene disruption	This study
81116 *0890*::Cm	*Cj0890* gene disruption	(Wösten et al., 2006)
81116 *rpoN*::Km	*rpoN* gene disruption	(Wösten et al., 2004)
81116 *fliA*::Cm	*fliA* gene disruption	(Wösten et al., 2004)
81116 *pta*::Cm	*pta* gene disruption	This study
81116 *ackA*::Km	*ackA* gene disruption	This study
81116 *dctA*:Cm	*dctA* gene disruption	(Wösten et al., 2017)
E. coli		
DH5a	General cloning strain	labstock
Plasmids		
pJET	cloning vector	Thermo
pAV35	contains Chloramphenicol resistance gene	(van Vliet et al., 1998)
pMW2	contains Kanamycin resistance gene	(Wösten et al., 2010)
p32f *aspA*::Km	p32 with *aspA* genedisruption	(Guccione et al., 2008)
pJET *cj1000*::Cm	pJET with *cj1000* gene disruption	This study
pJET *dksA*::Cm	pJET with *dksA* gene disruption	This study
pJET *spoT*::Cm	pJET with *spoT* gene disruption	This study
pJET *nssR*::Cm	pJET with *nssR* gene disruption	This study
pJET *cj1042*::Cm	pJET with *cj1042* gene disruption	This study
pJET *cj1387*::Cm	pJET with *cj1387* gene disruption	This study
pJET *cj0571*::Cm	pJET with *cj0571* gene disruption	This study
pJET *cj0883*::Cm	pJET with *cj0883* gene disruption	This study
pGEM *cj1491*::Cm	pJET with *cj1491* gene disruption	This study

pJET *cj1546*::Cm	pJET with *cj1546* gene disruption	This study
pJET *cj0440*::Cm	pJET with *cj0440* gene disruption	This study
pJET *cj1505*::Cm	pJET with *cj1505* gene disruption	This study
pJET *cj1507*::Cm	pJET with *cj1507* gene disruption	This study
pJET *pta*::Cm	pJET with *pta* gene disruption	This study
pJET *ackA*::Km	pJET with *ackA* gene disruption	This study

Chapter 6

General discussion



CAMPYLOBACTER JEJUNI

Chicken meat that is contaminated with *C. jejuni* remains a major burden to human health and the economy. Effective strategies that prevent *C. jejuni* from colonizing chicken flocks or contaminating meat during the slaughter process are still not available. This leads to an estimated 100 million cases of human campylobacterosis yearly world-wide (Havelaar et al., 2015). Better understanding of the fundamental biology of *C. jejuni* and the factors that determine chicken colonization of the pathogen is needed to bring solutions to combat this human pathogen. This thesis has laid a foundation for understanding of the regulation of metabolic and energy conservation pathways of *C. jejuni*. The major goal of the work described in this thesis was to unravel the regulatory mechanisms and environmental cues that help the bacteria to adapt its metabolism to thrive in the chicken gut. One *C. jejuni* mechanism described to be essential for efficient colonization of the chicken gut is the two-component signal transduction system Reduced Ability to Colonize (RacRS) (Brás et al., 1999). This system may thus be an appropriate target to limit colonization of chicken. However, the signals that activate the RacRS system and the complete set of genes regulated by this system are still an enigma. The first aim was to clarify the function of the RacRS regulatory system.

FUNCTION OF RACRS

The RacRS system is composed of the sensory protein RacS and the response regulator RacR. Activation of the sensor histidine kinase results in phosphorylation of RacR. Once phosphorylated, RacR binds to a specific promoter region in front of RacR regulated genes (Chapter 2), influencing the transcription of the RacRS regulon. In search for conditions that activate RacRS, we initially tested for *racR* mutant phenotypes with different bacterial growth environments, mimicking the oxygen-limiting conditions of the chicken gut. To identify possible environmental cues we used bacterial growth as read-out assuming that an activated RacR would result in a change in bacterial growth. The experiments showed that in the presence of alternative electron acceptors (e.g. nitrate instead of oxygen) the *racR* mutant strain showed a growth defect. Transcriptome analysis demonstrated that the RacR regulon consisted of genes encoding for metabolic, respiratory and regulatory proteins. *C. jejuni* survives in the absence of oxygen by generating fumarate as endogenous electron acceptor. This necessary change in metabolism is achieved via RacR-independent upregulation of the enzyme AspA. The use of endogenous fumarate as electron acceptor comes at the expense of the excretion of succinate causing less carbon being available for bacterial growth (Chapter 2) (van der Stel et al., 2015). Succinate is not used in the absence of oxygen as carbon source/electron donor, since its oxidation consumes the pmf (Chapter 4). Under these conditions, *C. jejuni* operates the TCA-cycle in a horse-shoe manner, with an oxidative and a reductive branch, both ending at succinate (Guccione et al., 2010). In the presence of alternative electron acceptors (e.g. nitrate or TMAO), RacR is activated, which strongly represses the aspartase activity and the respiration with fumarate with associated succinate excretion (Chapter 2). RacR does not influence the expression of alternative reductases (e.g. the nitrate reductase NapA) as most of the genes coding for these enzymes are upregulated by the

absence of oxygen rather than by the presence of alternative electron acceptors (Chapter 4). Overall, the results indicate that RacR is a key modulator of the reductive branch of the TCA cycle in *C. jejuni* and ensures optimal carbon utilization in the absence of oxygen.

A second group of genes which transcription is regulated by the RacRS system are involved in nitrogen metabolism. Phosphorylated RacR increases the expression of the glutamate producing enzymes GOGAT and GGT (Chapters 2&3). GOGAT is mostly expressed during exponential growth phase and utilizes NADPH as co-factor (Chapter 5). GOGAT, together with glutamine synthetase incorporates free ammonium and generates glutamate, which is needed for anabolic purposes. RacR increases the transcription of *gltB* (encoding the major subunit of GOGAT) under conditions that allow rapid growth (Chapter 4) and thus demand anabolic precursors. GGT is a versatile enzyme that is expressed by many organisms in all kingdoms of life. GGT transfers or hydrolyses gamma-glutamyl groups (e.g. glutathione to glutamate+Cys-Ala or glutamine to glutamate+NH_3). In many organisms, GGT has a role in glutathione (GSH) homeostasis. However, *C. jejuni* does not synthesize GSH itself and not all strains possess the *ggt* gene, indicating another role for this enzyme. Indeed, multiple studies highlight functions of GGT in carbon, nitrogen and sulphur metabolism, as well as pathogenicity (Barnes et al., 2007; Floch et al., 2014; Hofreuter et al., 2006; Vorwerk et al., 2014). Glutamate is the most important nitrogen source in cells; more than 80% of all nitrogen originates from glutamate (e.g. during the synthesis of amino acids and nucleotides) (Reitzer, 2003). Apart from being a nitrogen source, glutamate is also an important carbon source of *C. jejuni* due to the lack of this organism to metabolize sugars. Our studies confirm a role for GGT in carbon metabolism under oxygen limiting conditions and under microaerophilic conditions (Chapters 3&5). More in-depth experimental approaches are needed to truly elucidate the metabolic fate of glutamate in *C. jejuni* and to differentiate between its use as a carbon and nitrogen source under different environmental conditions. This could be studied by feeding and following the incorporation of ^{13}C and/or ^{15}N radio-labelled glutamate. Our results indicate that the increased production of GGT and GOGAT and the decreased AspA activity caused by the activation of RacRS system leads to an increase in anabolic precursors (i.e. glutamate) needed during the faster growth of *C. jejuni* under these conditions.

Other genes regulated by the RacRS system include the cyt *c* peroxidase (*cj358c*), the two-component system (*cj1491/cj1492*) and the chemotactic transducer-like protein Tlp6 (Chapter 2). The expression of these genes is repressed by activated RacR. The cyt *c* peroxidase (CCP) Cj0358 can metabolize the toxic compound hydrogen peroxide. The CCP of *E. coli* and *Salmonella* was recently shown to be able to sustain growth by using hydrogen peroxide as electron acceptor (Khademian and Imlay, 2017). A similar role can be imagined for the *C. jejuni* CCP, especially since no protective phenotype has been found (Bingham-Ramos and Hendrixson, 2008). The function of the regulatory system Cj1491/Cj1492 is also unclear (Luethy et al., 2015). The histidine kinase Cj1492 contains a PAS domain. This domain family is known respond to oxygen, redox potential and light (Taylor and Zhulin, 1999). Although experiments in our lab showed that Cj1492 does not regulate the response towards oxygen-limiting conditions (data not shown), it may still act as a sensor for high oxygen tensions.

To compete with the microbiota and thrive under oxygen-limiting conditions, *C. jejuni* needs to efficiently conserve energy for growth and survival. One of its tactics is to use chemo- and energy taxis to evade non-favorable locations and to find nutrients. *C. jejuni* reacts on carbon sources by the transducer-like proteins Tlp1 and Tlp3, on electron donors by Tlp7 and on energy taxis by Tlp9. The cytoplasmic protein Tlp6 is most likely also an energy taxis protein that might react to the metabolic status of the bacterium. Tlp6 does not contain a periplasmic sensing domain and could be activated by interacting with metabolic proteins, just like the orthologues TlpD protein of *H. pylori*. The *H. pylori* TlpD interacts with the TCA-cycle enzyme AcnB and the catalase KatA (Behrens et al., 2016; Schweinitzer et al., 2008). The fact that the expression of Tlp6 is repressed by RacR, suggests that under RacR inducing conditions (i.e. in the presence of alternative electron acceptors), Tlp6-dependent energy taxis has no priority.

Overall, our discovery that RacR regulates respiratory pathways, carbon/nitrogen metabolic pathways, regulatory machineries and energy taxis, likely reflects the need of this organism to adapt to changing spatial/temporal conditions found in the host (e.g. chicken or human). The strategy of *C. jejuni* to regulate multiple aspects of its biology by a single regulatory input, rather than highly specific sensor/regulator systems, demonstrates the unique adaptive mechanisms of this peculiar organism.

What is the signal of RacS?

The true ligand of RacS is an intriguing unresolved question that arises from the studies described in this thesis. RacS has a classical histidine kinase topology, with a periplasmic domain, which most likely acts as the sensory input domain. The periplasmic sensor domain virtually excludes intracellular compounds as a RacS activating signal. Unlike other histidine kinases, the periplasmic domain of RacS does not belong to one of the super-family domains, like the PAS or CHASE domains present in many other histidine kinases (Cheung and Hendrickson, 2010).

As the presence of nitrate results in the activation of the RacR protein, nitrate might be a good candidate as inducing ligand. However, also the presence of TMAO results in a RacR-dependent repression of AspA activity (Chapter 2). Moreover, experiments with a *napA* mutant strain showed that the repression of AspA and induction of GGT is dependent on actual nitrate respiration rather than the presence of nitrate in the medium. This indicates that nitrate is not the ligand that activates RacS (data not shown). Another putative signal for RacS might be the redox state of the bacterium that senses the respiratory activity of *C. jejuni*. Redox dependent histidine kinases have been identified to sense respiration and indirectly adjust for a lack of suitable electron acceptors. These histidine kinases are responsive to either the (ubi/mena)quinone pools (e.g. ArcB) or to the cyt cbb_3 oxidase activity (e.g. RegB) (Bekker et al., 2010; Georgellis et al., 2001; Swem et al., 2003). The mechanism of activation depends on oxidation or reduction of intracellular cysteine residues. RacS also has two cysteine residues located seven amino acids apart. However, these residues are situated in the periplasmic sensing domain, unlike the cytoplasmic localization in ArcB and RegB sensor proteins. We performed site-directed mutagenesis experiments to substitute the individual or both cysteine residues, but this had no effect on the function of the RacRS system. Nor were inter- or intramolecular disulphide bonds

detected using a biotin switch assay using a HA-tagged RacS construct (data not shown). Other attempts to identify a RacS ligand encompassed the use of a RacS/PhosS chimeric protein (containing the N-terminal sensing domain of RacS and the C-terminal signaling domain of PhosS), a fluorescence thermal-shift assay, a co-IP experiment and autophosphorylation experiments. Despite these attempts, no ligands or regulatory input mechanisms of RacS were identified. Therefore, we can only speculate what the ligand of RacS might be. Possible ligands, other than elements from the electron transport chain, are metabolites such as succinate or acetate that are excreted under oxygen-limiting conditions by *C. jejuni*. These metabolites can be sensed directly or indirectly by interaction with their transporters as is seen for the *E. coli* C4-dicarboxylate sensor DcuS. The sensor DcuS interacts with the C4-dicarboxylate transporters DcuB and DctA (Witan et al., 2012). RacS could interact with the aspartate and succinate transporters DcuA or DcuB (Wösten et al., 2017). These transporters are directly regulated by RacR, creating a feed-back loop (Chapter 2). Other possible clues could be inferred from the functions and ligands of the cj1491/cj1492 TCS and the Tlp6 energy taxis protein, as these are directly regulated by RacS (Chapter 2). It is therefore likely that the specific ligands of RacS, Cj1492 and Tlp6 are correlated; either similar (in the case of Tlp6) or opposite (in the case of Cj1492).

ENERGY CONSERVATION BY RESPIRATION

Compared to other bacteria, epsilonproteobacteria have extensive and branched electron transport chains, which enable the utilization of many different molecules as electron donor and – acceptor (Chapter 4; Figure 7) (Kern and Simon, 2009; Sellars et al., 2002; Zhang and Sievert, 2014). Nevertheless, only a handful of redox enzymes have a topology that could lead to charge separation (Chapter 4). Experiments described in Chapter 4 of this thesis showed that indeed none of the periplasmic reductases (except for the cyt bc_1 complex) contribute to the generation of the membrane potential and appears to function solely as electron sink. Furthermore, on the donor side, only the formate dehydrogenase and hydrogenase contribute to the membrane potential. The *C. jejuni* Nuo complex, which re-oxidizes flavodoxin proteins and acts as proton pump, yielded a low membrane potential. This is in contrast with the Nuo complex of other bacteria that use NADH instead of flavodoxin as substrate. The lower efficiency of the Nuo complex might explain why other epsilonproteobacteria with the same protein complex are unable to grow without hydrogen or formate. This is remarkable as in most organisms, electrons liberated through the TCA cycle are shuttled to the Nuo complex (aka Complex I) to generate the major part of the membrane potential (Brandt, 2006). *E. coli* contains two protein complexes that can oxidize NADH. Besides the Nuo-homologue NADH dehydrogenase1 (NDH1), it contains the non-proton pumping NDH-2 protein complex that can only reduce ubiquinone and not menaquinone (Tran et al., 1997). It can be imagined that the Nuo complex of *C. jejuni* is only capable to reduce MK and not mMK. In-depth biochemical experiments are needed to understand the Nuo complex of epsilonproteobacteria at the molecular level. The recently identified methyltransferase of *Wolinella succinogenes* and other epsilonproteobacteria, which produces mMK from MK, may be instrumental in future experiments to address these questions (Hein et al., 2017). Altogether, the results

indicate that in its natural environment *C. jejuni* will encounter or search for the important respiratory substrates formate, hydrogen and oxygen. The gastrointestinal tract has a steep gradient of oxygen caused by bacterial respiration, high at the epithelial cell layer, low in the lumen (Espey, 2013). Therefor it is tempting to speculate that a lack of electron acceptors in the gut can contribute to *C. jejuni* pathogenicity, aided by energy taxis (guiding the bacterium to the oxygen-rich epithelial cells) and its motility, which enables penetration of the mucus-layer.

Oxidative stress defense

The electron transport chain of *C. jejuni* can protect the organism from reactive oxygen species (ROS) and reactive nitrogen species (RNS). *C. jejuni* encounters multiple forms of oxidative stress in its life cycle and it is equipped with multiple protective enzymes. A superoxide dismutase (SodB) and a catalase (KatA) are the main enzymes that break down radical oxygen species as well as hydrogen peroxide. These enzymes are regulated by the transcriptional regulators Fur and PerR (Atack and Kelly, 2009). *C. jejuni* also encodes two periplasmic cyt *c* peroxidases (*cj0020* and *cj0358c*), which are coupled to menaquinone oxidation (Atack and Kelly, 2006). The transcription of *cj0358c* is down-regulated by RacR, together with the *mfrABC* operon (Chapter 2). The periplasmic facing MfrABC complex generates hydrogen peroxide via the Fenton reaction (Kassem et al., 2014). Together, this suggest that the function of Cj0358c is to metabolize the hydrogen peroxide formed by the MfrABC complex under low oxygen conditions, while the superoxide dismutase and catalase handle exogenous ROS, that enter the cytoplasm. *C. jejuni* expresses also two oxidases (Jackson et al., 2007), a proton pumping cyt *cbb3* oxidase (*cconNOPQ*), which is fully responsible for oxygen respiration and generation of the membrane potential (Chapter 4) and the CioAB oxidase that does not participate in oxygen respiration, but is upregulated in the presence of nitrate (Chapter 4). The CioAB of *C. jejuni* might be involved in the protection against reactive nitrogen species formed during nitrate respiration, as also has been observed for the CioAB orthologues in other bacteria (Borisov et al., 2015). Overall, our results indicate that the electron transport chain is regulated in such a way that it aids in reducing harmful oxidative compounds.

ADAPTATION TO LOW OXYGEN

Oxygen is the most potent and preferred electron acceptor for *C. jejuni*, since it yields the most energy (Chapter 4). In the absence of oxygen, *C. jejuni* can utilize other molecules as electron acceptor. Many of the reductases that are responsible for the reduction of these molecules are regulated independent of RacR (Chapter 2 & 4). The expression of the alternative reductases for the utilization of fumarate, nitrate, nitrite, TMAO/DMSO and tetrathionate is increased in the absence of oxygen (Figure 1). This upregulation occurs independent of the actual presence of the corresponding alternative electron acceptor molecules. The formate dehydrogenase (FdhABC) and the hydrogenase (HydABC) electron donor enzymes are the major contributors of the proton motive force generation in the absence of oxygen (Chapter 4). These enzyme complexes are also upregulated under oxygen-limiting conditions (Chapter 4). The expression of the alternative electron reductases and donor enzymes is also increased when *C. jejuni* colonizes the chicken gut

or when the bacteria invade mammalian cells (Liu et al., 2012; Woodall et al., 2005). This indicates that these environments are probably also oxygen-limiting (or fully anaerobic) rather than microaerobic. These results emphasize the relevance to culture *C. jejuni* in the laboratory under oxygen-limiting conditions (in the presence of an alternative electron acceptor) rather than the conventional microaerobic growth conditions when studying aspects of its colonizing or virulent behavior.

Does *C. jejuni* have an oxygen sensor?

Hot questions are what is the signal that causes the observed adaptation under low oxygen conditions and via which mechanisms does this occur? Although some regulators of *C. jejuni* are described in detail (Wösten et al., 2008), a regulator that directly or indirectly responds to oxygen concentrations has not yet been identified. We constructed multiple null mutants of putative regulatory proteins. These mutant strains were screened for aspartase activity under microaerobic and oxygen-limiting conditions, as this enzyme is highly regulated by oxygen availability (Chapter 2). However, all mutants still displayed a strong increase in aspartase activity under oxygen-limiting conditions (data not shown), therefore none of these regulators was considered as an oxygen responsive protein. Although not all predicted regulators of *C. jejuni* have been mutated and screened for aberrant phenotypes, it should be considered that adaptation to oxygen levels is not necessarily regulated by a classical transcription factor. Most epsilonproteobacteria are oxygen-sensitive bacteria and normally grow only under already reduced oxygen-tensions (Kendall et al., 2014; Nakagawa and Takaki, 2001). The observed changes in gene expression in response to different oxygen concentrations could also be directed by multiple (redundant) regulators. Phenotypic difference might therefore not be observed by mutation of a single regulator. Alternatively, it is possible that other mechanisms than transcription factors participate in the adaptation towards oxygen availability. Phenomena like DNA-methylation, post-transcriptional regulation, regulation by anti-sense RNA or ribo-switches could be worth studying. Although it is evident that adaptation towards oxygen-limiting conditions acts on the transcriptional level, indicating that post-transcriptional or post-translational regulation is less likely.

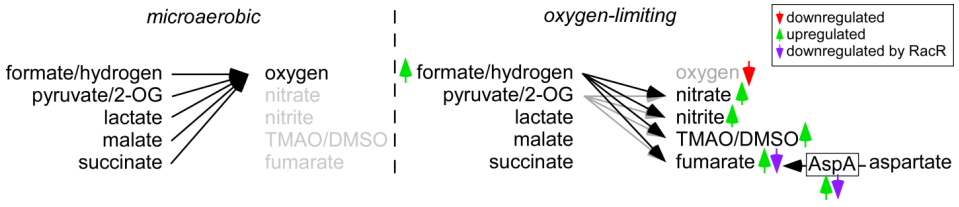


Figure 1: Model of the regulation of the electron transport chain towards oxygen-limiting conditions in *C. jejuni*. The black arrows indicate the electron transport routes from an electron donor to an electron acceptor that generate a membrane potential (Chapter 4). The coloured arrows indicate regulation in oxygen-limiting conditions; red arrows indicate downregulation of the corresponding protein complexes; green arrows indicate upregulation of the corresponding protein complexes; purples arrows depict proteins that are downregulated by activated RacR in the presence of nitrate (Chapter 2).

Intracellular succinate regulates carbon source utilization hierarchy

C. jejuni feeds on a select number of amino acids and organic acids. Of these carbon and energy sources serine and aspartate are described as preferred nutrients (Leach et al., 1997; Wright et al., 2009). This is based on the rapid depletion of these amino acids from rich growth medium, as well as their high abundance in their preferred niche of the chicken cecum. Our study indicates that serine is preferred over aspartate and that other carbon sources that are metabolized via the acetyl-CoA pathway (i.e. lactate and pyruvate) are equally preferred. These molecules are channeled through the same metabolic pathways and subsequently exert similar transcriptional repression on other catabolic pathways.

Glucose oxidizing organisms usually prefer glucose above other carbon substrates, via catabolite repression control (Gosset et al., 2004; Nam et al., 2005). During the glycolysis, which yields ATP, glucose is converted to acetyl-CoA, which enters the TCA cycle. In the TCA cycle, further oxidation yields electrons, which are shuttled to the Nuo complex and contribute to the pmf and the generation of more ATP. In the case of insufficient electron acceptor, the glycolysis still functions and carbon is redirected to lactate, acetate or ethanol.

C. jejuni does not encode a full glycolysis pathway, but can produce acetate, which yields ATP via substrate level phosphorylation (Chapter 4). This might determine the preference for serine over aspartate. Another advantage of the serine/pyruvate pathway over the aspartate/fumarate pathway is the flavodoxin oxidizing enzyme POR. Both pathways will pass the OOR complex, but the oxidation of pyruvate yields additional oxidized flavodoxin. This result in more electrons flowing through the Nuo complex, and possibly a higher growth yield, when serine/pyruvate is used instead of aspartate/fumarate. We identified the accumulation of intracellular succinate as a possible regulatory cue for metabolic reprogramming (Chapter 5). Accumulation of succinate coincides with the repression of many catabolic pathways. When carbon becomes scarce, intracellular succinate levels drop and catabolic pathways are upregulated to scavenge carbon from the environment.

During the exponential growth phase the addition of serine (or pyruvate/lactate) to the medium leads to the down-regulation of many catabolic pathways (Chapter 5). Despite the screening of many regulatory protein mutants, we found no regulator to be fully responsible for this metabolic adaptation. However, we were unable to knock-out all putative regulators as several have already been described to be lethal to the bacterium. Therefore, as an alternative approach, a pull-down experiment with the *ggt* promotor as bait was performed to identify potential candidate transcription regulators. Although some proteins specifically bound to the DNA-bait, no obvious hits were obtained (data not shown). Therefore, it remains enigmatic how the central metabolism of *C. jejuni* is regulated by different carbon sources. Recently, it has been shown that *C. jejuni* adapt its metabolism to the present of extracellular acetate (Luethy et al., 2017). Upon sensing acetate, *C. jejuni* alter the gene expression of some metabolic pathways. However, the usage of acetate itself is not altered, indicating that acetate serves as spatial cue for a specific host environment. Another possible scenario is that the vast amount of anti-sense RNA (asRNA) (Dugar et al., 2013; Shaw et al., 2012) produced by *C. jejuni* plays a role.

These RNA species are likely to have regulatory functions. The presence of these regulatory asRNAs might explain the small number of regulatory proteins in the genome of *C. jejuni*. Another interesting RNA transcript is the 6S RNA, which folds into a double helix, of which the sequence resembles the RpoD recognition sequence (Porcelli et al., 2013). The amount and alternative folding of this specific RNA could regulate the transcription of all RpoD-dependent genes. In the absence of alternative sigma-factors, such a mechanism might regulate a metabolic down-shift.

FUTURE PERSPECTIVES

Awareness is growing that metabolic features of pathogenic bacteria are crucial during infection and even led the concept of 'nutritional virulence' to become a paradigm (Rohmer et al., 2011). This is also seems to hold true for *C. jejuni* (Gao et al., 2017). The highly regulated metabolic adaptation strategies of *C. jejuni* may well compensate for the lack of obvious classical virulent factors in *C. jejuni.* The virulent behavior of *C. jejuni* might be nothing more than an attempt of the bacterium to gain access to nutrients, like electron acceptors. Similar results are found for other pathogenic bacteria. *E. coli*, for instance, stimulates the immune system to release nitric oxide, which spontaneously is converted to the electron acceptor nitrate. Furthermore, *Salmonella* stimulates the host to produce ROS, which catalyzes the production of the electron acceptor tetrathionate. Lastly, *Citrobacter* causes oxygen-rich hyperblasia to oxygenate its microenvironment (Lopez et al., 2016; Winter et al., 2010, 2013). All these pathogens are fighting for electron acceptors to gain energy to replicate. This could be a shared feature of all pathogens in the heavily populated gastro-intestinal tract and is worth further investigating. By studying *C. jejuni* in cell-based assays and constructed model-organisms these mechanisms can be identified and could help to better understand the human pathogenicity of this bacterium.

The present results provide several new leads towards to treat severe cases of campylobacteriosis in humans or, as important, to develop intervention strategy in chickens to prevent human disease. We identified several proteins that are essential for energy generation or efficient bacterial adaptation towards changing environmental conditions. By targeting these protein complexes or regulatory mechanisms, bacteria like *C. jejuni* are limited in their colonization potential. Additionally, both the respiratory chain and the ROS/RNS defense proteins are considered as attractive therapeutical targets, because of their impact on the organism and the specificity that can be achieved (Cook et al., 2014). Another application strategy would be to target the regulation of critical metabolic enzymes, or deprive the bacterium of co-factors essential for these enzymes (Barrett and Hoch, 1998; Silva et al., 2011). Co-factors that may be targeted include amongst others iron, copper and nickel. Many redox enzymes rely on a prosthetic heme group, which harbors an iron ion. This makes iron accessibility and its regulation attractive targets (Thompson et al., 2012). However, the host organism also requires iron and already has strategies to keep free iron concentration extremely low to prevent bacterial growth. The metallic co-factors copper and nickel are needed by the cyt cbb_3 complex and the hydrogenase, respectively. The enzyme formate dehydrogenase utilizes the amino

acid selenocysteine, which contains a selenium atom rather than sulphur (Carvalheda and Pisliakov, 2017; Howlett et al., 2012; Kruse et al., 2017; Shaw et al., 2012). Making the essential co-factors unavailable will prevent the bacterium from employing these crucial metabolic systems but may also effect host biology and thus cause undesired adverse effects.

Approaches that do not need specific protein targets might be more feasible, as bacteria will always evolve resistance to compounds against specific proteins. By combining the knowledge of the microbiota, together with specific metabolic requirements of a pathogen as described in this thesis, eventually probiotic or prebiotic therapies could be developed to diminish the niche of unwanted bacteria, such as *C. jejuni*. Especially with the upcoming antibiotic crisis, the need for new antibacterials is needed (Wright, 2015). Since classical antibiotics highly disturb the microbiota in a way that is harmful for the host, a more subtle approach as suggested here would yield a great advantage (Blaser, 2011). A possible disadvantage of pre- and probiotica can also be listed. The treatment might only work when given as a preventive measure, which is feasible for livestock as well as for humans, when exposure to pathogenic bacteria is anticipated (e.g. traveling to a high-risk area).

Overall, there are multiple paths ahead that can be taken into preventing and curing bacterial infections. Based on the fundamental knowledge acquired about the molecular mechanisms and metabolic requirements of bacteria, pathogens can be combatted in the future. By either targeting specific metabolic proteins or regulators, chelating metal co-factors or adding pre- and probiotica to the diet, we can shape our microbiota to become more resilient towards pathogenic bacteria or treat against a specific infection.

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Addenda


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Inleiding

Met ongeveer 80.000 besmetting per jaar is *Campylobacter jejuni* (*C. jejuni*) de meest voorkomende bron van bacteriële voedselvergiftiging in Nederland. Besmetting met *C. jejuni* leidt tot episodes van (bloedige) diarree die gepaard gaan met heftige buikkrampen. Hoewel besmetting normaal gesproken zelflimiterend is, kunnen er complicaties optreden met ernstige gevolgen. Bij een kleine groep leidt besmetting met *C. jejuni* tot de ontwikkeling van het auto-immuun syndroom van Guillain-Barré, waarbij antilichamen die gericht zijn tegen *C. jejuni*, ook het centrale zenuwstelsel van de mens aanvallen. Jaarlijks leidt in Nederland een besmetting met *C. jejuni* bij ongeveer 30 mensen tot de dood.

Kippenvlees is de meest voorkomende bron van humane *C. jejuni* besmetting, ofwel doordat het niet goed gegaard is of via kruisbesmetting met andere voedselproducten in de keuken. *C. jejuni* komt in de kippendarmen voor als commensaal en is niet pathogeen voor dieren. Voornamelijk in de cecum (blinde darm) groeit *C. jejuni* uit tot dichtheden van wel één miljard bacteriën per gram darminhoud. Vrijwel alle pluimvee wordt gekoloniseerd met *C. jejuni,* die tijdens de slacht overgebracht kan worden van de darm op het kippenvlees. Ten gevolge hiervan is een hoog percentage van het kippenvlees in de supermarkten besmet met *C. jejuni*.

Meerdere maatregelen om besmetting van de voedselketen te voorkomen zijn reeds onderzocht, maar tot nu toe is er geen effectieve preventie methode gevonden. Onder andere vaccinatie van kippen, het toevoegen van additieven aan het kippenvoer of strikte 'biosecurity' van de stallen heeft niet geleidt tot afname van de kolonisatie van *C. jejuni* in kippen. Daarom is er meer kennis nodig over de fysiologie van *C. jejuni* en de omgevingsfactoren die leiden tot efficiënte groei en kolonisatie van *C. jejuni*, alsmede de factoren en mechanismen die dit organisme gebruikt om zich aan zijn omgeving aan te passen. Deze kennis kan vervolgens worden gebruikt om doelgerichte maatregelen te nemen tegen deze hardnekkige bacterie.

Een factor die het onderzoek ernstig belemmerd is het feit dat *C. jejuni* fysiologisch gezien afwijkt van andere veel bestudeerde bacteriën. Dit zorgt ervoor dat kennis over het metabolisme en de regulatie hiervan niet één-op-één kan worden toegepast op *C. jejuni*. Een van de meest in het oog springende voorbeelden is dat *C. jejuni*, in tegenstelling tot veel andere darm pathogenen, geen suikers kan gebruiken om op te groeien. In plaats daarvan gebruikt *C. jejuni* aminozuren en andere organische moleculen, die minder energie opleveren.

Dit proefschrift

In **hoofdstuk 1** geef ik een overzicht van de beschikbare literatuur over het metabolisme en de enzymen die verantwoordelijk zijn voor de energievoorziening van de bacterie. *C. jejuni* groeit het beste bij verlaagde zuurstof concentraties en in de aanwezigheid van koolstofdioxide. Zuurstof wordt door *C. jejuni* wel gebruikt voor respiratie. In de afwezigheid van zuurstof kan *C. jejuni* gebruik maken van alternatieve elektronenacceptoren om te respireren. Om dit te bewerkstelligen heeft *C. jejuni* een

uitgebreide elektronentransportketen, die uiteindelijk een elektrische potentiaal over het binnen membraan genereert. Deze membraanpotentiaal wordt gebruikt als drijvende kracht voor veel biologische processen. *C. jejuni* kan niet op suikers groeien, in plaats hiervan worden andere kleinere moleculen, zoals aminozuren gebruikt als koolstofbron. Deze koolstofbronnen kunnen ook worden verbrand, wat de benodigde energie levert om van te groeien.

Omdat de omgeving van bacteriën sterk kan veranderen hebben deze eencellige organismen adaptatiemechanismen ontwikkeld om zich snel aan de nieuwe situatie te kunnen aanpassen. Deze mechanismen bestaan uit eiwitten die in staat zijn om een bepaalde omgevings- of cellulaire factor te registeren, en die er vervolgens voor zorgen dat er een adequate response optreed. Een dergelijke response bestaat vaak uit het veranderen van de genexpressie, het uitlezen van genen op het DNA, die coderen voor specifieke eiwitten. Dit zorgt ervoor dat de eiwitten, die stuk voor stuk een unieke functie hebben, alleen worden aangemaakt wanneer deze nodig zijn. Deze adaptatiemechanismen kunnen worden gebruikt om de bacterie te beschermen tegen kwalijke invloeden, of om het metabolisme optimaal aan te passen aan de voorradige nutriënten.

C. jejuni heeft relatief weinig van dit soort regulatoire eiwitten. Bovendien zijn deze eiwitten dusdanig anders dan veel bestudeerde eiwitten uit andere bacteriën dat vooraf niet kan worden voorspeld wat de specifieke functie zal zijn van deze regulatoreiwitten. Tot dusver is er erg weinig kennis over zowel het afwijkende centrale metabolisme, evenals hoe de bacterie aan zijn energiebehoefte komt en op welke wijze dit wordt gereguleerd.

In **hoofdstuk 2** wordt onderzoek beschreven naar de functie van het RacR/RacS twee-component systeem. Dit systeem bestaat uit twee samenwerkende eiwitten. RacS is het sensor eiwit, dat gedeeltelijk in de periplasmatische ruimte en gedeeltelijk in het cytoplasma van de bacterie is gelokaliseerd. Wanneer RacS in de periplasmatische ruimte wordt geactiveerd draagt het dit signaal over aan het cytoplasmatische RacR, door er een fosfaat groep aan te bevestigen. Geactiveerd RacR voorzien van een fosfaat groep kan vervolgens op specifieke plekken aan het DNA van de bacterie binden, wat een verandering van de genexpressie teweegbrengt. In dit hoofdstuk worden de door RacR gereguleerde genen geïdentificeerd en laten we zien wat de functie van dit regulator eiwit systeem is. In de afwezigheid van zuurstof en andere elektronenacceptoren kan *C. jejuni* intracellulair fumaraat aanmaken, dat vervolgens kan worden gebruikt om te respireren. Dit gaat echter wel ten koste van het groeivermogen van de bacterie, omdat er koolstof wordt uitgescheiden in de vorm van succinaat, wat anders gebruikt kan worden om biomassa mee te genereren. In de aanwezigheid van andere elektronenacceptoren (bijv. TMAO of nitrate) wordt het RacR/RacS systeem actief en dit verhinderd de aanmaak van intracellulair fumaraat en de uitscheiding van succinaat. Als gevolg hiervan groeit *C. jejuni* beter en produceert de bacterie meer biomassa, een observatie die vrijwel niet zichtbaar is indien het *racR* gen is gemuteerd. Deze resultaten kunnen grotendeels verklaren waarom het RacR/RacS twee-component systeem belangrijk is voor de aanpassing van *C. jejuni* in de natuurlijke omgeving, de kippendarm. In deze omgeving is weinig tot geen

zuurstof aanwezig en zullen bacteriën andere manieren moeten gebruiken om aan hun energiebehoefte te voldoen.

Hoofdstuk 3 is een vervolg studie van de experimenten beschreven in hoofdstuk 2. In dit hoofdstuk wordt de rol van het RacR/RacS twee-component systeem op de aanmaak van glutamaat beschreven. Glutamaat is een belangrijke precursor voor de aanmaak van aminozuren, eiwitten en DNA in de bacterie. De activatie van RacR/RacS leidt tot een verhoogde genexpressie van de genen die coderen voor enzymen die glutamaat produceren. Geactiveerd RacR bindt aan de DNA sequentie die voor deze genen liggen (de zogenaamde promotor). De RacR-afhankelijke verhoogde genexpressie van deze glutamaat producerende eiwitten draagt bij aan betere groei van *C. jejuni*.

Om te kunnen groeien gebruiken bacteriën redox reacties om een potentiaal over het membraan op te bouwen. Tijdens deze reacties stromen elektronen van elektronendonor moleculen naar elektronenacceptor moleculen. De energie die hierbij vrijkomt wordt gebruikt om de membraanpotentiaal op te bouwen. Deze potentiaal wordt vervolgens gebruikt voor biologische processen. In **hoofdstuk 4** worden de experimenten beschreven waarbij de bijdrage van verschillende elektronentransportroutes is onderzocht. Hier kwam als belangrijkste bevinding uit dat zuurstof de voornaamste elektronenacceptor is die in *C. jejuni* bijdraagt aan de membraanpotentiaal en formiaat de belangrijkste elektronendonor. Aanwezigheid van één van deze stoffen is cruciaal voor snelle groei en bewegelijkheid van *C. jejuni*. Vervolgens hebben we ontdekt dat in de afwezigheid van zuurstof, de aanmaak van de enzymen die gebruikt worden om formiaat te gebruiken sterk wordt verhoogd. Deze aanpassing heeft als doel om de opbouw van de membraanpotentiaal te garanderen. De efficiënte groei en bewegelijkheid van de *C. jejuni* bacterie zijn essentieel voor de kolonisatie, pathogenese en competitie met de overige microbiota in zijn natuurlijke leefomgeving. Omdat deze factoren afhankelijk zijn van de membraanpotentiaal is dit een vruchtbaar onderwerp voor verder onderzoek naar de bestrijding van deze en andere bacteriën.

C. jejuni gebruikt hoofdzakelijk aminozuren als groeisubstraat en heeft een duidelijke voorkeur voor serine en aspartaat. Deze aminozuren en de tussenproducten komen via twee verschillende routes in het centraal metabolisme terecht. In **hoofdstuk 5** worden experimenten beschreven die laten zien dat *C. jejuni* zijn metabolisme aanpast aan de hand van het aanwezige groeisubstraat. Dit gebeurt niet aan de hand van de afzonderlijke substraten, maar wordt bepaald door welke van de twee voornaamste routes actief gebruikt wordt om substraten af te breken. Wanneer serine of andere substraten die via dezelfde route worden afgebroken aanwezig zijn, gaat de bacterie minder van de overige substraten gebruik maken. GGT is een sterk door metabolisme gereguleerd enzym dat helpt in het afbreken van het groeisubstraat glutamine. Aanwezigheid van serine leidt onder andere tot minder messenger-RNA (mRNA) en enzymactiviteit van GGT. Eén van de regulatie mechanismen die wij hebben ontdekt is dat ophoping van de centrale metaboliet succinaat in de cel, omgekeerd evenredig is met de aanmaak van het GGT enzym. De aanmaak van metabolische eiwitten wordt mogelijk dus gereguleerd door centrale metabolieten in de bacterie. Ophopingen van dergelijke metabolieten worden ook gebruikt door andere bacteriën om de activiteit van regulator eiwitten te bepalen. Tot

op heden is er echter nog geen regulatoreiwit gevonden dat aan de hand van succinaat, het metabolisme in *C. jejuni* reguleert.

Discussie en toekomst perspectief

Als laatste worden in **hoofdstuk 6** alle experimenten bediscussieerd en in een breder perspectief geplaatst. De implicaties voor het *Campylobacter* onderzoek worden besproken, net als de nieuwe vragen die zijn ontstaan naar aanleiding van dit onderzoek. Meerdere nog niet beantwoorde vragen zijn het bespreken waard. Ten eerste, heeft *C. jejuni* een zuurstof gevoelige regulator, en zo ja welk eiwit is dat dan. Experimenten uit dit proefschrift en uit meerdere laboratoria laten zien dat *C. jejuni* zich aanpast aan veranderende zuurstof concentraties. Deze effecten worden deels beïnvloed door het RacR/RacS regulator systeem, maar ook de genexpressie van genen buiten het RacR regulon worden beïnvloed door de zuurstof concentratie. Bovendien vindt er nog steeds zuurstofafhankelijke regulatie plaats in bacteriën waarin het *racR* gen is verwijderd. Meerdere pogingen zijn gedaan om deze zuurstofafhankelijke regulator te identificeren, maar tot nog toe zonder succes. Het is daarom goed mogelijk dat de waargenomen zuurstofafhankelijke regulatie niet wordt bewerkstelligd door een eiwit, maar door een ander mechanisme. Alternatieve mechanismen zoals DNA methylatie of RNA-RNA interactie zijn de moeite waard om te onderzoeken. Soortgelijke mechanismen zouden mogelijk ook de substraat-afhankelijke regulatie, zoals beschreven in hoofdstuk 5, kunnen verklaren. Een andere vraag die opduikt naar aanleiding van de hier beschreven experimenten is de aard van het signaal van het RacS eiwit. Alhoewel het RacR/RacS systeem actief wordt in de aanwezigheid van nitraat hebben experimenten aangetoond dat nitraat zelf niet activerend werkt. Het is daarom aannemelijk dat RacS fungeert als een redoxsensor of mogelijk de activiteit van de ademhalingsketen gebruikt om actief te worden.

Steeds meer onderzoek duidt op de belangrijke rol van het metabolisme in de pathogenese van bacteriën. Dit lijkt ook het geval te zijn voor *C. jejuni*. Het gedrag van de bacterie dat wij als pathogeen beschouwen, zou niets anders kunnen zijn dan een poging van de bacterie om aan zijn eigen energie behoefte te voldoen. Recentelijk zijn er onderzoeken verschenen waarbij is aangetoond dat virulentie van *Escherichia coli*, *Salmonella* en *Citrobacter* bacterie stammen, het immuunsysteem stimuleren waarna er voor deze bacteriën elektronenacceptoren werden geproduceerd. *C. jejuni* heeft ook behoefte aan elektronenacceptoren om te overleven. Tevens heeft *C. jejuni* de beschikking over een een sterk bewegelijkheid apparaat en is aangetoond dat het actief naar omgevingen zwemt waar de respiratie optimaal is. Dit kan een verklaring zijn waarom *C. jejuni* de zuurstofrijke darmwand binnendringt.

Mogelijke toekomstige manieren om *C. jejuni* en andere bacteriën te bestrijden kunnen worden gezocht in het maken van moleculen gericht tegen de regulatiesystemen van de bacterie (bijv. RacR/RacS, of de mogelijke succinaatgevoelige regulator). Door de werking van deze eiwitten te verhinderen, of door over-stimulatie kan kolonisatie van een gastheer worden voorkomen. Andere doelwitten zijn de enzymen uit de ademhalingsketen, die de membraanpotentiaal opbouwen. Wanneer deze enzymen niet meer werken kan de bacterie niet meer overleven. De werking van veel van deze enzymen

vereist specifieke cofactoren, vaak met een metaalion, zoals ijzer, koper, nikkel of de beschikbaarheid van selenium. Het weglaten of wegvangen van deze elementen uit het dieet, kan ook worden gebruikt als tactiek om kolonisatie van specifieke bacteriën tegen te gaan. Als laatste zou toekomstig onderzoek kunnen leiden tot het aanpassen van de microbiota met behulp van pro- of prebiotica. Dit kan dan gebruikt worden om de specifieke niche van *C. jejuni* te verkleinen, of op te vullen met andere competerende organismen. Alles bij elkaar genomen zijn er meerdere mogelijkheden om ongewenste bacteriën, zoals *C. jejuni* te bestrijden (zonder gebruik te maken van antibiotica). De nadruk ligt hierbij op het aanvallen van de metabolische- en regulatiemechanismen van deze bacterie, zodat deze niet meer in staat is om zich efficiënt te vermenigvuldigen.

CURRICULUM VITAE

Anne-Xander van der Stel was born on June 5th in Vlissingen, The Netherlands. He grew up in Haarlem, were he finished secondary education at Het Stedelijk Gymnasium Haarlem in 2006. Thereafter he started the bachelor program Life Science and Technology at both Delft University of Technology and Leiden University. During this time, an internship was performed to localize different proteins in *Streptomyces coelicolor* using immuno-fluorescence in the research group of Prof. dr. Gilles van Wezel. During his master internship, he studied the protein ligand interactions using isothermal titration calorimetry in the research group of Prof. dr. Marcellus Ubbink. After completing the master program Life Science and Technology at Leiden University with a specialication in biochemistry in 2011, Anne-Xander worked as a guest researcher at the VU University in Amsterdam under the supervision of Prof. dr. Jan Lankelma. This project concerned the metabolism of cancer cells, which was studied using low-cost analytical devices. In October 2012, he started his PhD research at Utrecht University under the supervision of Dr. Marc Wösten and Prof. dr. Jos van Putten in the Infection Biology research group on the regulation of metabolism and respiration in the pathogenic bacterium *Campylobacter jejuni*. The result of this research is presented in this thesis.

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