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Catabolite repression in *Campylobacter jejuni* correlates with intracellular succinate levels

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Summary

Bacteria have evolved different mechanisms to catabolize carbon sources from nutrient mixtures. 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 sources. In the presence of serine as well as lactate and pyruvate *C. jejuni* inhibits 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*, depended on intracellular succinate levels.

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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 conserve 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 (Hueck and Hillen, 1995; Brückner and Titgemeyer, 2002; Görke and Stülke, 2008; Rojo, 2010). To maximize growth rates, bacteria can employ energy inefficient pathways (Valgepea *et al.*, 2010; Peebo *et al.*, 2015). 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* favours 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 (Nam *et al.*, 2005; Sauer and Eikmanns, 2005; Kochanowski *et al.*, 2013). 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 (Jiang

and Ninfa, 2009; You *et al*., 2013; Chubukov *et al*., 2014; Millard *et al*., 2017).

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 (Leach *et al*., 1997; Guccione *et al*., 2008). *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 (Velayudhan *et al*., 2004; Thomas *et al*., 2011). 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* (Pérez-Rueda and Collado-Vides, 2000), the *C. jejuni* genome encodes a small number of regulators; ten response regulators and less than 20 additional transcriptional regulators from different protein families (Parkhill *et al*., 2000). Because of its atypical 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 (Wright *et al*., 2009; Line *et al*., 2010). 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 and oxygen availability as cues to adjust its carbon metabolism (Luethy *et al*., 2017; Guccione *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 with *C. jejuni* strains 81116, 11168 and 81176. Hereto cultures were grown in Heart 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 and Supporting Information Fig. S1A). At different time points, the bacterial cell suspensions of strain 81116 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 h of growth). The amount of TTC reduction was different for each carbon source tested. For instance, serine and pyruvate led to more TTC reduction than aspartate and glutamine (Fig. 1 and Supporting Information Fig. S2), indicating a higher utilization rate. Supplementation of the medium with serine led to a decrease of the utilization of most carbon sources at exponential growth phase compared to the growth conditions without serine in all *C. jejuni* strains tested (Fig. 1, Supporting Information Figs S1B and S2). However, at the 15-h time-point an increase was measured in the utilization capacity of the carbon sources aspartate, glutamine, fumarate, asparagine, glutamate and proline (Fig. 1E and F and Supporting Information Fig. S2), 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.

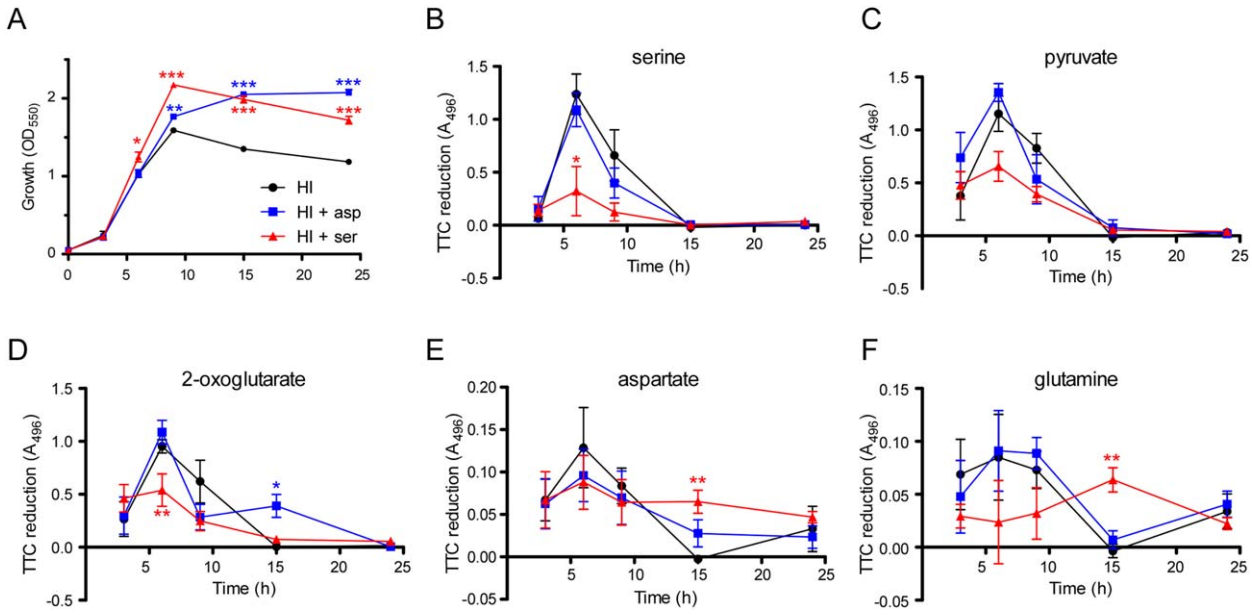

Fig. 1. Carbon source utilization of *C. jejuni*. TTC reduction was measured during microaerophilic batch cultures in HI-medium (black circles) or with the addition of 25 mM serine (red triangles) or aspartate (blue squares).
A. Growth of the batch cultures during a 24-hour period.
B–F. Carbon source 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 h) or early stationary (12 h) 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 (Fig. 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 harbour genes required for methionine biosynthesis. The first operon encodes MetC, which converts cystathionine into homocysteine. The second operon harbours 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.

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*,

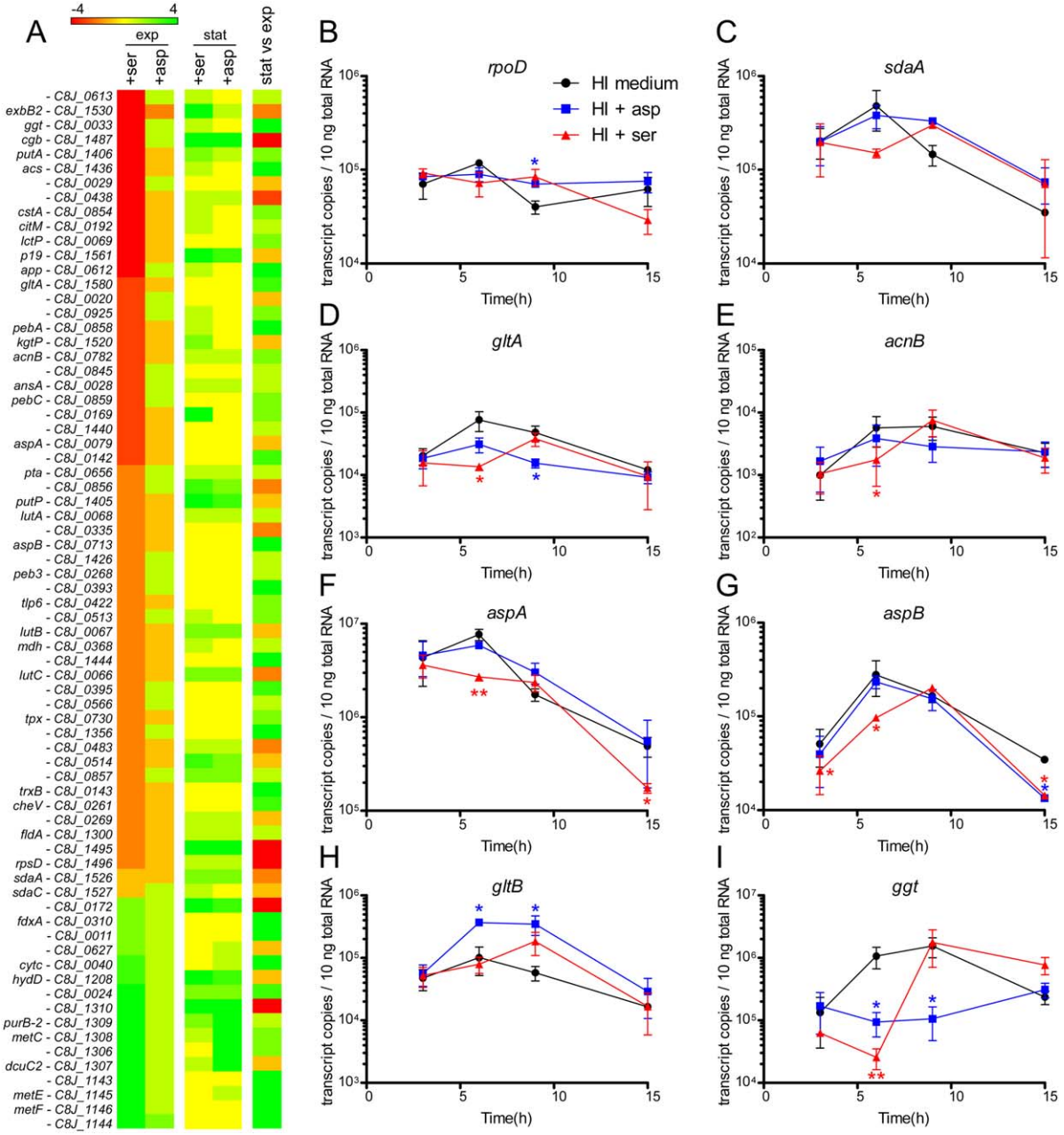

Fig. 2. Transcriptional regulation of *C. jejuni* by carbon sources. RNA seq analysis was performed on bacteria grown in microaerophilic batch-culture in HI-medium with or without the addition of 25 mM serine or aspartate.

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 versus exp). The displayed genes were selected by filtering for greater than twofold 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. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

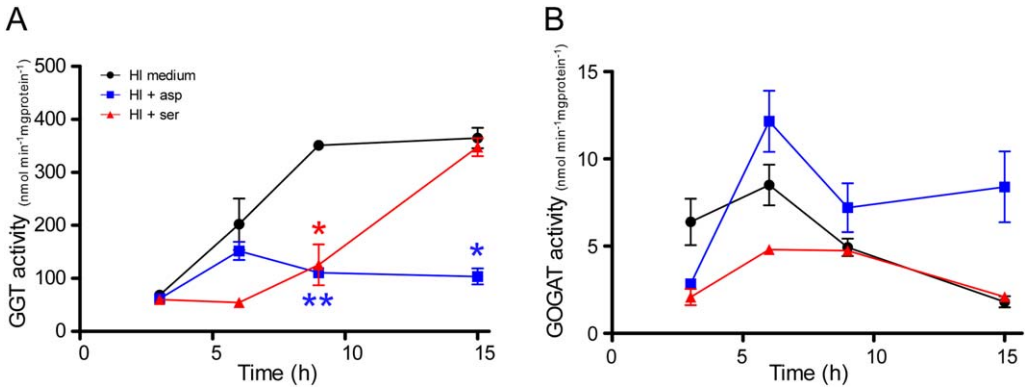

Fig. 3. Enzyme activities of GGT and GOGAT in *C. jejuni* in batch cultures.
A. GGT activity of *C. jejuni* grown in microaerophilic batch cultures in HI-medium (black circles) or with the addition of 25 mM serine (red triangles) or aspartate (blue squares), harvested at different time points.
B. GOGAT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

acnB, *ansA*, *pebC* and *aspB*. Hereto RNA was harvested from *C. jejuni* cultures grown for 3, 6, 9 and 15 h 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 Supporting Information Fig. S3). 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 h). The central metabolic genes *gltA* (sixfold), *acnB* (threefold) and *acs* (12-fold) were downregulated as well as the amino acid catabolism genes *aspA* (threefold), *putP* (10-fold), *ggt* (42-fold), *aspB* (threefold) and *sdaA* (threefold). At the early stationary growth phase (9 h), 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; threefold), *putP* (9 h; fivefold) and *sucD* (9, 15 h; fourfold) 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 and Supporting Information Fig. S3). The transcriptional data confirm that metabolic regulation, as observed in the phenotypic TTC assays, takes (at least partially) 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 and Supporting Information Fig. S3). 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 h. 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.

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

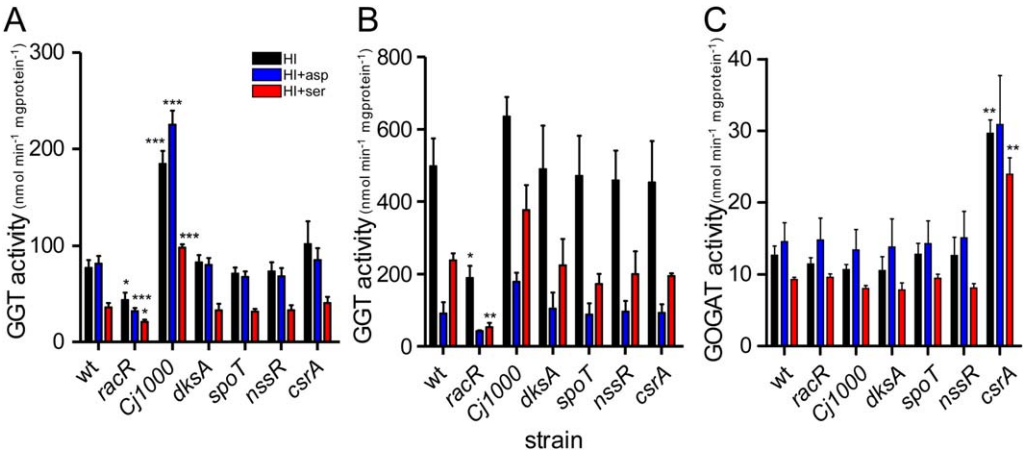

Fig. 4. Metabolic enzyme activity of *C. jejuni* wt and metabolic regulator mutant strains. *C. jejuni* was grown in microaerophilic HI medium (black bars) or with the addition of 25 mM aspartate (blue bars) or serine (red bars).
A and **B**. GGT activity of *C. jejuni* strains in exponentially (6 h)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

GGT activity (Fig. 4A). The *cj1000* mutant strain showed higher GGT activity under all conditions, but most strongly in the exponential growth phase (6 h). The *racR* mutant strain had lower GGT activity than the wt under all conditions (Fig. 4A and 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 and 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*.

Metabolic gene expression is regulated by intracellular cues

Bacteria can sense and change their metabolism in response to the available carbon sources inside 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 and 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 and 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

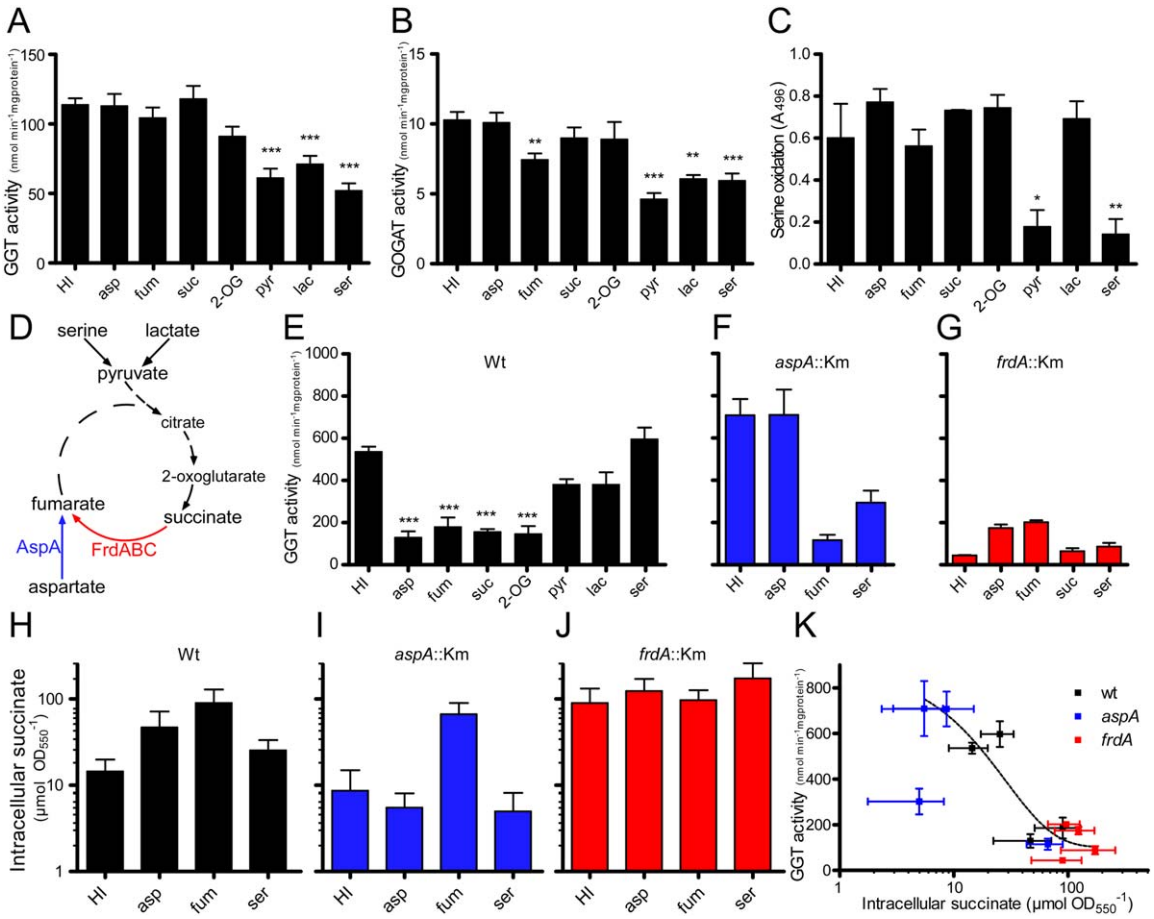

Fig. 5. Metabolic enzyme activity correlates with intracellular succinate.
A–**C**. GGT activity (A), GOGAT activity (B), and serine oxidation (determined by TTC-coupled reduction) (C), in exponentially growing *C. jejuni* (6 h batch culture) grown in HI medium or with the addition of 25 mM carbon source.
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. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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 (Supporting Information Fig. S3). 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 correlates with C. jejuni *metabolic regulation*

To identify the intracellular metabolite(s) to which *C. jejuni* adapts its carbon metabolism, we measured their concentrations in 6-h old cultures of the wt and the *aspA* and *frdA* mutant strains. The concentration of several intracellular

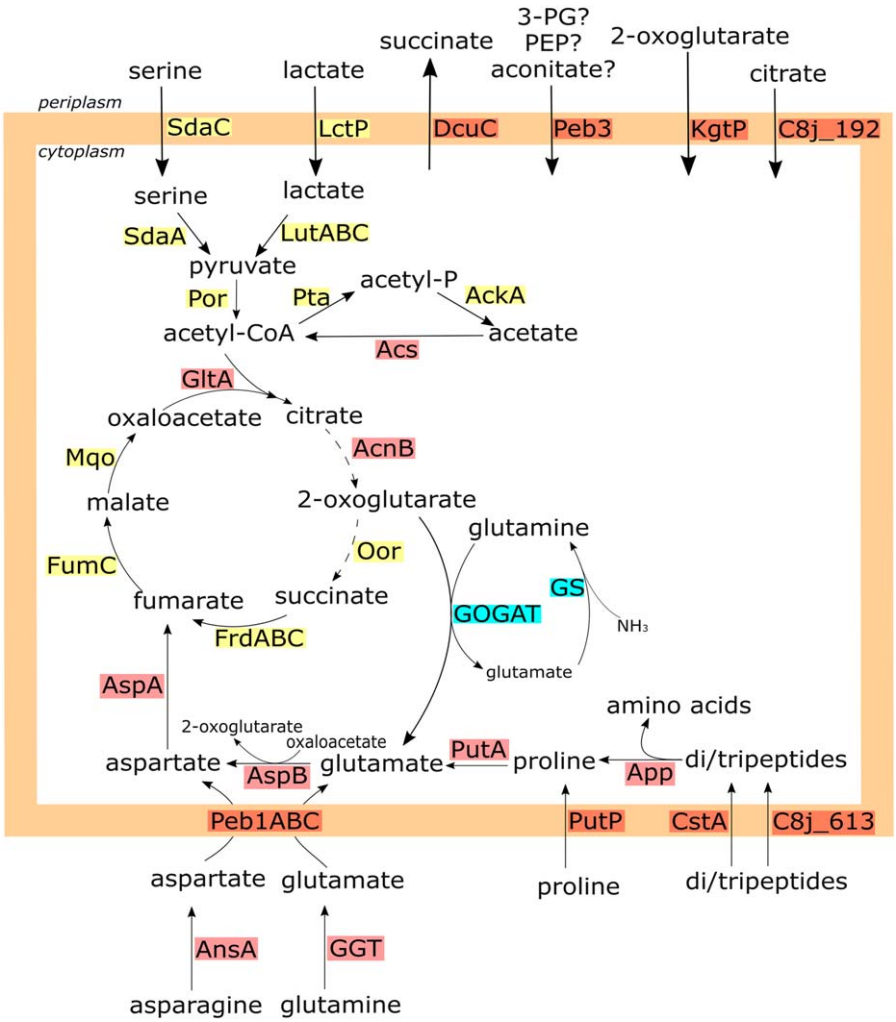

Fig. 6. Model of central metabolic regulation in *C. jejuni*. Upon the presence of serine, lactate or pyruvate, a metabolic down-shift takes place; enzymes and transporters shaded in red are down-regulated, while yellow shaded proteins are not differential regulated. Blue shaded indicate enzymes, which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

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 (Supporting Information Fig. S5). Addition of serine to the medium, which had no influence on the GGT activity, increased intracellular 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 (Supporting Information Fig. S5) (Howlett *et al.*, 2014). Only when fumarate was added to the medium a high intracellular 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 GGT activity low (Fig. 5G and 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.

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.

A first indication of regulation of metabolism in *C. jejuni* was the increase of the growth rate of several C. *jejuni* strains when grown in media with addition of serine but not addition of aspartate (Fig. 1A, Supporting Information Fig. S1). 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 and Supporting Information Fig. S1) and by a lower expression of metabolic genes (Fig. 2 and Supporting Information Fig. S3). 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 and Supporting Information Fig. 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 (Supporting Information Fig. S2). 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* (Figs 1 and 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. Therefore, 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 (Supporting Information Fig. S1). 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 and 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 and Supporting Information Fig. S2). 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*. Although not all strains harbor the *ggt* gene (de Haan *et al.*, 2012), it is likely that this global regulatory mechanism is present in all *C. jejuni* strains. The adaptation mechanism regulates not only GGT but a large number of genes. Moreover, the preference for serine as carbon source and serine dependent regulation is conserved in *ggt* and non *ggt* containing strains (Supporting Information Fig. S1). On the basis of our results a scenario is unfolding 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 (Figs (1 and 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). Comparable transcriptional adaptation has been described for many organisms indicating that such a mechanism acts sufficiently fast in order to compete with other micro-organisms (Covert *et al.*, 2001; Barua *et al.*, 2010; Rijsewijk *et al.*, 2011; Chubukov *et al.*, 2014). 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 (Sonnleitner *et al.*, 2009; 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).

Recently studies were published, which showed that extracellular acetate, butyrate and propionate, and oxygen availability are used as cues by *C. jejuni*. These cues regulate the central metabolic pathways and could help the bacterium to determine its spatial localization in the host (Guccione *et al.*, 2017; Luethy *et al.*, 2017). Our results indicate a mechanism via which the central metabolism is 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 exact mechanisms of metabolic awareness of *C. jejuni* are not clarified yet. Multiple signals could potentially lead to the activation or repression of the same regulatory factors. 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. Although the similarity between the transcriptional and functional results suggest transcriptional regulation, other regulatory events cannot be excluded at this point. It has been shown that post-transcriptional and post-translational regulation can have major effects on gene regulation (Waters and Storz, 2009; Commichau and Stülke, 2015; Seip and Innis, 2016). Moreover, *C. jejuni* transcribes a high number of asRNA (Dugar *et al.*, 2013; Porcelli *et al.*, 2013), which likely have regulatory functions and will a fruitful subject of future studies.

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.

Experimental procedures

General growth conditions

Strains and plasmids used in this study are listed in Supporting Information Table S2. *Campylobacter jejuni* strains 81116, 81–176 and 11168 and derivatives were grown on saponin plates (Biotrading, Mijdrecht, The Netherlands) supplemented (when appropriate) with the antibiotics chloramphenicol (20 μg ml^{-1}) or kanamycin (50 μg ml^{-1}), 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°C.

Mutagenesis

To disrupt the genes encoding regulatory or metabolic proteins in *C. jejuni* strain 81116 (Supporting Information 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 (Supporting Information 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 (Supporting Information 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* strains 81116, 11168 and 81176 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 h under microaerobic conditions at 42°C, 160 rpm. 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 μl) was mixed with 15 μl of 1% TTC and 15 μl 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).

RNA-seq

To identify genes that are differentially expressed upon addition of carbon source, *C. jejuni* strain 81116 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). Briefly, the total RNA for each sample was rRNA depleted using the Ribozero Magnetic Kit for-Gram negative bacteria (Illumina) following option 1 manufacturer's instructions. Libraries for the Illumina MiSeq 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 min at 85°C. Standard desalted TruSeq LT primers (Integrated DNA Technologies, Coralville, IA) were used at 50–100 nM final concentration based on starting RNA amount. The PCR step was reduced to six cycles. Libraries were quantified using the KAPA Library Quantification Kit (Kapa), except with 10 μl volume and 90 s 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 2 × 76 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. The fastq sequences were trimmed to remove poor quality bases and assembled to reference genome CP000814 using Bowtie within Geneious 10.1. Geneious software was used to calculate the normalized transcripts per million (TPM) and to compare expression levels between the control growth conditions.

RT-qPCR

Precultures of *C. jejuni* strain 81116 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 (Supporting Information 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 h. The pellet was resuspended in 250 μl of 50 mM Tris/HCl (pH 7.6) and 1 μg ml^{-1} of lysozyme, and incubated for 30 min on ice. The bacteria were disrupted by sonication. After centrifugation (10 min at 12 000 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, 5000 × *g* at −9°C and washed in 1 ml of ice-cold saline (0.9% NaCl). After centrifugation for 1 min, 12 000 × *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 four 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^{-1}. 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). The mass scanning range was m/z 50–500, at an orbitrap resolution of 120 k, 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 MS^2 spectra were recorded of the five 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.

Accession numbers

Gene expression ratios from the RNA-seq experiments are deposited in the GEO database under accession number GSE104231.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Serine enables the *C. jejuni* strains 81116 (ggt^+), 11168 (ggt^-) and 81-176 (ggt^+) to growth faster by causing a metabolic downshift during the exponential growth phase. **A**. Growth curves of the *C.jejuni* strains were generated in HI medium with or without supplementation of 25 mM serine or aspartate and 1mM methionine in a 96-wells plate (175 μl well^{-1}) (Costar) using a Synergy HTX multi-mode reader (Biotek, Winooski), placed inside a hypoxic chamber (Coy labs, Grass Lake) set to 10% CO_2 and 10% O_2, balanced with N_2The 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 25 h. For clarity reasons not all measured time point are shown. The results of three independent experiments are presented as mean ± SEMs.**B**. Serine oxidation as determined by TTC reduction. *C. jejuni* strains were grown for 6 hours in HI medium or in HI medium with 25 mM serine, harvested and subjected to the TTC assay to determine the serine oxidation capacity. The results of three independent experiments are presented as mean ± SD.

Fig. S2. Carbon source utilization of *C. jejuni* 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. ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Fig. S3. 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. ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Fig. S4. GGT activity in the *dctA* mutant strain is not susceptible to aspartate and succinate addition. Bacteria were grown for 15 h 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. (*$p < 0.05$, **$p < 0.01$).

Fig. S5. Intracellular metabolite levels in *C. jejuni*. Bacteria (wt: black; *aspA*: blue; *frdA*: red) 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.

Table S2. Strains and plasmids used in this study