

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

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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 CO₂, NADH 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; Jausch 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_m nitrate/nitrite +430 mV) is preferred over TMAO

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(E_m TMAO/TMA +160 mV), which is preferred over fumarate (E_m 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 Cj0358 (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. Auto-phosphorylation 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.

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

RacS	-	+	+	+	+	+	+	+	+
RacR	+	-	+	+	+	+	+	+	+
Time (min)	16	0	0.25	0.5	1	2	4	8	16

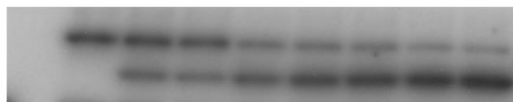


Fig. 1. Phosphate transfer between the cytoplasmic domain of the sensor RacS and its cognate regulator protein RacR. *In vitro* phosphate transfer assay between the recombinant truncated Histidine-tagged RacS and recombinant Histidine-tagged RacR protein was visualized using 12% SDS-polyacrylamide gels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

an antibiotic resistance cassette. Inactivation of *racR* in strain 81116 has previously been reported to result in a decreased growth rate at $\geq 42^{\circ}\text{C}$ 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).

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*).

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 *mfrXABE* 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*

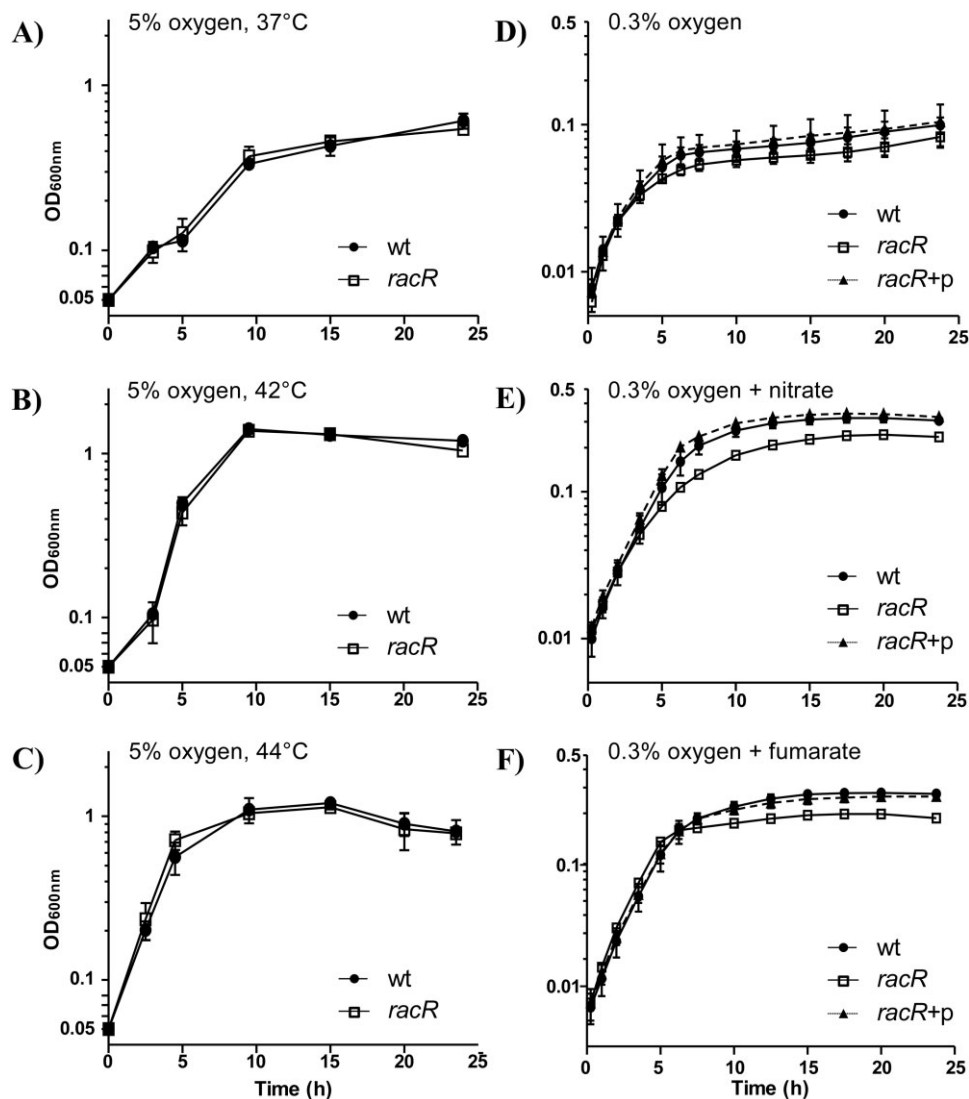


Fig. 2. Growth curves of *C. jejuni* strain 81116 (filled circle), the *racR* mutant (open square) and the *racR* mutant complemented with pMA1-1261-1263 (triangle up). Growth curves were generated in HI medium in a microaerophilic atmosphere (5% O₂) at:

A. 37°C.

B. 42°C.

C. 44°C.

D. Under oxygen-limited (0.3% O₂) conditions at 42°C.

E. With 50 mM NaNO₃.

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 \pm SD.

(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 *c₅₅₁* 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

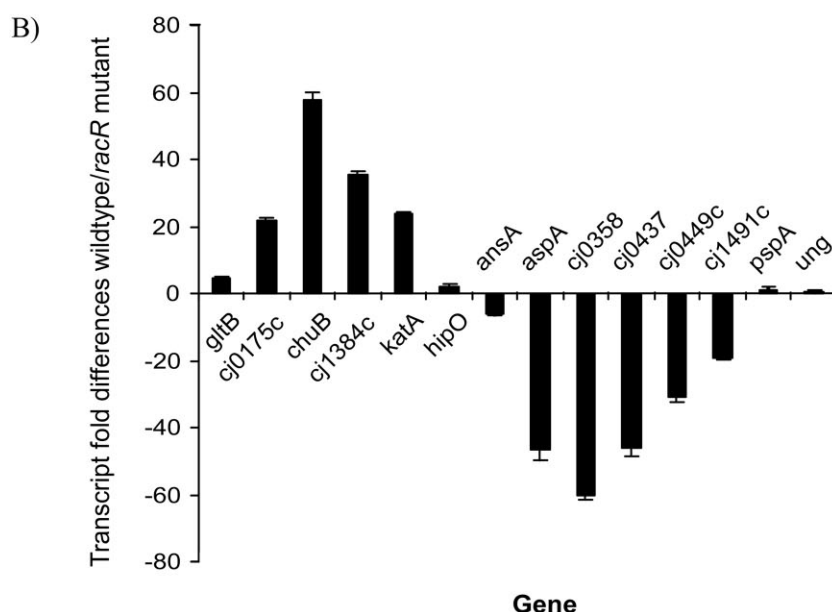
To investigate whether RacR is able to directly bind to DNA promoter regions, electrophoretic mobility shift assays (EMSA) were performed. Recombinant RacR or

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

Fig. 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 NaNO₃ 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.



phosphorylated RacR (accomplished by the addition of RacScto and ATP) was incubated with [γ -³²P]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 ³²P-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

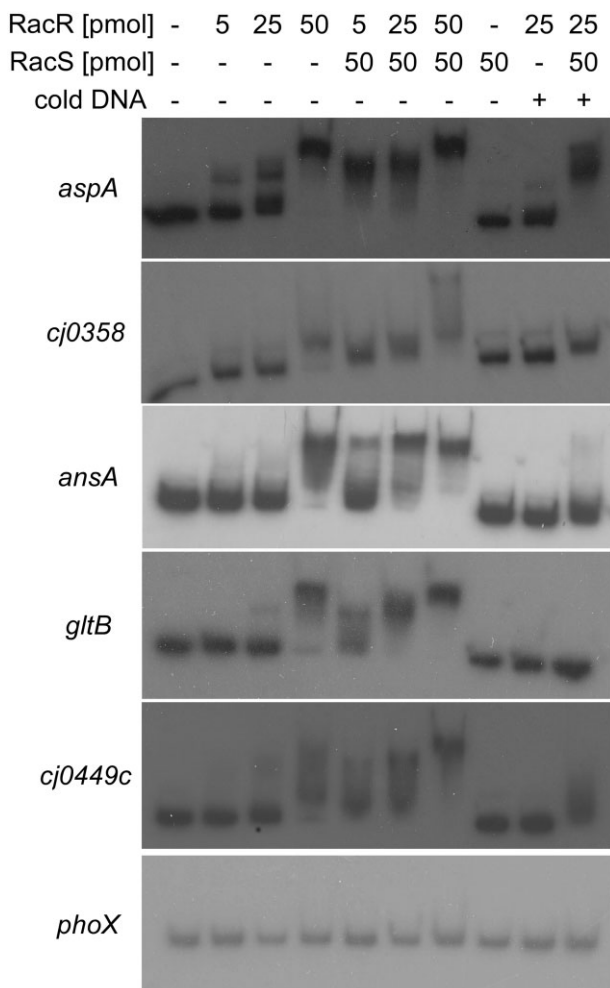


Fig. 4. Identification of genes directly regulated by RacR. Electrophoretic mobility shift assays showing that recombinant Histidine-tagged RacR binds to the [γ - 32 P]ATP labelled promoter regions (~25 pmol) upstream the *gltB*, *aspA*, *ansA*, *cj0358* and the *cj0449c* promoter regions but not to 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.

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.

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.

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 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.

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

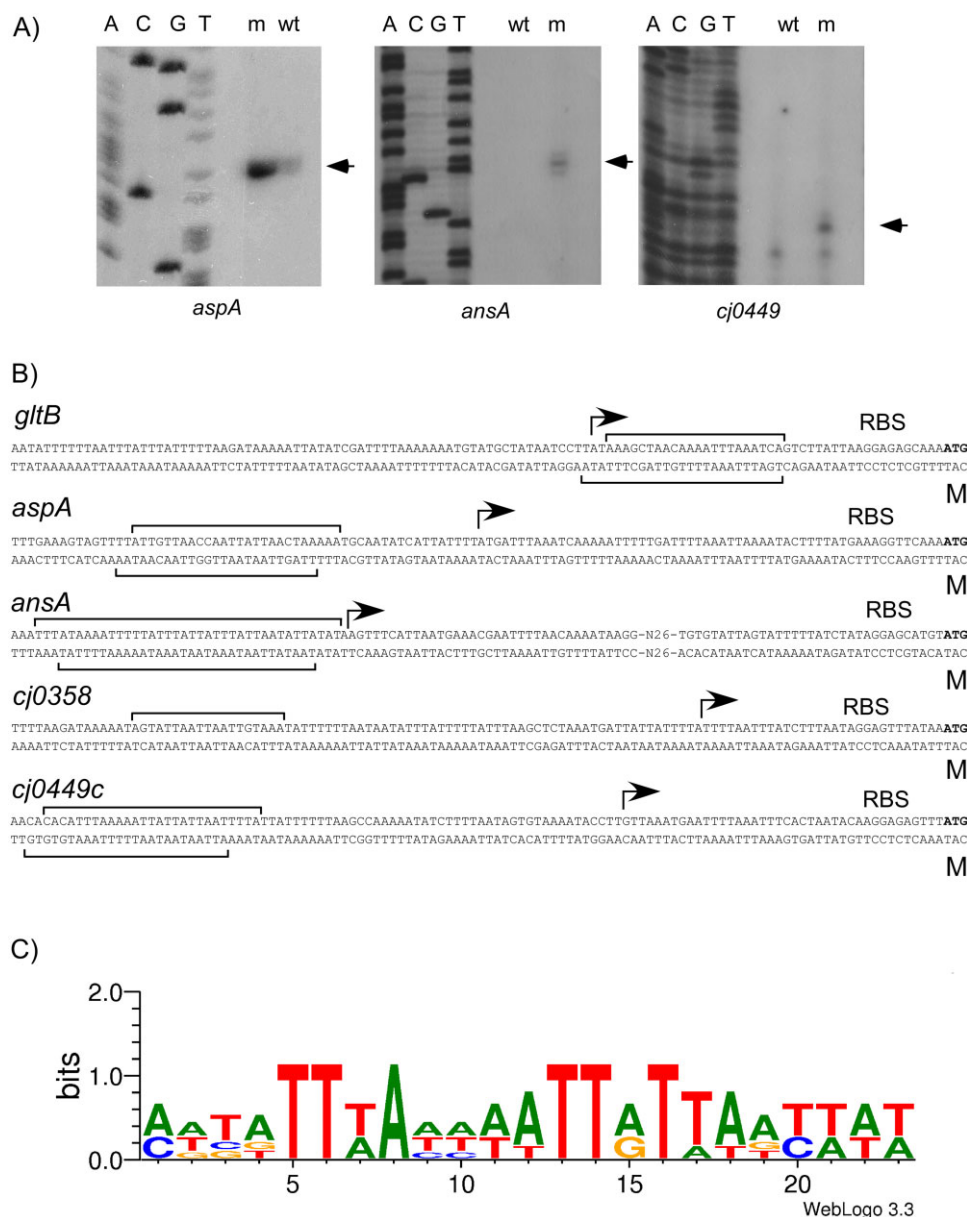


Fig. 5. Characterization of the promoter regions directly regulated by RacR.

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.

investigated whether the *racR* mutant is able to grow in media with fumarate as sole carbon source. For this purpose we used the defined media 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

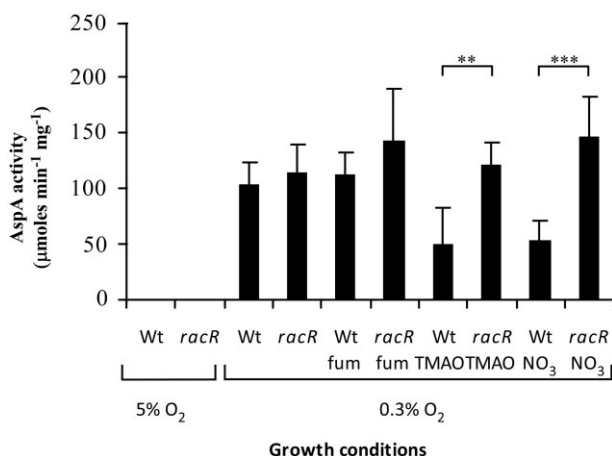


Fig. 6. Influence of the *RacR* on AspA activity. AspA enzyme activity was measured of strains grown in DM under high (10%) or low (0.3%) oxygen conditions with aspartate as main carbon source and with or without 50 mM nitrate, 50 mM TMAO or 5 mM fumarate as alternative electron acceptors. Aspartase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

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% O₂) 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 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% O₂) conditions for 24 h and then incubated for another 8 h under standard microaerobic (5% O₂) 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

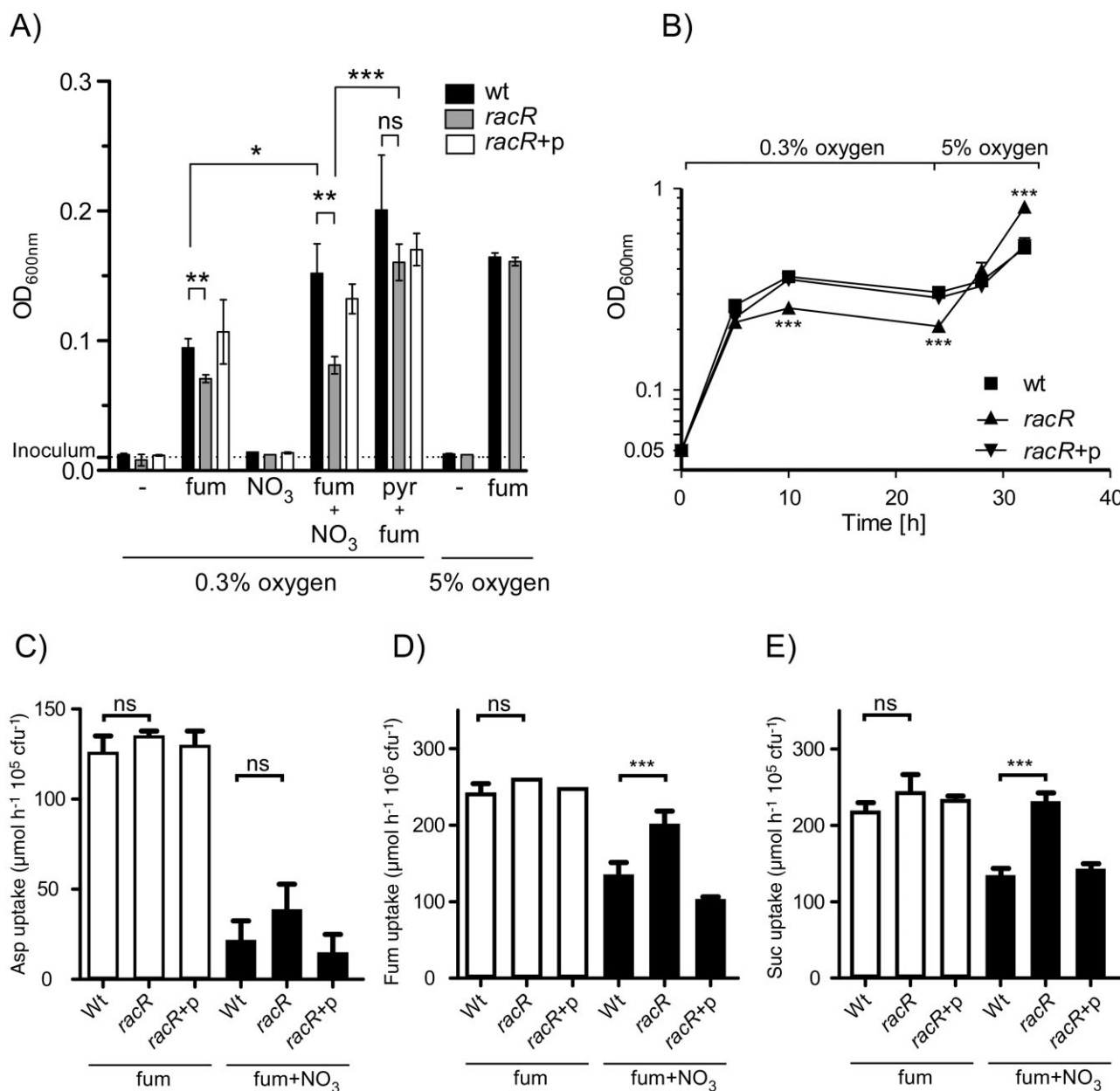


Fig. 7. The *racR* mutant is less able to make optimal use of nutrients under reduced oxygen concentrations in the presence of nitrate.

A. Final optical density OD_{600nm} 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% O₂) or oxygen-limited (0.3% O₂) 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⁻¹ 10⁵ cfu⁻¹).

D. Fumarate consumption (μmol h⁻¹ 10⁵ cfu⁻¹).

E. Succinate secretion (μmol h⁻¹ 10⁵ 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).

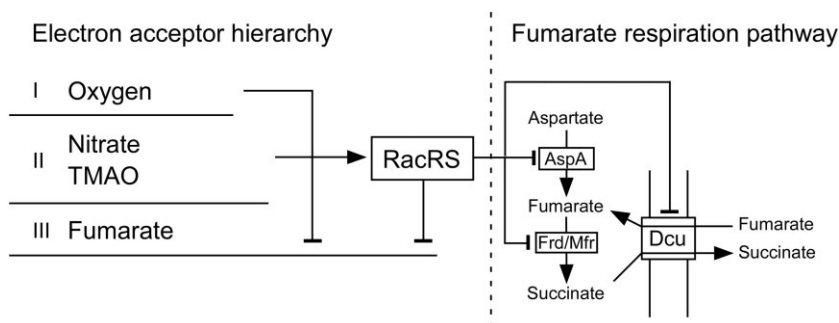


Fig. 8. Role of the RacR/RacS system in *C. jejuni* strain 81116. The RacR/RacS system represses the use of fumarate as electron acceptor when more favorable electron acceptors (nitrate or TMAO) are available under oxygen-limited conditions. This is accomplished by repressing the aspartate–succinate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

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 Cj81116 (Fig. 2B and C), Cj11168 or Cj81-176 (data not shown). These different phenotypic results may be due to temperature-dependent oxygen solubility, the use of other lab strains of Cj81116 and Cj81176, the design of *racR* mutation, microaerobic conditions (with or without H₂) or slight differences in growth conditions (media/vessels/volume) 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.

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 *cj0175c-cj0176c*, *chuABCD* and *fldA-cj1384c* encode respectively for a Fe³⁺- and heme 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 (Figs 4 and 5). A single retarded DNA–RacR complex was detected indicating that these promoters 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/Per 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_m +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_m +430 mV) and TMAO (E_m +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 hierarchical 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 anaerobiosis 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.

Experimental procedures

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% O₂, 10% CO₂, 10% H₂ and 75% N₂) 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% O₂, 10% CO₂, 10% H₂ and 75% N₂), overnight cultures (O/N) of *Campylobacter* grown in HI were diluted to OD₆₀₀ of 0.05 in fresh 5 ml HI media in vertical standing 25 cm² 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% O₂, 10% CO₂, 10% H₂, and 79.7% N₂) 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 OD₆₀₀ 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% O₂, 10% CO₂, 10% H₂ and 79.7% N₂) at 42°C were generated in jars using the Anoxomat system (MART Microbiology BV). Overnight cultures in DM were diluted to OD₆₀₀ 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 OD₆₀₀ 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 histidine-tagged RacS (50 pmol) was performed at room temperature (RT) for 15 min in the presence of 10 μ Ci [γ -³²P]ATP (MP Biomedicals, The Netherlands) and 100 μ l of phosphorylation buffer (50 mM Tris-HCl, 75 mM KCl, 2 mM MgCl₂, 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 μ l 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 NaNO₃ 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 ($2^{-\Delta\Delta C_t}$) (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 -³²P]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-H6 for 20 min at RT in binding buffer containing 20 mM Tris, pH 7.4, 5 mM MgCl₂, 50 mM KCl, 2 mM ATP, 50 μ g ml⁻¹ of bovine serum albumin, 10 μ g ml⁻¹ of poly-(dl-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 μ l 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 μ l 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 -³²P]ATP labelled primers. A total of 5 pmol of primer *aspARpromprex*, *ansA-R* or *Cj0449c* (Supporting Information Table S2) was annealed to 20 μ g of RNA extracted from *Campylobacter* grown in defined medium with 0.08 mM [P_i]. 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 *aspARpromprex*, *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 \times 2.0 mm, particle size of 4 μ m) 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 μ A 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 OD₆₀₀ 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 (OD₆₀₀ 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 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$.

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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References

- Aguilar, P.S., Hernandez-Arriaga, A.M., Cybulski, L.E., Erazo, A.C., and de Mendoza, D. (2001) Molecular basis of thermosensing: a two-component signal transduction thermometer in *Bacillus subtilis*. *EMBO J* **20**: 1681–1691.
- Apel, D., Ellermeier, J., Pryjma, M., Dirita, V.J., and Gaynor, E.C. (2012) Characterization of *Campylobacter jejuni* RacRS reveals roles in the heat shock response, motility, and maintenance of cell length homogeneity. *J Bacteriol* **194**: 2342–2354.
- Bekker, M., Alexeeva, S., Laan, W., Sawers, G., Teixeira de Mattos, J., and Hellingwerf, K. (2010) The ArcBA two-component system of *Escherichia coli* is regulated by the redox state of both the ubiquinone and the menaquinone pool. *J Bacteriol* **192**: 746–754.
- Bezy, R.P., and Kehoe, D.M. (2010) Functional characterization of a cyanobacterial OmpR/PhoB class transcription factor binding site controlling light color responses. *J Bacteriol* **192**: 5923–5933.
- Bordi, C., Ansaldi, M., Gon, S., Jourlin-Castelli, C., Iobbi-Nivol, C., and Mejean, V. (2004) Genes regulated by TorR, the trimethylamine oxide response regulator of *Shewanella oneidensis*. *J Bacteriol* **186**: 4502–4509.
- Bott, M. (2007) Offering surprises: TCA cycle regulation in *Corynebacterium glutamicum*. *Trends Microbiol* **15**: 417–425.
- Bras, A.M., Chatterjee, S., Wren, B.W., Newell, D.G., and Ketley, J.M. (1999) A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonization. *J Bacteriol* **181**: 3298–3302.
- Braun, Y., Smirnova, A.V., Weingart, H., Schenk, A., and Ullrich, M.S. (2007) A temperature-sensing histidine kinase: function, genetics, and membrane topology. *Methods Enzymol* **423**: 222–249.
- Cho, H.S., Pelton, J.G., Yan, D., Kustu, S., and Wemmer, D.E. (2001) Phosphoaspartates in bacterial signal transduction. *Curr Opin Struct Biol* **11**: 679–684.
- Dugar, G., Herbig, A., Forstner, K.U., Heidrich, N., Reinhardt, R., Nieselt, K., and Sharma, C.M. (2013) High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. *PLoS Genet* **9**: e1003495.
- Everiss, K.D., Hughes, K.J., Kovach, M.E., and Peterson, K.M. (1994) The *Vibrio cholerae* *acfB* colonization determinant encodes an inner membrane protein that is related to a family of signal-transducing proteins. *Infect Immun* **62**: 3289–3298.
- Gaynor, E.C., Cawthraw, S., Manning, G., MacKichan, J.K., Falkow, S., and Newell, D.G. (2004) The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. *J Bacteriol* **186**: 503–517.
- Goh, E.B., Bledsoe, P.J., Chen, L.L., Gyaneshwar, P., Stewart, V., and Igo, M.M. (2005) Hierarchical control of anaerobic gene expression in *Escherichia coli* K-12: the nitrate-responsive NarX-NarL regulatory system represses synthesis of the fumarate-responsive DcuS-DcuR regulatory system. *J Bacteriol* **187**: 4890–4899.
- Golby, P., Davies, S., Kelly, D.J., Guest, J.R., and Andrews, S.C. (1999) Identification and characterization of a two-component sensor-kinase and response-regulator system (DcuS-DcuR) controlling gene expression in response to C4-dicarboxylates in *Escherichia coli*. *J Bacteriol* **181**: 1238–1248.
- Goransson, M., Sonden, B., Nilsson, P., Dagberg, B., Forsman, K., Emanuelsson, K., and Uhlin, B.E. (1990) Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli*. *Nature* **344**: 682–685.
- Gripp, E., Hlahla, D., Didelot, X., Kops, F., Maurischat, S., Tedin, K., *et al.* (2011) Closely related *Campylobacter jejuni* strains from different sources reveal a generalist rather than a specialist lifestyle. *BMC Genomics* **12**: 584–2164–12-584.

- Guccione, E., Leon-Kempis Mdel, R., Pearson, B.M., Hitchin, E., Mulholland, F., van Diemen, P.M., *et al.* (2008) Amino acid-dependent growth of *Campylobacter jejuni*: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate. *Mol Microbiol* **69**: 77–93.
- Guccione, E., Hitchcock, A., Hall, S.J., Mulholland, F., Shearer, N., van Vliet, A.H., and Kelly, D.J. (2010) Reduction of fumarate, mesaconate and crotonate by Mfr, a novel oxygen-regulated periplasmic reductase in *Campylobacter jejuni*. *Environ Microbiol* **12**: 576–591.
- Gunsalus, R.P., and Park, S.J. (1994) Aerobic-anaerobic gene regulation in *Escherichia coli*: control by the ArcAB and Fnr regulons. *Res Microbiol* **145**: 437–450.
- Hartley-Tassell, L.E., Shewell, L.K., Day, C.J., Wilson, J.C., Sandhu, R., Ketley, J.M., and Korolik, V. (2010) Identification and characterization of the aspartate chemosensory receptor of *Campylobacter jejuni*. *Mol Microbiol* **75**: 710–730.
- Hofreuter, D., Novik, V., and Galan, J.E. (2008) Metabolic diversity in *Campylobacter jejuni* enhances specific tissue colonization. *Cell Host Microbe* **4**: 425–433.
- Iuchi, S., and Lin, E.C. (1987) The *narL* gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamine N-oxide reductase operons in *Escherichia coli*. *Proc Natl Acad Sci USA* **84**: 3901–3905.
- Janausch, I.G., Zientz, E., Tran, Q.H., Kroger, A., and Udden, G. (2002) C4-dicarboxylate carriers and sensors in bacteria. *Biochim Biophys Acta* **1553**: 39–56.
- Kelly, D.J. (2008) Complexity and versatility in the physiology and metabolism of *Campylobacter jejuni*. In *Campylobacter*. Nachamkin, I., Szymanski, C.M., and Blaser, M.J. (eds). Washington, DC, USA: ASM Press, pp. 41–61.
- Kumar, R., and Shimizu, K. (2010) Metabolic regulation of *Escherichia coli* and its *gdhA*, *glnL*, *gltB*, *D* mutants under different carbon and nitrogen limitations in the continuous culture. *Microb Cell Fact* **9**: 8–2859-9-8.
- Labigne-Roussel, A., Harel, J., and Tompkins, L. (1987) Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. *J Bacteriol* **169**: 5320–5323.
- Leach, S., Harvey, P., and Wali, R. (1997) Changes with growth rate in the membrane lipid composition of and amino acid utilization by continuous cultures of *Campylobacter jejuni*. *J Appl Microbiol* **82**: 631–640.
- Levanon, S.S., San, K.Y., and Bennett, G.N. (2005) Effect of oxygen on the *Escherichia coli* ArcA and FNR regulation systems and metabolic responses. *Biotechnol Bioeng* **89**: 556–564.
- Liu, X., Gao, B., Novik, V., and Galan, J.E. (2012) Quantitative proteomics of intracellular *Campylobacter jejuni* reveals metabolic reprogramming. *PLoS Pathog* **8**: e1002562.
- Liu, Y.-W., Denkmann, K., Koscirov, N., Dahl, C., and Kelly, D.J. (2013) Tetrathionate stimulated growth of *Campylobacter jejuni* identifies a new type of bi-functional tetrathionate reductase that is widely distributed in bacteria. *Mol Microbiol* **88**: 173–188.
- MacKichan, J.K., Gaynor, E.C., Chang, C., Cawthraw, S., Newell, D.G., Miller, J.F., and Falkow, S. (2004) The *Campylobacter jejuni* *dccRS* two-component system is required for optimal *in vivo* colonization but is dispensable for *in vitro* growth. *Mol Microbiol* **54**: 1269–1286.
- Mascher, T., Helmann, J.D., and Udden, G. (2006) Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* **70**: 910–993.
- Moore, J.O., and Hendrickson, W.A. (2009) Structural analysis of sensor domains from the TMAO-responsive histidine kinase receptor TorS. *Structure* **17**: 1195–1204.
- van Mourik, A., Bleumink-Pluym, N.M., van Dijk, L., van Putten, J.P., and Wosten, M.M. (2008) Functional analysis of a *Campylobacter jejuni* alkaline phosphatase secreted via the Tat export machinery. *Microbiology* **154**: 584–592.
- Nishijyo, T., Haas, D., and Itoh, Y. (2001) The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. *Mol Microbiol* **40**: 917–931.
- Palmer, S.R., Gully, P.R., White, J.M., Pearson, A.D., Suckling, W.G., Jones, D.M., *et al.* (1983) Water-borne outbreak of *Campylobacter* gastroenteritis. *Lancet* **1**: 287–290.
- Palyada, K., Sun, Y.Q., Flint, A., Butcher, J., Naikare, H., and Stintzi, A. (2009) Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. *BMC Genomics* **10**: 481–2164-10-481.
- Parke, D. (1990) Construction of mobilizable vectors derived from plasmids RP4, pUC18 and pUC19. *Gene* **93**: 135–137.
- Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., *et al.* (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**: 665–668.
- Pearson, B.M., Gaskin, D.J., Segers, R.P., Wells, J.M., Nuijten, P.J., and van Vliet, A.H. (2007) The complete genome sequence of *Campylobacter jejuni* strain 81116 (NCTC11828). *J Bacteriol* **189**: 8402–8403.
- Pittman, M.S., Elvers, K.T., Lee, L., Jones, M.A., Poole, R.K., Park, S.F., and Kelly, D.J. (2007) Growth of *Campylobacter jejuni* on nitrate and nitrite: electron transport to NapA and NrfA via NrfH and distinct roles for NrfA and the globin Cgb in protection against nitrosative stress. *Mol Microbiol* **63**: 575–590.
- Reid, C.J., and Poole, P.S. (1998) Roles of DctA and DctB in signal detection by the dicarboxylic acid transport system of *Rhizobium leguminosarum*. *J Bacteriol* **180**: 2660–2669.
- Reitzer, L. (2003) Nitrogen assimilation and global regulation in *Escherichia coli*. *Annu Rev Microbiol* **57**: 155–176.
- Rohs, R., Jin, X., West, S.M., Joshi, R., Honig, B., and Mann, R.S. (2010) Origins of specificity in protein-DNA recognition. *Annu Rev Biochem* **79**: 233–269.
- Schmittgen, T.D. (2001) Real-time quantitative PCR. *Methods* **25**: 383–385.
- Schreiber, K., Krieger, R., Benkert, B., Eschbach, M., Arai, H., Schobert, M., and Jahn, D. (2007) The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J Bacteriol* **189**: 4310–4314.
- Sellers, M.J., Hall, S.J., and Kelly, D.J. (2002) Growth of *Campylobacter jejuni* supported by respiration of fumarate,

- nitrate, nitrite, trimethylamine-N-oxide, or dimethyl sulfoxide requires oxygen. *J Bacteriol* **184**: 4187–4196.
- Stahl, M., Friis, L.M., Nothhaft, H., Liu, X., Li, J., Szymanski, C.M., and Stintzi, A. (2011) L-fucose utilization provides *Campylobacter jejuni* with a competitive advantage. *Proc Natl Acad Sci USA* **108**: 7194–7199.
- Stewart, V., and Bledsoe, P.J. (2003) Synthetic *lac* operator substitutions for studying the nitrate- and nitrite-responsive NarX-NarL and NarQ-NarP two-component regulatory systems of *Escherichia coli* K-12. *J Bacteriol* **185**: 2104–2111.
- Stintzi, A., Marlow, D., Palyada, K., Naikare, H., Panciera, R., Whitworth, L., and Clarke, C. (2005) Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. *Infect Immun* **73**: 1797–1810.
- Tabor, S., and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82**: 1074–1078.
- Taveirne, M.E., Theriot, C.M., Livny, J., and Dirita, V.J. (2013) The complete *Campylobacter jejuni* transcriptome during colonization of a natural host determined by RNAseq. *PLoS ONE* **8**: e73586.
- Tolla, D.A., and Savageau, M.A. (2010) Regulation of aerobic-to-anaerobic transitions by the FNR cycle in *Escherichia coli*. *J Mol Biol* **397**: 893–905.
- Unden, G., and Bongaerts, J. (1997) Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. *Biochim Biophys Acta* **1320**: 217–234.
- Unden, G., and Schirawski, J. (1997) The oxygen-responsive transcriptional regulator FNR of *Escherichia coli*: the search for signals and reactions. *Mol Microbiol* **25**: 205–210.
- van Vliet, A.H., Wooldridge, K.G., and Ketley, J.M. (1998) Iron-responsive gene regulation in a *Campylobacter jejuni* *fur* mutant. *J Bacteriol* **180**: 5291–5298.
- Weerakoon, D.R., and Olson, J.W. (2008) The *Campylobacter jejuni* NADH : ubiquinone oxidoreductase (complex I) utilizes flavodoxin rather than NADH. *J Bacteriol* **190**: 915–925.
- Weingarten, R.A., Grimes, J.L., and Olson, J.W. (2008) Role of *Campylobacter jejuni* respiratory oxidases and reductases in host colonization. *Appl Environ Microbiol* **74**: 1367–1375.
- Weingarten, R.A., Taveirne, M.E., and Olson, J.W. (2009) The dual-functioning fumarate reductase is the sole succinate : quinone reductase in *Campylobacter jejuni* and is required for full host colonization. *J Bacteriol* **191**: 5293–5300.
- Woodall, C.A., Jones, M.A., Barrow, P.A., Hinds, J., Marsden, G.L., Kelly, D.J., et al. (2005) *Campylobacter jejuni* gene expression in the chick cecum: evidence for adaptation to a low-oxygen environment. *Infect Immun* **73**: 5278–5285.
- Wösten, M.M., Wagenaar, J.A., and van Putten, J.P. (2004) The FlgS/FlgR two-component signal transduction system regulates the *fla* regulon in *Campylobacter jejuni*. *J Biol Chem* **279**: 16214–16222.
- Wösten, M.M.S.M., Parker, C.T., van Mourik, A., Guilhabert, M.R., van Dijk, L., and van Putten, J.P.M. (2006) The *Campylobacter jejuni* PhosS/PhosR operon represents a non-classical phosphate-sensitive two-component system. *Mol Microbiol* **62**: 278–291.
- Wösten, M.M.S.M., van Mourik, A., and van Putten, J.P.M. (2008) Regulation of genes in *Campylobacter jejuni*. In *Campylobacter*. Nachamkin, I., Szymanski, C.M., and Blaser, M.J. (eds). Washington DC, USA: ASM Press, pp. 611–624.
- Zhou, Y.F., Nan, B., Nan, J., Ma, Q., Panjikar, S., Liang, Y.H., et al. (2008) C4-dicarboxylates sensing mechanism revealed by the crystal structures of DctB sensor domain. *J Mol Biol* **383**: 49–61.

Supporting information

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

Fig. S1. DNase I footprinting analysis of 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.

Table S2. Primer sequences used in this study.

Table S3. Microarray data showing genes with more-than-twofold higher transcript amounts in the *racR* mutant compared with Wt.

Table S4. Microarray data showing genes with more-than-twofold-higher transcript amounts in the Wt compared with *racR* mutant.