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

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Summary

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

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Introduction

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

Campylobacter jejuni is the leading cause of bacterial food-borne diarrhea in humans causing approximately 400 million cases each year worldwide. This microaerophilic organism is often present as a commensal in the oxygen-limiting environment of the gastro-intestinal tract of birds. *C. jejuni* utilizes amino acids and TCA-cycle intermediates as main carbon sources as it is unable to metabolize glucose due to the lack of the glycolytic enzymes glucokinase and 6-phosphofructokinase. *C. jejuni* possesses a branched electron transport chain with multiple redox-enzymes that utilize an array of molecules as electron donor or acceptor (Sellars *et al.*, 2002). Electrons of the carbon sources, pyruvate, succinate and malate are donated to the menaquinone (MK) pool, as this microaerophilic organism lacks ubiquinone, as is present in *Escherichia coli*. Pyruvate is oxidized to acetyl-CoA by pyruvate oxidoreductase (POR; *cj1476*), the electrons derived from pyruvate are subsequently donated to the NADH ubiquinone oxidoreductase (Nuo) complex, which in *C. jejuni* uses flavodoxin (FldA) and menaquinone instead of NADH and ubiquinone (Fig. 1A) (Hughes *et al.*, 1998; Maurice *et al.*, 2007; Weerakoon and Olson, 2008). The Nuo complex is predicted to be a proton pump (Smith *et al.*, 2000). Malate, via Malate:quinone oxidoreductase (Mqo; Cj0393), and

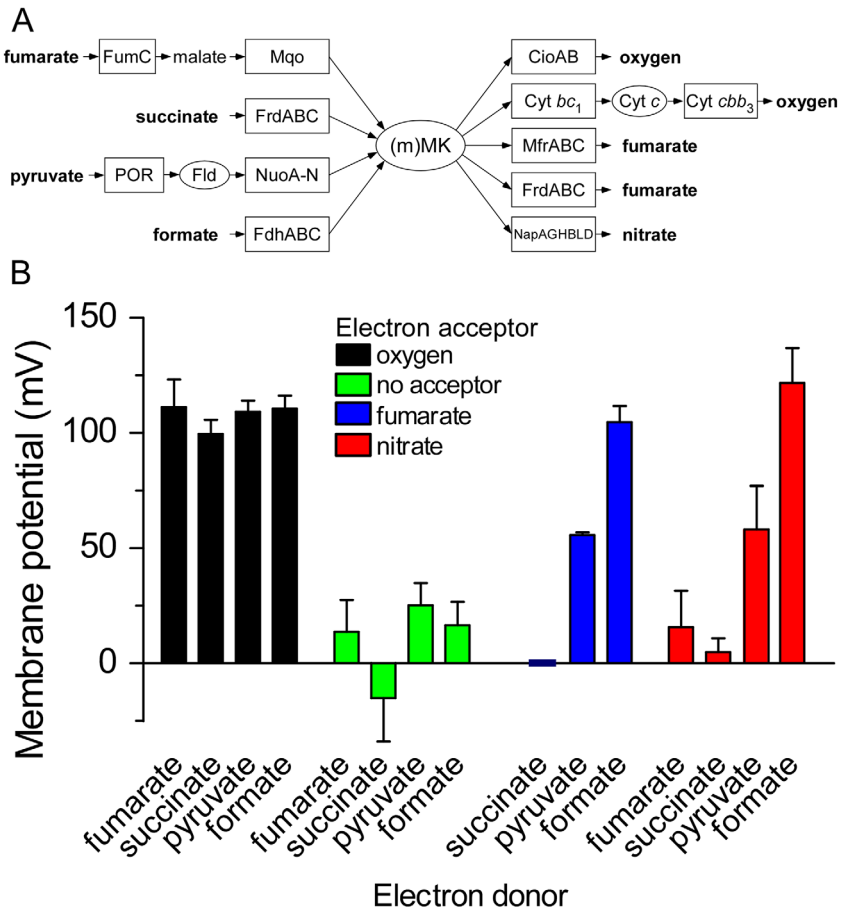

Fig. 1. Membrane potential ($\Delta\psi$) generated by *C. jejuni*.
A. Schematic model of the electron transport chain of *C. jejuni*, showing the possible combinations of the electron-donor and -acceptor complexes of the here studied compounds.
B. $\Delta\psi$ values measured in microaerobic grown *C.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

succinate, via Fumarate reductase (FrdABC), donate their electrons directly to the menaquinone pool (Fig. 1A). Besides these carbon sources, *C. jejuni* can use formate and hydrogen as electron donor. Formate and hydrogen are oxidized by the periplasmic formate dehydrogenase (*fdhABC*) and hydrogenase (*hydABC*) respectively (Weerakoon *et al.*, 2009).

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

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

Results

Generation of $\Delta\psi$ *in* C. jejuni

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

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

Association between respiration and $\Delta\psi$ *generation*

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

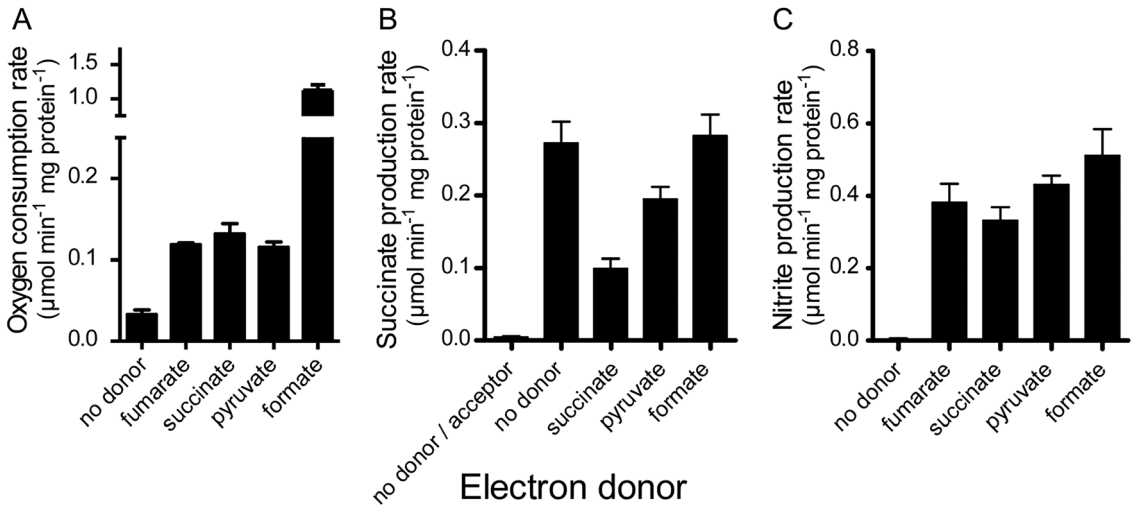

Fig. 2. Respiration rates of microaerobic grown *C. jejuni* in cell suspensions, obtained with different electron donors.
A. Oxygen respiration rate as determined with a Clark-type oxygen electrode. Oxygen consumption was measured in parallel with the $\Delta\psi$ measurements.
B. Fumarate respiration rates. Succinate accumulation was measured using LC/MS/MS.
C. Nitrate respiration rates. Nitrite accumulation was determined using the Griess-assay. Bars show the mean of three biologically independent experiments ± SEM.

of a $\Delta\psi$ (Figs 1B and 2). Altogether, the data indicate that although respiration is necessary for $\Delta\psi$ formation, respiration and the respiration rate are not obligatory correlated with the formation and amplitude of the $\Delta\psi$ respectively.

Motility is dependent on oxygen or formate

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

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

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

ATP is the most important energy carrier in organisms and drives a multitude of reactions inside the cell. In general, ATP can be generated either by oxidative phosphorylation where the pmf functions as the driving force or by substrate-level phosphorylation coupled to certain enzymatic reactions. Although *C. jejuni* harbors the enzyme AckA, which converts acetyl-P and ADP to acetate and ATP, so far no ATP generation independent of respiration has been described for *C. jejuni*. To study the influence of the respiration on ATP synthesis, the ATP levels were determined in bacterial cell suspensions incubated with different electron donor/acceptor combinations. Without an electron acceptor, ATP levels were low, except when pyruvate was present (Fig. 4). This indicates that ATP can be generated independent of the pmf. To investigate whether this was caused by

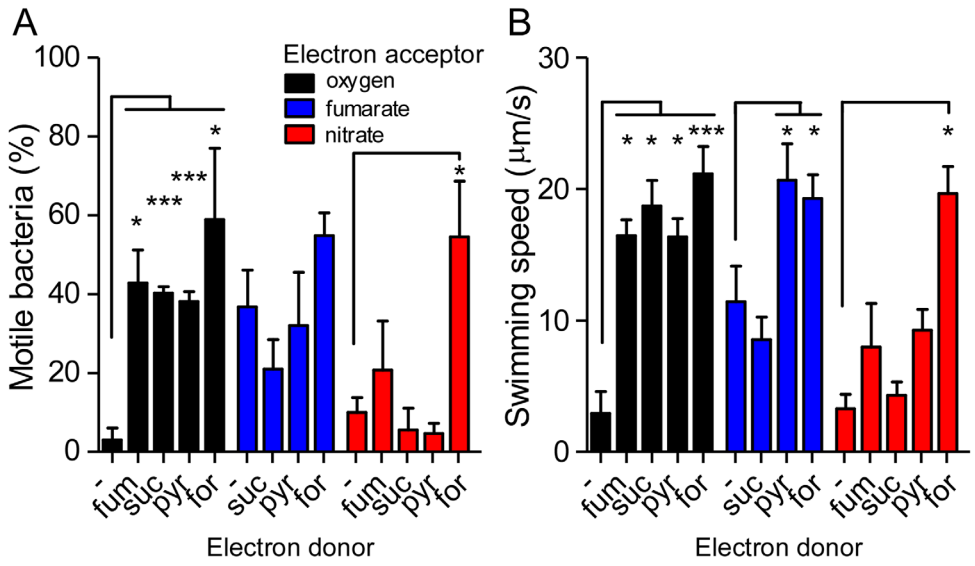

Fig. 3. The influence of electron acceptor/donor couple on the motility of *C. jejuni* wt bacteria. *C. jejuni* was grown in HI-medium under microaerobic conditions and subsequently diluted into HEPES buffer with or without an electron donor and acceptor and incubated for 30 min at 42°C before the motility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

the AckA enzyme we measured the ATP levels in an *ackA* mutant. ATP levels did not increase when this mutant was incubated with pyruvate in the absence of an electron acceptor, indicating that ATP in the wild type is produced via AckA (Supporting Information Fig. S3A). To ensure that the ATP generation was not caused by traces of oxygen, a *ccoN/cioA* double mutant was constructed, which is defective in both oxidases and therefore unable to utilize oxygen as electron acceptor. This double mutant strain produced small colonies and did not reach high densities in liquid cultures (HI-medium with formate and pyruvate and nitrate or fumarate under oxygen limitation). Also, no growth was observed under microaerobic conditions, similar as has been described previously (Weingarten *et al*., 2008). The ATP levels of this double mutant strain gave identical results as the parent strain (Supporting Information Fig. S3), confirming that *C. jejuni* is able to generate ATP by substrate-level phosphorylation. These data (Fig. 3 and Supporting Information Fig. S3) show that *C. jejuni* is able to generate ATP by both oxidative phosphorylation using the pmf and substrate-level phosphorylation via AckA.

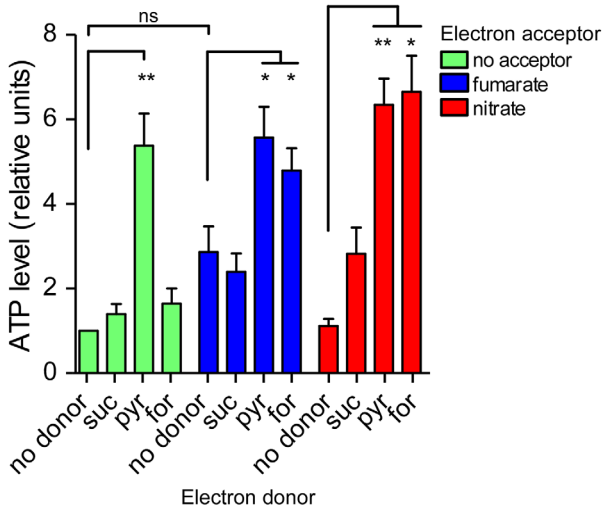

Fig. 4. ATP level of *C. jejuni* cell suspensions, measured in the presence of different electron acceptor/donor couples. *C. jejuni* was grown in HI-medium under microaerobic conditions, whereafter the cells were harvested and resuspended in HEPES buffer. After incubation under oxygen-limiting conditions,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

Growth rate correlates to the $\Delta\psi$

To determine the effect of the presence or absence of the membrane potential on the growth characteristics of *C. jejuni*, growth curves were recorded in the presence of different electron donor/acceptor combinations in an atmosphere of 10% CO_2 with either 10% or 0.5% oxygen. Hereto, bacteria were grown in a defined medium supplemented with 2 mM aspartate as nitrogen source and 1 mM pyruvate to ensure reproducible growth. In the presence of 10% oxygen, poor growth of *C. jejuni*

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

Electron acceptor	Carbon source	Growth rate (h^{-1}) (±SEM) Without formate	With formate
Oxygen	No carbon source	0.07 (0.15)	0.08 (0.01)
	Fumarate	0.29 (0.02)	0.38 (0.05)
	Succinate	0.18 (0.02)	0.17 (0.01)
	Pyruvate	0.31 (0.04)	0.38 (0.02)
No acceptor	No carbon source	*nc*	*nc*
	Fumarate	0.14 (0.03)	0.25 (0.04)
	Succinate	0.06 (0.02)	*nc*
	Pyruvate	0.12 (0.03)	0.09 (0.03)
Fumarate	No carbon source	0.07 (0.1)	0.12 (0.05)
	Fumarate	0.18 (0.05)	0.37 (0.07)
	Succinate	0.11 (0.04)	0.28 (0.09)
	Pyruvate	0.18 (0.03)	0.37 (0.11)
Nitrate	No carbon source	*nc*	0.05 (0.03)
	Fumarate	0.03 (0.01)	0.31 (0.05)
	Succinate	*nc*	0.09 (0.06)
	Pyruvate	0.10 (0.02)	0.32 (0.06)

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

was observed in this medium (Table 1). Addition of 25 mM of fumarate, succinate or pyruvate as electron donor/carbon source increased the specific growth rate and the production of biomass (Table 1 and Supporting Information Table S1). Addition of the electron donor formate led to an increased growth rate with fumarate and pyruvate as carbon source. Formate also strongly reduced the lag phase as observed with all carbon sources, but slightly reduced the biomass production. These results indicate that formate has a strong influence on the growth performance of *C. jejuni*.

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

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

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

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

As only certain electron donor/acceptor couples generated a $\Delta\psi$, we wondered whether the available electron acceptor influenced the transcription of genes involved in these processes. Hereto, we compared the transcriptomes of exponentially growing *C. jejuni* cells grown in HI medium under three different conditions; microaerobic, oxygen-limiting and oxygen-limiting in the presence of the alternative electron acceptor nitrate. RNA-seq analysis revealed that under oxygen-limiting conditions, the oxygen electron acceptor genes *cioAB*, *ccoNOPQ*, as well as *petABC* were downregulated (Fig. 5A). However, all alternative electron acceptor reductase genes (*NapAGHBLD*, *TorA/Cj0265c*, *mfrABC* and *nrfAH*) were upregulated, independent of the availability of their corresponding substrates. Similar results were seen in the presence of the alternative electron acceptor nitrate, however for the alternative electron acceptor reductase genes less transcript differences were observed. Apart from electron acceptor complexes, transcription of genes encoding components of electron donor complexes were also affected by the availability of oxygen. Under oxygen-limiting conditions, the formate dehydrogenase encoding genes (*fdhABC*) and the hydrogenase

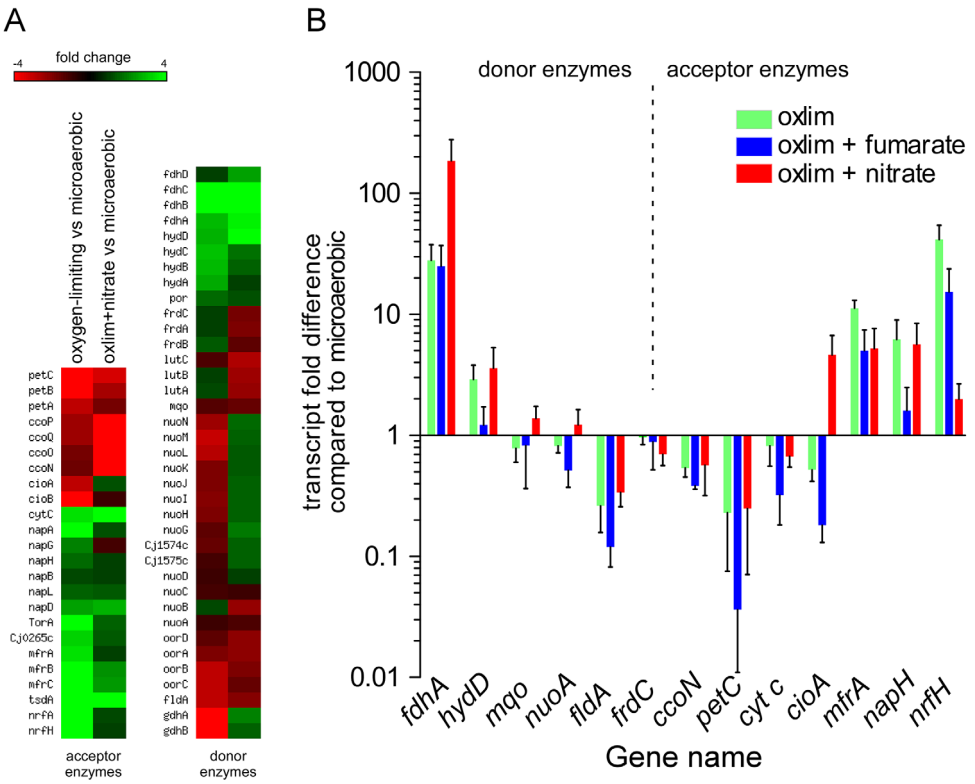

Fig. 5. Transcriptional adaptation toward low oxygen. A. Heat map of RNA-seq data of *C. jejuni* grown under oxygen limitation with or without 10 mM nitrate compared with the microaerobic grown cultures. Genes are clustered per regulatory operon unit and subsequently ordered on the basis of expression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

operon (*hydABCD*) were upregulated, while the gluconate dehydrogenase (*gdhAB*), 2-oxoglutarate reductase (*oorABCD*), NADH ubiquinone oxidoreductase (*nuoA-N*), as well as flavodoxin (*fldA*) were downregulated. The presence of nitrate increased the expression of the *fdhABC* operon even further, but in contrast to the oxygen-limiting conditions transcription of *gdhAB* genes was upregulated. These transcriptomic data suggest that dependent on the availability of oxygen as electron acceptor, the gene transcription of electron donor and acceptor complexes that allow for the generation of a high $\Delta\psi$ are upregulated.

To confirm the transcriptomic results and to investigate the transcriptional response when fumarate is used as electron acceptor instead of nitrate an RT-qPCR experiment was performed on the electron transport genes. The RT-qPCR results confirmed the outcome of the transcriptomic data as even larger differences in transcript abundance were observed for the investigated genes (Fig. 5B). The genes necessary for oxygen utilization, *petC*, *ccoN* and *cioA*, were downregulated under oxygen-limiting conditions and the alternative electron acceptor reductase genes *mfrA*, *napH* and *nrfH* were upregulated, just like the electron donor complexes *fdhA* and *hydD*. In contrast to the RNA-seq data *Cj0037* (putative Cyt *c*) was found to be slightly downregulated instead of upregulated. No obvious difference was observed between cultures grown under oxygen-limitation with or without fumarate or nitrate, except for the *cioA* gene, which was upregulated in the presence of nitrate. These results indicate that the gene regulation of the electron acceptor and electron donor genes is almost exclusively dependent on the availability of oxygen.

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

Under oxygen-limiting conditions, the electron donors formate and hydrogen are crucial for the generation of a membrane potential (Fig. 1B). The transcription of especially not only the formate dehydrogenase genes, but also of the hydrogenase genes, is strongly upregulated under these conditions (Fig. 5). To investigate whether this upregulation also led to higher formate dehydrogenase and hydrogenase activity in bacteria grown under oxygen-limiting conditions and whether this is conserved among *C. jejuni* strains, a 2,3,5-Triphenyl-tetrazolium chloride (TTC) reduction assay was performed. Therefore, six wild type strains were grown under microaerobic or oxygen-limiting conditions. Next, TTC reduction, dependent on substrate oxidation, was measured in cell suspensions in the presence of the electron donors, formate, hydrogen or pyruvate. All strains showed significantly more formate

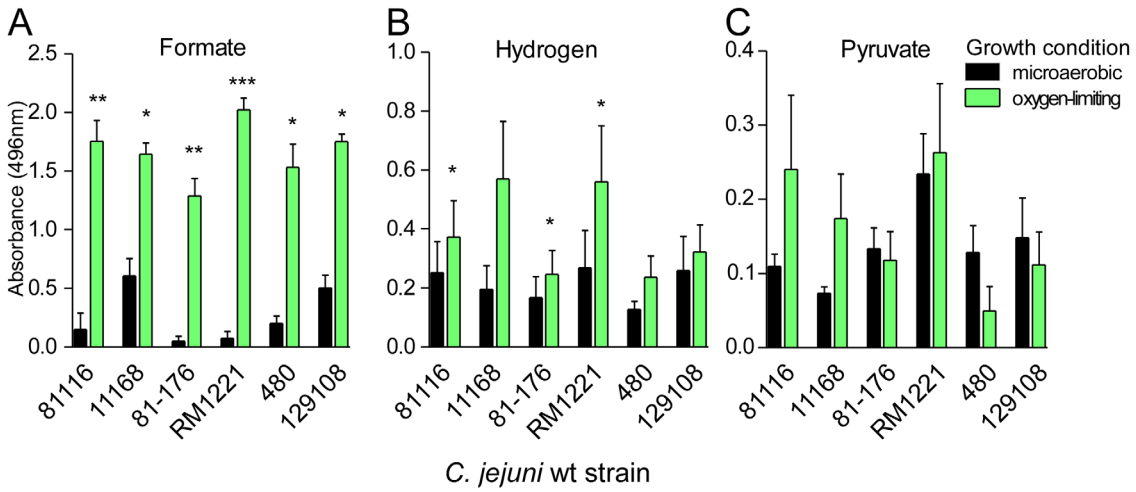

Fig. 6. Formate, hydrogen and pyruvate dependent TTC reduction showing the formate dehydrogenase (A), hydrogenase (B) and pyruvate oxidation (C) activity of six *C. jejuni* wt strains. TTC reduction was measured at the absorbance of 496 nm in bacterial suspensions from cultures grown in HI medium under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

dependent TTC reduction (3- to 25-fold) in oxygen-limiting compared with microaerobic grown cultures, indicating increased formate dehydrogenase activity (Fig. 6A). Similar but less pronounced results were observed for the hydrogenase activity (1.2- to 2.9-fold) (Fig. 6B). Pyruvate dependent TTC reduction was independent of the growth condition (Fig. 6C) which is in agreement with the unaltered transcript levels of the *por*, *nuo* and *fldA* genes that are needed to use pyruvate as electron donor. These data suggest a strong preference for formate and hydrogen utilization when oxygen cannot be used as electron acceptor.

Discussion

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

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

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

Oxygen is the preferred electron acceptor for *C. jejuni* (Table 1 and Supporting Information Table S1). The bc_1 complex and the cbb_3-type oxidase are both proton translocating enzymes and this route is thus

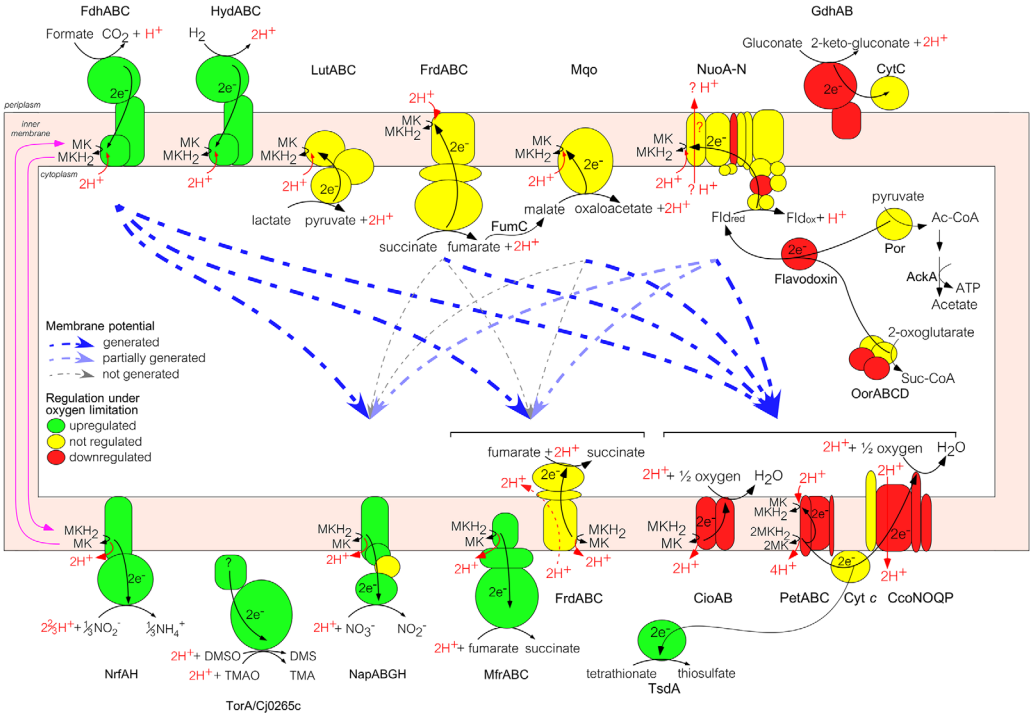

Fig. 7. Model of electron/proton flow in the *C. jejuni* electron transport chain. Respiratory donor (top) and acceptor (bottom) complexes are shown, including electron movements (black arrows) between donor, carrier—MK(MKH_2), cyt *c*, flavodoxin—and acceptor molecules. Red arrows show proton translocations. A membrane potential ($\Delta\psi$) is generated either by proton pumping enzymes or through translocations of protons by menaquinones via a redox loop, as depicted by the pink arrows. The generation of a $\Delta\psi$ in *C. jejuni* with different substrates (as shown in Fig. 1B), is indicated with blue dot dashed arrows (dark blue: high $\Delta\psi$; light blue: partial $\Delta\psi$; gray: no $\Delta\psi$). The transcriptional response toward oxygen limitation of the genes coding for the electron transport proteins (as shown in Fig. 5) is indicated as follows: green fill indicates upregulation upon oxygen limitation, red indicates downregulation and yellow indicates equal expression.

electrogenic (Fig. 7 and Table 2) (Hoffman and Goodman, 1982; Rauhamäki *et al.*, 2012). With a mid-point redox potential of +820 mV, oxygen is the strongest electron acceptor *C. jejuni* can use. Because oxygen is such a potent electron acceptor, all electron donors yield comparable membrane potentials, growth yields and motility performance (Figs 1 and 3; Table 1). The growth yields on all carbon sources/electron donors are significantly higher with oxygen than with other electron acceptors (Supporting Information Table S1). A *cioA* mutant had similar growth characteristics as the wt (data not shown) and it was previously reported that the

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

Acceptor	Donor	$\Delta E_0'$ (mV)	theoretical $q^+/e-$	experimental $\Delta\psi$ (mV)
Oxygen	Formate	1250	3	110
	Pyruvate	~1220[a]	~4[b]	109
	Fumarate[c]	986	2	111
	Succinate	790	1	100
Fumarate	Formate	460	1	105
	Pyruvate	~430[a]	~2[b]	56
	Fumarate[c]	196	0	n.d.
	Succinate	0	−1	0
Nitrate	Formate	850	1	122
	Pyruvate	~820[a]	~2[b]	58
	Fumarate[c]	586	0	15
	Succinate	390	−1	5

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

CioAB complex is not involved in oxygen respiration under microaerobic conditions, despite its relative high affinity toward oxygen (Jackson *et al.*, 2007). This suggests that the pathway through the bc_1 complex and cbb_3-type oxidase is solely responsible for pmf generation with oxygen respiration. CioAB homologues of other bacteria have repeatedly been implicated in ROS and RNS resistance (Borisov *et al.*, 2015). Our observation that the transcription of *cioA* is upregulated in the presence of nitrate (Fig. 5) supports the notion that the *C. jejuni* CioAB might have a similar role in nitrosative stress resistance.

When oxygen is not available as electron acceptor, the transcription of all genes coding for alternative electron acceptor complexes are upregulated independent of the presence of their corresponding substrates (Fig. 5B). Besides these genes, also the genes coding for the electron donor complexes, formate dehydrogenase and hydrogenase are upregulated, indicating that these electron donor/acceptor combinations are preferred under these conditions. Indeed, formate (hydrogen was not tested due to safety duties) together with the alternative electron acceptors generates a high $\Delta\psi$, while with pyruvate a moderate and with succinate no $\Delta\psi$ is generated (Fig. 1B), even though respiration is observed in all cases. Although the different expression of respiratory complexes does not lead to altered $\Delta\psi$ values (data not shown), it does influence substrate kinetics (Fig. 6) and this will likely give an advantage to the bacterium *in vivo* where substrate might be scarce. Other donor complexes, which do not contribute to the pmf, were not upregulated, such as Mqo and FrdABC. Malate, produced from fumarate by the enzyme FumC, donates its electrons to the menaquinone pool via the electroneutral enzyme Mqo (Fig. 7 and Table 2). Despite not being coupled to proton translocation, this reaction still has a role in carbon assimilation in the TCA cycle. Succinate donates electrons to the MK-pool via the FrdABC complex (Fig. 7). However, the mid-point redox potential of succinate (+35 mV) is more positive than that of both types of menaquinones of *C. jejuni*—menaquinone (−74 mV) and methylmenaquinone (−124 mV)—consequently, this reaction is endergonic and consumes pmf to use succinate as electron donor (Fig. 7 and Table 2) (Dietrich and Klimmek, 2002; Juhnke *et al.*, 2009). This likely explains the poor growth observed with succinate as carbon source in the absence of oxygen as electron acceptor, even in the presence of the electron donor formate (Table 1).

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

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

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

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

Our results show that formate plays a significant role in the motility, growth rate and ATP production of *C. jejuni* in an oxygen-limiting environment. Therefore, *C. jejuni* would have difficulties colonizing chickens or cause disease in humans without the electron donor formate (or hydrogen). A chemotactic response toward formate has been reported, through the transducer-like protein Tlp9 (Tareen *et al*., 2010), emphasizing the preference of *C. jejuni* for formate as electron donor. Mixed acid fermentation by the gut microbiota leads to the formation of formate and hydrogen in the intestine, as can be assumed from the expression of formate- and hydrogen-generating enzymes by the gut microbiota (Sergeant *et al*., 2014; Polansky *et al*., 2016). Induction of formate dehydrogenase is observed when *C. jejuni* is grown in a rabbit ileal-loop *ex vivo* (Stintzi *et al*., 2005), but not during chicken colonization (Woodall *et al*., 2005; Taveirne *et al*., 2013). Moreover, formate utilization is crucial for piglet colonization (Vries *et al*., 2017), efficient colonization of chickens (Weerakoon *et al*., 2009) and pathology in mouse models (Bereswill *et al*., 2011; Kassem *et al*., 2012). Formate also acts as invasion signal for the pathogenic bacterium *Salmonella enterica* serovar Enteritidis (Van Immerseel *et al*., 2003; Huang *et al*., 2008). This all indicates that without formate metabolism, *C. jejuni* loses its potential to colonize the gut and cause pathogenicity.

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

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

Experimental procedures

General growth conditions

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

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

The genes including the flanking regions were amplified using primers cioAF/cioAR, cconF/cconR, mfrAF/mfrAR, frdAF/frdAR or ackAF/ackAR (Supporting Information Table S3) respectively, and subsequently cloned into plasmid pJET (Thermo, Waltham, USA) or for *mfrA* into plasmid pGEM (Promega, Madison, USA). The obtained plasmids were used as template to partially delete the targeted genes by PCR using the primers CioAstart/CioAend, CcoNstart/CcoNend, mfrAstart/mfrAend, frdAstart/frdAend or ackAstart/ackAend. These PCR products were digested with BamHI (Thermo) or BglII for the Δ*mfrA* PCR product, and were subsequently ligated to a BamHI digested chloramphenicol or kanamycin resistance gene, from pAV35 or pMW2 respectively.

Campylobacter jejuni 81116 was naturally transformed using the knockout plasmids as described before (van der Stel *et al*., 2015). Colonies with the *ccoN* mutation were recovered on fresh saponin plates supplemented with chloramphenicol (17 μg/ml) and 10 mM formate, pyruvate, fumarate and nitrate under oxygen-limiting conditions (0.3% O_2, 10% CO_2, 10% H_2). Gene knockouts were verified with PCR.

Membrane potential measurements

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

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

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

Respiration rates

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

Motility

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

Measuring the ATP level

Campylobacter grown until mid-exponential phase in HI medium were harvested and resuspended in HEPES buffer (10 mM HEPES pH 8.0, 100 mM NaCl) to an OD_{550} of 1.0. Handling was performed inside a hypoxic glovebox with an atmosphere set to 0.5% O_2, 10% CO_2. The bacterial suspension was incubated for 30 min on ice and subsequently aliquots (180 μl) were incubated with or without 10 mM electron donor for 30 min at 30°C. Next, if appropriate, 5 mM electron acceptor was added, and the suspension was further incubated for 30 min. Of each reaction, 20 μl was lysed with 10 μl trichloroacetic acid (final concentration 1%) for 10 min and subsequently neutralized with NaOH. Finally, the lysed samples were diluted 10-fold in TRIS buffer (100 mM TRIS/HCl, pH 7.5). ATP concentration was determined by mixing equal amounts of sample and Bactiter-GLO (Promega, Madison, USA) and measuring relative light units (RLU) on a Tristar^2 multimode reader (Berthold Technologies, Wildbad, Germany). The RLU of the sample without electron donor and electron acceptor was set to 1.

Growth curves

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

calculated by subtracting the starting OD_{550} from the maximally obtained OD_{550}. Growth experiments were conducted three times independently.

RNA-isolation

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

RNA sequence and transcriptomic analysis

RNAseq was performed using an Illumina MiSeq sequencer (Illumina, San Diego, CA). Initially, total RNA samples were rRNA depleted using the Ribozero Magnetic Kit for-Gram negative bacteria (Illumina) following manufacturer's instructions (option 1). Illumina libraries were prepared using the KAPA stranded RNA-seq kit (Kapa Biosystems, Wilmington, MA), following manufacturer's instructions except for the following changes: 159–400 ng RNA was sheared for 6 min at 85°C. Standard desalting TruSeq LT primers were ordered from Integrated DNA Technologies (Coralville, IA) and used at 50–100 nM final concentration based on starting RNA amount. The PCR step was reduced to 6 cycles. Libraries were quantified using the KAPA Library Quantification Kit (Kapa), except with 10 μl volume and 90 sec annealing/extension PCR. Libraries were pooled and normalized to 4 nM. Pooled libraries were re-quantified by ddPCR on a QX200 system (Bio-Rad), using the Illumina TruSeq ddPCR Library Quantification Kit and following manufacturer's protocols. The libraries were sequenced in two 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. Subsequent processes were performed with Geneious 9.1. The fastq sequences were trimmed, mapped to remove poor quality bases and then assembled to reference genome CP000814 using Bowtie within Geneious. Geneious software was used to calculate the normalized transcripts per million (TPM) and to compare expression levels between the control growth condition (microaerobic) and experimental conditions (oxygen-limiting + nitrate and oxygen-limiting).

RT-qPCR

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

Formate, hydrogen and pyruvate utilization assays

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

Statistical analysis

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

Accession numbers

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

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Author contributions

AXS, FB and MW performed the conception and design of the study; AXS, SH, CP and LD performed the experiments; AXS, FB, SH, CP, JP and MW performed analysis or interpretation of the data; AXS, FB and MW wrote the manuscript.

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