Sulphite : cytochrome *c* oxidoreductase deficiency in *Campylobacter jejuni* reduces motility, host cell adherence and invasion

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Campylobacter jejuni lacks the enzyme phosphofructokinase and, consequently, is incapable of metabolizing glucose. Instead, the pathogen uses a number of other chemicals to serve as electron donors. Like chemolithotrophic bacteria, *C. jejuni* is able to respire sulphite in the presence of a sulphite : cytochrome *c* oxidoreductase (SOR) that is encoded by the genes *cj0004c* and *cj0005c*; the former encodes a monohaem cytochrome *c* oxidoreductase and the latter a molybdopterin oxidoreductase. After screening of a transposon-based mutant library, we identified a mutant with an insertion in gene *cj0005c* that was strongly reduced in its capacity to infect Caco2 cells. Further characterization of a corresponding non-random knockout mutant together with a complemented mutant and the parental strain showed the *cj0005c*-deficient mutant to exhibit clearly reduced motility and diminished adherence to host cells. Furthermore, the transcription of genes responsible for the synthesis of, in particular, legionaminic acid was downregulated and the mutant had a reduced capacity to autoagglutinate. In contrast, neither the proliferation of the mutant, nor its intracellular ATP content, was altered compared to the parental strain.

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INTRODUCTION

Campylobacter jejuni is the most important cause of bacterial diarrhoea of humans in developing and developed countries (Altekruse *et al.*, 1999; Friedman *et al.*, 2000; Mead *et al.*, 1999). The spectrum of the disease can vary from mild watery diarrhoea to bloody diarrhoea and is characterized by fever and abdominal cramps. In addition, the Guillain–Barré syndrome might emerge as a post-infection complication (Allos, 2001). At the beginning of the infection, *C. jejuni* colonizes the small intestine before moving to the colon, which represents the target organ. *C. jejuni* is generally considered to be invasive; the level of invasiveness varies depending on the strain (Black *et al.*, 1988; van Vliet & Ketley, 2001). In contrast to the infection of humans, *C. jejuni* is only a commensal organism in chickens and other birds, where the pathogen preferentially resides in the caecum, which contains many anaerobic fermentative bacteria (Barnes *et al.*, 1972; Lu *et al.*, 2003). Within those natural habitats, *C. jejuni* is able to metabolize products like free amino and keto acids generated by these accompanying bacterial species or from

the host itself (Lee & Newell, 2006). Since *C. jejuni* lacks the glycolytic enzyme phosphofructokinase, glucose cannot be catabolized, and other products serve as electron donors for respiration, for instance succinate, malate, formate, D-lactate, hydrogen and NAD(P)H (Hoffman & Goodman, 1982; Parkhill *et al.*, 2000; Kelly, 2001; Sellars *et al.*, 2002; Velayudhan & Kelly, 2002). It has also been shown that sulphite can be exploited as a respiratory electron donor. Like chemolithotrophic bacteria, *C. jejuni* possesses a sulphite : cytochrome *c* oxidoreductase (SOR), which is encoded by the genes *cj0005c* and *cj0004c*. The *cj0004c* gene encodes a monohaem cytochrome *c* oxidoreductase, while *cj0005c* encodes a molybdopterin oxidoreductase (Myers & Kelly, 2005). The possession of a sulphite oxidation system may be important for the pathogen to survive in foodstuffs that have been treated with sulphite for preservative reasons, or in the human host, since it has been shown that sulphite is also released by neutrophils as part of the host defence (Mitsuhashi *et al.*, 1998). Thus a potential antimicrobial chemical can be used by *C. jejuni* for the generation of electrons, which are subsequently supplied to the respiratory chain of the pathogen and therefore serve for the provision of energy.

In this report we describe that the possession of SOR and the ability to utilize sulphite as an electron donor significantly influences bacterial motility, gene regulation,

Abbreviations: Cp, crossing point; LegAm, legionaminic acid; SOR, sulphite : cytochrome *c* oxidoreductase.

Three supplementary tables are available with the online version of this paper.

autoagglutination and the invasiveness of *C. jejuni* towards host cells. Bacterial proliferation and intracellular ATP level do not correlate with the presence of this enzyme.

METHODS

Bacterial strains, media and culture conditions. *C. jejuni* strain B2, isolated from a patient suffering from gastroenteritis, was grown on Columbia agar supplemented with 5% defibrinated sheep blood at 42 °C in microaerobic conditions (85% N₂, 10% CO₂, 5% O₂) (Schmidt-Ott *et al.*, 2005; Dasti *et al.*, 2007). When needed, kanamycin and chloramphenicol were added at concentrations of 50 µg ml⁻¹ and 30 µg ml⁻¹, respectively. Growth experiments were carried out at 42 °C in Mueller–Hinton (MH) broth under microaerobic conditions in the presence or absence of 10 mM Na₂SO₃. Cloning experiments were performed in *Escherichia coli* strain DH5α. The *E. coli* bacteria were grown on Luria Bertani (LB) agar or in LB broth at 37 °C. When necessary, ampicillin (100 µg ml⁻¹) was added.

Preparation of competent cells and electroporation. *E. coli* DH5α was grown in 500 ml LB broth to an OD₆₀₀ of 0.35–0.45. The culture was stored on ice for 10 min and centrifuged at 4000 g at 4 °C. After washing the bacteria three times in ice-cold water, the pellet was resuspended in 15 ml 10% (v/v) glycerol and centrifuged again. Finally, the pellet was dissolved in 1 ml 10% (v/v) glycerol and 100 µl aliquots were used for electroporation. *C. jejuni* cells were collected from Columbia agar plates. Then the cells were washed four times in ice-cold washing buffer containing 272 mM sucrose and 15% (v/v) glycerol. Finally, the bacteria were resuspended in 400 µl washing buffer and aliquots of 100 µl were used for electroporation.

For each electroporation of *E. coli* and *C. jejuni*, 0.5–3 µg plasmid DNA was added to the competent cells and the mixture was transferred into an ice-cold electroporation cuvette. After incubation on ice for 30 min, electroporation was carried out at 2.5 kV, 25 µF and 200 Ω with a BTX Electro Cell manipulator before 500 µl SOC medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄, 20 mM glucose] was added. In the case of *C. jejuni* electroporation, the cells were incubated on non-selective Columbia blood agar plates overnight at 37 °C under microaerobic conditions. Then, the cells were transferred onto plates supplemented with appropriate antibiotics and incubated under microaerobic conditions at 42 °C. Transformed *E. coli* bacteria were directly plated on selective LB agar and incubated at 37 °C overnight.

Cultivation of Caco2 cells. Human colon carcinoma Caco2 cells were cultivated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1 × non-essential amino acids, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

Invasion and adhesion assays. Bacterial invasion assays were carried out according to Everest *et al.* (1992). Caco2 cells were grown to approximately 80% confluence in a six-well plate, then the cells were washed with PBS and inoculated with 400 µl *C. jejuni* suspension adjusted to an OD₆₀₀ of 0.5, which represents an m.o.i. of 100. In the experiments in which the bacteria were brought into contact with the Caco2 cells, the six-well plates were centrifuged at 600 g for 15 min. Two hours post-infection, the bacterial suspension was removed and the cells were washed three times with PBS before further incubation with culture medium supplemented with 100 µg gentamicin ml⁻¹. Then the Caco2 cells were lysed by treatment with 1% Triton X-100 for 10 min and the number of viable bacteria was determined by counting c.f.u. on Columbia blood agar plates after incubation for 48 h at 42 °C under microaerobic conditions.

For the investigation of bacterial adhesion, Caco2 cells were incubated with the bacteria for only 30 min. Then the monolayer was washed with PBS, the cells were lysed and the bacteria were plated on Columbia blood agar plates, where the number of recovered bacteria was determined as described above. Every experiment was repeated six times.

ATP assays. To determine the intracellular ATP concentration of bacterial samples, the BacTiter-Glo assay from Promega was used. After growing the bacteria in MH broth at 42 °C under microaerobic conditions, cultures were adjusted to an OD₆₀₀ of 0.5. Then, serial dilutions of all samples were prepared according to the manufacturer's instructions. Following incubation of the samples at room temperature in a 96-well plate, luminescence was measured together with an ATP standard using a VICTOR³ V Multilabel Counter (Perkin Elmer).

Motility assays. One microlitre of an overnight culture that was adjusted to an OD₆₀₀ of 0.05 was stabbed onto the centre of a 0.4% MH agar plate. After incubation of the plate at 42 °C for 36 h, motility was judged by measuring of the diameter of the halo. Every experiment was carried out four times.

Autoagglutination assays. Autoagglutination assays were carried out as initially described by Misawa & Blaser (2000). Bacteria grown on plates were suspended in PBS and adjusted to an OD₆₀₀ of approximately 1.0. A 2 ml sample of each suspension was incubated under microaerobic conditions for 24 h at 37 °C. Then 1 ml of the bacterial suspension was carefully removed and the OD₆₀₀ was determined. The parental strain B2, its knockout mutant and the complemented mutant were tested five times each. The data obtained were normalized according to Howard *et al.* (2009): the OD₆₀₀ measured after 24 h was subtracted from the OD₆₀₀ from the start of the experiment; the values obtained were divided by the initial OD₆₀₀ and were finally multiplied by 100 in order to give the percentage autoagglutination.

Isolation of nucleic acids. Genomic DNA of *C. jejuni* was isolated with the QIAamp DNA Mini kit (Qiagen), following the manufacturer's instructions. Plasmid DNA was prepared using the GeneElute Plasmid Miniprep kit (Sigma) according to the manufacturer's protocol. Bacterial RNA from *C. jejuni* was extracted with the RiboPure Bacter kit (Ambion). DNA contaminations were removed by the treatment with DNase I (Ambion) as recommended by the protocol. Prior to RT-PCR analysis, PCR assays with the isolated bacterial RNA as a template were performed to exclude remaining traces of DNA.

Sequencing of genomic DNA. Genomic DNA sequencing was performed by SeqLab (Göttingen, Germany) using 10 µg genomic *C. jejuni* DNA and 10 pmol KanF primer (for all primer sequences, see Supplementary Table S1, available with the online version of this paper).

Insertional knockout of *cj0005c*. Primers Cj0005cF and Cj0005cR (Supplementary Table S1) were used to amplify a 1239 bp DNA fragment corresponding to *cj0005c*. The PCR fragment was *Xba*I digested and inserted into the *Xba*I-restricted and dephosphorylated plasmid vector pBluescript II KS (Stratagene) to obtain pBcj0005c. For the insertion of a kanamycin-resistance cassette, the *aphA-3* gene including a 5'-promoter was amplified from plasmid pSB1699 (Colegio *et al.*, 2001) using the 5'-phosphorylated primers Kan1 and Kan2 (Supplementary Table S1). A PCR containing 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, all four dNTPs (each 0.2 mM), 10 pmol primer and 1 U *Pfu* DNA polymerase was carried out to obtain a blunt-ended PCR product. Initial incubation at 95 °C for 1 min was followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s

and 72 °C for 1 min, with a final incubation at 72 °C for 10 min. After purification of the PCR product using the QIAquick PCR Purification kit (Qiagen), the kanamycin-resistance cassette was ligated into plasmid pBcj0005c, which was *Bsa*BI-restricted and dephosphorylated with Antarctic Phosphatase (New England Biolabs) to obtain pBcj0005cKan.

Cloning of *cj0005c* into pRRC. The *C. jejuni* gene *cj0005c* was amplified by PCR and cloned into *C. jejuni* expression vector pRRC (Karlyshev & Wren, 2005). The primers Cj0005cF and Cj0005cR with *Xba*I restriction sites are listed in Supplementary Table S1. PCR was carried out using a TRIO-Thermocycler (Biometra) with 10 ng genomic DNA of *C. jejuni* strain B2 as a template. The PCR mixture contained 0.02 U KOD Hot Start DNA Polymerase (Novagene), dNTPs (each 0.2 mM), 1.5 mM MgSO₄, 10 pmol of each primer and 1 × KOD Hot Start buffer. After initial incubation at 95 °C for 3 min, 40 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min were carried out with a final incubation at 72 °C for 5 min. After PCR, the PCR amplicon and the expression vector pRRC were *Xba*I digested. Following dephosphorylation of pRRC with Antarctic Phosphatase (New England Biolabs), the restricted PCR fragment was ligated into pRRC using the Quick Ligase kit (New England Biolabs), following the manufacturer's recommendations.

Real-time RT-PCR analysis. Semiquantitative real-time RT-PCR analysis was performed with the LightCycler 1.5 instrument (Roche) and the QuantiFast SYBR Green RT-PCR kit (Qiagen) as described previously (Tareen *et al.*, 2010). In brief, 10 ng samples of RNA isolated from *C. jejuni* B2 wild-type strain, its knockout mutant B2Δ:*cj0005c* and its complemented knockout mutant were used to compare the transcription levels of the genes summarized in Supplementary Table S1. Amplicons of the 23S gene were used to normalize the RNA pools under investigation based on the crossing points obtained. 23S RT-PCR was carried out as follows. Initial reverse transcription for 20 min at 50 °C and denaturation for 5 min at 95 °C was followed by 30 cycles of denaturation (95 °C; 10 s), annealing (50 °C; 10 s) and elongation (72 °C; 5 s). After adjustment of the RNA samples with regard to the crossing points of the 23S gene, real-time RT-PCR assays were undertaken in order to compare the transcription level of all other investigated genes. Initially, RNA was reverse transcribed for 20 min at 50 °C and denatured for 5 min at 95 °C. Subsequently, 28–35 cycles of denaturation (95 °C; 10 s), annealing (58 °C; 10 s) and elongation (72 °C; 10 s) were executed. The primers used are listed in Supplementary Table S1. Every assay was run in triplicate. Semi-quantified transcription levels of the respective genes were calculated as follows: $x=2^{\Delta C_p}$, where x represents the factor of altered transcription, and ΔC_p stands for the difference in crossing points ($C_{p1}-C_{p2}$) of the sample pools compared.

Statistical analysis. The STATISTICA software (StatSoft) was used to perform Student's *t*-tests. *P*-values <0.01 were considered to be significant.

RESULTS

Identification of *cj0005c*

In order to detect genes of *C. jejuni* that are important for the pathogen to invade host cells, we screened a transposon-based mutant library. A total of 660 mutants were examined for their capacity to infect Caco2 cells (Tareen *et al.*, 2010). One of the mutants that showed a clearly reduced infectivity carried a transposon insertion in the gene *cj0005c*, which was mapped by direct sequencing of genomic DNA using a

primer that binds directly to the 5' region of the kanamycin-resistance cassette (Supplementary Table S1). Gene *cj0005c* encodes a molybdopterine oxidoreductase, which, together with the adjacent gene *cj0004c* encoding the monohaem cytochrome *c* oxidoreductase, constitutes a sulphite:cytochrome *c* oxidoreductase (SOR). In three independent gentamicin protection assays, the percentage of recovered colonies of mutants compared to the parental strain B2 was 24.5, 26.5 and 25 %, respectively, with a mean value of 25.34 % ($P<0.001$).

Construction of a knockout mutant and complementation of the mutant phenotype

Since it could not be ruled out that the observed invasion-deficient phenotype of *C. jejuni* B2Δ*cj0005c* was not due to the transposon insertion into *cj0005c* but rather to genetic rearrangements in the genome, we created a knockout mutant to confirm the above-described observations. After transformation of *C. jejuni* B2 with pBcj0005cKan, PCR analysis was carried out by using genomic DNA from the obtained *C. jejuni* clones for the detection of a single band corresponding to the length of *cj0005c* including the *aphA-3* cassette (B2:: *cj0005c*) (Fig. 1b). Subsequent gentamicin protection assays with the generated knockout mutant B2:: *cj0005c* confirmed the observed phenotype of *C. jejuni* strain B2 harbouring the transposon within *cj0005c*, clearly demonstrating that the inactivation of *cj0005c* was responsible for the invasion-deficient phenotype (Fig. 1c). To restore the phenotype of the parental strain B2, we introduced the *C. jejuni* expression plasmid pRRC carrying *cj0005c* into B2:: *cj0005c* to obtain B2:: *cj0005c*-comp-*cj0005c*. Because in pRRC the chloramphenicol-resistance cassette and downstream gene *cj0005c* are flanked by 16S and tRNA sequences, a double recombination event leads to the integration of both genes into one of the three 16S gene clusters. After verification of the intact *cj0005c* gene by PCR (Fig. 1b), we repeated invasion assays with wild-type strain B2, both mutants and the complemented knockout mutant. Taking the number of recovered B2 colonies as 100 %, the mean values of invasion capacity of B2Δ*cj0005c*, B2:: *cj0005c* and B2:: *cj0005c*-comp-*cj0005c* were 25 %, 29.8 % and 85.6 %. The altered invasion capacities of both mutants were clearly significant ($P<0.0001$). In contrast, the *P*-value for the complemented knockout mutant was only 0.16, indicating no significant difference compared to the parental strain B2 (Fig. 1c). An overview of the genetic arrangement of *cj0005c* in the parental strain, the parental strain with the transposon insertion in *cj0005c*, the *cj0005c* knockout mutant and the complemented mutant is shown in Fig. 1(a).

The loss of a functional SOR leads to reduced growth only in the presence of sodium sulphite

To investigate whether the loss of a functional SOR affects the growth of *C. jejuni*, we compared the growth of the

Fig. 1. Key for all panels: 1, parental strain B2; 2, B2 with transposon insertion in *cj0005c* (B2Δ*cj0005c*); 3, *cj0005c* knockout mutant (B2::*cj0005c*); 4, complemented knockout mutant (B2::*cj0005c*-comp-*cj0005c*). (a) Genome arrangements of the bacterial strains under investigation. Primers Cj0005cF and Cj0005cR for the amplification of *cj0005c* are indicated as arrowheads. The primer Kan1 that binds to the 5'-end of the kanamycin-resistance cassette is shown as an arrow. (b) Verification of the native gene, the respective mutants and the complemented strain by PCR analysis. The native *cj0005c* gene from strain B2 has a size of 1239 bp (1). Transposon insertion and insertion of the kanamycin-resistance gene leads to PCR fragments of 2932 bp (2) and 2623 bp (3), respectively. PCR analysis of the complemented knockout mutant using primers Cj0005cF and Cj0005cR led only to the amplification of the wild-type gene, probably due to the higher efficiency for the amplification of smaller PCR fragments. To detect the native gene (1239 bp) and the one with the kanamycin-resistance cassette (2234 bp) a PCR analysis with the primer pair Cj0005cF/Cj0005cR and an additional third primer (Kan1) binding to the 5'-end of the *aphA-3* gene was carried out. (4). (c) Gentamicin protection assays after the infection of Caco2 cells. The invasion-

deficient phenotype of B2Δ*cj0005c* could be confirmed after knockout of *cj0005c* (B2::*cj0005c*). Complementation of the knockout mutant restored the parental phenotype. The data are means ± SD.

strains in the presence and the absence of sodium sulphite. While in the absence of sodium sulphite no differences in the proliferation of the parental strain B2, the knockout mutant B2::*cj0005c* and the complemented mutant were observed, the growth of the mutant was clearly reduced as compared to B2 and B2::*cj0005c*-comp-*cj0005c* when sodium sulphite was added to the medium at a concentration of 10 mM (Fig. 2).

ATP levels do not differ in the wild-type, mutant and complemented mutant

Since we could detect no variations in bacterial proliferation of the populations examined in a medium that did not contain the particular substrate for SOR, we asked whether also the ATP level is identical in the parental strain B2 and the *cj0005c*-deficient mutant. Upon measuring serial dilutions of the bacteria under investigation with regard to their intracellular ATP content, we could not detect any differences (Fig. 3), demonstrating that the ATP level is not

Fig. 2. Growth curves of parental strain B2 (◆), B2::*cj0005c* (▲) and complemented mutant B2::*cj0005c*-comp-*cj0005c* (■) (a) not supplemented and (b) supplemented with 10 mM Na₂SO₃. The growth experiments were repeated three times. Standard deviations are indicated.

altered even in the absence of an important enzyme, which is an integral part of the energy metabolism.

Reduced motility of *C. jejuni* in the absence of a functional SOR

We further asked if the motility of the pathogen is diminished by *cj0005c* mutation, since motility is an energy-consuming process. Upon comparing the parental strain B2, its knockout mutant and the complemented knockout mutant, we clearly detected a motility loss of the mutant B2::*cj0005c* that could be reverted completely to the level of the wild-type strain after introduction of a functional *cj0005c* gene into the mutant. In four independent motility assays, the mean diameter ($\pm$ SD) of the B2 motility zone was 40 ± 4.6 mm, while the mutant B2::*cj0005c* was significantly less motile (18.2 ± 1.7 mm, $P < 0.01$). Introduction of a functional copy of *cj0005c* restored the motility to wild-type level again (38.7 ± 4.1 mm). These findings implied that the reduced infectivity may be primarily due to a lack of motility in the absence of a functional SOR.

Downregulation of *flhA*, *fliR* and *rpoN* transcription in the *cj0005c* mutant

To gain an insight into how the lack of a functional SOR is linked to reduced motility, we investigated the regulation of genes which have been shown to be important for the composition of a functional flagellar apparatus (Hendrixson & DiRita, 2003). We carried out semiquantitative real-time RT-PCR assays to compare the transcription of genes *rpoN*, *flgR*, *flgS*, *flhA*, *flhB*, *fliP*, *fliR* and *maf7* in parental strain B2 and the mutant. While the transcription levels of *flgS*, *flgR*, *maf7*, *flhB* and *fliP* were not altered relevantly, we detected a significant downregulation of

rpoN, *fliR* and *flhA* in the mutant strain compared to strain B2; the downregulation of *flhA* was particularly marked (Table 1). Subsequent testing of the transcription levels of genes significantly downregulated in the mutant revealed only minor differences of the respective crossing points in the parental strain B2 compared to the complemented mutant. The results are shown in Supplementary Table S2.

Reduced motility contributes to decreased invasiveness

To investigate whether the reduced motility is the only reason for the clearly reduced invasion capacity of the *cj0005c*-deficient mutant, we performed modified gentamicin protection assays in which motility was mimicked by centrifugation of the bacteria onto the Caco2 host cells. Taking the number of colonies recovered in the case of wild-type strain B2 as 100 %, we detected only 48.6 % of colonies ($P < 0.01$) after centrifugation of the knockout mutant B2::*cj0005c*. The complemented mutant gave 87 % of recovered colonies, which was not significantly different compared to the parental strain (Fig. 4). As compared to the gentamicin protection assays without centrifugation (Fig. 1c) we could clearly observe an increase in recovered mutant colonies from 29.8 % up to 48.6 %. Nevertheless, this finding was surprising since one would have expected to shift the mutant invasiveness up to wild-type level after bypassing motility as influencing factor. In contrast, this result indicates that altered motility is one, but not the only factor responsible for the clearly reduced invasiveness of the *cj0005c*-deficient mutant.

Reduced invasiveness is also caused by diminished adherence

To characterize the process of invasion in more detail, we investigated whether the adherence capability of the *cj0005c*-deficient mutant was altered. For this, we carried out adhesion assays of the wild-type isolate B2, the mutant B2::*cj0005c* and the complemented mutant. After centrifugation of the bacteria onto the host cells, we could clearly determine an adhesion-deficient phenotype of the mutant, which could be restored to wild-type behaviour after complementation. Taking the number of recovered colonies from the parental strain B2 as 100 %, the mean value of colonies from the mutant B2::*cj0005c* was 67 % (± 5.1 , $P < 0.001$) in contrast to B2::*cj0005c*-comp-*cj0005c* with a recovery rate of 94 % (± 2.2) (Fig. 4).

The absence of a functional SOR downregulates the transcription of virulence-associated factors

After the confirmation that the reduced motility in combination with diminished adherence is a reason for the decreased invasiveness of the *cj0005c* mutant, we examined the transcription level of genes involved in virulence and, in particular, in the adherence of *C. jejuni* to host cells. The investigated genes were *groEL* and *dnaJ*,

Fig. 3. Intracellular ATP levels of parental strain B2, mutant B2::*cj0005c* and complemented mutant. ATP contents of four serial dilutions of the bacteria under investigation were measured. The results are shown as means $\pm$ SD of data from triplicate wells of a representative experiment. No differences in intracellular ATP content between the bacterial strains could be detected ($P > 0.1$).

Table 1. Transcription levels of genes important for flagellar synthesis in parental strain B2 and mutant strain B2::cj0005c

The crossing point data are means $\pm$ SD. Significantly downregulated genes are in bold.

Gene	Crossing point		Fold change (<i>t</i> -test <i>P</i> -value)	Function
	B2::cj0005c	B2 wild-type		
<i>flhA</i>	20.86 ($\pm$ 0.06)	17.98 ($\pm$ 0.32)	7.41 (0.0001)	Flagellar biosynthesis
<i>flhB</i>	19.08 ($\pm$ 0.10)	17.64 ($\pm$ 0.14)	2.71 (0.065)	Flagellar biosynthesis
<i>flgR</i>	16.16 ($\pm$ 0.10)	15.14 ($\pm$ 0.36)	2.02 (0.0051)	Signal transduction regulation
<i>flgS</i>	20.52 ($\pm$ 0.18)	19.92 ($\pm$ 0.19)	1.51 (0.062)	Signal transduction histidine kinase
<i>fliP</i>	20.80 ($\pm$ 0.06)	19.44 ($\pm$ 0.05)	2.56 (0.0051)	Flagellar biosynthesis
<i>fliR</i>	20.24 ($\pm$ 0.10)	18.66 ($\pm$ 0.13)	3.24 (0.0001)	Flagellar biosynthesis
<i>maf7</i>	24.46 ($\pm$ 0.01)	22.72 ($\pm$ 0.10)	3.34 (0.035)	Unknown
<i>rpoN</i>	24.07 ($\pm$ 0.24)	21.98 ($\pm$ 0.15)	4.25 (0.0001)	RNA polymerase σ^{54} subunit

encoding heat-shock proteins (Thies *et al.*, 1999; Konkel *et al.*, 1998), the chemotaxis regulator gene *cheY* (Yao *et al.*, 1997), the putative chemoreceptor *tlp7* (Marchant *et al.*, 2002), genes that encode proteins important for binding and adhesion of *C. jejuni*, like *cadF*, *peb1*, *jlhA*, *porA* and *flpA* (Konkel *et al.*, 1997; Pei & Blaser 1993; Jin *et al.*, 2001; Flanagan *et al.*, 2009; Konkel *et al.*, 2010) and genes known to be responsible for O-linked glycosylation of the flagella by the synthesis of pseudaminic acid and legionaminic acid (LegAm) (Logan *et al.*, 2008). In addition, we compared the transcription level of *Campylobacter* invasion antigen gene *ciaB* (Rivera-Amill *et al.*, 2001; Konkel *et al.*, 2004), the cytolethal distending toxin subunit B gene *cdtB* (Pickett & Whitehouse, 1999) and *flaA* (Guerry *et al.*, 1991) in the wild-type strain B2 and its mutant. Semiquantitative

real-time RT-PCR assays detected a statistically significant 5.16-fold downregulation of *dnaJ* transcription in the mutant compared to the transcription level of this gene in wild-type strain B2 ($P < 0.001$). In addition, the transcription of *ptmB*, *ptmC* and *ptmE*, which contribute to a set of genes responsible for the synthesis of LegAm were significantly downregulated by a factor of 4.31–7.67 ($P < 0.001$) as outlined in Table 2. The transcription of other genes, including genes of LegAm metabolism, were also downregulated significantly, but, to a lesser extent (*ptmA*, *ptmF*, *ptmG*). Other virulence factors investigated, e.g. for the biosynthesis of pseudaminic acid, were not controlled or only regulated by a factor of uncertain biological significance. The results are summarized in Supplementary Table S3.

Further, we tested in the *cj0005c*-complemented mutant the transcription of the genes judged to be significantly downregulated in the *cj0005c*-mutant. The results showed crossing points on the same transcription level as for the parental strain (Supplementary Table S2).

Autoagglutination deficiency of mutant B2::cj0005c

Howard *et al.* (2009) demonstrated that *C. jejuni* mutants lacking gene *cj1324* or genes *cj1321–1325/6*, known to be responsible for the synthesis of LegAm, had significantly lower levels of autoagglutination compared to the parental strain. By comparing the transcription profile of the mutant B2::cj0005c with that of the wild-type strain B2 we were able to detect a significant downregulation of transcription of *ptmG* (*cj1324*) as well as that of other genes involved in the synthesis of LegAm as described above. We therefore tested whether the competence of mutant B2::cj0005c is also affected in regard to autoagglutination. We found that the autoagglutination capability of the mutant is indeed decreased. Whereas the percentage autoagglutination of wild-type strain B2 and the complemented mutant was 74.24% (± 0.25) and 73.65% (± 0.22), respectively, the mutant B2::cj0005c had an

Fig. 4. Invasion and adhesion assays with wild-type strain B2, knockout mutant B2::cj0005c and complemented mutant B2::cj0005c-comp-cj0005c. Dark grey bars: gentamicin protection invasion assays after centrifugation of the bacteria onto Caco2 cells. Taking the number of *C. jejuni* strain B2 colonies recovered as 100%, the mean percentage of knockout mutant B2::cj0005c colonies was 48.6%. Complementation of the mutant partially restored the parental phenotype (87% recovery). See text for details. Light grey bars: adhesion assays after centrifuging of the bacteria onto Caco2 cells. The percentage of adhered colonies of the mutant B2::cj0005c was only 67% compared to wild-type isolate B2.

Table 2. Transcription level of genes responsible for the synthesis of LegAm and the gene encoding heat-shock protein DnaJ in *C. jejuni* wild-type strain B2 and mutant B2::*cj0005c*

The crossing point data are means $\pm$ SD. Strongly downregulated genes are in bold. See text for details.

Gene	Crossing point		Fold change (<i>t</i> -test <i>P</i> -value)	Function
	B2:: <i>cj0005c</i>	B2 wild-type		
<i>dnaJ</i> (<i>cj1260c</i>)	23.34 ($\pm$ 0.09)	20.97 ($\pm$ 0.05)	5.16 (0.0001)	Heat shock response
<i>ptmA</i> (<i>cj1332</i>)	22.29 ($\pm$ 0.55)	20.79 ($\pm$ 0.17)	2.82 (0.0001)	LegAm synthesis
<i>ptmB</i> (<i>cj1331</i>)	19.40 ($\pm$ 0.77)	16.83 ($\pm$ 0.01)	5.93 (0.0001)	LegAm synthesis
<i>ptmC</i> (<i>cj1327</i>)	18.31 ($\pm$ 0.47)	16.20 ($\pm$ 1.19)	4.31 (0.0001)	LegAm synthesis
<i>ptmD</i> (<i>cj1328</i>)	20.53 ($\pm$ 0.59)	20.16 ($\pm$ 0.72)	1.29 (0.39)	LegAm synthesis
<i>ptmE</i> (<i>cj1329</i>)	18.11 ($\pm$ 0.06)	15.17 ($\pm$ 0.38)	7.67 (0.0001)	LegAm synthesis
<i>ptmF</i> (<i>cj1330</i>)	19.74 ($\pm$ 0.50)	18.30 ($\pm$ 0.89)	2.71 (0.0001)	LegAm synthesis
<i>ptmG</i> (<i>cj1324</i>)	19.16 ($\pm$ 0.25)	17.64 ($\pm$ 0.15)	2.86 (0.0001)	LegAm synthesis
<i>ptmH</i> (<i>cj1325/6</i>)	20.17 ($\pm$ 0.26)	18.82 ($\pm$ 0.12)	2.54 (0.017)	LegAM synthesis

autoagglutination level of only 37.02 % ($\pm$ 0.61), which was significantly reduced compared to B2 ($P < 0.001$).

DISCUSSION

C. jejuni possesses a highly branched and complex respiratory chain that allows the pathogen to use a variety of electron donors such as D-lactate, hydrogen, formate, succinate, malate, NAD(P)H and sulphite (Kelly, 2001; Sellars *et al.*, 2002; Myers & Kelly, 2005; Weerakoon *et al.*, 2009). The enzyme responsible for the oxidation of sulphite to sulphate and the release of electrons applied into the respiratory chain is a molybdenum-containing sulphite:cytochrome *c* oxidoreductase (SOR) which is composed of *Cj0005c* and *Cj0004c* and has homology to the corresponding enzyme of *Starkeya novella*. The two subunits were shown to constitute a functional SOR which respire sulphite in the presence of oxygen. The SOR-generated electrons, in turn, enter the respiratory chain after the *bc₁* complex at the level of cytochrome *c* (Kappler *et al.*, 2000; Kappler & Dahl, 2001; Myers & Kelly, 2005).

In this report, we describe a *C. jejuni* *cj0005c* mutant that has strongly reduced motility and, therefore, a reduced capacity to invade host cells. In contrast to other motility-reduced mutants, no gene of the flagellar apparatus itself is affected, but SOR, an enzyme mediating the delivery of electrons into the respiratory chain of the pathogen. Motility was shown to be important for *C. jejuni* to colonize and invade the respective host. Several studies in recent years have described the impact of genes and proteins of the flagellar apparatus on host colonization or the invasion of host cells in particular. One of the first publications, by Morooka *et al.* (1985), described that non-motile *C. jejuni* strains were cleared from the intestinal tract of suckling mice, whereas a wild-type strain colonized the lower portion of the intestine, the caecum and the colon. Furthermore, the colonization of rabbits was shown to depend on the flagellum, since a non-flagellated mutant

was unable to survive inside the rabbit intestine (Pavlovskis *et al.*, 1991). In addition to the studies mentioned above, the chicken intestinal tract was shown to be colonized only by fully motile strains of *C. jejuni*, which demonstrated that an intact flagellum is an important colonization factor (Nachamkin *et al.*, 1993). Homologous recombination techniques were successfully applied to generate mutants that subsequently were investigated in cell culture experiments for their ability to invade host cells. Thereby, the importance of motility for the invasiveness of *C. jejuni* could be confirmed (Wassenaar *et al.*, 1991; Yao *et al.*, 1994; Szymanski *et al.*, 1995; Konkel *et al.*, 2004). Meanwhile, many genes and proteins have been characterized which contribute to an intact flagellum in order to guarantee motility and, therefore, infectivity (Hendrixson, 2008). In order to find a link between the absence of a functional SOR on the one hand, and the reduced motility of the mutant on the other hand, we investigated the transcription levels of genes known to be important for flagellar composition. We detected a significant down-regulation of genes *fliR* and *flhA*, which encode proteins shown to be essential for the expression of FlgD and FlgE2, and of *rpoN*, encoding the RNA polymerase σ^{54} subunit of the pathogen, known to be responsible for the expression of the *flgDE2* locus as well as for the expression of minor flagellin FlaB (Hendrixson *et al.*, 2001; Hendrixson & DiRita, 2003). These results indicate that the motility of the SOR mutant is negatively affected, although we have no data on protein level regarding the proteins whose expression is controlled by the gene products of *fliR*, *flhA* or *ropN*.

The finding that the loss of a gene which is responsible for the supply of electrons into the respiratory chain leads to reduced motility raised the question whether the reduced motility is linked to the impaired energy metabolism. However, when we compared the intracellular ATP level of B2::*cj0005c* with the wild-type strain and the *cj0005c*-complemented mutant, we detected no differences. In addition, the cell proliferation of the mutant was not

negatively affected when the bacteria were grown in MH broth. Only in the presence of sodium sulphite was the mutant's growth clearly impaired, probably due to the reduced ability of the mutant to clear this potentially antimicrobial chemical.

Surprisingly, when we eliminated motility as a virulence-associated factor by centrifuging the parental strain B2 and its mutant directly onto the host cells, we were able to shift the infectivity of the mutant from 29.8 % to only 48.6 %, indicating that not only reduced motility is responsible for the mutant's impaired invasiveness compared to the wild-type strain. To obtain a more detailed insight into the mechanism of host cell invasion, adhesion assays were carried out. These experiments detected a reduced capacity of mutant B2::*cj0005c* compared to the wild-type strain in this initial host-pathogen interaction. Since many *C. jejuni* genes which contribute to the virulence of the pathogen and to the process of adhesion in particular have been described, we investigated whether the loss of a functional SOR has an impact on the transcription level of these genes. *C. jejuni* genes involved in stress responses, motility or chemotaxis were shown to be essential for the invasion of host cells. In addition, genes important for binding and adhesion were examined. Associated with the gene encoding the heat-shock protein DnaJ, the transcription of several genes responsible for the synthesis of legionaminic acid (LegAm) were shown to be significantly downregulated in the mutant B2::*cj0005c*.

The acetoamidino form of LegAm together with pseudaminic acid are two distinct nine-carbon sugars, which are O-linked to flagellin in most *C. jejuni* strains (Logan *et al.*, 2008). Altogether eight genes have been identified which are required for the synthesis of LegAm. These so-called post-translational modification (*ptm*) genes are *cj1324* (*ptmG*), *cj1325/6* (*ptmH*), *cj1327-cj1330* (*ptmC*, *ptmD*, *ptmE*, *ptmF*), *cj1331* (*ptmB*) and *cj1332* (*ptmA*) (McNally *et al.*, 2007). Although knowledge of the importance of O-glycosylation by LegAm is limited, recent investigations by Howard *et al.* (2009) gave a first insight into its biological relevance. A *C. jejuni* *cj1324* mutant showed full motility, but less hydrophobicity and impaired autoagglutination and biofilm formation due to the absence of two LegAm glycan modifications. Furthermore, this mutant had a reduced capacity to colonize chickens. When we investigated the transcription level of the LegAm genes in the *cj0005c* mutant, we detected only a 2.86-fold downregulation of *cj1324* (*ptmG*). However, we also could clearly show reduced autoagglutination but no alterations in biofilm formation (data not shown). In contrast to Howard *et al.* (2009), we did not investigate the genes *cj1321-cj1323*, but we examined all genes required for LegAm synthesis with regard to altered transcription. This revealed transcription of *ptmB* (*cj1331*), *ptmC* (*cj1327*) and *ptmE* (*cj1329*) to be strongly downregulated in the *cj0005c* mutant, whereas the transcription of genes *ptmA* (*cj1332*), *ptmF* (*cj1330*) and *ptmG* (*cj1324*) was significantly downregulated but to a lesser extent. However, we have no data

regarding the corresponding proteins, and the data obtained so far and the resulting conclusions refer exclusively to our findings of reduced transcription. Since we have not performed transcription analysis covering the whole genome of the pathogen, alterations in the transcription level of other genes are likely. But the results obtained so far might indicate a correlation between the mutant's altered biological properties (in particular diminished adhesion and autoagglutination) and the downregulation of genes responsible for LegAm synthesis.

In this study, we have characterized an invasion-deficient mutant, which owes its particular phenotype not to a mutation in a gene directly responsible in the process of motility, adherence or infection, but to a mutation within a gene involved in the energy metabolism of the pathogen. Despite this, we demonstrated an influence on events that are necessary for host cell invasion. It cannot be excluded that the reduced motility and the downregulation of virulence-associated genes are connected to the attempt of the pathogen to maintain the intracellular ATP level even in the absence of an enzyme which provides electrons for the respiratory chain. Other *C. jejuni* mutants with restricted energy metabolism should be examined in the future in order to investigate if a constant ATP level accompanying reduced biological functions and downregulated transcription is a more general phenomenon in *C. jejuni*.

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