Campylobacter jejuni proteins Cj0952c and Cj0951c affect chemotactic behaviour towards formic acid and are important for invasion of host cells

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Campylobacter jejuni, an important food-borne bacterial pathogen in industrialized countries and in the developing world, is one of the major causes of bacterial diarrhoea. To identify genes which are important for the invasion of host cells by the pathogen, we screened altogether 660 clones of a transposon-generated mutant library based on the clinical *C. jejuni* isolate B2. Thereby, we identified a clone with a transposon insertion in gene *cj0952c*. As in the well-characterized *C. jejuni* strain NCTC 11168, the corresponding protein together with the gene product of the adjacent gene *cj0951c* consists of two transmembrane domains, a HAMP domain and a putative MCP domain, which together are thought to act as a chemoreceptor, designated Tlp7. In this report we show that genes *cj0952c* and *cj0951c* (i) are important for the host cell invasion of the pathogen, (ii) are not translated as one protein in *C. jejuni* isolate B2, contradicting the idea of a postulated read-through mechanism, (iii) affect the motility of *C. jejuni*, (iv) alter the chemotactic behaviour of the pathogen towards formic acid, and (v) are not related to the utilization of formic acid by formate dehydrogenase.

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INTRODUCTION

Campylobacter jejuni is a Gram-negative, spiral-shaped bacterium which is a major cause of bacterial diarrhoea in both developing and industrialized countries (Altekruse *et al.*, 1999; Friedman *et al.*, 2000). The infection is also characterized by fever and abdominal cramps, and in rare cases Guillain–Barré syndrome can emerge as a post-infection complication (Allos, 2001).

Despite its importance as a human pathogen, little is known about the mechanisms by which *C. jejuni* causes disease, although several publications have described potential virulence factors (Dasti *et al.*, 2010). Putative adhesion factors have been identified, e.g. the fibronectin-binding proteins CadF and FlpA (Konkel *et al.*, 1997, 2010), the autotransporter CapA, and a surface-exposed lipoprotein, JlpA (Jin *et al.*, 2001). In addition, the sialylated lipooligosaccharide outer core of *C. jejuni* has been demonstrated to be an important factor for the invasion of epithelial cells (Guerry *et al.*, 2000; Louwen *et al.*, 2008). Moreover, *C. jejuni* synthesizes a set of proteins which are secreted by the flagellar export apparatus during co-culture of the pathogen with epithelial cells, and

these are referred to as *Campylobacter* invasion antigens (Cia proteins) (Konkel *et al.*, 1999). Although the function of these proteins is still unknown, mutation of *ciaB* results in a significant reduction of competency to invade host cells (Konkel *et al.*, 2004).

Motility of *C. jejuni* has been shown to be an intestinal colonization factor (Morooka *et al.*, 1985), and O-linked glycosylation of flagellin is crucial for the attachment of the bacterium to intestinal epithelial cells (Yao *et al.*, 1994). In addition, the motility of the pathogen is linked to chemotaxis, which was initially described by Hugdahl *et al.* (1988). In the genome sequence of *C. jejuni* NCTC 11168, orthologues of the chemotaxis genes *cheA*, *cheW*, *cheV*, *cheY*, *cheR* and *cheB* have been identified, in which *cheA* encodes a histidine protein kinase activated by chemoreceptors. CheW interacts with the histidine kinase CheA, mediating the signal from the receptor, while CheY represents a chemotaxis regulator that interacts with the flagellar motor in a phosphorylated form. Furthermore, the proteins CheR and CheB serve as methyltransferase and methylesterase, respectively, for reversible chemoreceptor methylation (Hazelbauer *et al.*, 2008). Finally, the presence of two aerotaxis genes and 10 putative chemoreceptor genes, designated Tlps for transducer-like proteins, has been revealed. These chemoreceptors can be further

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Abbreviation: MCP, methyl-accepting chemotaxis protein.

classified into different groups based on structural homologies (Marchant *et al.*, 2002). Thereby, group A receptors possess a similar structure to methyl-accepting chemotaxis proteins (MCPs) of *Escherichia coli* and to family A transducers of *Halobacterium salinarum* (Zhang *et al.*, 1996). Due to the presence of both transmembrane domains and a periplasmic ligand-binding domain, these group A receptors are thought to sense external ligands.

Performing BLASTX analysis, *C. jejuni* genes *cj0952c* and *cj0951c* are suggested to be pseudogenes in *C. jejuni* strain NCTC 11168, and it is thought that a read-through from upstream *cj0952c* into downstream *cj0951c* would create one functional chemoreceptor, termed Tlp7. The background of this assumption is that Cj0952c possesses two transmembrane domains followed by a C-terminal HAMP domain, whereas Cj0951c is a putative MCP-domain signal transduction protein, and together they would constitute a typical group A receptor protein of a chemotaxis system (Marchant *et al.*, 2002).

In this report, we describe the influence of the *C. jejuni* genes *cj0952c* and *cj0951c* on the ability of the pathogen to invade host cells. We also demonstrate that the gene products are not translated into a single protein, and consequently the corresponding genes cannot be characterized as pseudogenes in the clinical isolate B2. Furthermore, we demonstrate that these proteins act as a receptor that affects chemotaxis of the pathogen in the presence of formic acid and describe how the oxidation of formic acid by formate dehydrogenase does not depend on the presence of this chemoreceptor.

METHODS

Bacterial stains, media and culture conditions. The *C. jejuni* strains used in this study were B2, which was isolated in the University Medical Center Göttingen from a patient suffering from gastroenteritis (Schmidt-Ott *et al.*, 2005; Dasti *et al.*, 2007), and the *C. jejuni* strains 81-176 and NCTC 11168. Bacteria were routinely grown on Columbia blood agar supplemented with 5% defibrinated sheep blood under microaerophilic conditions (85% N₂, 10% CO₂, 5% O₂) at 42 °C for 24 h. When needed, appropriate antibiotics were supplemented at the following concentrations: kanamycin (50 µg ml⁻¹) or chloramphenicol (30 µg ml⁻¹). For growth experiments, bacteria were grown at 42 °C in Mueller–Hinton (MH) broth under microaerophilic conditions in the presence or absence of 10 mM formic acid. All growth experiments were done in triplicate. *E. coli* strain DH5 α , which was used for cloning experiments, was grown on Luria–Bertani (LB) agar or in LB broth at 37 °C. When necessary, the medium was supplemented with ampicillin (100 µg ml⁻¹).

Generation of competent cells and electroporation. A 10 ml volume of LB broth was inoculated with a single *E. coli* DH5 α colony and incubated overnight at 37 °C under shaking. Three millilitres of the overnight culture was grown in 500 ml LB broth at 37 °C to OD₆₀₀ 0.35–0.45. The culture was placed on ice for 10 min and centrifuged for 15 min at 4000 g at 4 °C. The pellet was gently resuspended in 50 ml cold water and again centrifuged for 15 min at 4000 g. This step was repeated three times. Then the pellet was resuspended in 15 ml 10% (v/v) glycerol and centrifuged again. Finally, the cell pellet was carefully dissolved in a final volume of 1 ml

10% (v/v) glycerol and 100 µl aliquots were used for each electroporation. For the preparation of competent cells of *C. jejuni*, cells were collected from Columbia blood agar plates and centrifuged at 5000 g at 4 °C for 10 min. The cells were resuspended in 1 ml ice-cold wash buffer containing 272 mM sucrose and 15% (v/v) glycerol at 4 °C. This step was repeated three times. Finally, the pellet was resuspended in 400 µl washing buffer and 100 µl aliquots were used for each transformation.

After the addition of 0.5–3 µg plasmid DNA, the mixture was transferred into an ice-cold electroporation cuvette and the cuvette was incubated on ice for 30 min. Electroporation was performed at 2.5 kV, 25 µF and 200 Ω using a BTX Electro Cell Manipulator. Then, 500 µl SOC medium was added to the cuvette and the suspension was transferred onto a non-selective Columbia blood agar plate and incubated overnight at 37 °C under microaerophilic conditions. Finally, the cells were transferred onto a selective plate and incubated at 42 °C under microaerophilic conditions for a further 2–3 days.

Construction of the transposon library. *Campylobacter coli* gene *aphA-3* can be used as an antibiotic marker in *C. jejuni*, in which it confers resistance to kanamycin. In an earlier study, *aphA-3* was cloned into plasmid pSB1699 (Colegio *et al.*, 2001). We digested pSB1699 with *Bam*HI, which resulted in the release of the *aphA-3* insert. After purification of the insert from the agarose gel, we ligated it into the *Bam*HI-digested pBluescript SKII vector, which resulted in plasmid pBSK-Kan. This plasmid was used as a template for PCR amplification of 1.8 kb DNA fragments that consisted of the *aphA-3* gene flanked by *Kpn*I restriction sites. These amplified fragments were then further subcloned into the *Kpn*I-digested and dephosphorylated transposon construction vector EZ::TN pMOD (Epicentre Biotechnologies), which resulted in EZ::TN pMODKan. The Tn5 transposon named EZ::TN Kan was finally released from pMODKan by digestion with *Pvu*II.

The transposition reaction was carried out by mixing 100 ng gel-purified EZ::TN Kan transposon DNA, 2 µl EZ::TN transposase (Epicentre Biotechnologies) and 2 µl 100% (v/v) glycerol, followed by incubation for 30 min at room temperature. To generate random insertional mutants of *C. jejuni*, the clinical isolate B2 was electroporated with 100 ng of the transposon and the bacteria were incubated for 5 h at 37 °C under microaerophilic conditions. The bacteria were subsequently harvested and transformants selected on Columbia blood agar supplemented with 5% sheep blood and kanamycin, by incubating at 42 °C under microaerophilic conditions. The same procedure was repeated several times, which produced a library of 660 individual mutants by using eight different signature tags. MH broth supplemented with 15% (v/v) glycerol was used to store the mutants at –80 °C. Random insertion of the transposons was verified for a fraction of 19 mutants. After preparation of genomic DNA and digestion with *Bsp*HI, a Southern blot analysis was carried out with a digoxigenin-11-UTP-probed kanamycin-resistance gene under conditions described previously (Dasti *et al.*, 2007).

Cultivation of cells. Human colon carcinoma Caco-2 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 1 $\times$ non-essential amino acids, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. The cells were routinely cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

Gentamicin protection assays. Bacterial invasion of host cells was initially described by Everest *et al.* (1992). Prior to the assay, 2 $\times$ 10⁶ Caco-2 cells were seeded to each well of a six-well plate. Then, cells were washed with PBS and inoculated with 400 µl *C. jejuni* suspension. To ensure that the number of bacteria was identical in every assay performed, the bacterial solution was adjusted to OD₆₀₀ 0.5, which represented an m.o.i. of 100. At 2 h post-infection, the

bacterial suspension was removed and the cells were washed three times with $1 \times$ PBS before further incubation with culture medium containing $100 \mu\text{g}$ gentamicin ml^{-1} . Afterwards, cells were lysed with 1 % Triton X-100 for 10 min to release intracellular bacteria. The number of viable bacteria was determined by plating serial dilutions of the lysate on Columbia blood agar and counting the number of bacteria grown after incubation for 48 h at 42°C under microaerophilic conditions. Means and sds of the number of colonies counted for the mutant and complemented strains are indicated.

Altogether 660 clones of the generated *C. jejuni* strain B2 transposon library were screened individually by performing gentamicin protection assays in which every clone was tested in triplicate. Clones that were shown to be less invasive were further investigated in 12 independent experiments to confirm their phenotype of reduced invasiveness.

Motility assay. To test for bacterial motility, $1 \mu\text{l}$ of an overnight culture adjusted to OD_{600} 0.025 was stabbed into the centre of a 0.4 % MH agar plate with the help of a suitable normalized inoculation loop. Plates were incubated at 42°C under microaerophilic conditions for 36 h. The low concentration of the agar allowed the bacteria to swarm, forming a visible halo within the agar. For judgement of motility the expansion of the haloes was examined by measuring the respective diameter. Each experiment was performed seven times.

Chemotaxis assays. The chemotaxis assay was performed as described by Vegge *et al.* (2009) with the following modifications. Bacteria were grown on Columbia blood agar plates overnight at 42°C under microaerophilic conditions. They were then suspended in PBS (pH 7.0), adjusted spectrophotometrically to OD_{600} 1 and mixed (1:1) with tempered soft agar (0.8 %, Becton Dickinson). Afterwards, 12 ml of the bacterial soft agar suspension was poured into a Petri dish and 6 mm filter discs (Oxoid) soaked with $20 \mu\text{l}$ of the test chemical were placed on the solidified agar (0.1 M and pH 7.0; all chemicals were obtained from Sigma-Aldrich). Zones of bacterial attraction or repulsion were measured after 4 h incubation at 42°C under microaerophilic conditions. The chemotaxis assays were carried out four times.

Isolation of nucleic acids. Genomic DNA of *C. jejuni* was isolated using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. Bacterial RNA was extracted from *C. jejuni* cells with the RiboPure Bacteria kit (Ambion) following the recommendations of the manufacturer's protocol. After RNA isolation, DNA contamination was removed by DNase I treatment (Ambion) and incubation at 37°C for 30 min, followed by denaturation of the enzyme for 15 min at 75°C . After DNase I treatment, PCR assays were performed to make sure that the RNA was free of remaining traces of DNA. DNA and RNA concentrations were determined with a NanoDrop ND 1000 spectrophotometer (Thermo Scientific).

Sequencing of genomic DNA of *C. jejuni*. Ten micrograms of genomic DNA was sequenced directly by SeqLab (Göttingen, Germany) using 10 pmol of the KanF primer listed in Table 1.

RT-PCR analysis. Conventional RT-PCR assays were carried out with the OneStep RT-PCR kit (Qiagen) as instructed by the manufacturer. After reverse transcription of 10 ng for 30 min at 50°C , 35 cycles of amplification were carried out according to the following protocol: cDNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 1 min, with a final incubation at 72°C for 10 min. PCR products were analysed on a 1 % agarose gel containing ethidium bromide at a final

concentration of $1 \mu\text{g ml}^{-1}$. The primers used for RT-PCR are listed in Table 1.

PCR and RT-PCR assays for nucleic acid alignment. To compare the DNA and RNA sequence between *cj0952c* and downstream *cj0951c*, PCR and RT-PCR assays were carried out using the primers Cj0952c-51cF and Cj0952c-51cR (Table 1) for the amplification of a 460 bp fragment encompassing the 3' domain of *cj0952c*, the intergenic region and the 5' domain of *cj0951c*. PCR was carried out using a TRIO-Thermoblock (Biometra) with genomic DNA of *C. jejuni* strain B2 as template. Each $50 \mu\text{l}$ of PCR mixture contained 40 ng genomic DNA, 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , all four dNTPs (0.2 mM each) and 2.5 U *Taq* DNA polymerase (Roche). Initial incubation at 95°C for 1 min was followed by 40 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. The RT-PCR assay was carried out with the OneStep RT-PCR kit as instructed by the manufacturer in a TRIO-Thermoblock. Briefly, 80 ng total RNA was reverse-transcribed for 30 min at 50°C . Following cDNA synthesis, HotStarTaq DNA polymerase (Qiagen) was initially activated for 15 min at 94°C . Then, 40 cycles of amplification were carried out according to the following protocol: cDNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 1 min, with a final incubation at 72°C for 10 min. After direct sequencing of the PCR amplicons by SeqLab with the primers used for amplification, derived sequences were compared by performing BLASTN analysis.

Real-time RT-PCR analysis. Semi-quantitative real-time RT-PCR analysis was carried out with a LightCycler 1.5 instrument (Roche) and the QuantiFast SYBR Green RT-PCR kit (Qiagen). Fifty nanograms of isolated RNA from *C. jejuni* B2 wild-type strain and from B2 Δ *cj0952c* complemented with *cj0952c* and *cj0952c-cj0951c* was used to compare the transcription levels of *cj0952c*. Amplicons of the 23S rRNA gene, which is transcribed constitutively, were used to adjust the *cj0952c* RT-PCR based on the crossing points obtained for this gene. 23S rRNA RT-PCR was performed as follows: after initial reverse transcription for 20 min at 50°C and denaturation for 5 min at 95°C , 40 cycles of denaturation (95°C , 10 s), annealing (50°C , 10 s) and elongation (72°C , 5 s) were carried out. After adjustment of the RNA samples with regard to the crossing points determined for the 23S rRNA gene, real-time RT-PCR assays for the amplification of *cj0952c* were run. The elongation time for the amplification of *cj0952c* was shortened to ensure that only wild-type *cj0952c*, and not *cj0952c* harbouring the transposon, was amplified. Reverse transcription for 20 min at 50°C and denaturation for 5 min at 95°C were followed by 40 cycles of denaturation (95°C , 10 s), annealing (55°C , 10 s) and elongation (72°C , 10 s). The primers used for both assays are shown in Table 1. Every assay was run in duplicate. The presence of the signal corresponding to wild-type *cj0952c* but not to transposon-interrupted *cj0952c* was ensured by melting-curve analysis and agarose gel electrophoresis (results not shown). Semi-quantitative measurement of transcription levels was calculated as follows:

$$x = 2^{\Delta\text{Cp}}$$

where x represents the factor of altered transcription, and ΔCp represents the difference in crossing points ($\text{Cp}_1 - \text{Cp}_2$) of two samples to be compared.

Cloning of *C. jejuni* genes into expression vector pRRC. The *C. jejuni* genes *cj0952c* and *cj0951c* alone, *cj0952c-cj0951c*, *cj0952c-cj0951c-cj0950c* and gene *cj0951c* from *C. jejuni* strain 81-176 were PCR-amplified and subsequently cloned into vector pRRC using primers harbouring restriction sites for *XbaI* (Table 1). The *Campylobacter* vector pRRC contains a *cam*^R gene cassette that is flanked by a 16S rRNA sequence and sequences for tRNAs for alanine and isoleucine. A single *XbaI* site is located immediately downstream

Table 1. Oligonucleotide primers used for sequencing, RT-PCR analysis and cloning of *C. jejuni* genes

*Xba*I restriction sites for cloning in pRRC are underlined, the start codon of *cj0952c* is shown in bold type and the putative SD sequence is shown in italic type.

Gene	Primer	Sequence (5'–3')
<i>aphA-3</i>	Sequencing	
	KanF	TATCACCTCAAATGGTTCGCT
<i>cj0951c</i>	RT-PCR	
	Cj0951F	GAGTTACCAAAGCCCTAGCATC
<i>cj0952c</i>	Cj0951R	CATGGTCAATCAAGCAGG
	Cj0952F	GCCACTTCCTTGAGC
<i>cj0953c</i>	Cj0952R	CGCCCTTTGATTGCAGATG
	Cj0953F	GGCTGAAATGATTACTTCTAC
23S	Cj0953R	CTAACCGATATTAACGCAGC
	Cj23SF	GTTTCGCCATTTAAAGCGGTA
	Cj23SR	TGCTCTTGGCAGAACACAG
<i>cj0952c-51c</i>	Alignment	
	Cj0952c-51cF	GCCAATTCATTTTCATTTTGAGA
	Cj0952c-51cR	TTTAAATGCTTAAAGTTGTTGTTGG
<i>cj0952c</i>	Cloning in pRRC	
	Cj0952cF	GCTCTAGATAGGAACTATGATGTTTAAACTA
<i>cj0951c</i>	Cj0952cR	GCTCTAGAAATTAATTGATATATCCACA
	Cj0951cF	NNNTCTAGAAATTTATCGAAAAAACTCATAAGG
<i>cj0952c/</i>	Cj0951cR	NNNTCTAGATTAAATTTGAAATTGGTTAAGTTTCGC
	Cj0952cF	GCTCTAGATAGGAACTATGATGTTTAAACTA
<i>cj0951c</i>	Cj0951cR	NNNTCTAGACCTTTAAATTTGAAATTGGTTAAGTTTCGC
<i>cj0952c/</i>	Cj0952cF	GCTCTAGATAGGAACTATGATGTTTAAACTA
<i>cj0951cHis</i>	Cj0951His	GGCTCTAGATTAGTGATGGTGATGGTGATGAATTTGAAATTGGTTAAGTTTCGC
<i>cj0952c/</i>	Cj0952F	GCTCTAGATAGGAACTATGATGTTTAAACTA
<i>cj0950c</i>	Cj0950R	NNNTCTAGAGGGCATTATTTAAAAAAGATTTTGATTTTC

of the *cam*^R gene cassette, and allows the expression of *Xba*I-cloned genes under the control of the constitutively expressed *cam*^R gene promoter. After introduction of the plasmid into competent cells of *C. jejuni*, the *cam*^R gene cassette together with the *Xba*I-cloned gene was integrated into one of the 16S rRNA loci of the recipient cell via highly efficient double recombination (Karlyshev & Wren, 2005).

PCR was carried out in a TRIO-Thermocycler (Biometra) with 10 ng genomic DNA of *C. jejuni* strain B2 as template. Each 50 µl of PCR mixture contained 0.02 U KOD Hot Start DNA Polymerase (Novagen), dNTPs (0.2 mM each), 1.5 mM MgSO₄, 10 pmol of each primer and 1 × KOD Hot Start buffer. Initial incubation at 95 °C for 3 min was followed by 40 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 5 min. The PCR products were purified with the QIAquick PCR Purification kit (Qiagen) and digested with *Xba*I (New England Biolabs). Plasmid pRRC was also *Xba*I-restricted and dephosphorylated using Antarctic Phosphatase (New England Biolabs). The restricted PCR fragments were ligated into pRRC using the Quick Ligase kit (New England Biolabs) according to the manufacturer's instructions. After transformation of competent *C. jejuni* B2Δ*cj0952c* cells, the complementation constructs were verified by PCR analysis for the detection of the respective genes.

Purification of His-tagged proteins and immunoblot analysis.

For the detection of His-tagged proteins, *C. jejuni* cells were grown in 500 ml MH broth at 42 °C for 36 h under microaerophilic conditions (85 % N₂, 10 % CO₂, 5 % O₂). All the following steps were carried out according to the protocol from The QIAexpressionist with the solutions recommended for purification of 6 × His-tagged proteins

under denaturing conditions (Qiagen). Briefly, after lysis of the cells, 1 ml 50 % Ni-NTA slurry was added and after shaking for 1 h, the lysate-resin mixture was loaded into an empty column. After removal of the flowthrough, the Ni-NTA slurry was washed twice with buffer C followed by elution of the His-tagged proteins with buffers D and E. Aliquots of 10 µl were separated on 15 % SDS-PAGE and transferred to a PVDF membrane (GE Healthcare) using a Semi-Dry Transfer system (Sartorius). The membrane was blocked for 1 h with 5 % milk powder in PBS containing 0.05 % Tween 20 and incubated in blocking solution. After incubation of the membrane with a 1:3000 diluted monoclonal mouse anti-His primary antibody (Qiagen) overnight at 4 °C, the immune complexes were labelled with a 1:3000-diluted horseradish peroxidase-conjugated anti-mouse secondary antibody (Dianova) for 1 h at room temperature and visualized by ECL chemiluminescence.

Statistical analysis. Significant differences between mean values were calculated by Student's *t* test using the STATISTICA software (StatSoft). *P* values of less than 0.01 were considered to be significant.

RESULTS

Invasion of Caco-2 cells by *C. jejuni* isolate B2 and *C. jejuni* strains NCTC 11168 and 81-176

To initially determine the capability of *C. jejuni* strain B2 to invade Caco-2 cells we compared this isolate with the well-characterized *C. jejuni* strains NCTC 11168 and 81-176.

Performing gentamicin protection assays, we could clearly show that isolate B2 is more invasive than both of these reference strains. While the mean number of colonies recovered from isolate B2 was 1.2×10^6 c.f.u.ml⁻¹, 0.8×10^5 and 4.1×10^5 c.f.u. ml⁻¹ were obtained for reference strains NCTC 11168 and 81-176, respectively. The results are shown in Fig. 1(a).

Identification of *cj0952c*

To identify genes which contribute to the ability of *C. jejuni* to invade Caco-2 cells, we screened individually altogether 660 clones of a transposon-based mutant library of *C. jejuni* strain B2 by performing gentamicin protection assays. Thereby, seven clones with a strongly decreased

invasiveness could be detected. The transposon insertion site of the respective *C. jejuni* clones was mapped by direct sequencing of genomic DNA using a primer that binds directly to the 5' region of the kanamycin-resistance cassette and therefore allows the identification of the affected *C. jejuni* genes. To confirm the decreased invasiveness, gentamicin protection assays with the respective clones and parental strain B2 were repeated five times. Thereby, the mean number of colonies recovered from B2 was 1.1×10^6 c.f.u. ml⁻¹. The mean c.f.u. ml⁻¹ values obtained for the respective mutants were as follows: *cj0005c*, 2.8×10^5 c.f.u. ml⁻¹; *cj0078c*, 3.4×10^5 c.f.u. ml⁻¹; *cj0093*, 4.1×10^5 c.f.u. ml⁻¹; *cj0268c*, 3.3×10^5 c.f.u. ml⁻¹; *cj0721c*, 3.2×10^5 c.f.u. ml⁻¹; *cj1439c*, 3×10^5 c.f.u. ml⁻¹; and *cj0952c*, 2.3×10^5 c.f.u. ml⁻¹. Since the *P* value for

Fig. 1. (a) Comparison of the capability of the *C. jejuni* strains B2, NCTC 11168 and 81-176 to invade Caco-2 cells. The mean values of colonies recovered from NCTC 11168 and 81-176 were 0.8×10^5 and 4.1×10^5 c.f.u. ml⁻¹. In contrast, the number of colonies obtained from strain B2 was 1.2×10^6 c.f.u. ml⁻¹, demonstrating the increased infectivity of this clinical isolate. (b) A general view of genes detected in our study that mediate reduced invasiveness of *C. jejuni* strain B2. (c) Description of the detected genes concerning function and localization as far as known. See text for details.

every mutant tested was less than 0.001, the reduced invasion capacities were judged to be significant. An overview of the genes mapped is given in Fig. 1(b, c). One of the clones with strongly decreased invasion capacity exhibited insertion of the transposon in gene *cj0952c* at position 106 according to *C. jejuni* strain NCTC 11168, and we chose this mutant as the object of further investigations.

Insertion of the transposon in *cj0952c* does not affect transcription of either the upstream gene *cj0953c* or the downstream gene *cj0951c*

To find out whether the insertion of the transposon into *cj0952c* has any impact on the transcription of the adjacent genes, we carried out rRT-PCR assays with the wild-type strain B2 and the mutant B2Δ*cj0952c*. Since the non-coding sequence between *cj0953c* and *cj0952c* has a length of 96 bp, and the distance between *cj0952c* and *cj0951c* is 263 bp in the clinical isolate B2, transcription of these three genes in a polycistronic manner cannot be excluded. Accordingly, insertion of a transposon in one gene may interrupt transcription, particularly of genes located downstream. As shown in Fig. 2, transcription of *cj0952c* including the transposon, but also of *cj0953c* and *cj0951c*, could clearly be detected by RT-PCR in the mutant B2Δ*cj0952c*, indicating that the invasion-deficient phenotype is exclusively mediated by the insertion of the transposon into *cj0952c*.

Functional complementation of *C. jejuni* B2Δ*cj0952c*

In order to restore the invasiveness of the B2Δ*cj0952c* mutant, *cj0952c* and *cj0951c* alone and *cj0952c* together with *cj0951c* were cloned into vector pRRC and introduced into mutant B2Δ*cj0952c* by electroporation. Performing gentamicin protection assays on Caco-2 cells, the number of *C. jejuni* colonies recovered demonstrated that *cj0952c*–*cj0951c*, but not *cj0952c* or *cj0951c* alone, altered the mutant phenotype to that of wild-type B2. The results of five independent experiments are shown in Fig. 3(a, b). In contrast to wild-type strain B2, transcription of *cj0952c* in the complemented mutants is under control of a *cam*^R promoter. To compare the transcription levels of *cj0952c*, we carried out real-time RT-PCR analysis. After adjustment of the RNA samples according to the crossing points of the 23S rRNA transcripts, we investigated the *cj0952c* transcription level of the wild-type strain and of the mutant B2Δ*cj0952c* complemented with *cj0952c* alone and with *cj0952c*–*cj0951c*. Surprisingly, while the mean crossing points of both complemented mutants were 20.49 (*cj0952c*) and 20.18 (*cj0952c*–*cj0951c*), the mean crossing point for the detection of *cj0952c* with RNA from the wild-type strain was 32.44, demonstrating a more than 4000-fold upregulation of *cj0952c* in the complemented mutants compared with the wild-type strain. Accordingly, lower transcription of *cj0952c* could be excluded as a reason for the incomplete invasion phenotype.

Fig. 2. (a) Genome arrangement of *cj0953c*, *cj0952c*, *cj0951c* and *cj0950c* in the clinical *C. jejuni* isolate B2 (GenBank accession no. GU799572). The direction of the arrows denotes the direction of transcription. Primers are shown as arrowheads. The insertion site of the transposon is indicated. (b) RT-PCR analysis for the investigation of *C. jejuni* genes *cj0953c*, *cj0952c* and *cj0951c* in the wild-type B2 strain and the mutant strain B2Δ*cj0952c*. The PCR amplicons show the expected size for *cj0953c* (225 bp), *cj0952c* wild-type (267 bp), *cj0952c* with transposon insert (2091 bp) and *cj0951c* (356 bp). PCR analysis of the RNA templates under investigation yielded no amplicons, demonstrating that DNA contamination was completely removed (not shown). Primers used for RT-PCR assays are listed in Table 1.

Fig. 3. (a) Infection of Caco-2 cells. Restoration of invasiveness to the level of the *C. jejuni* wild-type B2 strain was successful by complementation of B2 Δ cj0952c with cj0952c–cj0951c but not with cj0952c or cj0951c alone. The additional expression of Cj0950c did not influence the extent of restoration. Complementation of B2 Δ cj0952c with the corresponding gene of *C. jejuni* strain 81-176 (B2 Δ cj0952c-comp-81-176) gave the same extent of restoration as complementation of the mutant with cj0952c–cj0951c from the parental isolate B2. (b) Mean numbers of colonies recovered after five independent experiments; SDs are indicated. While the reduced invasiveness of the mutant B2 Δ cj0952c and the mutants complemented with cj0952c or cj0951c was significant compared with the parental strain B2 ($P < 0.001$), the variation in recovered colony numbers after invasion assays with mutants complemented with cj0952c–cj0951c, cj0952c–cj0950c or 81-176 was not significant ($P > 0.001$).

In contrast to the clinical isolate B2 and *C. jejuni* strain NCTC 11168, in which the putative chemoreceptor Cj0952c–Cj0951c is composed of two genes, the corresponding protein in *C. jejuni* strain 81-176 is encoded by only one gene that covers the transmembrane domain, the HAMP domain as well as the MCP domain. To investigate whether the single gene of *C. jejuni* 81-176 is able to complement B2 Δ cj0952c, we cloned it into pRRC and introduced the recombinant plasmid into the mutant. Subsequent invasion assays with Caco-2 cells restored the invasiveness to wild-type levels, and this is summarized in Fig. 3(a, b). In a further attempt to fully restore the invasiveness of the B2 Δ cj0952c mutant, we cloned a PCR fragment encompassing cj0952c–cj0951c–cj0950c into vector pRRC. The cj0950c gene is located downstream of cj0951c and encodes a putative lipoprotein of unknown function. It contains a predicted signal peptide and shows similarities to heat-shock protein HSLJ of *E. coli*. Since

nothing is known about the role of this gene, a functional correlation of Cj0950c with Cj0952c and Cj0951c could not be excluded. After introduction of the recombinant plasmid carrying all three genes, gentamicin protection assays with Caco-2 cells were again carried out. In these assays, the recovery rate of colonies compared with the mutant complemented with cj0952c–cj0951c was not significantly different, indicating that Cj0950c does not have a functional relationship with Cj0952 and Cj0951c. The results are summarized in Fig. 3(a, b).

***C. jejuni* genes cj0952c and cj0951c of the clinical isolate B2 are not translated as one protein**

C. jejuni genes cj0952c and cj0951c in the clinical isolate B2 as well as in strain NCTC 11168 are thought to be pseudogenes, and a read-through mechanism is postulated to create one functional protein. We carried out PCR and

RT-PCR with DNA and RNA of the parental strain B2 to answer the question of whether the synthesized RNA is altered during the process of transcription in such a way that, for example, the stop codon of *cj0952c* is replaced to create one ORF for both genes. PCR and RT-PCR assays were performed with the primers Cj0952c-51cF and Cj0952c-51cR (Table 1), which span the 3' region of *cj0952c*, the intergenic region and the 5' domain of *cj0951c*. After sequencing of the PCR and RT-PCR products and subsequent alignment we could not detect any differences between the two amplicons. The stop codon of *cj0952c* at position 891 663 of the genome was not converted and nor could other changes of the RNA sequence be detected, which clearly demonstrated that no read-through mechanism can be postulated based on information obtained from the RNA sequence (data not shown). However, by the detection of an RT-PCR amplicon, we could verify that *cj0951c* and *cj0952c* are cotranscribed on one mRNA. To determine whether Cj0952c and Cj0951c are translated as one protein or separately as single proteins, we cloned both genes together into pRRC. For the detection of the protein expressed *in trans*, the primer corresponding to the 3' end of *cj0951c* was expanded by nucleotides representing a 6 × His-tag (Table 1). After introduction of the recombinant plasmid into the mutant B2Δ*cj0952c*, we lysed cells of the complemented mutant and of parental strain B2, which served as a negative control in the following immunoblot experiment. The proteins of both pools were purified on a Ni-NTA matrix, separated by SDS-PAGE, blotted and finally stained using a monoclonal antibody against the His-tag. Thereby, we could clearly detect a band corresponding to approximately 25 kDa, which agrees with the predicted protein size of Cj0951c but not with the expected size for Cj0952c–Cj0951c (58 kDa), indicating that no read-through mechanism takes place in *C. jejuni* strain B2 (Fig. 4). We repeated the experiment with other randomly chosen clones obtained after electroporation and

confirmed the results of the immunoblot analysis. While in most of the clones expression of the recombinant protein could not be detected, two clones showed a band corresponding to 25 kDa after immunostaining. A recombinant protein with a size of approximately 58 kDa was never observed (data not shown).

***C. jejuni* mutant B2Δ*cj0952c* possesses decreased motility**

Since it is well known that chemotaxis of the pathogen is linked to motility (Hugdahl *et al.*, 1988), we used motility assays to determine whether the B2Δ*cj0952c* mutant has an altered phenotype. Thereby, we could ascertain that the motility of the mutant was clearly reduced compared with that of the parental strain B2. While the mean diameter of the motility zone of B2 was 47.4 ± 3.13 mm, the mutant was significantly less motile (30.6 ± 2.4 mm, $P < 0.0001$). After complementation of the mutant with *cj0952c* or *cj0951c*, the motility was not altered (30.6 ± 2.9 mm or 29.4 ± 2.3 mm, respectively) and remained significantly reduced compared with that of the parental strain ($P < 0.0001$). In contrast, when *cj0952c* together with *cj0951c* was introduced into the mutant, the complementation restored the motility to the wild-type level (46.6 ± 3.2 mm). We were also successful in restoring the parental phenotype after introduction of the corresponding gene from *C. jejuni* 81-176. The motility level of this revertant was 46.2 ± 2.8 mm. The results are shown in Fig. 5.

Cj0952c–Cj0951c alters chemotactic behaviour of *C. jejuni* in the presence of formic acid

In order to investigate the function of Cj0952c–Cj0951c, we compared the chemotactic behaviour of the B2Δ*cj0952c* mutant with that of the wild-type strain B2. For both, we could detect attraction towards L-asparagine, L-aspartate, L-cysteine, fumarate, L-glutamate, D-lactate, L(–)-malate, pyruvate, L-serine and succinate. Repulsion was demonstrated from cholic acid, deoxycholic acid, glycocholic acid and taurocholic acid, while no taxis response could be detected from PBS, L-fucose and citrate. No significant differences in the extent of attraction or repulsion between wild-type strain B2 and the mutant B2Δ*cj0952c* could be identified (results not shown).

In contrast, a clear difference could be observed for formic acid. Formic acid has recently been described as chemoattractant for *C. jejuni* (Vegge *et al.*, 2009). In accordance with the results of Vegge and co-workers, we were able to demonstrate a significant attraction halo around the fomite-soaked paper disc (Fig. 6a). In contrast, a biphasic halo with an inner repulsion zone surrounded by a faint ring was observed for B2Δ*cj0952c*. Complementation of the mutant with *cj0952c* or *cj0951c* alone did not reconstitute the chemotactic behaviour, while complementation with *cj0952c*–*cj0951c* or the corresponding gene from *C. jejuni* 81-176 did reconstitute chemotactic behaviour (Fig. 6a).

Fig. 4. Immunoblot for the detection of the recombinant protein in *C. jejuni*. Lanes: 1, molecular mass marker (molecular masses are indicated in kDa); 2, wild-type strain B2; 3, mutant B2Δ*cj0952c*-comp-*cj0952c*-*cj0951c*His. The stained molecule exhibits a molecular mass of approximately 25 kDa, which is in accordance with the size of Cj0951c. In contrast, a protein assembled from Cj0952c and Cj0951c would have a predicted molecular mass of 58 kDa.

Fig. 5. (a) Motility of wild-type B2 strain, B2Δ*cj0952c*, B2Δ*cj0952c*-comp-*cj0951c*, B2Δ*cj0952c*-comp-*cj0952c*, B2Δ*cj0952c*-comp-*cj0952c*-*cj0951c* and the mutant complemented with the corresponding gene from strain 81-176 (B2Δ*cj0952c*-comp-81-176). The reduced motility of B2Δ*cj0952c* compared with wild-type strain B2 was not affected by the introduction of *cj0952c* or *cj0951c* alone, but was affected by the combination of *cj0952c* and *cj0951c* or by the introduction of the corresponding gene of *C. jejuni* strain 81-176. Diagonal lines show the diameters of the bacterial spread on the agar plates. (b) Diameters of the motility zones of wild-type strain B2, the mutant B2Δ*cj0952c* and the complemented mutants. Motility assays were performed on 0.4% MH agar plates inoculated with the indicated strains and incubated at 42 °C under microaerophilic conditions for 36 h. Each experiment was performed seven times.

After four independent experiments, the attraction zones from B2 and the mutant complemented with both genes and the gene from *C. jejuni* 81-176 were 26.5 ± 2.6 mm, 25 ± 2.5 mm and 25.5 ± 2.6 mm, respectively, whereas the attraction zones of the mutant and the mutant complemented with *cj0952c* or *cj0951c* alone were 10.2 ± 0.9 mm, 10.0 ± 1.4 mm and 10.2 ± 1.7 mm, as shown in Fig. 6(b). This finding indicates that the *cj0952c* and *cj0951c* genes together encode a receptor that is involved in the chemotactic recognition of formic acid.

The utilization of formic acid is not altered by the presence or absence of Cj0952c–Cj0951c

Recombinant expression of Cj0952c–Cj0951c and detection of the protein by immunoblotting indicated that both

proteins are translated separately and not as a single chemoreceptor. To investigate whether this unusual composition of the chemoreceptor Cj0952c–Cj0951c is related to the utilization of formic acid by formate dehydrogenase, we measured the growth of the wild-type strain B2, the mutant, the *cj0952c*–*cj0951c*-complemented mutant and the mutant harbouring the single gene from *C. jejuni* 81-176 in the presence of 10 mM formic acid. As a negative control, the B2 strain with a transposon insertion in one of the genes encoding the subunits of formate dehydrogenase (B2Δ*fdh*) was used. In these experiments, we could not detect a relationship between chemoreceptor presence or composition and formic acid utilization. Wild-type strain B2, the mutant and the complemented mutant showed identical growth, while the growth of B2Δ*fdh* was clearly reduced (Fig. 7a). The growth of the same strains in

Fig. 6. Chemotactic behaviour in the presence of formic acid. 1, Wild-type strain B2; 2, B2Δcj0952c; 3, B2Δcj0952c-comp-cj0951c; 4, B2Δcj0952c-comp-cj0952c; 5, B2Δcj0952c-comp-cj0952c-cj0951c; 6, B2Δcj0952c-comp-81-176. (a) Whereas wild-type strain B2 and the mutant strain B2Δcj0952c complemented with *cj0952c-cj0951c* or the corresponding gene from *C. jejuni* strain 81-176 showed a significant attraction zone around the formate-soaked paper (green arrows), mutant B2Δcj0952c and the mutant complemented with *cj0952c* or *cj0951c* alone demonstrated a divergent chemotactic response: an inner repulsion zone (blue arrow) can be observed surrounded by a dense chemoattractant ring (green arrow). (b) Chemoattractant zones indicated in millimetres. The chemotactic responses of wild-type B2 and the mutant B2Δcj0952c complemented with *cj0952c-cj0951c* or the respective gene from strain 81-176 were almost identical, whereas *cj0952c* or *cj0951c* did not restore the parental phenotype and the complemented mutants showed significantly reduced chemotaxis ($P < 0.001$) towards formic acid.

the absence of formic acid gave comparable results, with the exception of the growth of B2Δ*fdh*. This was probably due to the presence of a small amount of formic acid in the MH broth which could not be metabolized by B2Δ*fdh* (Fig. 7b).

DISCUSSION

Since the introduction of transposon mutagenesis techniques to *C. jejuni* research (Colegio *et al.*, 2001; Golden *et al.*, 2000), this method has proven to be a powerful tool for the identification of many genes related to the pathogenicity of the bacterium. Motility-deficient mutants have been

described that have transposon insertions not only in genes with a strong homology to motility genes of known function but also in genes responsible for the chemotactic competence of *C. jejuni*, e.g. *cheA*, *cheY* and *cheB* (Golden & Acheson, 2002; Hendrixson *et al.*, 2001). Also, a reporter assay in combination with transposon mutagenesis for the investigation of flagellar regulation has identified genes which are at the beginning of the flagellar transcription cascade (Hendrixson & DiRita, 2003). Furthermore, a signature-tagged transposon library of *C. jejuni* has detected genes which are involved in the commensal colonization of the gastrointestinal tract of chickens, among them the gene encoding the MCP *docB* (Tlp10) and the adjacent gene *docA*, encoding a putative cytochrome *c* peroxidase (Hendrixson

Fig. 7. Growth curves of parental strain B2 (◆), B2Δ*cj0952c* (▲), B2Δ*cj0952c*-comp-*cj0951c* (■), B2Δ*cj0952c*-comp-81-176 (●) and B2Δ*fdh* (large filled squares) in MH broth supplemented (a) or not supplemented (b) with 10 mM formic acid. Error bars show SD. See text for details.

& DiRita, 2004). Signature-tagged transposon mutagenesis has shown *Campylobacter* colonization in chickens to be complex and dynamic (Grant *et al.*, 2005), and furthermore, screening of a transposon-based mutant library has identified *C. jejuni* genes that are related to polymyxin resistance and to acid adaptation of the pathogen (Lin *et al.*, 2009; Reid *et al.*, 2008). Additionally, transposon mutagenesis in a clinical isolate of *C. jejuni* has detected 26 mutants with a clearly reduced invasion capacity (Javed *et al.*, 2010).

In order to find genes which contribute to the ability of *C. jejuni* to infect Caco-2 cells we screened 660 clones of a transposon-based mutant library of the clinical *C. jejuni* isolate B2 for altered invasiveness. One of the clones with significant invasion reduction turned out to exhibit transposon insertion in gene *cj0952c*. *C. jejuni* gene *cj0952c* shows similarity to genes encoding MCPs. Depending on the *C. jejuni* strain investigated, *cj0952c* is thought to be a pseudogene, since the adjacent downstream gene *cj0951c* encodes a signalling domain, which, together with Cj0952c, would constitute a complete chemoreceptor. Examples of *C. jejuni* strains in which this chemoreceptor is composed of two single genes are NCTC 11168, CF93-6 and 84-25. Also, in the clinical isolate B2, as in strain NCTC 11168, the

two genes are separated. In contrast, in strains 81-176 and 81116, the corresponding chemoreceptor is encoded by a single gene that encompasses the MCP domain, the transmembrane domain as well as the signalling domain. So far, *cj0952c* has only been described to be upregulated during colonization of the chick caecum (Woodall *et al.*, 2005), although not with regard to its biological function. As for many other bacteria, *C. jejuni* possesses genes essential for chemotaxis, such as *cheA*, *cheW*, *cheY* and MCP receptors. Moreover, chemotactic-mediated motility has been shown to be important for *Campylobacter* colonization (Takata *et al.*, 1992; Yao *et al.*, 1994). Altogether, 10 genes encoding chemotaxis receptor proteins have been detected in the genome of *C. jejuni*, designated Tlps for transducer-like proteins. These 10 Tlps can further be classified into three different groups (A–C) according to their predicted domain organization (Marchant *et al.*, 2002).

The group A chemoreceptors, to which Cj0952c–Cj0951c (Tlp7) belong, are MCPs and are composed of N-terminal transmembrane domains and a periplasmic ligand-binding domain followed by a HAMP domain and a cytoplasmic signalling domain at the C-terminal end. In this way, the HAMP domain serves as a linker region that is suggested to be conserved in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins and phosphatases, and converts ligand-induced conformational changes into kinase-controlling signals (Aravind & Ponting, 1999; Butler & Falke, 1998; Le Moual & Koshland, 1996; Williams & Stewart, 1999). Cj0951c shows strong homologies to the cytoplasmic signalling domains of MCPs, being composed of an adaptation region with methylation sites, a flexible region with a conserved glycine hinge, and a protein-interaction region for binding of the chemotaxis protein CheW and the kinase CheA (Hazelbauer *et al.*, 2008).

Although we could clearly demonstrate that transcription of downstream *cj0951c* was not interrupted, despite the transposon insertion in upstream gene *cj0952c*, introduction of *cj0952c* alone could not restore the parental phenotype. This might be due to the fact that transcription and translation of both genes have to take place at the same time on the same RNA to allow aggregation of both subunits into one functional chemoreceptor.

To confirm *cj0952c* and *cj0951c* to be pseudogenes, which are translated into a single functional chemoreceptor by a read-through mechanism, we cloned both genes in the *C. jejuni* vector pRRC by PCR. For the detection of the *in trans*-synthesized protein, the 3' end of the *cj0951c* primer was expanded by nucleotides encoding a 6 × His-tag. However, immunoblot analysis showed only one protein band of 25 kDa, which represents exactly the size of Cj0951c but not the size of a protein composed of Cj0952c and Cj0951c, indicating that Cj0952c and Cj0951c are translated separately in the clinical isolate B2, which corresponds with its genetic composition. Based on our

findings, at least in *C. jejuni* strain B2, a non-covalent interaction of both subunits must be postulated for the creation of a functional receptor.

As a prerequisite for infection, *C. jejuni* has to interact with its host. This process is mediated by the chemotaxis system of the pathogen as it guides the pathogen towards the host cells at the respective site of infection. Mutations of the chemotaxis apparatus strongly reduce the ability of *C. jejuni* to invade human epithelial and also chicken cells. Mutant strains that lack *cj0019* (Tlp10, *docB*) or *cj0262* (Tlp4, *docC*) show a decreased colonization of the chicken intestine, and mutagenesis of the chemoreceptors Tlp1 (*cj1506*), Tlp2 (*cj0144*), Tlp3 (*cj1564*), Tlp4 and Tlp10 has demonstrated these receptors to be important for the pathogen to invade host cells (Golden & Acheson, 2002; Hendrixson & DiRita, 2004; Vegge *et al.*, 2009). To date, less is known about the chemoreceptors of *C. jejuni*. Recently, Hartley-Tassell *et al.* (2010) were able to identify Cj1506c (Tlp1) as the aspartate chemosensory receptor of *C. jejuni*, and thus characterized the first known chemoreceptor of this pathogen responsive to a particular ligand.

In this report we describe how a transposon insertion within *cj0952c* clearly alters the chemotactic behaviour of *C. jejuni* towards formic acid. While the wild-type is attracted by formic acid, B2Δ*cj0952c* shows a biphasic halo with an inner repulsion zone surrounded by a faint ring. The reduced but not completely eliminated attraction towards formic acid might be explained by the energy taxis system of the pathogen. *C. jejuni* possesses two proteins (CetA, CetB) that together constitute an energy taxis receptor. Thereby, CetB senses changes in the redox state of the electron transport system and transmits this signal to CetA via direct interaction. From CetA, the signal is transduced to the chemotactic machinery, which in turn changes the direction of motility (Hendrixson *et al.*, 2001; Elliott & DiRita, 2008). Energy taxis has been shown to be an important force driving *C. jejuni* towards attractants that serve as carbon sources, electron acceptors and, as in the case of formic acid, electron donors (Vegge *et al.*, 2009). The formation of an inner repulsion zone was surprising and might indicate the existence of another chemosensory system that detects formic acid as a chemorepellent. However, when we complemented the mutant with *cj0952c-cj0951c* or the corresponding gene of *C. jejuni* strain 81-176, the attraction of B2 towards formic acid could be restored completely.

The genome sequence of *C. jejuni* encodes a formate dehydrogenase which is located on the *fdhABCD* operon encompassing the genes *cj1511c-cj1508c*. Formate dehydrogenase serves as an electron donor enzyme by the oxidation of formate to CO₂, protons and electrons. The released electrons, in turn, enter the electron transport chain to contribute to the proton ion gradient (Weerakoon *et al.*, 2009). The discovery that Cj0952c–Cj0951c mediates the chemotaxis of *C. jejuni* in the presence of formic acid raised the question of whether the particular composition

of this receptor in the clinical isolate B2 is related to the utilization of formic acid by formate dehydrogenase. However, growth curves in the presence and absence of formic acid for wild-type strain B2, the mutant B2Δ*cj0952c* and the complemented mutant were identical, negating the idea of a combination of the receptor and the enzyme formate dehydrogenase. Interestingly, the presence of formic acid in the medium significantly promoted the growth of all strains (except of B2Δ*fdh*), indicating that metabolism of formic acid represents an important source of electrons for the pathogen.

The observation that Cj0952c–Cj0951c (Tlp7) *in vitro* mediates chemotaxis of *C. jejuni* towards formic acid, and that this is related to the infectivity of the pathogen, must now be confirmed in animal models. Experiments are currently under way to investigate this complex within *in vivo* systems that represent the natural habitat of *C. jejuni*.

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