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.

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 CdgF 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 methyl esterase, respectively, for reversible chemoreceptor glycosylation (Hazellbauer 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 cjo952c and cjo951c are suggested to be pseudogenes in *C. jejuni* strain NCTC 11168, and it is thought that a read-through from upstream cjo952c into downstream cjo951c would create one functional chemoreceptor, termed Tlp7. The background of this assumption is that Cjo952c possesses two transmembrane domains followed by a C-terminal HAMP domain, whereas Cjo951c 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 cjo952c and cjo951c 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 strains, 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 in LB agar or in LB broth at 37°C. The bacterial inoculation of LB medium was supplemented with ampicillin (100 µg ml⁻¹) and chloramphenicol (30 µg ml⁻¹) to prevent the growth of contaminating bacteria. To prevent the growth of contaminating bacteria, the media were supplemented with ampicillin (100 µg ml⁻¹) and chloramphenicol (30 µg ml⁻¹). For growth experiments, bacteria were 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), chloramphenicol (30 µg ml⁻¹), and gentamicin (10 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 DH5α, 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* DH5α 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 pSB199 (Colegio et al., 2001). We digested pSB199 with BamHI, which resulted in the release of the aphA-3 insert. After purification of the insert from the agarose gel, we ligated it into the BamHI-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 KpnI restriction sites. These amplified fragments were then further subcloned into the KpnI-digested and dephosphorylated transposon construction vector EZ::TN (Epicentre Biotechnologies), which resulted in EZ::TN pMODKan. The Tn5 transposon named EZ::TN Kan was finally released from pMODKan by digestion with PvuII.

The transposon 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 insertion 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 transfectants 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 BspHI, 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× 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 × 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 × PBS before further incubation with culture medium containing 100 μg 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 μl of an overnight culture adjusted to OD₆₀₀ 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 OD₆₀₀ 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 μl 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 μg 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 μl of PCR mixture contained 40 ng genomic DNA, 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 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 B2Δcj0952c complemented with cj0952c and cjo952c-cj0951c was used to compare the transcription levels of cjo952c. 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 cjo952c were run. The elongation time for the amplification of cjo952c was shortened to ensure that only wild-type cjo952c, and not cjo952c 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 (35 °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 cjo952c but not to transposon-interrupted cjo952c was ensured by melting-curve analysis and agarose gel electrophoresis (results not shown). Semi-quantitative measurement of transcription levels was calculated as follows:

\[ x = 2^{ΔCp} \]

where \( x \) represents the factor of altered transcription, and \( ΔCp \) represents the difference in crossing points (Cp1–Cp2) of two samples to be compared.

**Cloning of C. jejuni genes into expression vector pRRC.** The C. jejuni genes cjo952c and cjo951c alone, cjo952c–cjo951c, cjo952c–cjo951c–cjo950c and gene cjo951c 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 camB gene cassette that is flanked by a 165 rRNA sequence and sequences for tRNAs for alanine and isoleucine. A single XbaI site is located immediately downstream of
of the cam<sup>B</sup> gene cassette, and allows the expression of XbaI-cloned genes under the control of the constitutively expressed cam<sup>B</sup> gene promoter. After introduction of the plasmid into competent cells of C. jejuni, the cam<sup>B</sup> gene cassette together with the XbaI-cloned gene was integrated into one of the 165 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 MgSO<sub>4</sub>, 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 5 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 XbaI (New England Biolabs). Plasmid pRRC was also XbaI-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 B2Acj0952c cells, the complementation constructs were verified by PCR analysis for the detection of the respective genes.

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

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<th>Gene</th>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
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<td><em>aphA-3</em></td>
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<td></td>
<td>Gj0952R</td>
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**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 % N<sub>2</sub>, 10 % CO<sub>2</sub>, 5 % O<sub>2</sub>). 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 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 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 under denaturing conditions (Qiagen).

**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 $1.2 \times 10^6$ c.f.u. ml$^{-1}$, $0.8 \times 10^5$ and $4.1 \times 10^5$ 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 $1.1 \times 10^6$ c.f.u. ml$^{-1}$. The mean c.f.u. ml$^{-1}$ values obtained for the respective mutants were as follows: cj0005c, $2.8 \times 10^5$ c.f.u. ml$^{-1}$; cj0078c, $3.4 \times 10^5$ c.f.u. ml$^{-1}$; cj0093, $4.1 \times 10^5$ c.f.u. ml$^{-1}$; cj0268c, $3.3 \times 10^5$ c.f.u. ml$^{-1}$; cj0721c, $3.2 \times 10^5$ c.f.u. ml$^{-1}$; cj1439c, $3 \times 10^5$ c.f.u. ml$^{-1}$; and cj0952c, $2.3 \times 10^5$ c.f.u. ml$^{-1}$. Since the $P$ value for

![Graph](http://mic.sgmjournals.org)
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 \textit{cj0952c} at position 106 according to \textit{C. jejuni} strain NCTC 11168, and we chose this mutant as the object of further investigations.

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

To find out whether the insertion of the transposon into \textit{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 B2\textbackslash \textit{cj0952c}. Since the non-coding sequence between \textit{cj0953c} and \textit{cj0952c} has a length of 96 bp, and the distance between \textit{cj0952c} and \textit{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 \textit{cj0952c} including the transposon, but also of \textit{cj0953c} and \textit{cj0951c}, could clearly be detected by RT-PCR in the mutant B2\textbackslash \textit{cj0952c}, indicating that the invasion-deficient phenotype is exclusively mediated by the insertion of the transposon into \textit{cj0952c}.

**Functional complementation of \textit{C. jejuni} B2\textbackslash \textit{cj0952c}**

In order to restore the invasiveness of the B2\textbackslash \textit{cj0952c} mutant, \textit{cj0952c} and \textit{cj0951c} alone and \textit{cj0952c} together with \textit{cj0951c} were cloned into vector pRRC and introduced into mutant B2\textbackslash \textit{cj0952c} by electroporation. Performing gentamicin protection assays on Caco-2 cells, the number of \textit{C. jejuni} colonies recovered demonstrated that \textit{cj0952c}–\textit{cj0951c}, but not \textit{cj0952c} or \textit{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 \textit{cj0952c} in the complemented mutants is under control of a \textit{cam} promoter. To compare the transcription levels of \textit{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 \textit{cj0952c} transcription level of the wild-type strain and of the mutant B2\textbackslash \textit{cj0952c} complemented with \textit{cj0952c} alone and with \textit{cj0952c}–\textit{cj0951c}. Surprisingly, while the mean crossing points of both complemented mutants were 20.49 (\textit{cj0952c}) and 20.18 (\textit{cj0952c}–\textit{cj0951c}), the mean crossing point for the detection of \textit{cj0952c} with RNA from the wild-type strain was 32.44, demonstrating a more than 4000-fold upregulation of \textit{cj0952c} in the complemented mutants compared with the wild-type strain. Accordingly, lower transcription of \textit{cj0952c} could be excluded as a reason for the incomplete invasion phenotype.

**Fig. 2.** (a) Genome arrangement of \textit{cj0953c}, \textit{cj0952c}, \textit{cj0951c} and \textit{cj0950c} in the clinical \textit{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 \textit{C. jejuni} genes \textit{cj0953c}, \textit{cj0952c} and \textit{cj0951c} in the wild-type B2 strain and the mutant strain B2\textbackslash \textit{cj0952c}. The PCR amplicons show the expected size for \textit{cj0953c} (225 bp), \textit{cj0952c} wild-type (267 bp), \textit{cj0952c} with transposon insert (2091 bp) and \textit{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.
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 strain 81-176 is able to complement B2Dcj0952c, 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).

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\(\times\) His-tag (Table 1). After introduction of the recombinant plasmid into the mutant B2\(\Delta cj0952c\), 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 B2\(\Delta cj0952c\) 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 \(B2\Delta cj0952c\) 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\(\pm\)3.13 mm, the mutant was significantly less motile (30.6\(\pm\)2.4 mm, \(P<0.0001\)). After complementation of the mutant with \(cj0952c\) or \(cj0951c\), the motility was not altered (30.6\(\pm\)2.9 mm or 29.4\(\pm\)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\(\pm\)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\(\pm\)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 \(B2\Delta cj0952c\) 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 \(B2\Delta cj0952c\) could be identified (results not shown).

In contrast, a clear difference could be observed for formic acid. Formic acid has recently been described as chemottractant 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 fomate-soaked paper disc (Fig. 6a). In contrast, a biphasic halo with an inner repulsion zone surrounded by a faint ring was observed for \(B2\Delta cj0952c\). 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).

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**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 \(B2\Delta cj0952c\)-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.
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 strain 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 (B2Δfdh) 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 B2Δfdh was clearly reduced (Fig. 7a). The growth of the same strains in...
the absence of formic acid gave comparable results, with the exception of the growth of B2\textit{Dfdh}. This was probably due to the presence of a small amount of formic acid in the MH broth which could not be metabolized by B2\textit{Dfdh} (Fig. 7b).

**DISCUSSION**

Since the introduction of transposon mutagenesis techniques to \textit{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 \textit{C. jejuni}, e.g. \textit{cheA}, \textit{cheY} and \textit{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 \textit{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 \textit{docB} (Tlp10) and the adjacent gene \textit{docA}, encoding a putative cytochrome \textit{c} peroxidase (Hendrixson...
Also, in the clinical isolate B2, as in strain NCTC 11168, the C. jejuni would constitute a complete chemoreceptor. Examples of genes encoding a signalling domain, which, together with Cj0952c, pseudogene, since the adjacent downstream gene cj0951c shows similarity to genes encoding MCPs. Depending on the transposon insertion in gene cj0952c, introdution 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 previous findings, 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 isolate B2 for altered invasiveness. One of the clones with significant invasion reduction turned out to exhibit transposon insertion in upstream 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, 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, adenyl 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 & Koshand, 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.
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 (Tlp4), 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, B2Acj0952c 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 B2Δcj0952c 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 B2A<_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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