INTRODUCTION


Participation of CheR and CheB in the chemosensory response of Campylobacter jejuni

Doungjit Kanungpean, Tsutomu Kakuda and Shinji Takai

Campylobacter jejuni is a leading cause of bacterial gastroenteritis in humans and a commensal bacterium of the intestinal tracts of animals, especially poultry. Chemotaxis is an important determinant for chicken colonization of C. jejuni. Adaptation has a crucial role in the gradient-sensing mechanism that underlies chemotaxis. The genome sequence of C. jejuni reveals the presence of genes encoding putative adaptation proteins, CheB and CheR. In-frame deletions of cheB, cheR and cheBR were constructed and the chemosensory behaviour of the resultant mutants was examined on swarm plates. CheB and CheR proteins significantly influence chemotaxis but are not essential for this behaviour to occur. Increased mobility of two methyl-accepting chemotaxis proteins (MCPs), DocC and Tip1, during SDS-PAGE was detected in the mutants lacking functional CheB in the presence of CheR, presumably resulting from stable methylation of receptors. In vitro studies using tissue culture revealed that deletion of cheR resulted in hyperadherent and hyperinvasive phenotypes, while deletion of cheB resulted in nonadherent, noninvasive phenotypes. Furthermore, the ΔcheBR mutant showed significantly reduced ability to colonize chick caeca. Our data suggest that modification of chemoreceptors by the CheBR system is involved in regulation of chemotaxis in C. jejuni although CheB is apparently not controlled by phosphorylation.

Abbreviation: MCP, methyl-accepting chemotaxis protein.
mediated by two enzymes, methyltransferase CheR and methylesterase CheB (Yonekawa et al., 1983). The methylation of the chemoreceptors by CheR causes the associated CheA kinases to become more active. Feedback is provided by CheB phosphorylation through CheA that increases CheB activity (Lupas & Stock, 1989). Diverse adaptation systems have been reported in many bacteria (Szurmant & Ordal, 2004). The methylation system of CheR and CheB is the only known adaptational mechanism in E. coli, although another, undescribed mechanism may exist. In B. subtilis, the chemotaxis pathway seems to use three adaptation systems (Rao et al., 2008): one involves reversible receptor methylation and the other two involve CheC, CheD and CheV, which are chemotaxis proteins not found in E. coli. Helicobacter pylori, a bacterium closely related to C. jejuni, lacks both CheB and CheC but possesses three CheV homologues (Jiménez-Pearson et al., 2005). The genome sequence of C. jejuni reveals that C. jejuni possesses three putative adaptation proteins, CheB, CheR and CheV (Marchant et al., 2002). However, C. jejuni CheB has no response regulator domain and consists of the methylesterase domain only. This structural feature of CheB is unique to C. jejuni.

In this report, we constructed in-frame deletion mutants of cheB, cheR and cheBR of C. jejuni. We found that CheB and CheR proteins modify some of the chemoreceptors and that this modification might influence the chemotactic behaviour of C. jejuni. We also found that deletion of cheR resulted in hyperadherent and hyperinvasive phenotypes but that deletion of cheB resulted in nonadherent and noninvasive phenotypes. Furthermore, C. jejuni lacking the cheBR locus demonstrated deficient colonization in a chick model.

**METHODS**

**Strains and plasmids.** C. jejuni 81-176 is a clinical isolate from a patient with gastroenteritis that has since been shown to promote gastroenteritis in humans and commensal colonization of the chick gastrointestinal tract (Black et al., 1988; Hendrixson, 2006; Hendrixson & DiRita, 2004; Krolath et al., 1985). C. jejuni was routinely grown on Mueller–Hinton (MH) agar containing 10 µg trimethoprim ml⁻¹ in microaerobic conditions (10 % CO₂, 5 % O₂ and 85 % N₂) at 37 °C. Antibiotics for C. jejuni growth were added to MH agar when necessary at the following concentrations: 50 µg kanamycin ml⁻¹, 15 µg chloramphenicol ml⁻¹, 30 µg cephoperazone ml⁻¹ or 2 mg streptomycin ml⁻¹. All C. jejuni strains were stored at −80 °C in an 85 % MH broth−15 % (v/v) glycerol solution. E. coli DH5α and BL21(DE3) were grown in Luria–Bertani (LB) agar or broth. Antibiotics were used when needed at the following concentrations: 50 µg kanamycin ml⁻¹, 15 µg chloramphenicol ml⁻¹ or 50 µg ampicillin ml⁻¹. All E. coli strains were stored at −80 °C in an 85 % LB broth−15 % (v/v) glycerol solution. All strains and plasmids used in this study are listed in Table 1.

**Construction of bacterial strains.** To construct in-frame deletion mutants, we employed Cre-loxP based mutagenesis (Abremski & Hoess, 1984). A cat−rpsL fragment (Hendrixson et al., 2001) flanked by loxP sites was amplified from pDR1265 by PCR using primers with 5’ Smal and loxP sites. This fragment was cloned into pGEM-T Easy vector (Promega) to create pTK529. To construct in-frame deletion mutants of cheB, cheR or both genes, a 2.4 kb fragment that includes approximately 500 nt upstream of cheB and downstream of cheR was amplified from the chromosome of C. jejuni 81-176 by PCR. This fragment was cloned into pGEM-T Easy vector to create pDK247. Pfu mutagenesis was used to create deletions of the desired gene locus (Weiner et al., 1994), then pDK247 was used as the template to delete cheB, cheR or both genes and replaced with a Smal site to create pDK310, pDK270 and pDK319. These deleted regions in cheB, cheR and cheBR genes were codons 4−156, codons 13−249 and codon 4 of cheR to 249 of cheR, respectively. The cat−loxP sequence flanked by loxP sites was cut out from pTK529 with Smal and ligated into the Smal site of pDK310, pDK270 and pDK319 to create pDK313, pDK311 and pDK332, respectively. Strain DH212 (81-176 Smα, Hendrixson et al., 2001) was transformed with pDK313, pDK311 or pDK332 by electroporation and then grown on MH agar with chloramphenicol to select for transformants with insertions of cat−loxP flanked by loxP sites. The chromosomal DNA was extracted from each transformant and used for in vitro recombination by Cre recombinase (New England BioLabs) to delete cat−rpsL from chromosomal DNA. The deletion of cat−rpsL from each DNA sample was confirmed by PCR. Original transformants from which DNA was derived were then transformed by natural transformation with Cre-treated DNA and grown on MH agar with streptomycin to select for in-frame deletion mutants (Wiesner et al., 2003). The elimination of the cat−rpsL cassette by Cre recombination leaves behind a 48 nt scar (5’-CCCGGGATACCCGTTAATTATGTACGTAAGTTTACGGGGG-3’, where the italic and bold text indicates loxP and Smal sites, respectively) in place of the deleted gene, creating in-frame deletions in which the scar encodes a new 16 nt internal peptide (PGTSTSYNVCYTKLCPG). To complement the ΔcheBR mutant, we attempted to replace the coding region of the astA gene with the cheBR gene containing its own putative promoter. To achieve this, we first amplified the cat gene by PCR with a forward primer containing 5’ BamHI−Kpd−XhoI−SmaI sites and cloned it into pGEM-T Easy vector (pTK801). We then amplified fragments of approximately 1.0 kb upstream and downstream of the astA gene by PCR with primers containing 5’ SplI and PstI sites, respectively. The upstream and downstream fragments were cloned into the SplI and PstI sites of pTK801 to construct pTK802. A fragment of the cheBR gene containing its own putative promoter was amplified by PCR with forward and reverse primers containing 5’ BamHI and 5’ XhoI sites, respectively, and then cloned into the BamHI and XhoI sites of pTK802 to construct pDR433. pDR433 was electroporated into the ΔcheBR mutant and selected on MH plates containing chloramphenicol. Point mutations in the cheB (S9T) and cheR (G109A, D133A) coding sequence in pDK433 were made using Pfu mutagenesis.

Ceta::solo was selected as a mutant exhibiting altered motility phenotype on semisolid motility agar from a C. jejuni 81-176 transposon library, constructed as previously described (Hendrixson et al., 2001).

**Motility assay.** C. jejuni strains from frozen stocks were grown on MH agar with trimethoprim for 48 h in microaerobic conditions at 37 °C. After growth, strains were streaked on MH agar with trimethoprim and grown for an additional 16 h under microaerobic conditions at 37 °C. Strains were resuspended from agar plates and diluted to OD₆₀₀ 0.4. For agar-based assays for motility, bacterial strains were stabbed into semisolid MH agar plates containing 0.4 % agar as described previously (Hendrixson et al., 2001). Motility phenotypes were assessed approximately 24 h after inoculation and incubation at 37 °C in microaerobic conditions.

**Growth assay.** Defined Dulbecco’s modified Eagle’s medium (DMEM) supplemented with ferrous sulfate was used for the growth assay. Specific amino acids for supplementation of the DMEM were
Table 1. Strains and plasmids used in this study

<table>
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used in concentrations of 20 mM. *C. jejuni* suspensions were adjusted to OD<sub>600</sub> 0.1 in appropriate liquid medium and then 1.2 ml of each bacterial solution was added into a well of a 24-well plate containing 300 μl 1% agar-containing DMEM supplemented with specific amino acids. The biphasic culture was carried out under microaerobic conditions at 37 °C for 24 h. Bacterial growth was monitored by measuring the OD<sub>600</sub> of the culture with a spectrophotometer.

**Preparation of antisera.** Primers were designed to amplify the coding sequences of cheB from codon 1 to the stop codon, cheR from codon 1 to the stop codon, docC from codons 94–266, tlp1 from codons 65–248 and peb1A from codon 27 to the stop codon. In-frame *NdeI* and *XhoI* restriction sites were added to the 5’ ends of the forward and reverse primers, respectively. After amplification from the chromosome of *C. jejuni* strain 81-176, the gene fragments were digested with *NdeI* and *XhoI* and then ligated into *NdeI* and *XhoI* double-digested pET16b (Merck). BL21(DE3) was transformed with the resultant plasmids. Bacteria were grown in 200 ml LB broth with ampicillin to mid-exponential phase, incubated with 1 mM IPTG and then incubated for another 5 h at 37 °C. Bacteria were harvested and disrupted by sonication. The soluble fraction was obtained by removing the insoluble material by centrifugation at 20 000 g for 30 min at 4 °C. The protein was purified with Ni-affinity column chromatography (Merck). After purification, recombinant protein was dialysed against PBS for 12 h and then stored at −30 °C until use. Anti-CheB (α-CheB), α-CheR, α-Peb1A, α-DocC and α-Tlp1 antisera were generated by immunizing five mice. Anti-CetA antiserum was purchased from Calbiochem (Merck).

**Immunoblot analysis.** Bacteria were resuspended from plates and diluted to OD<sub>560</sub> 0.1. Whole-cell lysates were prepared by pelleting 1 ml bacteria, washing once with PBS and then resuspending the bacteria in 50 μl SDS–PAGE loading buffer. Aliquots (10 μl) of protein samples were separated by SDS–PAGE using 12.5% or 4–12% acrylamide gels and proteins were then transferred to a nitrocellulose membrane. Membranes were incubated with a 1:200 dilution of α-CheB antiserum, a 1:100 dilution of α-CheR antiserum, a 1:1000 dilution of CetA antiserum, a 1:200 dilution of α-DocC antiserum, a 1:1000 dilution of α-Tlp1 antiserum or a 1:3000 dilution of α-Peb1A antiserum for 1 h, washed and then incubated with a 1:10 000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse antiserum for 1 h. For detection, an ECL Plus Western blotting detection kit (GE Healthcare) was used according to the manufacturer’s instructions.
RT-PCR. *C. jejuni* strains were grown on MH plates for 16 h. RNA extractions were performed using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. To eliminate contaminating DNA, RNA was treated with 10 U RNase-free DNase for 30 min at 37 °C. The DNase was then inactivated by incubating the mixture for 5 min at 75 °C. A total of 2.5 μg RNA was mixed with random hexamers and cDNA was synthesized by using PrimeScript RT-PCR kit (TaKaRa) according to the manufacturer’s instructions. Control reactions with reverse transcriptase omitted were performed simultaneously to detect any contaminating DNA. Equal amounts of cDNA products were then used as a template for PCR. RT-PCR products were separated on a 0.8% agarose gel and visualized with ethidium bromide.

Adherence and invasion assay. The adherence and invasion assay was carried out as described previously (Kakuda & DiRita, 2006). INT407 cells were seeded into 24-well plates at semiconfluency (approximately 1 × 10^5 per well) approximately 16 h prior to infection. Bacteria were grown in MH biphasic medium. One milliliter of culture medium containing 2 × 10^7 bacteria was added to each well (m.o.i. 200). A 5 min centrifugation step at 300 g was done when indicated. Infected monolayers were incubated for 2 h at 37 °C in 5% CO_2–95% air atmosphere to allow invasion to occur. Following the invasion period, wells for assaying adhesion and invasion (total cell-associated bacteria) were washed three times with DMEM and lysed with 0.1% Triton X-100 in PBS for 15 min at room temperature. At this time, wells for assaying invasion were washed three times with DMEM and incubated for another 2 h in fresh tissue culture medium containing gentamicin (100 μg ml^-1) to kill extracellular bacteria. After the gentamicin kill period, the infected monolayers were washed three times with DMEM and lysed as described above. The number of viable bacteria released from the cells was assessed after serial 10-fold dilutions of the lysates on MH plates.

Chick colonization assay. The chick colonization assay was carried out as described previously (Kakuda & DiRita, 2006). White leghorn chicken eggs were supplied by a local farm and maintained in an egg incubator for 21 days at 37.5 °C with appropriate humidity and rotation of eggs according to the manufacturer’s instructions until the chicks hatched (Showa Furanki). For testing the caecal colonization capacity of *C. jejuni* 81-176 derivatives, each strain was streaked on MH agar and grown at 37 °C under microaerobic conditions for 16 h. Approximately 12 h after hatch, chicks were divided into groups of 10–12 and infected orally with 100 μl inoculum. Dilutions were placed on MH agar to determine the number of bacteria in each inoculum. Each group of chicks was housed separately in brooders and given water and food *ad libitum*. Chicks were killed at day 7 post-infection and caeca were collected, weighed and resuspended in PBS to a final concentration of 0.1 g caecal contents ml^-1. Tenfold serial dilutions of each sample were made and plated on MH agar containing 10 μg trimethoprim ml^-1 and 30 μg cefoperazone ml^-1 to determine the number of *C. jejuni* per gram of caecal contents.

RESULTS

**cheB and cheR are transcribed monocistronically**

Because CheB and CheR are encoded by adjacent genes on the *C. jejuni* chromosome and there are 15 bp between the *cheB* and *cheR* genes, we assumed that they would be cotranscribed. To determine whether *cheB* and *cheR* form an operon, RT-PCR was performed with RNA extracted from wild-type *C. jejuni* using primers spanning the putative operon. Each RT-PCR gave a product of the expected size, as shown in Fig. 1. Based on these results, we concluded that *cheB* and *cheR* are cotranscribed. To characterize the role of CheB and CheR proteins in *C. jejuni*, we constructed in-frame deletion mutants of *cheB* and/or *cheR* genes. Immunoblot analysis revealed that each mutant had the expected pattern of CheB and CheR expression (Fig. 2a).

Downstream of the *cheBR* operon, there is a four-gene operon of an ABC transporter that plays a role in the utilization of aspartate and glutamate (del Rocio Leon-Kempis et al., 2006). In addition, Peb1A, the periplasmic-binding protein component of this transport system, has been reported to possess another role as a surface-exposed adhesin (Pei et al., 1998). Because growth and adhesion defects caused by the polar effect might disturb further characterization of the mutants, we tried to confirm whether mutations of the *cheBR* locus might influence expression of downstream genes. Immunoblot analysis

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**Fig. 1.** RT-PCR analysis of *cheBR* locus. (a) The *cheBR* locus and locations of primers used for RT-PCR. (b) DNA fragments generated after RT-PCR analysis. Three sets of PCRs were performed, each using the indicated primer pair. Each PCR set included a reaction mixture containing cDNA generated from *C. jejuni* 81-176 RNA after *in vitro* growth (+) and a reaction of the same RNA incubated in the absence of RT (−) to verify the absence of DNA in the RNA preparation.
with antisera against Peb1A and growth assay in DMEM supplemented with aspartate or glutamate demonstrated that deletion of cheB and/or cheR did not affect expression of the downstream operon (Fig. 2b).

CheR and CheB influence chemotactic behaviours of C. jejuni in a swarm plate

To examine chemotactic behaviours of cheB, cheR and cheBR mutants, we carried out a semisolid agar motility assay. In this assay, mutants affected in metabolism, motility, or chemotaxis fail to form a chemotactic ring. CetA and CetB are a bipartite energy taxis system in C. jejuni. This system is proposed to sense environments providing high electron transport and ATP generation, possibly using FAD as a redox sensor to monitor electron transport. Both mutants of CetA and CetB display a reduced motility phenotype on semisolid motility agar, suggesting that they are defective in energy taxis, thus hindering their ability to migrate to new environments and maintain maximal electron transport and ATP generation (Hendrixson et al., 2001). Therefore, we used cetA mutant as negative control of chemotaxis in this experiment. Analysis of the ΔcheB, ΔcheR and ΔcheBR mutants in semisolid motility agar revealed that the swarm sizes of these mutants were significantly decreased compared with that of the wild-type (Fig. 3). Complementation of ΔcheBR with chromosomal insertion of the cheBR gene restores the motility phenotype to wild-type level (Fig. 3b). The degree of decrease in swarm size was less than that of the cetA mutant but constant in six independent determinations. The ΔcheB, ΔcheR and ΔcheBR mutants showed similar growth rates in MH broth and amino-acid-supplemented DMEM (Fig. 2b). Furthermore, the phase-contrast microscopic observation revealed that the swimming speeds of these mutants in MH broth were similar to that of the wild-type. These results suggest that CheR and CheB influence the chemotactic behaviour of C. jejuni although they are not necessary for chemotaxis to occur under the conditions tested.

Amino acid sequence comparison of C. jejuni CheR with CheR proteins from E. coli, S. enterica and B. subtilis using CLUSTAL W revealed that the C. jejuni CheR shows a low overall level of similarity with other CheRs, but there are functionally important shared residues throughout the sequence. The AdoMet binding site of C. jejuni CheR is predicted to be PCSSG (residues 105–109) although the first residue does not agree with the signature sequence (G/A)(G/A/S)XG of AdoMet binding sites. Two residues R80 and D133, previously reported as critical residues for methyltransferase activity, are both conserved (Shiomi et al., 2002). In contrast, the overall sequence of CheB is similar to the methylesterase domain of other CheBs and the catalytic triad (S9, H35, D126) is present although the N-terminal response regulator domain is completely missing (West et al., 1995). To examine whether mutations of critical residues for enzymic activities of CheB and CheR resulted in reduced motility, we performed site-directed mutagenesis. We replaced S9 of CheB with threonine and replaced G109 and D133 of CheR with alanine. The resulting mutant proteins were expressed in the ΔcheBR strain at levels similar to those of wild-type proteins (Fig. 4a). The mutants expressing either mutated cheB/cheR or wild-type genes resulted in significantly decreased swarm size compared with the mutant complemented with wild-type genes (Student’s t test, P<0.01) with the exception of CheR(G109A) whose
reduction in swarm size was smaller than those of the other mutants (Fig. 4b). These results suggest that S9 of CheB and D133 of CheR are critical residues for their enzymic activities.

**DocC and Tlp1 are modified by CheR**

It has been reported that MCP molecules methylated by CheR run faster than unmethylated forms in SDS-PAGE, while CheB-dependent deamidation and demethylation of MCP molecules result in reduced mobility (Bibikov et al., 2004). We looked for MCP modifications in the form of CheR- or CheB-dependent band shifts in SDS-PAGE. The putative methylation sites, similar to the methylation site motif of *E. coli* MCPs, are found in Tlp1, Tlp2, Tlp3, DocC and CetA (Marchant et al., 2002). Because the C-terminal cytoplasmic signalling domains of Tlp2, Tlp3 and DocC are identical (Marchant et al., 2002), we chose Tlp1, DocC and CetA in this study. In the cheR mutant, mobility of MCPs examined in this study was indistinguishable from that of corresponding MCPs in the ΔcheBR mutant. These results indicate that deamidation does not occur in these MCPs or this modification cannot be detectable by the method we used. On the other hand, DocC and Tlp1 migrated as a single band with fast mobility in a mutant that lacked CheB but had CheR (Fig. 5). In contrast, CetA mobility shifts were not detectable in any mutant. These results suggest that DocC and Tlp1, but not CetA, are modified by CheR, although we cannot exclude the possibility that CetA undergoes CheR modifications that are not detectable as band shifts in the gel electrophoresis system we used. The mobility shift of DocC was detected more easily

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**Fig. 3.** Semisolid agar motility assay. (a) Motility phenotypes of *C. jejuni* strains in MH semisolid agar 24 h after inoculation. The strains used included wild-type strain 81-176 SmR (DRH212), ΔcheB, ΔcheR, ΔcheBR and cetA::solo. (b) Motility assay performed on wild-type, ΔcheB, ΔcheR, ΔcheBR, cetA::solo and ΔcheBR complemented with cheBR. Plates were incubated for 24 h under microaerophilic conditions. The diameter of the outermost motility ring was measured. The mean and sd of five or six replicates are indicated. *P < 0.01, significant difference between wild-type and mutant strains by Student’s t test.

**Fig. 4.** (a) Expression of CheB and CheR. Whole-cell extracts were prepared from wild-type, ΔcheBR and ΔcheBR complemented with various combinations of wild-type and mutant cheB/cheR genes. These were separated by SDS-PAGE using a 12 % gel. CheB and CheR were detected by immunoblotting. (b) Influence of point mutations on the function of CheB and CheR in motility. Relative motility of the ΔcheBR mutant expressing various combinations of wild-type and mutant CheB/CheR compared with motility of ΔcheBR mutant complemented with wild-type cheBR is shown. The means ± sd of 4–6 replicates are indicated. *P < 0.01, significant difference between ΔcheBR mutant complemented with wild-type cheBR gene and the other mutant strains by Student’s t test.
than that of Tlp1. We chose DocC for further analysis using site-directed mutagenesis. The mobility shift of DocC was detected in a mutant expressing CheB(S9T) in the presence of CheR function. The CheR mutation D133A but not G109A abolished the mobility shift of DocC in the presence of CheB(S9T) (Fig. 6). Together with the results shown in Fig. 4(b), these results suggest that G109 of CheR is unnecessary for CheR function in C. jejuni.

### Analysis of the adherence and invasion phenotypes of cheB and/or cheR deletion mutants

It has been reported that mutation in components of the chemotaxis pathway results in an altered phenotype of cellular adherence and invasion (Hartley-Tassell et al., 2010; Takata et al., 1992; Yao et al., 1997). To examine the influence of CheB and CheR on these phenotypes, we carried out an invasion assay using cell culture of INT-407 human intestinal cells. The ΔcheB mutant displayed a reduced number of adherent and/or invasive bacteria (Table 2). In contrast, ΔcheR and ΔcheBR mutants showed increased numbers of adherent and/or invasive bacteria. To reduce the contribution of motility to adhesion and invasion in this experiment, the bacteria were brought into contact with the INT407 cells by centrifugation at the beginning of the assay. By using this procedure, the difference in the number of adherent bacteria between the wild-type and ΔcheB or ΔcheBR mutant was reduced although it was still significant (Table 2). On the other hand, the difference in the number of invasive bacteria was maintained. The level of adhesion and invasion by ΔcheB mutant remained lower than that of the wild-type. These results indicate that the mutations of cheB and cheR have influence on the ability to adhere to and/or invade cells.

The complemented mutant of ΔcheBR restored the adhesion and invasion phenotype to wild-type levels. As expected, the mutant expressing CheB(S9T) with wild-type CheR or CheR(G109A) showed a reduced ability to adhere to and/or invade cells. In contrast, CheR(D133A), but not (G109A), resulted in an increased level of adherence and/or invasion (Fig. 7). These results suggest that this phenotype is closely related to the enzymic activities of CheB and CheR.

### CheB and CheR are required for chick colonization

To analyse whether CheB and CheR are required by C. jejuni for efficient colonization of chicks, the ability of the ΔcheBR mutant to colonize the chick caecum was compared with that of the wild-type by orally infecting 1-day-old chicks with an inocula of approximately 10^6 organisms. Wild-type C. jejuni colonizes the chick caecum at approximately 10^6 c.f.u. (g caecal content)^{-1} at day 7

![Fig. 5. Band shifts of MCPs in SDS-PAGE. Whole-cell extracts were prepared from wild-type, ΔcheB, ΔcheR and ΔcheBR. These were separated by SDS-PAGE using 4–12 % gradient gel (DocC and Tlp1) or 12 % (CetA) gel. DocC, Tlp1 and CetA were detected by immunoblotting. Band shift is indicated by arrows. Lanes: 1, wild-type; 2, ΔcheB; 3, ΔcheR; 4, ΔcheBR.](http://mic.sgmjournals.org)

![Fig. 6. DocC band shift in SDS-PAGE. Whole-cell extracts were prepared from ΔcheBR and ΔcheBR expressing various combinations of wild-type and mutant CheB/ CheR. These samples were separated by SDS-PAGE using 4–12 % gradient gel. DocC was detected by immunoblotting. Band shift is indicated by arrows.](http://mic.sgmjournals.org)
post-infection (Fig. 8). The ΔcheBR mutant colonizes at a level 100-fold lower than wild-type bacteria and the complemented mutant demonstrates levels of colonization similar to those of the wild-type. These results suggest that CheB and CheR are required for efficient colonization of chick caeca.

**DISCUSSION**

In this study, we found that deletion of CheB and/or CheR homologues in C. jejuni influenced its motility in MH-based semisolid agar. These mutants are fully flagellated and showed the same growth kinetics as the wild-type.
These results suggest that the motility phenotype observed results from a change in chemotactic behaviour. We also found that in the ΔcheB mutant, band shifts of Tlp1 and DocC were detected by SDS-PAGE. In this ΔcheB mutant, the receptors would be fully methylated and therefore exhibit faster mobility. Band shifts of these proteins were not observed in the wild-type. Presumably, methylation of receptors is transient in the presence of CheB activity.

The mutant lacking CheB and CheR retained chemotaxis in semisolid motility agar, which suggested that the chemotaxis pathway of C. jejuni uses methylation-independent adaptation. Methylation-independent adaptation has also been reported in many bacterial species (Sockett et al., 1987; Stephens et al., 2006). In B. subtilis, it is known that apart from reversible receptor methylation, the chemotaxis pathway uses two adaptation systems that involve the CheC–CheD–CheYp and CheV systems (Rao et al., 1987; Stephens et al., 2006). In E. coli, aerotaxis, one of the energy taxes mediated by Aer, has been shown to be methylation-independent (Bibikov et al., 2004). Therefore, the same mechanism of adaptation may exist in the energy taxes of both organisms.

The chemotaxis assay using semisolid agar is a spatial gradient assay that requires metabolism and growth of bacteria (Miller et al., 2009). In other bacteria, a discrepancy between the results obtained with a spatial gradient assay and those obtained with a temporal gradient assay, another type of chemotaxis assay, has been reported (Stephens et al., 2006). The cheB and cheR mutants of Azospirillum brasilense retained the ability to form a chemotactic ring on semisolid agar but did not respond to stimulation with the attractants in a temporal gradient assay. Therefore, the temporal gradient assay may show different effects of CheB and CheR on the chemotactic behaviours of C. jejuni.

The CheB protein of C. jejuni apparently lacks the response regulator domain found in CheB of other bacteria. To be involved in adaptation, the activities of CheB and/or CheR must be controlled by mechanisms other than CheB control by phosphorylation. This is unlikely to involve the ratio of their expression levels being transcriptionally regulated because both genes are transcribed monocistronically. In B. subtilis, truncated cheB encoding the enzymic domain complements a null cheB mutant: this strain releases enhanced levels of methanol on both addition and removal of attractant (Bunn & Ordal, 2004). This result implies that a conformational change in the receptor influences its susceptibility to the action of methyltransferase. The same mechanism may also be involved in controlling CheB of C. jejuni.

In vitro study using tissue culture revealed that ΔcheR and ΔcheBR mutants had an increased level of adhesion and/or invasion but that the ΔcheB mutant had a decreased level of adhesion and/or invasion. Site-directed mutagenesis revealed that this phenotype is closely related to the enzymic activities of CheB and CheR. Recently, Novik et al. (2010) reported that transposon insertion of cheB exhibited an approximately threefold decrease in cell entry. The degree of decrease is smaller than that for the ΔcheB mutant in our study (approximately 10-fold decrease), but differences in the protocols, growth conditions and/or cell lines used may account for this difference. Hartley-Tassell et al. (2010) reported that a tlp1 mutant showed hyper-adherent and hyperinvasive phenotypes. These phenotypes of the tlp1 mutant and those of ΔcheR and ΔcheBR mutants might be caused by a common mechanism because Tlp1 would not be methylated and its function would also probably be modified in ΔcheR and ΔcheBR mutants. In a semisolid agar motility assay, we could not demonstrate directly opposing effects of methylation and demethylation of the receptor. In contrast, the invasion assay apparently showed that in C. jejuni, methylation of receptors could also be involved in functional regulation of chemoreceptors. A previous study also found that a cheY mutant had increased adhesion and invasion but that a diploid cheY strain had decreased adhesion and invasion in vitro (Yao et al., 1997). Novik et al. (2010) reported that motility per se is required for C. jejuni entry even when bacteria–cell contact is facilitated by centrifugation during the assay. Therefore, the directly opposing effects of methylation and demethylation of the receptor on bacterial adhesion and invasion might result from their opposing effects on motility: e.g. run- or tumble-biased phenotype. On the other hand, as was suggested by the authors of several studies (Elliott et al., 2009; Hartley-Tassell et al., 2010; Yao et al., 1997), there may be co-ordinated regulation of motility and virulence genes in C. jejuni. Further study is necessary to clarify the mechanism that underlies these phenomena.

The ΔcheBR strain has a severe defect in its ability to colonize the gastrointestinal tract of chicks. The chromosomal insertion of cheBR genes complemented this phenotype. Some chemoreceptors have been demonstrated to be required for colonization (Hartley-Tassell et al., 2010; Hendrixson & DiRita, 2004). DocC is one of these colonization determinants and also undergoes methylation (Hendrixson & DiRita, 2004). Therefore, a defect in signalling through DocC may be responsible for the colonization defect of the ΔcheBR mutant. Alternatively, given the link between regulation of chemotaxis and other virulence traits, lack of regulation of traits other than chemotaxis may also be involved in the colonization defect.
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REFERENCES


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