Methyl-accepting chemotaxis proteins 3 and 4 are responsible for *Campylobacter jejuni* chemotaxis and jejuna colonization in mice in response to sodium deoxycholate

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Methyl-accepting chemotaxis proteins (MCPs), also termed transducer-like proteins (Tlps), serve as sensors in bacterial chemotactic signalling, and detect attractants and promote bacterial movement towards suitable sites for colonization. *Campylobacter jejuni* is a leading cause of human enteritis, but the mechanisms responsible for bacterial chemotaxis and early colonization in the jejunum of hosts are poorly understood. In the present study, we identified several types of bile and sodium deoxycholate (SDC) acting as chemotactic attractants of *C. jejuni* strain NCTC 11168-O *in vitro*, in which SDC was the most efficient chemoattractant. In mice with bile duct ligation, the wild-type strain displayed a markedly attenuated ability for colonization. Blockage of Tlp3 or Tlp4 protein with antibody or disruption of the *tlp3* or *tlp4* gene (Δ*tlp3* or Δ*tlp4*) caused a significant inhibition of SDC-induced chemotaxis and attenuation for colonization on jejunal mucosa in mice of the bacterium. Disruption of both the genes (Δ*tlp3*/Δ*tlp4*) resulted in the absence of bacterial chemotaxis and colonization, while the *tlp*-gene-complemented mutants (CΔ*tlp3* and CΔ*tlp4*) reacquired these abilities. The results indicate that SDC is an effective chemoattractant for *C. jejuni*, and Tlp3 and Tlp4 are the SDC-specific sensor proteins responsible for the bacterial chemoattraction.

INTRODUCTION

Campylobacter jejuni is a global major foodborne pathogen causing human enteritis (Zilbauer *et al.*, 2008). The disease has a 5 to 7 day course of infection, with typical clinical signs and symptoms, such as fever and inflammation, severe abdominal cramping, watery diarrhoea and bloody stools (Allos, 2001; Young *et al.*, 2007). Sequelae of

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Abbreviations: ECR, extracellular region; MCP, methyl-accepting chemotaxis protein; qPCR, quantitative PCR; RT, reverse transcription; SDC, sodium deoxycholate; TCS, two-component signalling; Tlp, transducer-like protein.

A supplementary table is available with the online version of this paper.

campylobacteriosis include arthropathies and Guillain–Barré syndrome (Nielsen *et al.*, 2010; van Doorn *et al.*, 2008).

C. jejuni can be found in numerous environmental niches, such as surface water, and in food sources including cattle, sheep and poultry (Allos, 2001; Zilbauer *et al.*, 2008; Young *et al.*, 2007). The bacterium naturally colonizes the gastrointestinal tract of many birds and animals with a harmless commensal relationship (Bingham-Ramos & Hendrixson, 2008; Chang & Miller, 2006; Hanning *et al.*, 2009). Human beings are infected with *C. jejuni* through either consumption of undercooked poultry meat or contact with animal excrement that is contaminated by the pathogen (Jørgensen *et al.*, 2002; Kramer *et al.*, 2000; Nadeau *et al.*, 2002). After *C. jejuni* enters the human gastrointestinal tract, the microbe utilizes its flagellar chemotactic motility to penetrate the mucosal layer on the tract for colonization on the surface of jejunal epithelial cells (Takata *et al.*, 1992; Wallis, 1994). However, the molecular basis for the chemotaxis of *C. jejuni* remains poorly understood.

Transmembrane methyl-accepting chemotaxis proteins (MCPs) of motile bacteria are responsible for sensing chemoattractants in the environment (Fernando *et al.*, 2007; Zhulin, 2001). The combination of MCPs with chemoattractants causes the activation of intracellular CheA histidine kinase to trigger the MCPs-dependent signalling pathway, in which the CheA activates the CheY response regulator that controls the flagellar rotation direction (Hansen *et al.*, 2008; Stecher *et al.*, 2004). Subsequently, the bacteria depend on flagellar directional movement to reach suitable sites in tissues for colonization (Moisi *et al.*, 2009; Williams *et al.*, 2007). Chemotaxis towards jejunal mucous membrane has also been shown to be aprerequisite for colonization of *C. jejuni* (Hendrixson & DiRita, 2004). When the CheA or CheY protein in the chemotaxis-signalling pathway was inhibited, *C. jejuni* was no longer able to establish colonization in mice, chickens and ferrets (Yao *et al.*, 1997).

The genome of *C. jejuni* strain NCTC 11168 contains at least ten genes that encode potential MCPs, which are also called transducer-like proteins (Tlps) (Parkhill *et al.*, 2000). The Tlps of *C. jejuni* have been classified into A, B and C groups, in which the Tlps in group A (Tlp1 to Tlp4) have structures similar to the well-characterized MCPs of *Escherichia coli* (Marchant *et al.*, 2002). However, a previous report showed that the deletion of some Tlp-encoding genes in group A of *C. jejuni* strain NCTC 11168 resulted in attenuation of the ability to invade human epithelial and chicken embryo cells, but did not affect chemotactic behaviour *in vitro* (Vegge *et al.*, 2009). Thus, the Tlps of *C. jejuni* responsible for chemotaxis *in vitro* and colonization *in vivo* have not been fully characterized.

In the group A Tlps of *C. jejuni* strain NCTC 11168-GS, Tlp2, Tlp3 and Tlp4 comprise a subgroup that contains the whole MCP domain such as periplasmic ligand-binding, signalling and methylation sites (Marchant *et al.*, 2002; Parkhill *et al.*, 2000). Previous studies reported that some specific amino acids and organic acids, such as L-serine and pyruvate, and porcine enteric mucus components, such as mucin, act as attractants to induce the chemotaxis of *C. jejuni in vitro* (Hugdahl *et al.*, 1988; Khanna *et al.*, 2006; Vegge *et al.*, 2009). In these reports, bovine or chick bile was found to induce the bacterial chemotaxis (Hugdahl *et al.*, 1988). However, the chemotactic movement could be repelled by some major components of bile such as cholic acid, deoxycholic acid or sodium deoxycholate (SDC), and taurocholic acid or sodium taurocholate (Hugdahl *et al.*, 1988; Khanna *et al.*, 2006; Vegge *et al.*, 2009). Therefore, the contradiction between the attraction of bile and repulsion of bile components in the chemotaxis of *C. jejuni* needs to be clarified. Moreover, the function of *tlp2*, *tlp3* and *tlp4* genes of *C. jejuni* in the sensing of the chemoattractants also needs to be determined.

In the present study, we determined the ability of 13 chemoattractant candidates to attract the chemotaxis system of *C. jejuni*, and the roles of the *tlp2*, *tlp3* and *tlp4* genes in the bacterial chemotaxis *in vitro* and in the colonization in mice. The results of this study identified bile and SDC as effective chemotactic attractants, and the Tlp2 and Tlp3 proteins for sensing chemotactic attractants and promoting colonization by *C. jejuni*.

METHODS

Bacterial strains and growth conditions. *C. jejuni* strain NCTC 11168-O, an original clinical strain with high pathogenicity, was provided by the Institute of Infectious Disease Prevention and Control, National Disease Prevention and Control Center of China. The bacterium was grown on Mueller–Hinton (MH) agar (Oxoid) plates supplemented with 8 % sheep blood and 10 mg trimethoprim ml^{-1} (Sigma) under a microaerobic atmosphere (85 % N_2, 10 % CO_2 and 5 % O_2). *E. coli* BL21(DE3) (Novagen) and *E. coli* DH5α (Novagen) were grown in Luria–Bertani (LB) medium (Oxoid).

Animals. Female BALB/c-ByJ mice (18 ± 1 g) and New Zealand white rabbits (3.0 to 3.5 kg) were provided by the Laboratory Animal Center of Zhejiang University. All the animal experimental protocols were approved by the Ethics Committee for Animal Experiment of Zhejiang University (Zhejiang, China).

Primers. All the primers used in this study were synthesized by Invitrogen and are listed in Table S1 (available in the online Supplementary Material).

Amplification and sequence analysis of *tlp* genes. Genomic DNA of *C. jejuni* strain NCTC 11168-O was extracted using a bacterial DNA extraction kit (BioColour). Several PCRs were performed to amplify entire *tlp2*, *tlp3* or *tlp4* genes from the bacterial DNA template with the primers T2A-F/T2A-R, T3A-F/T3A-R or T4A-F/T4A-R, respectively (Table S1), using a high fidelity PCR kit (TaKaRa). The PCR products were examined in 1.5 % ethidium bromide pre-stained agarose gels after electrophoresis. Subsequently, the target amplification segments with the expected sizes were cloned into pMD18-T plasmid using a T-A cloning kit (TaKaRa) for sequencing by Invitrogen. The sequence conservation, methylation motif and transmembrane domain in the actual Tlp sequences were analysed using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

and TMHMM server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Alexander & Zhulin, 2007). In addition, extracellular region (ECR) segments of the *tlp2*, *tlp3* and *tlp4* genes were amplified by PCR using the primers T2A-F/T2H-R, T3A-F/T3H-R and T4A-F/T4H-R, respectively (Table S1), and then the ECR segment products were cloned into pMD18-T for sequencing.

Expression and purification of target recombinant proteins. The recombinant pMD18-T plasmids containing the entire gene or ECR segments of *tlp2*, *tlp3* and *tlp4* and pET42a vector (Novagen) were digested with *Nde*I and *Xho*I endonucleases (TaKaRa). Each of the recovered segments was linked with the linearized pET42a using T4 DNA ligase (TaKaRa) and then used to transform *E. coli* BL21(DE3). The engineered bacteria were cultured in kanamycin-containing LB liquid medium to express the recombinant proteins rTlp2, rTlp3, rTlp4, rTlp2-ECR, rTlp3-ECR and rTlp4-ECR under induction with 0.5 mM IPTG (Sigma). The expressed proteins were examined by SDS-PAGE plus a gel image analyser (Bio-Rad), and then purified using a Ni-NTA affinity chromatographic column (Promega).

Preparation of rTlp-antisera, IgGs and IgG F(ab′)$_2$s. Rabbits were immunized intradermally on days 1, 7, 14 and 21 with each of the purified recombinant proteins, which had been pre-mixed with Freund's adjuvant. Fifteen days after the last immunization, the sera were collected and the IgGs separated by ammonium sulfate precipitation plus a DEAE-52 column (Sigma) using 10 mM phosphate buffer (pH 7.4) for elution. The IgG F(ab′)$_2$ of each of the IgGs was prepared with pepsin digestion plus a Sephadex G-100 column using 10 mM phosphate buffer, 140 mM NaCl solution (pH 7.4) for elution (Boushaba *et al.*, 2003). The rTlp-IgG and rTlp-IgG F(ab′)$_2$ in the eluted solutions were enriched with polyethylene glycol at 4 °C and then dialysed against 0.01 M PBS (pH 7.4). The titres of IgG and IgG F(ab′)$_2$ were determined using an immuno-diffusion test.

Western blot assay. The concentration of the purified rTlp2, rTlp3, rTlp4, rTlp2-ECR, rTlp3-ECR or rTlp4-ECR was measured using BCA reagent (Thermo Scientific), and then the recombinant proteins with equal concentration were electro-transferred onto PVDF membrane (Millipore) after SDS-PAGE. Using each of the rTlp-IgGs as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit-IgG (Jackson ImmunoResearch) as the secondary antibody, several Western blot assays were performed to detect the cross-immunoreactivity of the rTlp-IgGs.

Generation of the *tlp* gene-disrupted mutants. pBluescript-II-SK plasmid is commonly used to knockout target genes in *C. jejuni* (Bachtiar *et al.*, 2007). According to the sequencing data about the *tlp2*, *tlp3* and *tlp4* genes of *C. jejuni* strain NCTC 11168-O, there are the two endonuclease *Hin*dIII sites at 1305–1310 and 1461–1466 bp in the *tlp2* gene sequence, an endonuclease *Hin*dIII and a *Spe*I site at 461–466 and 1570–1575 bp, respectively, in the *tlp3* gene sequence, and an endonuclease *Sca*I and *Spe*I site at the 618–623 and 1579–1584 bp, respectively, in the *tlp4* gene sequence. These endonuclease sites can be used to insert Km^R or Cm^R sequences for disruption of the *tlp* genes. Briefly, the entire *tlp2*, *tlp3* and *tlp4* genes were amplified by PCR with the primers T2B-F/T2B-R, T3B-F/T3B-R and T4B-F/T4B-R, respectively (Table S1), and then cloned into the *Xho*I and *Xba*I sites of pBlueskrit-II-SK to form pBlueskrit-II-SK^{tlp2}, pBlueskrit-II-SK^{tlp3} and pBlueskrit-II-SK^{tlp4}. The Km^R sequence (kan) in the pKD4 plasmid was amplified by PCR using the primers K1-F/K1-R, K2-F/K2-R or K3-F/K3-R with different endonuclease sites (Table S1). After digestion with the corresponding endonucleases, the kan1, kan2 or kan3 segment was inserted into the *Hin*dIII/*Hin*dIII, *Hin*dIII/*Spe*I or Sca I/*Spe*I sites in the *tlp2*, *tlp3* or *tlp4* gene of the recombinant pBlueskrit-II-SK plasmids to form suicide plasmids pBlueskrit-II-SK$^{tlp2\text{-kan}}$, pBlueskrit-II-SK$^{tlp3\text{-kan}}$ and pBlueskrit-II-SK$^{tlp4\text{-kan}}$ using T4 DNA ligase (TaKaRa). The suicide plasmids were used to transform *E. coli* DH5α for amplification in LB medium supplemented with 50 μg kanamycin ml^{-1} (Sigma) and were then extracted for sequencing. The suicide plasmids with the excepted sequences were denatured using the alkali denaturing method as described elsewhere (Hinds *et al.*, 1999), and the electrocompetent *C. jejuni* strain NCTC 11168-O were prepared according to Wassenaar's protocol (Wassenaar *et al.*, 1993). The competent bacterial cells were mixed with 2 μg of the denatured suicide plasmid DNAs on ice for 10 min for electrotransformation (1.8 kV, 200 Ω, 25 μF pulse) on ice. The mixtures were transferred onto kanamycin-containing MH blood plates for screening the resistant colonies in order to isolate *tlp2*, *tlp3* and *tlp4* gene-disrupted mutants (Δ*tlp2*, Δ*tlp3* and Δ*tlp4*) (Yao *et al.*, 1997). Moreover, the amplification of Cm^R sequence (cm) from the pCP20 plasmid with the primers C1-F/C1-R (Table S1), generation of the suicide plasmid pBlueskrit-II-SK$^{tlp4\text{-cm}}$, transformation of the competent Δ*tlp3* mutant with pBlueskrit-II-SK$^{tlp4\text{-cm}}$, and screening for the *tlp3* and *tlp4* gene-disrupted mutant (Δ*tlp3*/Δ*tlp4*) with 50 μg kanamycin ml^{-1} plus 30 μg chloramphenicol ml^{-1} (Sigma) were performed as described above. The steps to generate the *tlp* gene-disrupted mutants are summarized in Fig. 1(a).

Generation of the *tlp* gene-complemented mutants. To generate *tlp3* and *tlp4* gene-complemented mutants (CΔ*tlp3* and CΔ*tlp4*), four separate PCRs were performed to amplify segments of the *tlp3* gene plus its upstream 5′-homologous arm (5′ arm-*tlp3*, 2539 bp), the 3′-homologous arm downstream of the *tlp3* gene (*tlp3*-3′ arm, 553 bp), the *tlp4* gene plus its upstream 5′-homologous arm (5′ arm-*tlp4*, 2549 bp), and the 3′-homologous arm downstream of the *tlp4* gene (*tlp4*-3′ arm, 553 bp) from the chromosomal DNA of wild-type *C. jejuni* strain NCTC 11168-O using primers T3E-F/T3E-R, T3F-F/T3F-R, T4E-F/T4E-R and T4F-F/T4F-R (Table S1), respectively. In addition, two Cm^R cassette segments (*tlp3*-cm and *tlp4*-cm) were amplified from the pCP20 plasmid by PCR using the primers C2-F/C2-R and C3-F/C3-R (Table S1). Subsequently, two special PCRs with linking primers were performed to obtain two fusion DNA segments (5′ arm-*tlp3*-cm-3′ arm and 5′ arm-*tlp4*-cm-3′ arm). The reaction mixture contained all the PCR reagents except for the primers, and 300 ng equimolar DNAs for each of the 5′ arm-*tlp3*, *tlp3*-cm and *tlp3*-3′ arm DNA segments, or 5′ arm-*tlp4*, *tlp4*-cm and *tlp4*-3′ arm DNA segments as the templates. The reaction was initiated by incubation at 94 °C for 5 min, followed by 10 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 210 s, and incubation at 72 °C for 15 min to form compound templates, and then the primers T3E-F/T3F-R or T4E-F/T4F-R (Table S1) were added into the mixture for amplification with 30 cycles at 50 °C annealing temperature. The recovered products were digested with *Xho*I and *Xba*I endonucleases and then inserted into the *Xho*I and *Xba*I sites in pBluescript-II-SK using T4 DNA ligase (TaKaRa) to form pBluescript-II-SK$^{5'\ arm\text{-}tlp3\text{-}cm\text{-}3'\ arm}$ and pBluescript-II-SK$^{5'\ arm\text{-}tlp4\text{-}cm\text{-}3'\ arm}$. The recombinant plasmids were used to transform competent Δ*tlp3* or Δ*tlp4* mutants, as described above for *tlp3* or *tlp4* gene complementation, using the *tlp3*-cm or *tlp4*-cm segment in the plasmids to replace the disrupted *tlp3* or tlp4 gene based on allelic exchange of the 5′- and 3′-homologous arms, and these were transferred onto chloramphenicol-containing MH blood plates for screening for resistant colonies in order to isolate the *tlp3* or *tlp4* gene-complemented mutant (CΔ*tlp3* or CΔ*tlp4*). The steps for generating the *tlp3* or *tlp4* gene-complemented mutants are summarized in Fig. 1(b).

Identification of the *tlp* gene-disrupted or complemented mutants. The growth kinetics in 8% sheep blood MH liquid medium of each of the *tlp* gene-disrupted or complemented mutants were examined. The *tlp* gene disruption or complementation in the mutants was detected by PCR using the primers T2C-F/T2C-R, T3C-F/T3C-R, T4C-F/T4C-R, T3G-F/T3G-R or T4G-F/T4G-R (Table S1)

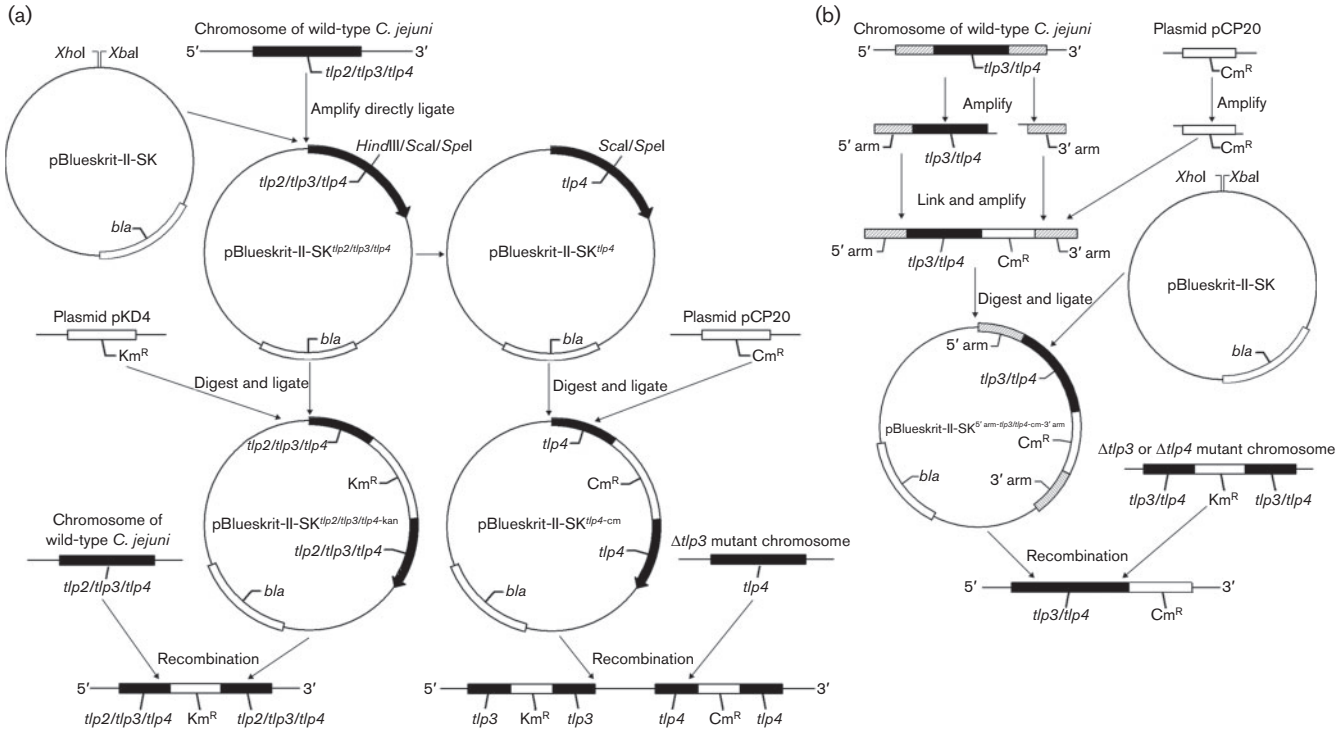

Fig. 1. Strategy for the generation of the *tlp* gene-disrupted mutants (a) and *tlp* gene-complemented mutants (b). See Methods for details.

and sequencing of the PCR products as above. In addition, total RNAs of each of the *tlp* gene-disrupted or complemented mutants were extracted using Trizol reagent (Invitrogen), and then treated with RNase-free DNase I (Novagen) at 37 °C for 30 min. Reverse transcription (RT) using each of the DNA-free RNAs as the template was carried out to synthesize cDNA using a M-MLV reverse transcriptase cDNA synthesis kit (TaKaRa). Subsequently, several real-time fluorescent quantitative PCRs (qPCRs) with the primers T2D-F/T2D-R, T3D-F/T3D-R or T4D-F/T4D-R (Table S1) were performed using a SYBR Premix Ex-*Taq* II kit (TaKaRa) in an ABI 7500 real-time PCR system (ABI) to detect the mRNAs of *tlp2*, *tlp3* and *tlp4* genes. The RT-qPCR data were analysed using both the ΔΔCt model and the randomization test in REST 2005 software (Pfaffl *et al.*, 2002). In the RT-qPCR, the bacterial 16 S rDNA gene was used as the inner reference.

Motility assay. Motility of the Δ*tlp2*, Δ*tlp3*, Δ*tlp4*, Δ*tlp3*/Δ*tlp4tlp*, CΔ*tlp3* and CΔ*tlp4* mutants was determined as described elsewhere (Brøndsted *et al.*, 2005). Briefly, the mutants were grown overnight in MH broth, and then diluted with MH broth to OD_{600} 0.1. A pipette tip that was dipped into each of the bacterial suspensions was stabbed into the centre of a MH motility plate (0.4 % agar). The plates were incubated at 37°C and the diameter of the motility ring was measured every 12 h for 3 days. In the assay, wild-type *C. jejuni* strain NCTC 11168-O was used as the control.

Chemotaxis assay. A soft-agar-based chemotaxis assay was used to detect the ability of 13 chemoattractant candidates to induce chemotaxis of *C. jejuni in vitro* (Khanna *et al.*, 2006; Vegge *et al.*, 2009). Briefly, wild-type *C. jejuni* strain NCTC 11168-O in Brucella broth (Oxoid) containing 0.3 % sodium succinate (Sigma) and 0.01 % L-cysteine-HCl (Sigma) was centrifuged at 2400 ***g*** for 15 min at 15 °C. After washing with PBS and further centrifugation, the bacterial pellet was suspended in PBS. The bacterial number in the suspension was spectrophotometrically adjusted to 2×10^9 c.f.u. ml^{-1} and then the suspension mixed with the same volume of heat-melted 0.8 % agar in 10 mM PBS (pH 7.0) at 45 °C. Filter discs (Whatman) with a 6 mm diameter, which were pre-saturated with each of the chemoattractants, were tightly placed on the surface of the soft agar plates. Among these chemoattractants was a human bile specimen from the gallbladder of a cholecystolithiasis patient after cholecystectomy; the patient had been enrolled with informed consent according to a protocol approved by the Ethics Committee of Zhejiang University. After incubation at 37 °C for 6 h, the accumulative bacterial rings towards each of the attractants in the plates were observed by the naked eye and then the diameters of rings were measured. In the assay, the PBS pre-saturated filter disc was used as the control.

Chemotaxis blocking assay. The protein concentration of rTlp2-, rTlp3- or rTlp4-IgG $F(ab')_2$ was determined by UV spectrophotometry (Zhao *et al.*, 2009). Each of the IgG $F(ab')_2$ types (100 $\mu g\ ml^{-1}$) was mixed with the same volume of PBS containing 4×10^9 c.f.u. wild-type *C. jejuni* strain NCTC 11168-O ml^{-1} for a 30 min incubation at 37 °C. After centrifugation at 2400 ***g*** for 10 min at 15 °C, and washing twice with PBS, the bacterial pellets were suspended in PBS (2×10^9 c.f.u. ml^{-1}). Subsequently, the chemotactic movement of each of the different IgG $F(ab')_2$-blocked bacteria in response to 200 mM SDC, which had been confirmed as a powerful and stable chemoattractant inducing the chemotaxis of wild-type *C. jejuni* strain NCTC 11168-O in the chemotaxis assay, was detected in the soft agar plates as described above. In the assay, the IgG $F(ab')_2$-unblocked wild-type *C. jejuni* strain NCTC 11168-O and the PBS-saturated filter disc were used as the controls.

Determination of role of the Tlp proteins in bacterial chemotaxis. Using 200 mM SDC as the chemoattractant, the chemotaxis assay was performed to assess the chemotactic migration of the Δ*tlp2*, Δ*tlp3*, Δ*tlp4*, Δ*tlp3*/Δ*tlp4*, CΔ*tlp3* and CΔ*tlp4* mutants as described above. In the assay, wild-type *C. jejuni* strain NCTC 11168-O and the PBS pre-saturated filter disc were used as the controls.

Mouse colonization assay. BALB/c-ByJ mice were used, which have been employed frequently to study the colonization of *C. jejuni* (Baqar *et al.*, 1995). All the tested mice were orally given 500 μl 5 % sodium bicarbonate per animal to neutralize gastric acid. Subsequently, according to the results of preliminary experiments, the mice (n=5) were orally infected with a m.o.i. of 1×10^7 c.f.u. per mouse of Δ*tlp2*, Δ*tlp3*, Δ*tlp4*, Δ*tlp3*/Δ*tlp4*, CΔ*tlp3* or CΔ*tlp4* mutants or wild-type *C. jejuni* strain NCTC 11168-O, and then conventionally maintained for 5 days. On the sixth day post-infection, jejunum tissue specimens were collected from the mice, and then were fixed, embedded, sectioned and stained by the silver staining method to allow observation of the bacteria attaching on the jejunal mucosa as described elsewhere (Williams *et al.*, 2007). In an additional assay, the jejunal contents were eluted and then serially diluted with PBS. An aliquot (100 μl) of each of the dilutions was spread on a MH blood plate and incubated for 72 h at 37 °C for counting bacterial numbers. The combination of the two assays could determine the changes in the colonization between the mutants and the wild-type strain. In addition, the mice (n=5) were subjected to bile duct ligation as described elsewhere (Rodríguez-Garay, 2003), and then orally challenged with the wild-type strain. The bacterial numbers in the jejunal contents and attaching on the jejunal mucosa were examined as described above.

Statistical analysis. Data from a minimum of three independent experiments were averaged and presented as the mean ± SD. One-way ANOVA followed by the Dunnett's multiple comparisons test were used to determine significant differences. Statistical significance was defined as a P value $\leqslant 0.05$.

RESULTS

Methylation motif and functional domain in the Tlp proteins

The sequencing data indicated that the PCR products of the *tlp2*, *tlp3* and *tlp4* genes of *C. jejuni* strain NCTC 11168-O had 100 % sequence identities compared to those of *C. jejuni* strain NCTC 11168-GS, a genome-sequenced strain with no or low pathogenicity (GenBank accession no. NC_002163). According to the bioinformatic analysis, the Tlp2, Tlp3 and Tlp4 proteins had an N-intracellular region, two transmembrane regions, an extracellular ligand-binding domain and an intracellular signalling domain (Fig. 2a). The signalling domains in all the Tlp proteins showed high sequence identities (99.4–100 %) with a methylation motif A-A-X2-E-E-X2-SS (Fig. 2b) as reported elsewhere (Alexander & Zhulin, 2007), but the sequence identities among the extracellular ligand-binding domains (33–43 to 286–297 aa) were low (15.0–34.8 %).

Expression and purification effects of the rTlp proteins

The engineered *E. coli* BL21(DE3) strains could efficiently express the rTlp2, rTlp3, rTlp4, rTlp2-ECR, rTlp3-ECR or rTlp4-ECR protein under induction by IPTG, and each of the recombinant proteins purified by Ni-NTA affinity chromatography showed a single band in gels after SDS-PAGE (Fig. 2c).

Titres of IgGs and IgG F(ab′)$_2$s against the rTlps

The rTlp2, rTlp3 and rTlp4 proteins induced the production of specific serum antibodies in the immunized rabbits. Immunodiffusion titres of the rTlp2-, rTlp3- and rTlp4-IgG, or rTlp2-, rTlp3- and rTlp4-F(ab′)$_2$ against rTlp2, rTlp3 and rTlp4, respectively, were 1 : 16 or 1 : 4.

Specificity of immunoreactivity of the ECRs

The Western blot assays showed that the rTlp2-, rTlp3- or rTlp4-IgG displayed cross-immunoreaction among the three rTlp proteins, but no cross-immunoreaction could be found among the three rTlp ECRs (rTlp2-ECR, rTlp3-ECR or rTlp4-ECR) (Fig. 2d), which is consistent with the low amino acid sequence identities among the ECRs.

Characterization of the *tlp* gene-disrupted or complemented mutants

All the *tlp* gene-disrupted and *tlp* gene-complemented mutants grew persistently on kanamycin and/or chloramphenicol-containing MH blood plates, with growth kinetics similar to wild-type *C. jejuni* strain NCTC 11168-O (data not shown). According to the results of PCR and sequencing, the target *tlp* gene in the Δ*tlp2*, Δ*tlp3*, Δ*tlp4* and Δ*tlp3*/Δ*tlp4* mutants was disrupted by the insertion of Km^R or Cm^R sequences (Fig. 3a, b), which resulted in the deletion of a 40 aa segment (450–489) in the signalling domain of the Tlp2 sequence, and a 373 aa segment (153–525) or a 323 aa segment (206–528) in both the ligand-binding and signalling domains of the Tlp3 or Tlp4 sequence (Fig. 2a). The PCR and sequencing data also confirmed that the disrupted *tlp3* or *tlp4* gene in the CΔ*tlp3* or CΔ*tlp4* mutant was complemented with the correct ORF compared to the wild-type strain (Fig. 3a, c). In this study, the sequences of the primers for identification of the *tlp* gene-disrupted or complemented mutants were derived from chromosomal DNA of the wild-type strain and located upstream (forward primers) or downstream (reverse primers) of each of the *tlp* genes with an interval of 120 bp (5′ side or 3′ side). According to the sequencing data, the sequences of both the 5′ side and 3′ side segments were absolutely homologous with those from GenBank (accession no. NC_002163), indicating that the disrupted *tlp2*, *tlp3* or *tlp4* gene, or the complemented *tlp3* or *tlp4* gene, was located in the chromosomal DNA of the *tlp* gene-disrupted or complemented mutants (Fig. 3b, c). Furthermore, the RT-qPCRs confirmed that the *tlp*-mRNAs in the *tlp* gene-disrupted mutants were undetectable, while the CΔ*tlp3* or CΔ*tlp4* mutant presented *tlp3*- or *tlp4*-mRNA at a level similar to the wild-type strain (data not shown).

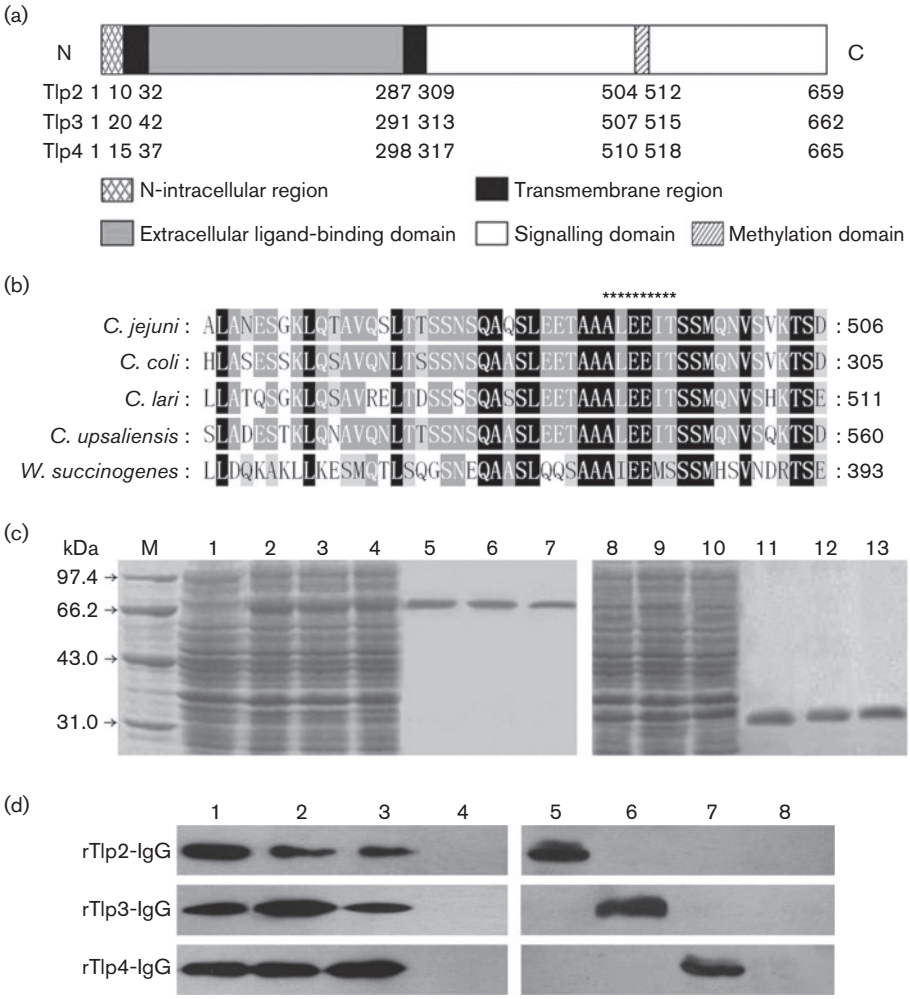

Fig. 2. Functional domains and methylation motif in the Tlp2, Tlp3 and Tlp4 proteins of *C. jejuni* strain NCTC 11168-O. (a) Conserved sequences and functional domains shared among the Tlp proteins. The Tlp2, Tlp3 and Tlp4 proteins have a N-intracellular region, two transmembrane regions, an extracellular ligand-binding domain and an intracellular signalling domain. (b) Methylation motif in the signalling domains of the Tlp proteins. The amino acid residues shown as white letters with black or grey shadow indicate that these amino acids are identical to the amino acids in all or most, respectively, of the methylation motif-containing domains of other bacteria. The amino acid residues shown as black letters with grey shadow or without shadow indicate that these amino acids are isogeneric or non-isogeneric, respectively, compared to the amino acids in the methylation motif-containing domains of other bacteria. The asterisks indicate the methylation motif (A-A-X2-E-E-X2-S-S) in the signalling domains of Tlp2, Tlp3 and Tlp4 proteins. (c) Expression and purification of the rTlp and rTlp-ECR proteins. Lanes: M, protein marker (BioColour); 1, wild-type pET42a plasmid; 2 to 4, the rTlp2, rTlp3 and rTlp4 proteins, respectively, expressed by the different engineered *E. coli* BL21(DE3) strains; 5 to 7, the rTlp2, rTlp3 and rTlp4 proteins, respectively, purified by Ni-NTA affinity chromatography; 8 to 10, the rTlp2-ECR, rTlp3-ECR and rTlp4-ECR proteins, respectively, expressed by the different engineered *E. coli* BL21(DE3) strains; 11 to 13, the rTlp2-ECR, rTlp3-ECR and rTlp4-ECR proteins, respectively, purified by Ni-NTA affinity chromatography. (d) Immunoreactive specificity of the rTlp2-, rTlp3- and rTlp4-IgGs. Lanes: 1 to 3, the immunoblotting bands of the rTlp2-, rTlp3- and rTlp4-IgGs with the Tlp2, rTlp3 and rTlp4 proteins, respectively; 4 and 8, blank controls; 5 to 7, the immunoblotting results of the rTlp2-, rTlp3- and rTlp4-IgGs with the rTlp2-ECR, rTlp3-ECR and rTlp4-ECR, respectively.

Motility of the *tlp* gene-disrupted mutants

The motility assay demonstrated that all the *tlp* gene-disrupted or complemented mutants displayed motility similar to wild-type *C. jejuni* strain NCTC 11168-O (data not shown). The data suggest that the disruption of *tlp2*, *tlp3* and/or *tlp4* genes did not affect the mobility of *C. jejuni*.

SDC as a powerful chemotactic attractant of *C. jejuni*

In soft agar plates, *C. jejuni* could migrate towards efficient chemoattractants. Previous studies reported that some chemicals, especially pyruvate, L-cysteine, L-serine and L-fucose, stimulate the chemotaxis of *C. jejuni in vitro*, but

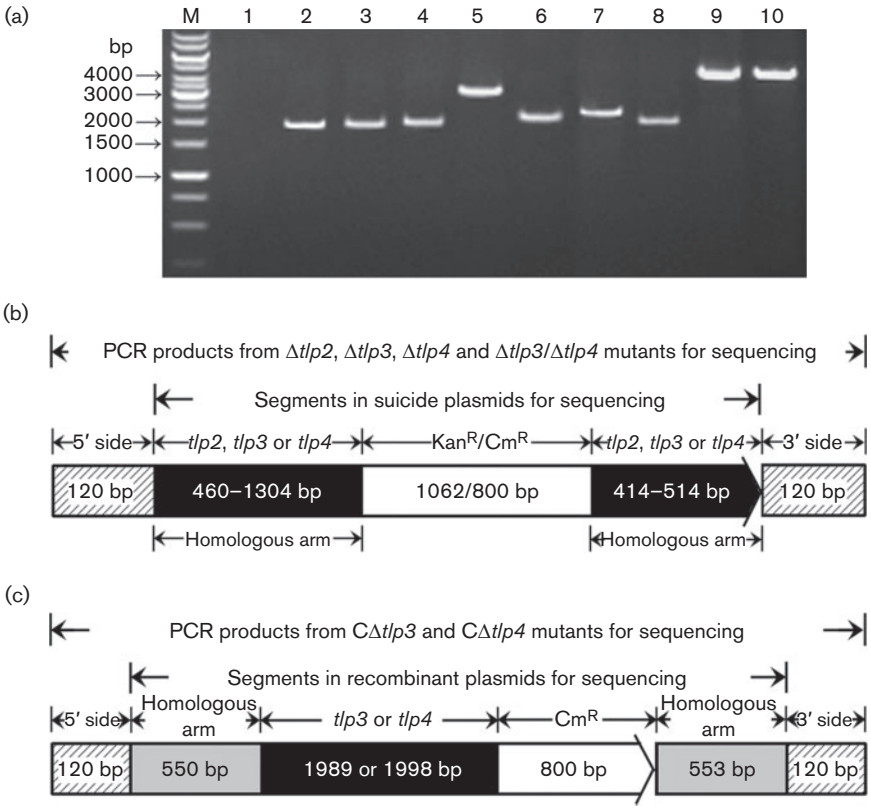

Fig. 3. Confirmation of the *tlp* gene-disrupted or complemented mutants. (a) PCR results for identification of the *tlp* gene-disrupted or complemented mutants. Lanes: M, DNA marker (TaKaRa); 1, blank control; 2 to 4, amplicons of the entire *tlp2* (1980 bp), *tlp3* (1989 bp) and *tlp4* (1998 bp) genes, respectively, from wild-type *C. jejuni* strain NCTC 11168-O; 5 to 7, amplicons of the Km^R-inserted *tlp2*, *tlp3* and *tlp4* genes plus the two 120 bp extending regions from the Δ*tlp2*, Δ*tlp3* and Δ*tlp4* mutants, respectively (3174, 2230 and 2387 bp, respectively); 8, amplicon of the Cm^R-inserted *tlp4* gene plus the two 120 bp extending regions from the Δ*tlp3*/Δ*tlp4* mutant (2125 bp); 9 and 10, amplicons of the complemented *tlp3* gene-cm and *tlp4* gene-cm segment plus the two 120 bp extending regions from the CΔ*tlp3* and CΔ*tlp4* mutants, respectively (4132 and 4146 bp, respectively). (b) Schematic diagram of sequencing results of the Δ*tlp2*, Δ*tlp3* and Δ*tlp4* mutants. (c) Schematic diagram of sequencing results of the CΔ*tlp3* and CΔ*tlp4* mutants.

that some bile components, such as cholic acid, deoxycholic acid or SDC, taurocholic acid or sodium taurocholate, and glycocholic acid, are chemorepellents (Hugdahl *et al.*, 1988; Khanna *et al.*, 2006; Vegge *et al.*, 2009). Unexpectedly, we found that only the bile from human, murine or bovine origin and SDC acted as attractants in the chemotaxis of wild-type *C. jejuni* strain NCTC 11168-O (Table 1). Among the two efficient attractants, SDC could attract the bacterium to form larger chemotactic rings than bile (Table 1).

Chemotaxis-blocking effects of rTlp3-IgG F(ab')$_2$ and rTlp4-IgG F(ab')$_2$

The rTlp3-IgG F(ab′)$_2$ and rTlp4-IgG F(ab′)$_2$ (anti-Tlp3 and anti-Tlp4) inhibited the chemotactic migration of wild-type *C. jejuni* strain NCTC 11168-O towards 200 mM SDC, and the combination of the two IgG F(ab′)$_2$s resulted in a higher ability to inhibit the bacterial chemotaxis (Figs 4a, b). However, the rTlp2-IgG F(ab′)$_2$ (anti-Tlp4) did not inhibit the SDC-induced chemotaxis of the wild-type strain. The data suggest that Tlp3 and Tlp4, but not Tlp2, act as the *C. jejuni* sensor proteins for SDC.

Decreased chemotaxis of the *tlp3* and *tlp4* gene-disrupted mutants

Wild-type *C. jejuni* strain NCTC 11168-O could respond to 200 mM SDC to form a chemotactic ring (Figs 4c, d). However, compared to the wild-type strain, the diameters of the chemotactic rings of the Δ*tlp3* and Δ*tlp4* mutants in response to SDC were reduced by 39 and 31 %, respectively, while the Δ*tlp3*/Δ*tlp4* mutant failed to show any chemotactic movement towards the SDC (Figs 4c, d). In contrast, the Δ*tlp2* mutant presented a chemotactic ring similar to the wild-type strain. When the Δ*tlp3* or Δ*tlp4* mutant was complemented with the *tlp3* or *tlp4* gene, the two mutants (CΔ*tlp3* and CΔ*tlp4*) regained the

Table 1. Effects of the tested attractants on the chemotaxis of *C. jejuni*

n=3 per experiment. +, Chemotactic attraction; −, no chemotactic attraction or repulsion.

Attractant	Concentration	Chemotaxis	Diameter of chemotactic ring (mm)*
Bovine bile	0.1 %	−	0
	1 %	+	2.0 ± 0.5†
	5 %	+	6.0 ± 0.5†
	10 %	+	10.0 ± 1.0†
Mouse bile	0.1 %	−	0
	1 %	+	2.2 ± 0.5†
	5 %	+	5.8 ± 0.6†
	10 %	+	11.5 ± 1.2†
Human bile	0.1 %	−	0
	1 %	+	1.8 ± 0.3†
	5 %	+	5.5 ± 0.5†
	10 %	+	10.5 ± 1.2†
SDC	0.05 M	−	0
	0.1 M	+	8.0 ± 0.5†
	0.2 M	+	15.0 ± 1.0†
	0.4 M	+	17.0 ± 1.5†
L-Cysteine	0.01–0.5 M	−	0
L-Serine	0.01–0.5 M	−	0
Glutamate	0.01–0.5 M	−	0
L-Fucose	0.01–0.5 M	−	0
Sodium pyruvate	0.01–0.5 M	−	0
Swine gastric mucin	0.1–1.0 %	−	0
$NaHCO_3$	0.01–0.5 M	−	0
Na_2HPO_4	0.01–0.5 M	−	0
K_2HPO_4	0.01–0.5 M	−	0
PBS (control)	0.01–0.5 M	−	0

*The diameter values are with the filter disc diameter (6 mm) subtracted.
†P<0.05 versus the control.

chemotactic ability (Fig. 4c, d). The data suggest that both the Tlp3 and Tlp4 proteins mediate the SDC-dependent chemotaxis of *C. jejuni in vitro*.

Attenuated colonization of the *tlp3* and *tlp4* gene-disrupted mutants in mice

Compared to wild-type *C. jejuni* strain NCTC 11168-O, the ability of the Δ*tlp3* or Δ*tlp4* mutant to colonize the jejunal mucosa of mice was significantly reduced, and the Δ*tlp3*/Δ*tlp4* mutant failed to colonize the mice (Fig. 5a, b). When the Δ*tlp3* or Δ*tlp4* mutant was complemented with the *tlp3* or *tlp4* gene, the mutants (CΔ*tlp3* and CΔ*tlp4*) displayed a colonizing ability similar to the wild-type strain (Fig. 5a, b). However, the Δ*tlp2* mutant behaved like the wild-type strain during colonization. In particular, when the bile ducts of mice were ligated, a much lower amount of the wild-type strain was found on the jejunal mucosa or in the jejunal contents, compared to that of the mice without ligation (Fig. 5a, b). The data suggest that the mouse bile acts as an environmental signal to induce the chemotaxis of *C. jejuni*, and the Tlp3 and Tlp4 proteins of the bacterium can sense the signal to trigger the bacterial directional chemotactic movement towards the jejunal epithelium.

DISCUSSION

Colonization is a prerequisite for the pathogenesis of bacterial pathogens, and the absence of colonization results in the loss of infective ability (Croxen *et al.*, 2006; Hendrixson & DiRita, 2004; Thompson-Chagoyán *et al.*, 2007). Chemotaxis towards the target tissues in hosts is critical for colonization for many pathogenic gastrointestinal bacteria with flagella, such as *C. jejuni*, *Helicobacter pylori*, *Yersinia enterocolitica* and *Vibrio cholerae* (Marchant *et al.*, 2002; Moisi *et al.*, 2009; Stecher *et al.*, 2004; Williams *et al.*, 2007). The bacteria depend on their flagellar directional movement to pass through the mucosal layer of the intestinal tract to adhere to intestinal cells for subsequent colonization (Baker *et al.*, 2006; Hazelbauer *et al.*, 2008). In general, chemotaxis includes attractive or repulsive movement, and both the two chemotactic patterns are important for bacteria during infection of hosts. The former contributes to the bacterial colonization and the

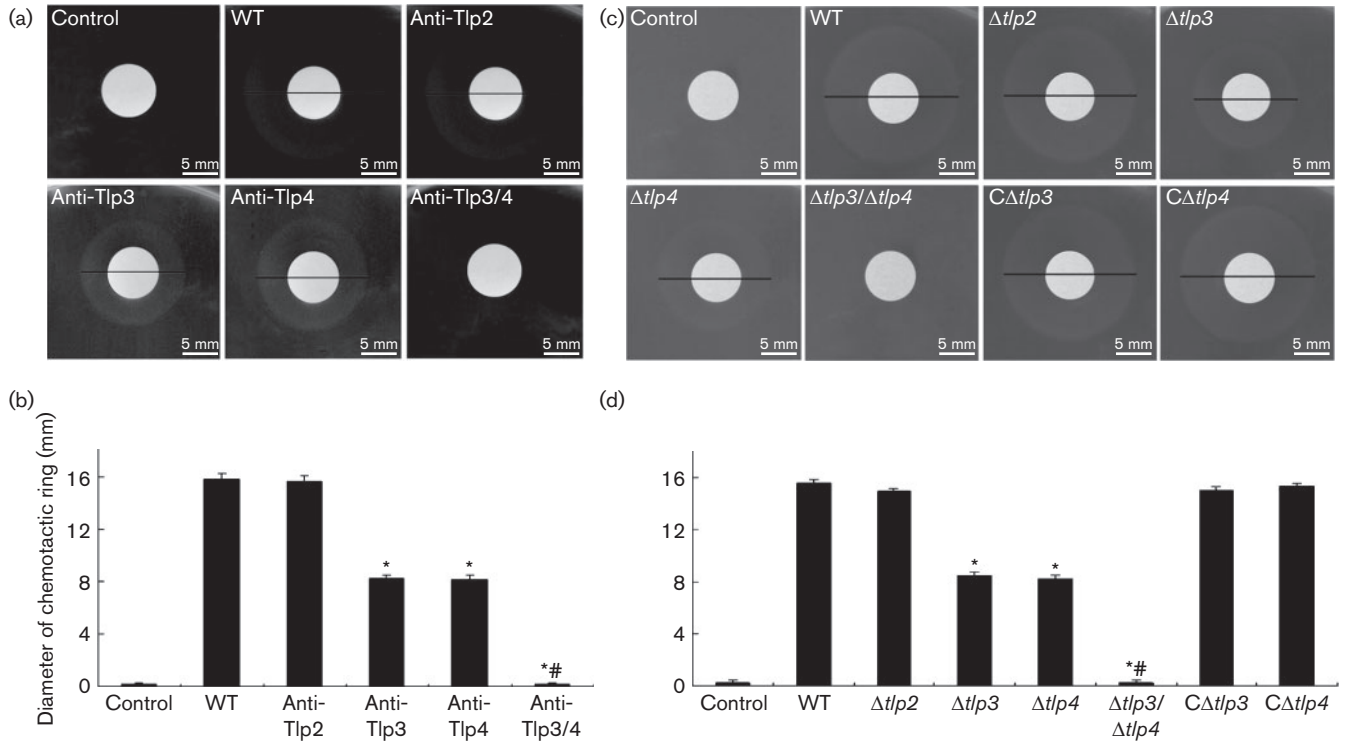

Fig. 4. Changes in the SDC-induced chemotaxis of *C. jejuni* after blockage with anti-Tlps or disruption of *tlp* genes. (a) Anti-Tlp3 and anti-Tlp4 block bacterial chemotaxis towards SDC. The changes in chemotaxis towards 200 mM SDC of wild-type *C. jejuni* strain NCTC 11168-O pre-blocked with the rTlp-IgG F(ab′)$_2$s (anti-Tlps) were detected using 0.4 % agar plates. Control, No chemotactic ring for the wild-type strain towards PBS; WT, the chemotactic ring of the wild-type strain towards SDC; anti-Tlp2, anti-Tlp3 or anti-Tlp4, the chemotactic ring of the anti-Tlp2-, anti-Tlp3- or anti-Tlp4-blocked wild-type strain towards SDC; anti-Tlp3/4, no chemotactic ring for the anti-Tlp3- and anti-Tlp4-blocked wild-type strain towards the SDC. (b) Quantitative analysis of chemotaxis blocking effects among the anti-Tlps. This shows the statistical data from experiments such as those shown in (a). Bars indicate the mean ± SD of three independent experiments. The sizes of the chemotactic rings towards 200 mM SDC of the anti-Tlp2-, anti-Tlp3- and/or anti-Tlp4-blocked wild-type *C. jejuni* strain NCTC 11168-O were quantified by the diameter after subtracting the diameter of the filter disc (6 mm). *$P<0.05$ versus the anti-Tlp2; #$P<0.05$ versus the anti-Tlp3 or anti-Tlp4. (c) Attenuated chemotaxis of the *tlp3* and *tlp4* gene-disrupted mutants towards SDC. The chemotaxis towards 200 mM SDC of wild-type *C. jejuni* strain NCTC 11168-O, the *tlp* gene-disrupted mutants (Δ*tlp2*, Δ*tlp3*, Δ*tlp4* and Δ*tlp3*/Δ*tlp4*) and the *tlp* gene-complemented mutants (CΔ*tlp3* and CΔ*tlp4*) were detected using 0.4 % agar plates. The control had no chemotactic ring for the wild-type strain towards PBS. WT shows the chemotactic ring of the wild-type strain towards SDC. The chemotactic ring of the Δ*tlp3* or Δ*tlp4* mutant towards SDC was decreased, while the Δ*tlp3*/Δ*tlp4* mutant did not show a chemotactic ring towards SDC. The Δ*tlp2*, CΔ*tlp3* and CΔ*tlp4* mutants presented chemotactic rings towards SDC similar to that of the wild-type strain. (d) Quantitative analysis of chemotactic ability among the *tlp* gene-disrupted mutants. This shows statistical data from experiments such as those shown in (c). Bars indicate the mean ± SD of three independent experiments. The sizes of the chemotactic rings towards 200 mM SDC of the different bacterial strains were quantified by the diameter after subtracting the diameter of the filter disc (6 mm). *$P<0.05$ versus the chemotactic ring sizes of the wild-type strain towards SDC; #$P<0.05$ versus the chemotactic ring sizes of the Δ*tlp3* or Δ*tlp4* mutant.

latter helps the bacteria to avoid harmful factors in hosts (Alexander & Zhulin, 2007; Kirby, 2009).

Chemoattractants are chemical and biological compounds that specifically induce bacterial chemotaxis, but there is a large diversity of chemoattractants for different bacteria (Cerda *et al.*, 2003; Liu & Parales, 2008; Terry *et al.*, 2005). For example, the substances that induce the chemotaxis of *C. jejuni* include amino acids, organic acid salts and bile (Hugdahl *et al.*, 1988; Khanna *et al.*, 2006; Vegge *et al.*, 2009). Among these substances, L-cysteine, L-serine, sodium pyruvate, mucin of swine and bile of bovines or chicken seem to be effective at attracting *C. jejuni*. In contrast, the major components of bile such as cholic acid, SDC and sodium taurocholate had no ability to induce the chemotactic movement of the bacteria (Hugdahl *et al.*, 1988; Khanna *et al.*, 2006; Vegge *et al.*, 2009). Unexpectedly, in the present study, we found that only the tested biles (human, mouse and cattle) and SDC could attract *C. jejuni*, and that SDC was the most effective chemoattractant (Table 1).

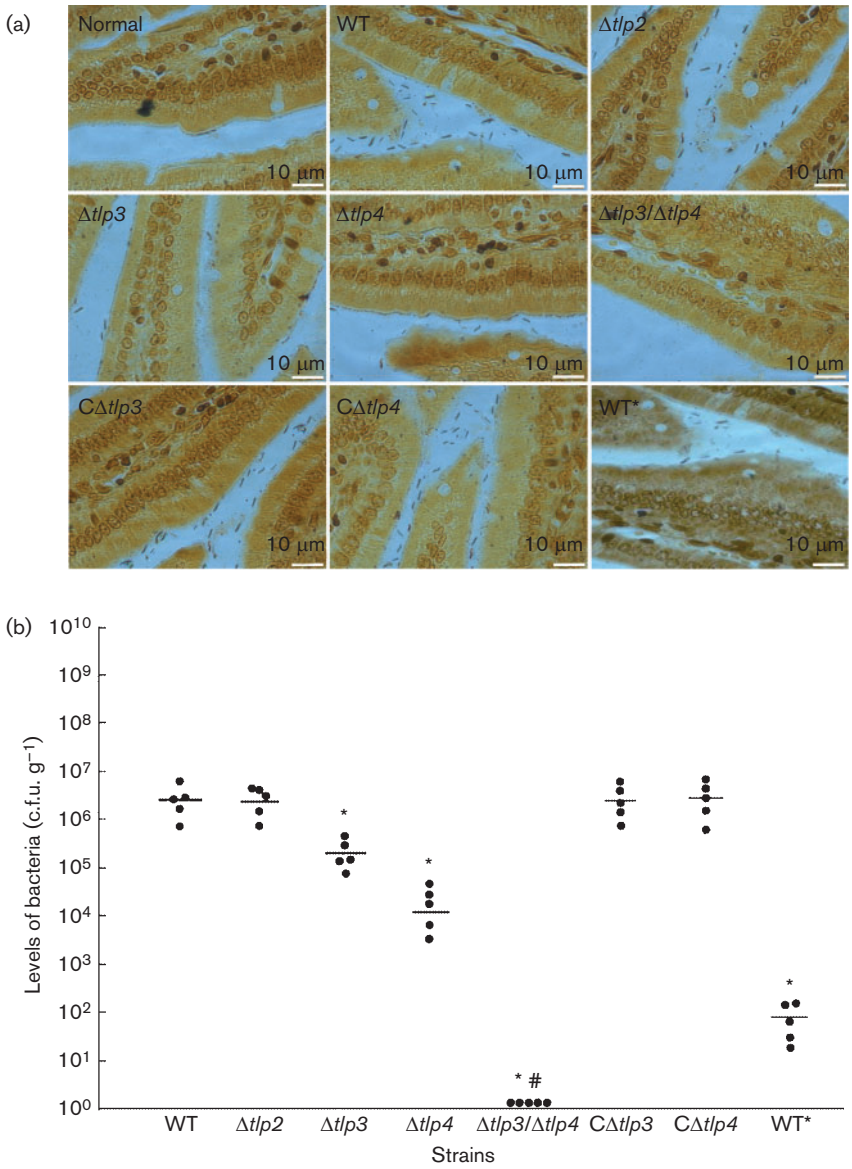

Fig. 5. Colonization by *C. jejuni* strains in mice and quantification of colony numbers. (a) Attenuated colonization in mice of the *tlp3* and *tlp4* gene-disrupted mutants (n=5). The mice were infected with wild-type (WT) *C. jejuni* strain NCTC 11168-O, the *tlp* gene-disrupted mutants (Δ*tlp2*, Δ*tlp3*, Δ*tlp4* and Δ*tlp3*/Δ*tlp4*) and the *tlp* gene-complemented mutants (CΔ*tlp3* and CΔ*tlp4*) at a m.o.i. of 1×10^7 c.f.u. Colonization of jejunal mucosa in mice by the wild-type strain and the mutants was examined using the silver staining method. (b) Number of c.f.u. in the jejunal contents of infected mice (n=5). The bacteria in the jejunal content specimens from wild-type *C. jejuni* strain NCTC 11168-O infected mice, or Δ*tlp2*, Δ*tlp3*, Δ*tlp4*, Δ*tlp3*/Δ*tlp4*, CΔ*tlp3* or CΔ*tlp4* mutant infected mice were grown on MH blood plates, and then quantified using the c.f.u. counting method. WT, The wild-type strain attached on the jejunal epithelium of normal mice; WT*, the wild-type strain attached on the jejunal epithelium of bile duct-ligated mice. *P<0.01 versus the c.f.u. number of the wild-type strain; #P<0.01 versus the c.f.u. number of the Δ*tlp3* or Δ*tlp4* mutant.

However, the L-cysteine, L-serine, sodium pyruvate and swine mucin were not found to be efficient chemoattractants, which differs from previous reports (Hugdahl *et al.*, 1988; Khanna *et al.*, 2006; Vegge *et al.*, 2009). Mammalian bile is produced by the liver for digestion and absorption of nutritional lipids in food, and then effluxes from the bile common duct into the jejunum through the duodenal papilla. Therefore, it is reasonable that bile and its components, such as SDC, act as the chemotactic attractants of *C. jejuni* in the jejunum. Cerda *et al.* (2003) reported that *H. pylori* strain ATCC 43504 responds chemotactically to aspartic acid and serine, but not to arginine or sodium

bicarbonate; whereas, *H. pylori* strain ATCC 700392 showed chemotaxis in response to all four attractants. Thus, the apparent contradiction in the effective chemotaxis-inducing chemoattractants may be due to the difference in tested *C. jejuni* strains.

In bacteria, the two-component signalling (TCS) system, which is usually composed of a sensor protein and a response regulator protein, has the function of sensing and responding to environmental signals (Laub & Goulian, 2007; Stock *et al.*, 2000). The TCS responsible for bacterial movement towards favourable locations in hosts, i.e. chemotaxis, is referred to as Che-TCS (Baker *et al.*, 2006; Kirby, 2009). In the Che-TCS system, there are three major groups of functional proteins: MCPs to receive the signals of the chemoattractants, a histidine kinase to start intracellular signalling, and flagellar motor switch proteins to control the rotative orientation of flagella (Hazelbauer *et al.*, 2008; Marchant *et al.*, 2002; Zhulin, 2001). The Tlp2, Tlp3 and Tlp4 proteins, the members of *C. jejuni* MCPs (Parkhill *et al.*, 2000), had been predicted to share MCP functional domains in their molecules (Marchant *et al.*, 2002). A previous study reported that the methylation motif sequence in the Tlp proteins from different *C. jejuni* strains is A/S/T/G-A/S/T/G-X2-E/Q-E/Q-X2-A/S/T/G-A/S/T/G (Alexander & Zhulin, 2007). According to our sequencing and bioinformatic data, the Tlp2, Tlp3 and Tlp4 proteins of *C. jejuni* strain NCTC 11168-O share a sequence-conserved intracellular signalling domain with the same methylation motif (A-A-X2-E-E-X2 S-S) (Fig. 2). However, the lower sequence identities among the extracellular ligand-binding domains imply the possibility of the three Tlp proteins being used for sensing different environmental signals.

To determine the function of the Tlp proteins of *C. jejuni*, we generated the *tlp2*, *tlp3* and *tlp4* gene-disrupted mutants (Δ*tlp2*, Δ*tlp3* and Δ*tlp4*), the double *tlp3* and *tlp4* gene-disrupted mutant (Δ*tlp3*/Δ*tlp4*), and the *tlp3* or *tlp4* gene-complemented mutant (CΔ*tlp3* or CΔ*tlp4*). Vegge *et al.* (2009) reported that deletion of the *tlp* genes did not affect the motility of the mutants. In agreement, our motility assay showed that all the *tlp* gene-disrupted mutants had a motility similar to the wild-type strain. However, our chemotaxis assay demonstrated that the Δ*tlp3* and Δ*tlp4* mutants, but not the Δ*tlp2* mutant, showed significantly attenuated chemotaxis towards 200 mM SDC, while the Δ*tlp3*/Δ*tlp4* mutant lost the ability to move in response to SDC (Fig. 4c, d). However, the CΔ*tlp3* and CΔ*tlp4* mutants reacquired the SDC-induced chemotactic ability. The blockage of Tlp3 and Tlp4 proteins with antibodies also decreased the chemotaxis of the wild-type strain towards SDC (Fig. 4a, b). All these data indicate that the Tlp3 and Tlp4 proteins serve as SDC signal sensors in the chemotactic movement of *C. jejuni*.

Colonization is an essential step for bacterial pathogens to establish an infection in hosts (Alexander & Zhulin, 2007; Bachtiar *et al.*, 2007; Velayudhan *et al.*, 2004). In our mouse colonization assay, the wild-type *C. jejuni* strain NCTC 11168-O could colonize the surface of jejunal mucosal epithelium in mice. However, the Δ*tlp3* and Δ*tlp4* mutants showed a markedly attenuated ability to colonize, while the Δ*tlp3*/Δ*tlp4* mutant lost its colonizing ability (Fig. 5). When the *tlp3* or *tlp4* gene was complemented (CΔ*tlp3* and CΔ*tlp4* mutants), the ability to colonize mice was restored. In particular, we found that the numbers of the wild-type strain in the jejunal mucosa in the bile duct-ligated mice were decreased significantly compared to the mice without ligation (Fig. 5). Taken together, the data from this study demonstrate that bile and at least one of its components (SDC) are chemotaxis-inducing attractants of *C. jejuni*, and both the Tlp3 and Tlp4 proteins act as the SDC or bile sensors for chemotactic movement and jejunal colonization by this pathogen *in vitro* and *in vivo*.

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