ASSOCIATION OF CAMPYLOBACTER JEUNI SSP. JEUNI CHEMOTAXIS RECEPTOR GENES WITH MULTILOCUS SEQUENCE TYPES AND SOURCE OF ISOLATION

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Received: October 16, 2015; Accepted: April 19, 2016

Campylobacter jejuni’s flagellar locomotion is controlled by eleven chemoreceptors. Assessment of the distribution of the relevant chemoreceptor genes in the C. jejuni genomes deposited in the National Center for Biotechnology Information (NCBI) database led to the identification of two previously unknown tlp genes and a tlp5 pseudogene. These two chemoreceptor genes share the same locus in the C. jejuni genome with tlp4 and tlp11, but the gene region encoding the periplasmic ligand binding domain differs significantly from other chemoreceptor genes. Hence, they were named tlp12 and tlp13.

Consequently, it was of interest to study their distribution in C. jejuni subpopulations of different clonality, and their cooccurrence with the eleven previously reported chemoreceptor genes. Therefore, the presence of all tlp genes was detected by polymerase chain reaction (PCR) in 292 multilocus sequence typing (MLST)-typed C. jejuni isolates from different hosts.

The findings show interesting trends: Tlp4, tlp11, tlp12, and tlp13 appeared to be mutually exclusive and cooccur in a minor subset of isolates. Tlp4 was found to be present in only 33.56% of all tested isolates and was significantly less often detected in turkey isolates. Tlp11 was tested positive in only 17.8% of the isolates, while tlp12 was detected in 29.5% of all isolates, and tlp13 was found to be present in 38.7%.

Keywords: Campylobacter jejuni, MLST, chemotaxis receptors, transducer-like proteins, Tlp4, Tlp5, Tlp7, Tlp11, Tlp12, Tlp13

Abbreviations: CC, clonal complex; CcaA, Campylobacter chemoreceptor for aspartate A (Tlp1); CcmL, Campylobacter chemoreceptor for multiple ligands (Tlp3); cj, gene numbering based on the genome sequence of C. jejuni strain NCTC 11168; MCP, methyl-accepting chemotaxis protein; MLST, multilocus sequence typing; ST, sequence type; tlp, transducer-like protein gene; UPGMA, unweighted-pair group method using average linkages

Introduction

Campylobacter jejuni ssp. jejuni is the most prevalent bacterial pathogen that causes acute enteritis in industrialized nations among human beings. This infection is known as campylobacteriosis and is characterized by watery or bloody diarrhea, fever, and abdominal cramps [1, 2]. In consequence of campylobacteriosis, postinfectious sequelae, namely, the Guillain–Barré syndrome, inflammatory bowel disease, and reactive arthritis may occur [3, 4].

C. jejuni ssp. jejuni has a wide range of hosts. These include wild birds and farm animals, namely, poultry, cattle, sheep, and, rarely, swine. It can also be found as contaminant in milk, meat, and surface water, but most studies have concluded that poultry is the natural host of C. jejuni ssp. jejuni and, hence, the major source of transmission to human beings and other farm animals [5].

Presently, it has been established that, upon entering the intestine of a host, C. jejuni ssp. jejuni has to overcome the intestinal microbiota barrier covering the epithelial cells and the mucin layer for successful colonization [6].
is driven by an inherent necessity to find niches with suitable optimal growth conditions such as amino acids and carboxylic acids which are the major carbon sources for \textit{C. jejuni ssp. jejuni} [7]. In order to locate and reach these niches, \textit{C. jejuni ssp. jejuni} utilizes a complex chemotaxis system, which controls its locomotion meditated by usually two flagella. This flagellar locomotion is controlled by different chemoreceptors comprising of – up to date – 13 transducer-like proteins (Tlp) and two aerotaxis genes [8–10]. These chemoreceptors are categorized into three groups, namely, A, B, and C [8].

Group A receptors consist of a periplasmic ligand-binding domain and a cytoplasmic signaling domain. Members of this group include: Tlp1, Tlp2, Tlp3, Tlp4, Tlp7, Tlp10, and Tlp11. Group B consists of one receptor, Tlp9, with two cytoplasmic ligand–proteins sensing tactic and chemotactic signals (Aer1 and Aer2). Group C receptors miss a periplasmic ligand-binding domain but contain a cytoplasmic signaling domain homologous to that of group A receptors. Their role is to sense internal cytoplasmic signals. Members of this group include Tlp5, Tlp6, Tlp8, and Tlp7, [9, 11]. To date, the chemoeffector proteins and the interaction mechanisms of the various receptors are only partially understood. The identification of specific chemoeffector proteins is complicated by the phenomenon where one chemoreceptor compensates the function of the other [12].

In spite of this challenge, a few chemoeffector proteins have been successfully identified: L-aspartate acts as a chemotactic signal towards formic acid is sensed [11, 14]. In contrast to that in particular \textit{C. jejuni ssp. jejuni} isolates, for example in strain 81-176, the tlp7 gene is translated as one RNA and expressed as one 58 kDa protein. In contrast to that in particular \textit{C. jejuni ssp. jejuni} isolates, for example in strain NCTC 11168, the tlp7 chemoreceptor gene is interrupted by a stop codon (at position 891 663 of the NCTC 11168 genome). This stop codon splits the gene in two open reading frames (ORFs), \textit{cj}0952\textit{c} and \textit{cj}0951\textit{c}, which are both transcribed and expressed as single proteins (25 kDa + 33 kDa). Only if both proteins \textit{Cj}0952\textit{c} and \textit{Cj}0951\textit{c} are expressed and interact as heterodimer, a chemotactic signal towards formic acid is sensed [11, 14].

To distinguish the three proteins, the cytoplasmic protein \textit{Cj}0951\textit{c} was named \textit{Tlp7}_c, protein \textit{Cj}0952\textit{c} containing the membrane-spanning regions, \textit{Tlp7}_\textit{ms}, and the 58 kDa protein containing the cytoplasmic as well as the membrane-spanning regions, \textit{Tlp7}_\textit{mc} [9]. Bile acids generally act as chemorepellants and are sensed by Tlp3 and Tlp4 [15]. It has been demonstrated that Tlp3 binds the chemoreceptors isolateuline, purine, malic acid, and fumaric acid and the chemorepellents lysine, glucosamine, succinic acid, arginine, and thiamine. Therefore, it has been named \textit{Campylobacter} chemoreceptor for multiple ligands (CcmL) [16]. \textit{Tlp9/CetA} (et is an abbreviation for \textit{Campylobacter} energy taxis), lacking an own specific ligand-binding domain, acts as redox-sensing receptor together with its two cytoplasmic ligand–proteins \textit{Aer1/CetC} and \textit{Aer2/CetB}, which are homologues of cytoplasmic redox-sensing proteins (Per-ARNT-Sim [PAS] domains) [8]. \textit{CetABC} acts as energy taxis system driving \textit{C. jejuni} cells towards high redox potentials and favorable conditions for energy generation, respectively. In contrast to \textit{CetABC}, the group C receptor Tlp8 (CetZ), a cytoplasmic sensor with two PAS domains, acts as an opponent to \textit{CetABC} driving cells away from high redox potentials [17].

In this study, we analyzed the currently known chemoreceptor genes in 142 \textit{C. jejuni ssp. jejuni} genome sequences which have been deposited in the NCBI genome database (32 complete genomes and 110 draft genomes in February 2015) and discovered two new unreported chemoreceptor genes, which we have named \textit{tlp12} and \textit{tlp13} as well as a \textit{tlp5} pseudogene.

In addition, we evaluated the distribution of \textit{tlp12}, \textit{tlp13}, the \textit{tlp5} pseudogene, and the 15 currently known chemoreceptor genes in 292 \textit{C. jejuni ssp. jejuni} strains that were isolated from humans, chicken, bovine, and turkey.

The phylogenetic relationship of these isolates was ascertained by multilocus sequence typing (MLST) followed by statistical analysis of the occurrence of the \textit{C. jejuni ssp. jejuni} chemoreceptors in each clonal complex.

**Materials and methods**

**Bacterial isolates and culture conditions**

In this study, a total of 292 \textit{C. jejuni ssp. jejuni} isolates (150 of human, 68 of chicken, 43 of bovine, 24 of turkey, three of ovine, two of wild bird, two of canine, and one of riparian origin) were included. The human isolates were isolated from stool samples of suspected cases of campylobacteriosis reported at the University Medical Center Göttingen, Germany during the period of 2000–2004. Ethical clearance for the analysis was obtained from the Ethics Committee at the University Medical Center Göttingen, Germany. However, these bacterial isolates have been used in some preliminary studies [14, 18–20]. Hence, no evaluation including personal patient data was performed; the Ethics Committee at the University Medical Center Göttingen waived the need for written informed consent from the donor or the next of kin. The study design, therefore, corresponds to a retrospective data analysis.

The chicken, bovine, and turkey isolates were obtained from the Bundesinstitut für Risikobewertung (BfR, Federal Institute for Risk Assessment, http://www.bf.bund.de/de/nationales_referenzlabor_fuer_campylobacter-8818.html) in Berlin, Germany.

Species identification was performed using the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany). Results with MALDI Biotyper identification score values ≥ 2.000 were considered correct. Additionally, multiplex polymerase chain reaction (PCR) was used to discriminate between \textit{C. jejuni} and \textit{Campylobacter coli} [21]. No isolates, featuring \textit{C. coli} or \textit{C. jejuni ssp. doylei} characteristic
MLST clonal complexes/sequence types, were used in this study.

The *C. jejuni* ssp. *jejuni* isolates were cultured on Columbia agar base (Merck) supplemented with 5% sheep blood (BA) and incubated at 42 °C under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) for 18 h prior to DNA extraction.

**DNA extraction**

Genomic DNA of all *C. jejuni* ssp. *jejuni* isolates was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA concentration was measured using a nanodrop 1000 spectrophotometer (peqlab, Thermo Fisher Scientific, Wilmington, USA).

**Evaluation of chemoreceptor distribution in relation to source of isolation and clonal complex PCR conditions**

For analysis of *tlp* and aerotaxis genes in each isolate based on source of isolation and clonal complex, 50 ng DNA and 1 μL of 10 μM of primers listed in *Table 1* were used. Primers to amplify the genes of *tlp1–3* and *tlp5–10* according to the manufacturer’s instructions. Genomic DNA concentration was measured using a nanodrop 1000 spectrophotometer (peqlab, Thermo Fisher Scientific, Wilmington, USA).

**Table 1. Primers used for amplification of *tlp* genes**

| Gene                  | Primer name        | Sequence 5′–3′       | Annealing temp. (°C) | Length (bp) |
|-----------------------|--------------------|----------------------|----------------------|-------------|
| *tlp1/ccaA* cj1506    | *tlp1-F02*         | AGCTAACTGCAAGTGTGCAAG | 60.0                 | 1382        |
|                       | *tlp1-R02*         | CCGCAAGCTGTCTTACCTCA |                      |             |
| *tlp2* cj0144         | *tlp2-F03*         | AAGAATTAGAGATGCTGGAGA | 59.0                 | 1191        |
|                       | *tlp2-R03*         | AGTGGTTAAGCTTTGAAAGCA |                      |             |
| *tlp3/ccmL* cj1564    | *tlp3-F06*         | CGTGAAGATTTCCGTTCCCAC | 59.0                 | 649         |
|                       | *tlp3-R06*         | AGCGCTTTCGTTAAATACAGC |                      |             |
| *tlp4/12-consensus*   | *tlp4-F02*         | TGGTTTGAATTATGTTGATT | 58.0                 | 1658        |
|                       | *tlp4-R03*         | CCTTACCATGTTCTCCAGC  |                      |             |
| *tlp5-consensus*      | *tlp5c-F02*        | GAAATGCAAATTTTAGTGGC | 57.0                 | 792/207*    |
|                       | *tlp5c-R02*        | AGCTAGAACATCTTACATGTC |                      |             |
| *tlp6* cj026c         | *tlp6-F01*         | GCAGGGAAATGACGGTGTCG | 57.0                 | 809         |
|                       | *tlp6-R01*         | GCATGCAATTTCGACACCCAG |                      |             |
| *tlp7*/ccfA* cj0951c   | *tlp7-F01*         | AGGTTCCTTGCATATTTTGTGTCG | 53.0                 | 880         |
|                       | *tlp7-R01*         | GCAGAATTTCCCAAGTCCGCA |                      |             |
| *tlp8* cj1110         | *tlp8-F01*         | TGCTGCTGCTAACTGTCATGGC | 58.0                 | 597         |
|                       | *tlp8-R01*         | GCACGTGCCGGCCCTCAATGCA |                      |             |
| *tlp9/cetA* cj1190    | *cetA-F01*         | TCGTAAAGCTTTCGCAAGGTC | 58.0                 | 470         |
|                       | *cetA-R01*         | CGCAGATCCGGCCCTCACATGC |                      |             |
| *tlp9/cetB/aer2* cj1899 | *cetB-F01*     | TGCAGGTTTACCATGCGGGTGTTGTGCTT | 57.0                 | 308         |
|                       | *cetB-R01*         | AGCCCTTTGCTGGTTGCTTCTT |                      |             |
| *tlp9/aer1* cj1191    | *aer1-F01*         | ACATGGAAGATATGCGCCACGCATG | 57.0                 | 186         |
|                       | *aer1-R01*         | GTGGTCACAGGAAAGACAATAA |                      |             |
| *tlp10* cj0019        | *tlp10-F01*        | AGAAGGCAATCTCACCCTGCTCAGT | 56.0                 | 532         |
|                       | *tlp10-R01*        | AAATCCACGCCCATGTTGCG |                      |             |
| *tlp11*               | *tlp11a-F02*       | AGCAATAGGAAATTGCTTATAGCATTGC | 59.0                 | 1770        |
|                       | *tlp11b-F02*       | GCAAGACCGTCCTCAATGCGC |                      |             |
| *tlp12*               | *tlp12-F01*        | TGCACCAATGCAATTCACAC | 58.0                 | 1015        |
|                       | *tlp4/12-R03*      | CCTTACCATGTTCTCCAGC  |                      |             |
| *tlp13*               | *tlp13-F01*        | TCGAGCGTTAGTTCAAAACTCT | 59.0                 | 1185        |
|                       | *tlp13-short-R01*  | ACCCCTTTGCGCCCTACATCA |                      |             |

*Primers amplify both *tlp5* variants. The intact gene results in an amplicon size of 792 bp. The disrupted gene results in an amplicon size of 207 bp.

†The interrupting stop codon was detected by *Ase*I restriction of the amplicon.
Table 2. Percentage distributions of tlp4, tlp5, tlp7m, tlp11, tlp12, and tlp13 among 292 *C. jejuni* isolates and their associations with host and CC/ST 

| Host or CC/ST | tlp4 | tlp5 | tlp7m | tlp11 | tlp12 | tlp13 |
|---------------|------|------|-------|-------|-------|-------|
| Host          |      |      |       |       |       |       |
| All           | 98/292 (33.6) | 165/292 (56.5) | 67/292 (22.9) | 52/292 (17.8) | 86/292 (29.5) | 113/292 (38.7) |
| Human         | 61/150 (40.7) | 84/150 (56.0) | 30/150 (20.0) | 20/150 (13.3) | 51/150 (34.0) | 69/150 (46.0)  |
| Chicken       | 18/68 (26.5) | 35/68 (51.5) | 4/68 (5.9)* | 4/68 (5.9)* | 23/68 (33.8) | 32/68 (47.1)  |
| Bovine        | 14/43 (32.6) | 31/43 (72.1)† | 27/43 (62.8)* | 24/43 (55.8)* | 5/43 (11.6)* | 2/43 (4.7)*   |
| Turkey        | 2/24 (8.3)*  | 9/24 (20.1) | 5/24 (20.8) | 3/24 (12.5) | 7/24 (29.2) | 9/24 (37.5)   |
| Other hosts   | 3/7 (42.9) | 6/7 (85.7) | 1/7 (14.3) | 1/7 (14.3) | 1/7 (14.3) |           |
| CC21          | 21/84 (25.0) | 84/84 (100)* | 50/84 (59.5)* | 35/84 (41.7)* | 32/84 (38.1) | 17/84 (20.2)* |
| ST21          | 10/31 (32.3) | 31/31 (100)* | 30/31 (96.8)* | 23/31 (74.2)* | 0/31 (0.0)*  | 2/31 (6.5)*   |
| ST53          | 1/8 (12.5) | 8/8 (100)* | 8/8 (100)* | 7/8 (87.5)* | 0/8 (0.0)*  | 7/8 (87.5)*   |
| ST50          | 2/17 (11.8) | 17/17 (100)* | 1/17 (5.9) | 0/17 (0.0)* | 17/17 (100)* | 5/17 (29.4)   |
| Other ST      | 8/28 (28.6) | 28/28 (100)* | 11/28 (39.3) | 5/28 (17.9) | 15/28 (33.6) | 3/28 (10.7)   |
| CC52          | 5/6 (83.3)*  | 1/6 (16.7) | 1/6 (16.7) | 0/6 (0.0)* | 0/6 (0.0)* | 5/6 (83.3)*   |
| CC446         | 1/5 (20.0) | 0/5 (0.0)* | 0/5 (0.0)* | 0/5 (0.0)* | 0/5 (0.0)* | 5/5 (100)*    |
| CC49          | 4/5 (80.0)† | 0/5 (0.0)* | 0/5 (0.0)* | 0/5 (0.0)* | 1/5 (20.0) | 1/5 (20.0)    |
| CC1034        | 0/7 (0.0)* | 0/7 (0.0)* | 0/7 (0.0)* | 0/7 (0.0)* | 3/7 (42.9) | 4/7 (57.1)    |
| CC354         | 0/6 (0.0)* | 0/6 (0.0)* | 0/6 (0.0)* | 0/6 (0.0)* | 5/6 (83.3)* | 3/6 (50)      |
| CC443         | 1/5 (20.0) | 0/5 (0.0)* | 0/5 (0.0)* | 0/5 (0.0)* | 1/5 (20.0) | 3/5 (60.0)    |
| CC206         | 12/21 (57.1)† | 14/21 (66.7)† | 0/21 (0.0)* | 0/21 (0.0)* | 9/21 (42.9) | 6/21 (28.6)   |
| ST46          | 0/3 (0.0)* | 3/3 (100)* | 0/3 (0.0)* | 0/3 (0.0)* | 2/3 (66.7) | 1/3 (33.3)    |
| ST122         | 5/5 (100)* | 5/5 (100)* | 0/5 (0.0)* | 0/5 (0.0)* | 2/5 (40.0) | 1/5 (20.0)    |
| ST572         | 5/5 (100)* | 0/5 (0.0)* | 0/5 (0.0)* | 0/5 (0.0)* | 0/5 (0.0)* | 0/5 (0.0)*    |
| ST2192        | 0/3 (0.0)* | 3/3 (100)* | 0/3 (0.0)* | 0/3 (0.0)* | 3/3 (100)* | 2/3 (66.7)    |
| Other ST      | 2/5 (40.0) | 3/5 (60.0) | 0/5 (0.0)* | 0/5 (0.0)* | 2/5 (40.0) | 2/5 (40.0)    |
| CC48          | 15/20 (75.0) | 4/20 (20.0) | 4/20 (20.0) | 5/20 (25.0) | 0/20 (0.0)* | 1/20 (5.0)*   |
| ST38          | 0/3 (0.0)* | 3/3 (100) | 3/3 (100) | 3/3 (100) | 0/3 (0.0)* | 0/3 (0.0)*    |
| ST48          | 7/7 (100)* | 0/7 (0.0)* | 0/7 (0.0)* | 0/7 (0.0)* | 0/7 (0.0)* | 1/7 (14.3)    |
| Other ST      | 8/10 (80.0) | 1/10 (10.0) | 1/10 (10.0) | 2/10 (20.0) | 0/10 (0.0)* | 0/10 (0.0)*   |
| CC257         | 0/10 (0.0)* | 0/10 (0.0)* | 0/10 (0.0)* | 0/10 (0.0)* | 8/10 (80.0)* | 9/10 (90.0)*  |
| CC464         | 1/8 (12.5) | 0/8 (0.0)* | 0/8 (0.0)* | 0/8 (0.0)* | 5/8 (62.5) | 8/8 (100)*    |
Table 2. (cont’d)

| Host or CC/ST | tlp4 | tlp5 | tlp7m | tlp11 | tlp12 | tlp13 |
|---------------|------|------|-------|-------|-------|-------|
| CC353         | 0/8 (0.0)* | 0/8 (0.0)* | 0/8 (0.0)* | 0/8 (0.0)* | 1/8 (12.5) | 1/8 (12.5) |
| CC658         | 1/4 (25.0) | 1/4 (25.0) | 0/4 (0.0)* | 0/4 (0.0)* | 0/4 (0.0)* | 4/4 (100)* |
| CC22          | 5/9 (55.6) | 9/9 (100)* | 0/9 (0.0)* | 2/9 (22.2) | 2/9 (22.2) | 5/9 (55.6) |
| CC1332        | 0/2 (0.0)* | 0/2 (0.0)* | 0/2 (0.0)* | 0/2 (0.0)* | 4/4 (100)* | 4/4 (100)* |
| CC2283        | 5/6 (83.3)* | 6/6 (100)* | 0/6 (0.0)* | 0/6 (0.0)* | 0/6 (0.0)* | 2/6 (33.3)* |
| CC42          | 6/7 (85.7)* | 7/7 (100)* | 1/7 (14.3) | 0/7 (0.0)* | 0/7 (0.0)* | 1/7 (14.3)* |
| CC61          | 0/11 (0.0)* | 0/11 (0.0)* | 10/11 (90.9)* | 10/11 (90.9)* | 2/11 (18.2) | 0/11 (0.0)* |
| Other         | 2/35 (5.7) | 6/35 (17.1) | 1/35 (2.3) | 0/35 (0.0) | 11/35 (31.4) | 24/35 (68.6) |

Chemoreceptor genes: tlp4, transducer-like protein 4; tlp5, transducer-like protein 5 (intact gene); tlp7m, transducer-like protein 7 membrane associated part (homologue to cj0952c); tlp11, transducer-like protein 11; tlp12, transducer-like protein 12; tlp13, transducer-like protein 13; other hosts: include three isolates of ovine, two of wild bird, two of canine, and one of riparian origin; other ST: include ST belonging to the particular CC not listed separately; other: include singletons and CCs with a very low number of isolates included in the study.

*p < 0.05; **p < 0.001 significance level in comparison to the remaining isolates not belonging to the corresponding clonal complexes/sequence types. Additionally, the values in isolate groups with above average numbers of receptor gene positive or negative isolates are given in bold numbers.

### MLST and phylogenetic analysis

The MLST-type was established using amplification and sequencing primers reported before [22]. The cycling conditions were 44°C for 1 min, followed by 35 cycles of 94°C for 30 s, 50°C for 60 s, and 72°C for 60 s of 94°C for 120 s, 50°C for 60 s, and 72°C for 60 s of 94°C for 90 s, 50°C for 60 s, and 72°C for 90 s, followed by a final elongation of 72°C for 5 min.

Amplicons of the seven genes included in the C. jejuni/C. coli MLST scheme were sent for sequencing to Seqlab Sequence Laboratories GmbH (Göttingen, Germany) using 10 pmol of the respective sequencing primer. MEGA6 software was used to construct the unweighted pair group method using average linkages (UPGMA)-tree [23]. The C. jejuni MLST website (http://pubmlst.org/campylobacter/) was consulted for assignment of sequence types and clonal complexes.

### Statistical analyses and multiple sequence alignment

The Statistica software (Statsoft, Tulsa, Oklahoma, USA) was used to perform statistical analysis. The χ² test was used to test for significant differences in the frequencies of the tlp genes within the defined groups. The obtained p values are shown in Table 2.
Multiple sequence alignment of the \textit{tlp} gene sequences was performed using the Clustal Omega package (http://www.ebi.ac.uk/Tools/msa/clustalo/) [25] hosted at the EMBL-EBI bioinformatics web and programmatic tools framework website [26].

\section*{Results}

\textbf{Analysis of chemoreceptor genes in \textit{C. jejuni} ssp. \textit{jejuni} genome sequences}

BLAST search of all 15 known chemoreceptor genes against the 142 assembled \textit{C. jejuni} ssp. \textit{jejuni} genome sequences deposited in the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome/genomes/149) showed that the genes \textit{tlp1}, \textit{tlp2}, \textit{tlp3}, \textit{tlp6}, \textit{tlp9}, and \textit{tlp10} were well conserved (coverage: ca. 100%; sequence identity ca. 97%). There was only limited sequence variability, and there were no pseudogenes close to the particular gene loci.

In contrast, significant variation was observed in genes \textit{tlp4}, \textit{tlp5}, \textit{tlp7}, and \textit{tlp11}.

\textbf{Gene sequence differences of \textit{tlp4} and \textit{tlp12}}

The chemoreceptor gene \textit{tlp4} (\textit{cj}0262c) was 1998 bp in strain NCTC 11168. It was flanked by gene \textit{cj}0261c

\begin{center}
\begin{figure}
\includegraphics[width=\textwidth]{figure1}
\caption{Genome arrangements of different bacterial strains at the gene loci of \textit{tlp4}, \textit{tlp11}, \textit{tlp12}, and \textit{tlp13}. \textit{tlp5} as well as \textit{tlp7}. A: The \textit{tlp4} gene is located downstream of \textit{zupT} in strain NCTC 11168. In strain IA3902, the \textit{tlp4} gene downstream of \textit{zupT} is replaced by gene encoding Tlp11. A SAM-dependent methyltransferase gene downstream of the \textit{tlp4} (NCTC 11168) or \textit{tlp11} (IA3902) gene is replaced by transcription initiation protein gene \textit{tat} in strain 00-6200. In strain R14, the gene encoding Tlp12 is located downstream of \textit{zupT}. Upstream of \textit{zupT}, a \textit{tlp13} gene is present. B: The \textit{tlp5} gene in NCTC 11168 \textit{cj}0246c is replaced by a disrupted pseudogene in strain S3. C: Tlp7 gene is interrupted by a stop codon at position 891 663 of the NCTC 11168 genome splitting the open reading frame into two parts \textit{cj}0952c and \textit{cj}0951c; in strain 81-176, \textit{tlp7} is one continuous gene.}
\end{figure}
\end{center}
encoding a S-adenosyl-l-methionine (SAM)-dependent methyltransferase and gene supT encoding a zinc transporter (Fig. 1A). Between these two genes, we discovered in the genome sequence of strain R14 a chemoreceptor gene (H730_01610) that was 1989 bp. The 173 bp at the 5′ end and the 1134 bp at the 3′ end of H730_01610 showed significant sequence identity to gene tlp4 (cj0262c) of NCTC 11168: 91% (158/173) and 96% (1085/1136), respectively (Fig. 2). In contrast, the 682 bp in between these two largely identical sequence regions shared no significant sequence identity. Therefore, we concluded that H730_01610 (in strain R14) is a new receptor and named homologues to chemoreceptor gene H730_01610 tlp12. Homologues to chemoreceptor gene cj0262c (tlp4 in strain NCTC 11168) were further referred to as tlp4. The overall sequence identity between tlp4 and tlp12 was 83% (1679/2023) for the DNA sequence.

Gene sequence differences of tlp11 and tlp13

Upstream of supT, we identified a further methyl-accepting chemotaxis protein gene, H730_01620, in the genome of strain R14 (Fig. 1A). H730_01620 was 2118 bp, and the 951 bp at the 3′ end encoding the cytoplasmic signaling domain was 98% (936/953) identical to the cytoplasmic signaling domain of tlp4 (cj0262c) and 94% (897/954) identical to tlp12 (H730_01610; Fig. 2). In contrast, the 3′ end of H730_01620 was 85% (1721/2018) identical to gene cjj8425_0287 of strain 84-25, which was designated as tlp11 [10]. Interestingly, the first 114 bp of H730_01620 differs from tlp11 (cjj8425_0287). Therefore, we concluded that H730_01620 was also a new receptor gene. Consequently, we named homologues to chemoreceptor gene H730_01620 in R14 as tlp13. Homologues to chemoreceptor gene cjj8425_0287 in strain 84-25 were further referred to as tlp13. The overall sequence identity between tlp11 and tlp13 was 85% (1703/2006) for the DNA sequence.

However, there was some variation in the flanking regions of tlp11. For example, in strain IA3902, tlp11 was located between SAM-dependent methyltransferase gene cjSA_0238 and zinc transporter gene supT, completely replacing tlp4, and in strain 00/6200, tlp11 was flanked by transcription initiation protein gene tat and zinc transporter gene supT (Fig. 1A).

Multiple sequence alignment of tlp2, tlp3, tlp4, tlp11, tlp12, and tlp13

Multiple sequence alignment of tlp4 (cj0262c), tlp11 (CJ18425_0287), tlp12 (H730_01610), tlp13 (H730_01620), tlp2 (cj0144), and tlp3 (cj1564) indicated that tlp4, tlp11, tlp12, and tlp13 form a phylogenetically related group, while tlp2 and tlp3 are distant and, hence, less related to tlp4, tlp11, tlp12, and tlp13.

The DNA sequence identity of tlp12 was 50.0% (994/1989) referring to tlp2 and 50.0% (994/1989) referring to tlp3.

Comparably, DNA sequence identity of tlp13 was 44.2% (936/2118) referring to tlp2 and 44.2% (936/2118) referring to tlp3. Therewith, the sequence identity was significantly lower in comparison to tlp4 and tlp11 (Figs. 2 and 3).

Tlp5 intact gene and tlp5 pseudogene

Intact gene tlp5 encompassed 1128 bp in strain NCTC 11168 (cj0246c). It was located between gene rplT that encodes the L20 ribosomal protein of the 50S subunit and cj0247 that encodes a chemotaxis sensory transducer protein. In five of the deposited genomes, namely, S3, 00-1597, F38011, CG8421, and R14, tlp5 gene was disrupted at position 504 of the gene, and at the same gene locus, a pseudogene of about 537 bp was found (annotated as disrupted methyl-accepting chemotaxis protein [MCP]-domain signal transduction protein pseudogene, Fig. 1B). The 503 bp at the 5′ end of the pseudogene shared 99% identity to the sequence of the periplasmic sensory domain of the complete tlp5 gene, but the cytosolic signaling domain was missing.

Detection of chemoreceptor genes in a phylogenetically diverse C. jejuni strain collection using PCR

Ubiquitous chemoreceptor genes

Analyzing a strain collection of 292 C. jejuni ssp. jejuni isolates for the so far well-described 13 chemoreceptor genes, two aerotaxis genes, tlp5 pseudogene, tlp7 two ORF variant, tlp12, and tlp13 using PCR, showed that a majority of the chemoreceptor genes were found to be ubiquitous. In detail, tlp1 (ccmA) was detected in 100% (292/292), tlp2 in 97.9% (286/292), tlp3 (ccmL) in 100% (292/292), tlp6 in 100% (292/292), tlp8 in 100% (292/292), tlp9/cteABC (including both cytoplasmic ligand–proteins: cetA, cetB/aer2, aer1) in 100% (292/292), and tlp10 in 97.9% (286/292) of the C. jejuni ssp. jejuni isolates (Fig. 4).

Six (2.1%; 6/292) tlp2 negative isolates were detected in the MLST clonal complex (CC) CC48-related CCs, and six (2.1%; 6/292) tlp10 negative isolates were detected in the MLST sequence type (ST) ST536, ST54, ST677, and CC45 isolates. However, these differences, in comparison to the remaining isolate population, were not significant (Table 2, Fig. 4).

Transducer-like protein genes tlp4, tlp11, tlp12, and tlp13

As shown in Fig. 1A, tlp4, tlp11, tlp12, and tlp13 shared nearly the same gene locus downstream (tlp4, tlp11, tlp12) or upstream (tlp13) of the supT gene. It seemed that these genes substitute each other at one site in the genome, but in some genomes, they were found in close proximity to each
Fig. 2. Multiple sequence alignment of tlp2 (cj0144), tlp3 (cj1564), tlp4 (cj0262c), tlp11 (CJJ8425_0287), tlp12 (H730_01610), and tlp13 (H730_01620). The multiple DNA sequence alignment was analyzed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). An asterisk indicates identical bases in all six sequences.
Fig. 2. (cont'd)

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| Gene          | Sequence                                                                 |
|--------------|---------------------------------------------------------------------------|
| tlp11_CJ8425 | TTTAGCTACTACCTAGAGATTTTCTTCGTACATTCATATTAACAGCACTTTAAAAATTCACTTTCTCTATC |
| tlp13_H730   | TTATGACATTTTATAGATTTTACATATTTTATGATTATTGATTTAAAATGCTCACTTCTCTATC       |
| tlp4_Cj0262c | CTTAGATTTTATGATTATTGATTTAAAATGCTCACTTCTCTATC                           |
| tlp12_H730   | ATGCTTTTATGATTATTGATTTAAAATGCTCACTTCTCTATC                            |
| tlp12_Cj0144 | ATGCTTTTATGATTATTGATTTAAAATGCTCACTTCTCTATC                            |
| tlp3_Cj1564  | ATGCTTTTATGATTATTGATTTAAAATGCTCACTTCTCTATC                            |

**Fig. 2. (cont'd)**
Fig. 2. (cont’d)
other (Fig. 1A, e.g., R14). Therefore, \( tlp4 \), \( tlp11 \), \( tlp12 \), and \( tlp13 \) have to be evaluated conjointly.

**Tlp4:** In 33.6% (98/292) of the isolates, the chemoreceptor gene \( tlp4 \) was detected. As shown in Table 2 and Fig. 4, \( tlp4 \)-positive isolates were predominantly present in eight clusters of isolates, namely, CC52, CC49, CC206 (ST122 and ST572), CC48 (ST48), CC22, CC45, CC283, and CC42 (Table 2). Noticeably, \( tlp4 \) was significantly (\( p < 0.001 \)) less often detectable in turkey isolates, i.e., 8.3% (2/24).

**Tlp11:** A percentage of 17.8% (52/292) of all isolates were \( tlp11 \) positive. Typical \( tlp11 \)-positive isolate clusters were CC21 (ST21 and ST53), CC48 (ST38), and CC61 (Table 2, Fig. 4). Noticeably, \( tlp4 \) was significantly (\( p < 0.001 \)) less often detectable in bovine isolates, i.e., 11.6% (5/43).

**Tlp12:** The PCR detecting chemoreceptor gene \( tlp12 \) was positive in 29.5% (86/292) of the isolates. In contrast to \( tlp4 \), \( tlp12 \) was predominantly present in the clonal complexes/sequence types: ST50, CC354, ST2192, CC257, ST464, and CC1332 (Table 2, Fig. 4). In the above-named Cs/STs positive for \( tlp4 \) or \( tlp11 \), \( tlp12 \) was significantly (\( p < 0.05 \)) less detected (9.4%; 9/96). Only 0.7% (2/292) of the isolates were \( tlp11 \) and \( tlp12 \) positive. Remarkably, \( tlp12 \) was significantly less often detected (\( p < 0.05 \)) in bovine isolates, i.e., 11.6% (5/43).

**Tlp13:** \( Tlp13 \) was detected in 38.7% (113/292) of all isolates. It was significantly more often observed in ST5, CC354, ST2192, CC257, ST464, and CC1332 (Table 2, Fig. 4). In the above-named Cs/STs positive for \( tlp4 \) or \( tlp11 \), \( tlp13 \) was significantly less less often detected (9.4%; 9/96). Only 0.7% (2/292) of the isolates were \( tlp11 \) and \( tlp13 \) positive. Remarkably, \( tlp13 \) was significantly less often detected (\( p < 0.05 \)) in bovine isolates, i.e., 11.6% (5/43).
Fig. 3. Phylogenetic tree based on the multiple sequence alignment of tlp2 (cj0144), tlp3 (cj1564), tlp4 (cj0262c), tlp11 (CJJ8425_0287), tlp12 (H730_01610), and tlp13 (H730_01620). Depicted is a neighbor-joining tree without distance corrections (http://www.ebi.ac.uk/Tools/msa/clustalo/). At the end of each branch, the designation of each aligned gene and the corresponding distance value is given. The phylogenetic tree indicates that tlp4, tlp11, tlp12, and tlp13 are more closely related than tlp2 and tlp3. Therefore, it can be assumed that these genes are paralogues, which may have arisen from an ancestral tlp gene.

Fig. 4. Circular depiction of the MLST-based UPGMA tree of all tested 292 C. jejuni ssp. jejuni isolates and the distribution of non-ubiquitous C. jejuni ssp. jejuni chemoreceptor genes. This figure shows a balanced MLST-based UPGMA tree of all tested 292 C. jejuni ssp. jejuni isolates. The innermost circle indicates the isolate source: human isolates = blue, chicken isolates = yellow, bovine isolates = red, turkey isolates = green, ovine isolates = orange, wild bird isolates = pink, riparian isolates = light blue, and canine isolates = laurel green. The superimposed circles represent a particular non-ubiquitous transducer-like protein encoding gene (tlp). These are arranged in numerical order including tlp4 (dark blue), tlp5 (medium blue), tlp7 uninterrupted gene (touquoise), tlp7 two ORF variant (bright green), tlp11 (dark green), tlp12 (yellow), and tlp13 (orange). Colored fields represent a chemoreceptor gene present in a given isolate, and white fields represent its absence (in the case of tlp5, the occurrence of tlp5 pseudogene is indicated by a white field). The outermost circle indicates the clonal complex (CC). Different CCs listed in Table 2 are displayed alternately in light and dark gray. White fields in this circle indicate singletons or STs that are currently not assigned to a CC.
to 31.0% (35/113) of the tlp13 positive isolates, were positive for the tlp13 gene but negative for tlp4, tlp11, or tlp12. Moreover, 14.4% (42/292) of all isolates were positive for both tlp12 and tlp13, but not tlp4 and tlp11. These isolates were especially found in ST257 and ST464. In 9.9% (29/292), the tlp13 gene cooccurs with tlp4, especially in CC45. Only 3.4% (10/292) were positive for both tlp11 and tlp13 (especially ST53 isolates).

Noticeably, 11.3% (33/292) of the isolates were neither positive for tlp4, tlp11, tlp12, nor for tlp13 (especially found in CC353 and CC45), whereas 2.4% (7/292) were positive for three genes of the tlp4–tlp11–tlp12–tlp13 group. None of the isolates was positive for all four genes of the tlp4/11 group.

Transducer-like protein gene tlp5

In addition to the intact tlp5 gene, a much shorter pseudogene that lacks the cytoplasmic signaling domain was demonstrated. Tlp5-PCR primers used in this study were designed to detect the tlp5 gene and the disrupted (pseudo-)gene. All tested isolates were either positive for one of these genes. 56.6% (165/292) of all tested isolates were positive for intact tlp5, and 43.4% (127/292) were positive for the pseudogene. Intact tlp5-positive isolates belonged predominantly to CC21, ST46, ST122, ST2192, ST38, CC22, CC45, CC283, and CC42 (Table 2). Additionally, it was significantly (p < 0.05) associated with bovine isolate origin (Table 2).

Transducer-like protein gene tlp7

Tlp7 was ubiquitous but occurred in a one-protein (58 kDa) and a heterodimeric two-protein variant (25 kDa + 33 kDa) as a result of an interrupting stop codon splitting the ORF into two (Fig. 1C). The two ORF variant was found in 22.9% (67/292) of the isolates, mainly, in isolates belonging to ST21, ST53, CC45, and CC46. Distribution of the tlp7 two ORF variant and tlp11 in the isolate collection reveals a correlative relation. According to our data, 71.6% (48/67) of tlp7m positive isolates were tlp11 positive. Further, 92.3% (48/52) of tlp11 positive isolates were positive for tlp7m. As shown in Fig. 4, most tlp7m and tlp11 positive isolates belong to ST21, ST53, CC45, and CC61.

The cooccurrence of both chemoreceptor genes tlp7m and tlp11 correlated significantly (p < 0.001) with bovine isolate origin. 62.8% (27/43) of the bovine isolates were tested positive for tlp7m and 55.8% (24/43) for tlp11. Accordingly, 51.2% (22/43) of the bovine isolates were tested positive for both tlp7m and tlp11.

In contrast, both chemoreceptor genes were significantly (p < 0.001) less detected in isolates of chicken origin. Only 5.9% (4/68) of the isolates of chicken origin were tested positive for tlp7m 5.9% (4/68) for tlp11, and 2.9% (2/68) contained both tlp7m and tlp11.

Generally, cooccurrence of tlp7m (and therewith tlp11) with tlp4 and tlp12 was rare. Only 6.8% (20/292) of the isolates were tested positive for the cooccurrence of both tlp7m and tlp4. Similarly, only 2.1% (6/292) were tested positive for the cooccurrence of both tlp7m and tlp12.

Discussion

Data obtained in our study revealed that most C. jejuni ssp. jejuni chemoreceptors were ubiquitous regardless of host, source of isolation, and clonal complex. This finding expands on a similar observation that was made in a previous exploratory study by Day and coworkers, which investigated the occurrence of group A receptor genes in 13 human isolates, seven chicken isolates, and 13 laboratory-maintained reference strains [10]. Day and colleagues revealed the following: ubiquitous occurrence of tlp1 (ccaA); a higher but non-ubiquitous occurrence of tlp2, tlp3 (ccmL), tlp4, tlp7, and tlp10; and the rare occurrence of tlp11. However, they did not consider association to factors such as MLST CC/ST; other hosts beyond chicken and human; group B and C receptor genes; genetic variants of tlp7, tlp4, and tlp11, tlp5 pseudogene; and mutual cooccurrence of the receptors. Also, the sample size of the isolate collection that was tested was too low for authoritatively deducing conclusions about distribution of C. jejuni ssp. jejuni chemoreceptors.

Data obtained in this study reveals an absolute ubiquitous occurrence of tlp1 (ccaA) and tlp3 (ccmL) and a near absolute ubiquitous occurrence of tlp2 (present in 97.3% of all tested isolates) and tlp10 (present in 97.9% of all tested isolates). The unexpected existence of near absolute tlp2 and tlp10 can arise from two scenarios. First, it may be attributed to the primers used in this study. The primers were designed to bind the consensus sequences of the genome-sequenced strains of the NCBI database. Therefore, it is likely that the primer binding sites of tlp2 and tlp10 negative tested isolates have undergone mutations, which may yield a negative test result though the gene is present. Second, the difference could be due to real absence of the genes in the negative tested isolates; hence, further studies should be carried out to understand if and how the functions of these possibly missing chemoreceptors are compensated, e.g., during host colonization.

Interestingly, our results show that the genomic region neighboring the zupT gene was the most variable region regarding the chemoreceptor genes. As shown in Fig. 1A, we found four different chemoreceptor genes in this region: tlp4, tlp11, tlp12, and tlp13. Because of their level of divergence and occurrence in the genomic region neighboring the zupT gene, we assume that these genes are paralogues, which may have arisen from an ancestral tlp gene as a response to niche adaptation. It should be noted that tlp12 and tlp13 have not yet been described outside of this study and their function remains uncharacterized. However, there were some isolates which were negative for all four of these receptor genes, indicating that they may not be crucial for the survival of C. jejuni ssp. jejuni.
Otherwise, we detected isolate groups that were positive for single genes or a combination of two or three of these four chemoreceptors.

The gene encoding *tlp4* was detected in only 33.6% of all isolates and, hence, non-ubiquitous. A clear reason for limited availability cannot be deduced because the function and chemoeffectors of *tlp4* remain unresolved. Importantly, *tlp4*-positive isolates were limited to eight clonal complexes vis-à-vis CC22, CC42, CC45, CC283 CC48, CC49, CC206, and CC52.

Similarly, *tlp11* was detected in a minority, i.e., 17.8%, of the tested isolates. The *tlp11*-positive isolates were predominantly found in three clonal complexes: CC21 (ST21 and ST33), CC48 (ST38), and CC61, which have been found to be the major cause of campylobacteriosis in man [14]. Interestingly, 55.8% of these isolates originate from the bovine host; hence, *tlp11* could be a marker of bovine-associated strains. Another interesting observation which we found was the cooccurrence of *tlp11* and the two ORF variant of *tlp7*. Data analysis showed that 92.3% (48/52) of all *tlp11* positive isolates were positive for the two ORF variant of *tlp7* gene. The biological significance of this cooccurrence remains unclear since function and chemoeffectors of Tlp11 are unknown.

The new described chemoreceptor gene *tlp12* was present in 29.5% of all tested isolates. In particular, isolates of ST50 were positive for *tlp12*. Due to the significant differences in the amino acid sequence of the sensory domain, it seems likely that there are functional differences of Tlp12 compared to Tlp4. Further studies are required to address this issue and to identify specific chemoeffectors.

The second new representative in the group A chemoreceptor group is *tlp13* that was present in 38.7% of the isolates. The homology between *tlp11* and *tlp13* was much higher than between *tlp4* and *tlp12*; functional differences due to variations in the sensory domain between Tlp11 and Tlp13 cannot be excluded. This question must also be answered by future experiments.

A more global view on the *tlp4*, *tlp11*, *tlp12*, and *tlp13* receptor distribution gives the impression that the individual receptors of this group were mutually exclusive to a certain degree.

*tlp9/cetA*, *aer1*, and *aer2* chemoreceptor genes of group B were ubiquitous in the entire test population. Similarly, group C receptor genes *tlp6* and *tlp8* were also ubiquitous in the surveyed isolates. This observation was attributed to the biological role that chemoreceptors of group B and C jointly play. For example, a recent study that evaluated the energy taxis system of *C. jejuni* ssp. *jejuni* established that *C. jejuni* ssp. *jejuni* is endowed with two energy taxis subsystems, namely, 1) CetABC (CetA = *tlp9*, CetB = *aer2* and CetC = *aer1*) and 2) CetZ = *tlp8* [17]. This energy taxis subsystem controls *C. jejuni* ssp. *jejuni* taxis in a well-coordinated manner, and also, the weakness or failure of one component is complemented by another. The only non-ubiquitous exception in the group C receptors is *tlp5* that occurs as intact gene and as disrupted pseudogene. The occurrence of the disrupted pseudogene in 43.5% of the tested isolates shows that Tlp5 is not crucial for survival of *C. jejuni* ssp. *jejuni* in some ecological niches. However, it is significant that the intact *tlp5* is more common in bovine isolates.

In conclusion, this study has shown that the chemoreceptor genes *tlp1*, *tlp2*, *tlp3*, *tlp6*, *tlp8*, *tlp9/cetA*, *aer1*, *aer2*, and *tlp10* are ubiquitous. *Tlp4*, *tlp11*, *tlp12*, and *tlp13* are non-ubiquitous; their occurrence is mutually exclusive (Fig. 4), and their distribution is related to specific MLST CCs/STs. Similarly, *tlp5* and its pseudogene present a mutually exclusive occurrence and an association to specific isolate groups. The findings of this study complete the picture of the complex chemoreceptor system of *C. jejuni*, which controls its flagellar driven taxis towards niches of favorable growth conditions while competing with the host’s microbiota.

**Funding sources**

The authors’ work was supported by the Deutsche Forschungsgemeinschaft (DFG GR906/13-1) and the Forschungsförderungsprogramm of the Universitätsmedizin Göttingen (UMG), Germany. This publication was funded by the Open Access support program of the Deutsche Forschungsgemeinschaft and the publication fund of the Georg August Universität Göttingen.

**Conflicts of interest**

All authors declare no conflicts of interests.

**Authors’ contributions**

A.E.Z. conceived the study idea, performed all mathematical analysis, and drafted the article. N.L.A.M., A.M.G., and W.O.M. performed bacterial culture, DNA isolation and PCR analysis, and MLST-PCR. R.L. performed DNA sequencing and assisted in drafting the article. U.G. participated in the study design and helped in drafting the article. All authors read, corrected, and approved the article.

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