A SpoT polymorphism correlates with chill stress survival and is prevalent in clinical isolates of Campylobacter jejuni

M. N. Nierop Groot,*1 A. G. de Boer,† W. van Pelt,‡ M. C. van der Hulst-van Arkel,† P. de Leeuw,* H. C. A. Widjaja,§ M. A. Smits,†§ and F. J. van der Wal†

*Wageningen UR Food and Biobased Research, PO Box 17, 6700 AA Wageningen, the Netherlands; †Central Veterinary Institute, Wageningen UR, PO Box 65, 8200 AB, Lelystad, the Netherlands; ‡Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), PO Box 3720 BA, Bilthoven, the Netherlands; and §Livestock Research, Wageningen UR, PO Box 65, 8200 AB, Lelystad, the Netherlands

ABSTRACT Resistance of Campylobacter jejuni to environmental stress is regarded as a risk factor for the transmission of C. jejuni from poultry or poultry products to humans. So far, the mechanisms underlying the capacity of C. jejuni to survive environmental stress conditions are not fully understood. In this study, we searched for polymorphisms in C. jejuni genes, potentially involved in resistance to chill stress. To this end, we assessed 3 groups of C. jejuni isolates (clinical, retail chicken meat, and feces) for survival of experimentally induced chill stress. For each isolate we sequenced 3 genes encoding the C. jejuni sigma factors FliA, RpoD, and RpoN as well as the genes for the transcriptional regulator SpoT and the periplasmic protein HtrA. Data suggest a higher prevalence of a specific polymorphism in spoT in clinical isolates compared with poultry meat or farm isolates. Moreover, this genotype correlated with enhanced survival of chill stress. The observation that the prevalence of this SNP is relatively high in clinical isolates, which most likely have been exposed to multiple forms of stress, suggest that this SNP may be a biomarker for enhanced survival of stress.

Key words: Campylobacter jejuni, chill stress, spot polymorphism, survival, real-time PCR

INTRODUCTION

Campylobacter jejuni is the leading cause of bacterial gastroenteritis in developed countries (Friedman et al., 2000), with an estimated number of 9 million cases per year in the European Union (EFSA, 2011). Campylobacteriosis has been associated with the consumption of raw milk and contaminated water (Olson et al., 2008), but the majority of cases can be attributed to poultry meat (20–30%) and the birds themselves (50–80%; EFSA, 2012). Many interventions to reduce Campylobacter prevalence in the poultry meat production chain have been proposed and tested, such as the use of fly screens in primary production to prevent infection, or treatment of carcasses and meat by irradiation, freezing, and decontamination with hot water or chemicals (Havelaar et al., 2007; Rosenquist et al., 2009; EFSA, 2011). Widespread implementation of such interventions could further assist in reducing Campylobacter on end products.

During broiler processing, bacteria on carcasses are subject to various forms of stress, including exposure to low temperature during carcass chilling (Bolder, 1998; Park, 2002). Even though C. jejuni is extremely sensitive to environmental conditions outside the poultry intestine, several mechanisms to survive such stress conditions are known (Jackson et al., 2009). A substantial reduction in numbers of C. jejuni on carcasses after chilling has been reported, but the effect is not consistent and high numbers of bacteria can still be present on the end product (Lindblad et al., 2006; Allen et al., 2007). Various studies suggest that strain-to-strain variation may play a role in the unpredictable capacity of C. jejuni to survive stress conditions. The genotypic characteristics responsible for this phenotypic diversity may be subtype-specific, and in particular cases have been detected by molecular methods such as fla-typing (Newell et al., 2001) or multi-locus sequence typing (Habib et al., 2010). However, when environmental stress selects for specific subtypes that better withstand suboptimal conditions (Colles et al., 2010; Habib et al., 2010), it is more likely that the genomic
alterations are present in functional genes than that they are part of a typing scheme (Duong and Konkel, 2009). Campylobacter jejuni genes of potential interest are those that code for proteins with a role in stress response. For the present study, a selection of candidate genes was made based on the literature: 3 genes encoding the sigma factors (fliA, rpoD, and rpoN; Parkhill et al., 2000), spoT (Gaynor et al., 2005), and htrA (Brondsted et al., 2005). The fliA gene, encoding sigma28, is required for flagellar biosynthesis and rpoN, encoding sigma54, regulates several functions including motility, protein secretion, and invasion (Hendrixson and DiRita, 2003; Carrillo et al., 2004; Hendrixson and DiRita, 2004). The rpoD gene encodes the primary sigma factor sigma70 (Wösten et al., 1998), with over 650 predicted binding sites within promoter regions of annotated genes (Petersen et al., 2003). SpoT is a global transcriptional regulator involved in the stringent response, which is a bacterial adaptation mechanism that affects survival under several conditions, including stationary phase and high oxygen tension (Gaynor et al., 2005). HtrA is a periplasmic protease/chaperone playing a role in stress tolerance at high temperature (Brondsted et al., 2005; Baek et al., 2011). A role in growth at low temperature has been proposed for HtrA in Escherichia coli (Skorko-Glonek et al., 2007; Skorko-Glonek et al., 2008).

The objective of this study was to identify polymorphisms in a selection of C. jejuni genes involved in stress response (i.e., fliA, rpoD, rpoN, spoT, and htrA) and to investigate whether the presence of these polymorphisms correlates with chill stress survival, origin of isolate, or both. Furthermore, by the use of a real-time PCR assay, we investigated whether selection for chill stress resistance occurs during the slaughtering process.

MATERIALS AND METHODS

Strains and Growth Conditions

The 33 C. jejuni strains used in this study were previously isolated from either cecal samples in the period 1997–1999 (strains F13 to F24; Jacobs-Reitsma et al., 2000), poultry retail products (2000–2001, strains R25 to R36; R. van der Hulst-van Arkel; unpublished), or humans suffering from campylobacteriosis (2002–2003, strains C1 to C12; Doorduyn et al., 2006) and were used for sequencing to identify genetic polymorphisms and the chill stress experiments. In addition, the original clinical NCTC 11168 isolate was used (Gaynor et al., 2004). To test the occurrence of the SpoT alleles in clinical and poultry isolates with the SpoT allelic discrimination assay developed in this study, an additional set of 86 clinical strains previously isolated was obtained (Doorduyn et al., 2006), as well as 452 C. jejuni field isolates originating from a large set of 917 nonspecified Campylobacters. The latter set was isolated on Campylobacter blood-free selective agar plates (CCDA, Oxoid, Badhoevedorp, the Netherlands) from fecal samples (transport crates and cecum) and chicken filets tracked through the abattoir in a parallel study (Nauta et al., 2009).

Routinely, C. jejuni strains were stored in Bacto Heart Infusion (HI) Broth (Becton Dickinson, Breda, the Netherlands) supplemented with 15% glycerol at −80°C. For the chill stress experiments, C. jejuni was inoculated from glycerol stocks on HI plates containing 5% sheep blood (HIS) and incubated under microaerobic conditions created by the CampyGen system (Oxoid) at 37°C.

Chill Stress Assay

Campylobacter jejuni cells, grown on HIS plates for 48 h, were suspended in 5 mL of HI broth, and the optical density at 600 nm was adjusted to 0.1 (corresponding to between 10^7 to 10^8 cfu per mL). The resulting suspension was used to inoculate 2 tubes containing 9 mL of HI broth with 1 mL of C. jejuni cells. These cultures were then incubated statically at 10°C under microaerobic conditions for 15 d to determine survival. Samples were taken at the start of the experiment (i.e., before the transfer to 10°C, and 15 d after incubation at 10°C). For each sample, bacterial cfu were determined by track dilution on HIS plates (Jett et al., 1997). Chill stress survival was expressed as the percentage of cfu after 15 d relative to the number of cfu at the start of the experiment i.e., \[\text{Survival} = \left( \frac{\text{cfu (t = 15)}}{\text{cfu (t = 0)}} \right) \times 100\%\].

Identification of Polymorphisms

To isolate genomic DNA, bacteria were harvested from a plate with one streak of an inoculation loop and subsequently resuspended in 300 μL of 20% Chelex 100 (Bio-Rad Laboratories, Veenendaal, the Netherlands). This suspension was heated at 95°C for 10 min, centrifuged, and the resulting supernatant containing genomic DNA was stored at −20°C until use. Amplification by PCR of the target genes was performed with primer designed using Primer3 (Rozen and Skaletsky, 2000; Table 1). Expand High Fidelity polymerase (Roche, Almere, the Netherlands) was used to amplify (partial) sequences of fliA, htrA, rpoD, and spoT, whereas SuperTaqPlus (Sphaero Q, Gorinchem, the Netherlands) was used to amplify rpoN. Amplified fragments were purified using a silica-based method (Qiagqick PCR purification kit, Qiagen, Venlo, the Netherlands) and sequenced (Sanger sequencing, BaseClear, Leiden, the Netherlands) using the primers shown in Table 1. Sequence data were analyzed using SeqMan II and MegaLign in Lasergene (DNASTAR, Madison, WI).

For fliA and rpoN, the complete genes were sequenced (Sanger sequencing, Baseclear, Leiden, the Netherlands). The rpoD gene was sequenced up to nucleotide 1848; consequently, the last 7 triplets are missing. The spoT gene was partially sequenced starting at nucleotide 898 (position corresponding to the NCTC11168
Typing Based on Amplified Fragment-Length Polymorphism

The 33 C. jejuni strains and NCTC11168 were subjected to genotyping based on amplified fragment-length polymorphisms (AFLP), essentially as described before (Duim et al., 1999). In short, bacteria were harvested from a plate with one streak of an inoculation loop and resuspended in 600 μL of lysis solution (Gen- trava Puregene Kit, Qiagen). Restriction fragments (HindIII and HhaI) of the isolated genomic DNA were used for a selective amplification to generate fluorescently labeled PCR fragments. The resulting amplicons were separated on a 3130 Genetic Analyzer (PoP7 polymer, 36 cm capillary array) using GeneScan 600 LIZ as internal marker (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands).

An AFLP analysis was performed for amplicons ranging from 100 to 500 bp using BioNumerics 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity of patterns was calculated using the Pearson product-moment correlation coefficient. For cluster analysis, the unweighted pair group method using arithmetic averages, and 1% optimization for position tolerance was used. Clusters were defined by calculating the cluster cut-off values in BioNumerics.

Real-Time PCR for Identification of Species and Polymorphisms

To isolate template DNA for PCR, colonies were picked and resuspended in 300 μL of 6% Chelex 100, heated 15 min at 56°C, boiled 8 min, and centrifuged at 14,000 × g for 5 min at room temperature. The supernatant containing genomic DNA was stored at −20°C, and 2 μL was used as template in real-time PCR.

For species identification of 917 Campylobacter field isolates, a published real-time PCR protocol (Best et al., 2003) was used to differentiate between C. jejuni and C. coli. This real-time PCR was performed in a volume of 20 μL with the TaqMan Fast Universal PCR Master Mix on a 7500 Fast Real-Time PCR System (Applied Biosystems) in Fast 7500 run mode, using the standard thermal cycler protocol (20 s at 95°C followed by 40 cycles of 3 s at 95°C/30 s at 60°C). Primers and probes were used with the final concentration of 12 and 4 pmol, respectively.

For identification of spoT genotypes, an allelic discrimination assay based on 2 minor groove binder (MGB) probes with nonfluorescent quencher (NFQ) labeled with 6-carboxyfluorescein (FAM) or 2’-chloro-7’-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) reporter dyes.

Table 1. Oligonucleotides designed for this study

<table>
<thead>
<tr>
<th>Application</th>
<th>Name</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>spoT_fwd4</td>
<td>GAATAGAAGAAGTAGCGGCAAAAG</td>
</tr>
<tr>
<td></td>
<td>spoT_rev4</td>
<td>CTTAATGAAACTCGGCGCTTGAT</td>
</tr>
<tr>
<td></td>
<td>htrA_fwd</td>
<td>AAACCTTATCAATCGTCGAATCATGAAG</td>
</tr>
<tr>
<td></td>
<td>htrA_rev</td>
<td>TTTTAAACGAAAGGAATCC</td>
</tr>
<tr>
<td></td>
<td>fliA_fwd1</td>
<td>GCCATTCGAGAAGAAGGAGAG</td>
</tr>
<tr>
<td></td>
<td>fliA_rev</td>
<td>CTTGTTGAGCGCCCTAAAATCA</td>
</tr>
<tr>
<td></td>
<td>rpoD_fwd</td>
<td>ATGGCGGCGATATTAGTAGGTA</td>
</tr>
<tr>
<td></td>
<td>rpoD_rev1</td>
<td>GCCGAACTTCGTCATGATAGA</td>
</tr>
<tr>
<td></td>
<td>rpoN5_fwd</td>
<td>ACCGATCTAATGTCGTTGA</td>
</tr>
<tr>
<td></td>
<td>rpoN7_rev</td>
<td>GCCCGGCTACCACTGAAGG</td>
</tr>
<tr>
<td>Sequencing</td>
<td>seq_spoT1</td>
<td>ATGCAAAGAGGATGCGTCTGGT</td>
</tr>
<tr>
<td></td>
<td>seq_spoT2</td>
<td>GCCGAAAGGAAGGAGGAG</td>
</tr>
<tr>
<td></td>
<td>seq_htrAF1</td>
<td>AGTCCCGGATATTAGTAGGTA</td>
</tr>
<tr>
<td></td>
<td>seq_htrAF3</td>
<td>CTTGCGGCTCGCTTTGAT</td>
</tr>
<tr>
<td></td>
<td>Fbseqev</td>
<td>GCTATGGGTGTGTCGTTG</td>
</tr>
<tr>
<td></td>
<td>RpDseqfor</td>
<td>TCAAAACATACATTAAACATTAAACCC</td>
</tr>
<tr>
<td></td>
<td>rpd1857R</td>
<td>GAGGGTTGCTCTCTTACCTCCAC</td>
</tr>
<tr>
<td></td>
<td>rpd550f</td>
<td>GATGAACTTGATAGATAGAAG</td>
</tr>
<tr>
<td></td>
<td>rpoN5_fwd</td>
<td>ACCGATCTAATGTCGTTGA</td>
</tr>
<tr>
<td></td>
<td>rpoN7_rev</td>
<td>GCCCGGCTACCACTGAAGG</td>
</tr>
<tr>
<td>Allelic discrimination</td>
<td>forw_spoT</td>
<td>GGAGTTGATAAAAAACAGAATTGAAACTTG</td>
</tr>
<tr>
<td></td>
<td>rev_spoT</td>
<td>TCTTGGCGCATTTTAAAGACCA</td>
</tr>
<tr>
<td></td>
<td>probe_spoT_C2</td>
<td>FAM-CGCTCTGCTAATTT-NFQ</td>
</tr>
<tr>
<td></td>
<td>probe_spoT_T2</td>
<td>VIC-CGCTCTGCTAATTT-NFQ</td>
</tr>
</tbody>
</table>

1Also used for sequencing.
2Minor groove binder probes with nonfluorescent quencher (NFQ) labeled with 6-carboxyfluorescein (FAM) or 2’-chloro-7’-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) reporter dyes.
the TaqMan Genotyping Master Mix (Applied Biosystems) was used, running the 7500 Fast Real-Time PCR System in 9600 emulation mode with the following protocol: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C/1 min at 60°C. The allelic discrimination assay for spoT was validated with the set of 33 strains (sequenced for spoT), and subsequently applied to 452 C. jejuni field strains.

**Statistical Analyses**

To investigate the biological relevance of the detected polymorphisms, experimental data were compared with the isolation source of strains and translated sequence data in statistical analyses. The correlation between chill stress survival and either the isolation source of strains or translated sequences, were analyzed for statistical significance using 1-way ANOVA and Student’s t-test, respectively. As an input for statistical analysis, the percentages of surviving cells as depicted in Figure 1 were used. All statistical values were considered significant when \( P \leq 0.05 \). All analyses were performed using the SPSS 15.0 software package (SPSS Inc., Chicago, IL).

**RESULTS**

**Identification of Polymorphisms and Correlation with the Origin of Strains**

Of 33 C. jejuni strains previously isolated from cecal samples, poultry retail products, or humans suffering from campylobacteriosis, the 5 stress-related genes were fully (fliA and rpoN), or partially (rpoD, spoT, and htrA) sequenced. The resulting sequences and the published sequence for NCTC11168 (Parkhill et al., 2000) were analyzed for polymorphisms that result in amino acid changes in translated sequences (Table 2), provided these were shared by 6 or more strains. For only 5 strains a polymorphism in FliA (amino acid residue 123) was detected; therefore, FliA was excluded for further analysis. All other translated sequences showed

![Figure 1](https://academic.oup.com/ps/article-abstract/93/11/2900/1575326). Survival of clinical, poultry, and retail-derived Campylobacter jejuni isolates under chill stress. Chill stress survival is expressed as the percentage of cells that survived after 15 d of static incubation at 10°C under microaerobic conditions. Percentage survival of chill stress is the average of duplicate experiments. Individual measurements did not vary by more than 9% from the mean (with the exception of strain R32; 12%). ND = not determined.

**Sequence Files**

Sequences of DNA have been assigned the following accession numbers in the GenBank database: KJ587863 to KJ587895 (fliA), KJ587896 to KJ587928 (htrA), KJ587962 to KJ587994 (rpoD), KJ587995 to KJ588027 (rpoN), and KJ588028 to KJ588060 (spoT).
2 or more amino acid polymorphisms that were shared by 6 or more strains (Table 2). For RpoN, with the exception of strain C12, the polymorphisms at amino acid position 101 and 122 were found to be linked and were therefore analyzed as one entity [RpoN-101Asn122Tyr (N/Y) or RpoN-101Ser122His (S/H)].

For SpoT, the amino acid at position 540 of the protein was found to be either a valine (Val540) or an alanine (Ala540), whereas the last residue of the protein (position 731) could be either a serine (Ser731) or a glycine (Gly731). The 9 analyzed isolates from poultry farms (strain F13 to F24; see Table 2) contained exclusively alanine and glycine at positions 540 and 731, respectively, which is a statistically relevant correlation between the Ala540/Gly731 genotype and poultry farms as source of isolation (binominal test, \( P = 0.004 \)).

For RpoN, a valine residue was present at position 70 (Val70) in all 13 clinical isolates (NCTC1168 and C1 to C12), and the presence of this genotype correlated with the source of isolation (binominal test, \( P < 0.001 \)). Retail and farm isolates carried either a valine or isoleucine at this position, but the distribution was not different between the 2 groups (binominal test, \( P > 0.05 \)). No statistically significant correlation with the origin of strains could be demonstrated for the polymorphisms at position 101/122 in RpoN or for any of the polymorphisms found in RpoD and HtrA.

### Polymorphisms and Correlation with Survival of C. jejuni Under Chill Stress

In chill stress experiments with 33 C. jejuni isolates, a high diversity in the survival capacity was observed (Figure 1). The decline in viable counts after 15 d ranged from 0.17 to 1.72 log cfu/mL (corresponding to 13% up to 88% reduction in viable counts), indicating that some strains survive exposure to low temperature moderately well. The capacity of the strains to survive...
chill stress did not correlate with their origin (1-way ANOVA, $P > 0.05$).

The sequence results were analyzed for correlations between the observed polymorphisms and the capacity of the strains to survive chill stress. A statistically significant positive correlation with chill stress survival was found for 2 polymorphisms [Figure 2; i.e., RpoN-70Val and SpoT-540Val (Student’s $t$-test $P = 0.003$ and $P = 0.022$, respectively)].

**AFLP Genotyping**

To examine the genetic relationship of *C. jejuni* strains carrying identical polymorphisms, strains used in the chill stress experiments were subjected to AFLP genotyping (Figure 3). In the resulting phylogenetic tree, the majority of strains were divided into 2 distinct clusters (I and II), whereas 2 strains did not belong to any cluster. The AFLP analysis showed that isolates from the 3 different sources were not grouped according origin, but were scattered throughout the phylogenetic tree, illustrating that (genetic) similarity of strains was not dictated by their source of isolation.

When the distribution of SpoT-540Val and RpoN-70Val in the phylogenetic tree is considered, it can be seen that the 12 SpoT-540Val strains (correlating with chill stress survival) were all positioned in a cluster of 24 strains (cluster I; Figure 3). Within cluster I, the genetic relationship between the SpoT-540Val strains varied from almost identical (98%; strains C4 and C6) or highly related (95%; strains C9 and C10) to only 54% (i.e., where the group containing strain C4 and C6 separates from the group with strain C9 and C10; Figure 3). The large majority of the RpoN-70Val strains belong to cluster I, with the exception of strain C8 and C7. Summarizing, the distribution of SpoT-540Val and RpoN-70Val strains in a phylogenetic tree showed that the presence of these SNP is not dictated by an overall genetic similarity of strains.

**Occurrence of SpoT Alleles in Clinical and Poultry Isolates**

The prevalence of the SpoT-540Val genotype appeared relatively high in clinical isolates compared with isolates of other origins (Table 2), but the number of isolates did not allow evaluation of statistical relevance. Therefore, a larger set of 86 clinical *C. jejuni* isolates (Doorduyn et al., 2006) was tested for the presence of the SpoT-540Val genotype and the prevalence of this genotype was compared with the prevalence in poultry isolates. To this end, DNA from the respective *C. jejuni* isolates was subjected to an allelic discrimination assay using a specific Taqman probe for each genotype (see Materials and Methods). Among the 86 clinical isolates, 32 (37%) carried the SpoT-540Val genotype. In parallel, 452 *C. jejuni* poultry isolates were tested using the same assay. These isolates were part of a set of 917 nonspecified *Campylobacter* isolates obtained from fecal samples and chicken files of broilers tracked through the slaughter process (Nauta et al., 2009). The species of these isolates were first identified with a *C. jejuni/C. coli* discriminative PCR. The resulting 452 *C. jejuni* isolates originated from 6 out of the 8 flocks investigated. Two flocks encompassed *C. coli* only; 16 isolates were not typeable with the *C. jejuni/C. coli* PCR (Table 3). The identified *C. jejuni* isolates were tested in the spoT allelic discrimination assay. A similar ap-
Approach for \textit{rpoN} SNP performed as a pilot experiment indicated that all strains contained identical sequences. Of the 452 poultry isolates, 59 (13\%) carried the SpoT-540Val genotype. The majority of these isolates (389; 86\%) contained the SpoT-540Ala genotype; 4 \textit{C. jejuni} strains could not be typed with the allelic discrimination assay and were excluded from further analysis. The prevalence of the SpoT-540Val genotype is considerably higher in clinical isolates (37\%) compared with poultry isolates (13\%). Remarkably, a mixed population of both SpoT-540 genotypes was never encountered in a single flock.

Table 3. Prevalence of SpoT genotypes in \textit{Campylobacter jejuni} isolated from fecal samples and chicken filets of 8 different flocks\textsuperscript{1}

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
<th>Flock number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>\textit{Campylobacter coli} (n = 449\textsuperscript{a})</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>\textit{C. jejuni} (n = 452\textsuperscript{2})</td>
<td>101</td>
</tr>
<tr>
<td>SpoT genotyping\textsuperscript{3}</td>
<td>Transport crates</td>
<td>— \textsuperscript{4}</td>
</tr>
<tr>
<td></td>
<td>Cecum</td>
<td>— \textsuperscript{4}</td>
</tr>
<tr>
<td></td>
<td>Filet</td>
<td>101 A</td>
</tr>
<tr>
<td>Prevalence of SpoT-540Val (%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{1}V (valine) refers to the SpoT-540Val genotype (T at nucleotide 1619); A (alanine) refers to the SpoT-540Ala genotype (C at nucleotide 1619).
\textsuperscript{2}Of the 917 \textit{Campylobacter} isolates, 16 were not typeable with the used \textit{C. jejuni}/\textit{C. coli} PCR.
\textsuperscript{3}Only performed for \textit{C. jejuni}.
\textsuperscript{4}Not sampled.
\textsuperscript{5}Not typeable with the allelic discrimination assay.

Figure 3. A phylogenetic tree based on amplified fragment-length polymorphism patterns showing the genetic relationship of the \textit{Campylobacter jejuni} strains used for chill stress experiments. The 2 clusters identified are indicated with solid black lines and labeled I and II, respectively. The genotypes related to enhanced chill stress survival are indicated.
DISCUSSION

In this study, we provide evidence that the prevalence of a specific polymorphism in \textit{spoT} is significantly higher in clinical isolates compared with poultry meat or farm isolates and that this \textit{spoT} genotype is significantly correlated with enhanced survival of chill stress. These results point to a potential role of this \textit{spoT} polymorphism at nucleotide position 540 in survival of chill stress and points to a possible role in selection for enhanced resistance to chill stress, which may play a role in the transmission of \textit{C. jejuni} to humans.

For this study, 3 groups of strains were used as it was hypothesized that these groups may have been exposed to different levels of environmental stress, including chill stress. Fecal isolates, obtained from ceca or transport crates, are likely to represent the intestinal microflora of chickens. Isolates from poultry meat products have undergone various forms of stress in the slaughterhouse, including temperature changes. Clinical isolates are likely to have encountered various forms of stress before infecting a human. Both clinical isolates and isolates from poultry meat may be enriched for more stress-resistant genotypes compared with isolates from fecal samples.

The data in the present study reveal that in \textit{C. jejuni} polymorphic sites exist in \textit{spoT}, \textit{rpoD}, \textit{rpoN}, \textit{htrA}, and \textit{flhA} and that identical nucleotide substitutions are shared by strains that have otherwise limited genetic similarity as observed by AFLP analysis. The prevalence of Spi-T-540Val, the genotype that is associated with chill stress survival, is considerably higher in clinical isolates (37%) than in poultry isolates (13%). Remarkably, the prevalence of Spi-T genotypes in the 6 flocks evaluated showed that mixed populations of genotypes (Ala540 and Val540) did not occur. This may suggest that each flock has undergone only one infection event. The flocks that were tracked through the production process (feces/filets) never carried both \textit{C. jejuni} genotypes simultaneously; thus, the hypothesis that one genotype survives the abattoir better could not be substantiated with this particular set of field samples.

During slaughter, bacteria in and on poultry are subjected to temperatures as low as 12°C (Bolder, 1998), which is a form of stress because \textit{Campylobacter} does not grow below 30°C (Park, 2002). Indeed, a review of literature on the effects of different processing steps in abattoirs discloses that chilling consistently decreases the numbers of \textit{Campylobacter} on carcasses (Guerin et al., 2010), indicating a reduction in survival due to chill stress.

To mimic circumstances of chill stress and the induction of stress response in \textit{Campylobacter}, strains were incubated in liquid cultures at low temperature. The experiments demonstrated a clear but diverse capacity of \textit{C. jejuni} strains to survive chill stress. Because the effect of low temperature on \textit{Campylobacter} viability is more severe at 10°C than at 5°C (Birk et al., 2004), the former temperature was chosen to give an optimal readout for survival. A second reason to select 10°C was that after evisceration carcasses are rinsed with tap water of approximately 10 to 12°C (Bolder, 1998). An earlier study reported a tendency of clinical isolates to be more tolerant to exposure to low temperature (4°C) than poultry-derived strains (Chan et al., 2001), but in the experiments presented here no clear relation was found between source of isolation and survival. The differences in incubation temperature between these studies may have obscured such a correlation, because \textit{C. jejuni} survives low temperature for longer at 5°C than at 10°C (Birk et al., 2004), at least in chicken juice.

For 2 polymorphisms, the results suggested a correlation with chill stress. A valine residue at position 540 of SpoT (Spi-T-540Val) was associated with chill stress survival. Because the amino acid at position 540 is not part of any of the predicted functional domains (Finn et al., 2008), the biological consequence of this sequence variation for Spi-T is not known. Nonetheless, the prevalence of the Spi-T-540Val genotype was higher in clinical isolates than in poultry isolates. A valine at position 70 of RpoN, instead of isoleucine, correlated with survival of chill stress. As for Spi-T, the mechanism by which the observed polymorphism affects the phenotype remains elusive because position 70 of RpoN is not part of the predicted functional domains (Finn et al., 2008). In the larger screening of 6 flocks only the RpoN-70Val variant was present, which calls into question the effect of RpoN polymorphism on chill stress survival and certainly eliminates RpoN as target for differentiation in relation to stress survival.

Several studies on the relation between genotype and survival of \textit{C. jejuni} have focused on molecular typing methods, such as AFLP, pulsed-field gel electrophoresis, multi-locus sequence typing, and FlaA typing (Newell et al., 2001; VanWorth et al., 2006; Coote et al., 2007; Peyrat et al., 2008). In the present study a different approach was followed, where the relevance of SNP for enhanced stress survival was assessed. Spi-T-540Val was found as a polymorphism potentially relevant for chill stress survival. Its occurrence did not depend on the position of the carrier strain in a phylogenetic tree constructed by using AFLP. An accompanying allelic discrimination assay was developed, but a potential role as biomarker for high-risk flocks, containing \textit{C. jejuni} with an increased capability to survive chill stress, could not be substantiated with the field strains used because all isolates within each flock carried the same genotype. Use of this assay would need further investigation with isolates taken before and after the slaughter process from a larger number of flocks carrying \textit{C. jejuni} (of both genotypes) to examine if the distribution of these polymorphisms alters during poultry processing. The current results point to a potential role of the Spi-T-540Val SNP in survival of chill stress by \textit{C. jejuni}. Although further research is needed to confirm this, the observation that the prevalence of this SNP is relatively high in clinical isolates, which most likely
have been exposed to multiple forms of stress, suggests that this SNP may be a biomarker for enhanced survival of stress.

ACKNOWLEDGMENTS

The authors thank Eric Boer (Wageningen UR Food and Biobased Research, Wageningen, the Netherlands) for assistance in the statistical analysis; Frans Putirulan, Nico Bolder, Wilma Jacobs-Reitsma, Linda van der Graaf-van Bloois, and Miriam Koene (Central Veterinary Institute, Wageningen UR, Lelystad, the Netherlands), and Mariette Helmond (WUR Food and Biobased Research, Wageningen, the Netherlands) for poultry strains and technical assistance; and Marc Wösthen and Jaap Wagenaar (Facility of Veterinary Medicine, Utrecht University, the Netherlands) for helpful discussions. The research was funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation in the strategic research program Food Safety.

REFERENCES


EFSA. 2011. Scientific opinion on Campylobacter in broiler meat production: Control options and performance objectives and/or targets at different stages of the food chain. EFSA J. 9:2105.


