Whole genome sequencing reveals extended natural transformation in *Campylobacter* impacting diagnostics and the pathogens adaptive potential

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*Campylobacter* is the major bacterial agent of human gastroenteritis worldwide and represents a crucial global public health burden. Species differentiation of *C. jejuni* and *C. coli* and phylogenetic analysis is challenged by inter-species horizontal gene transfer. Routine real-time PCR on more than 4000 *C. jejuni* and *C. coli* field strains identified isolates with ambiguous PCR results for species differentiation, in particular, from the isolation source eggs. K-mer analysis of whole genome sequencing data indicated the presence of *C. coli* hybrid strains with huge amounts of *C. jejuni* introgression. Recombination events were distributed over the whole chromosome. MLST typing was impaired, since *C. jejuni* sequences were also found in six of the seven housekeeping genes. cgMLST suggested that the strains were phylogenetically unrelated. Intriguingly, the strains shared a stress response set of *C. jejuni* variant genes, with proposed roles in oxidative, osmotic and general stress defence, chromosome maintenance and repair, membrane transport, cell wall and capsular biosynthesis and chemotaxis. The results have practical impact on routine typing and on the understanding of the functional adaption to harsh environments, enabling successful spreading and persistence of *Campylobacter*.

Since 2005, *Campylobacter* is the major zoonotic agent in the European Union, causing 250,161 confirmed campylobacteriosis cases in 2017. Around one third of the cases can be directly attributed to handling, preparation and consumption of broiler meat. Measures for *Campylobacter* reduction focus on virulence mechanisms and persistence factors, enabling the pathogen to successfully circulate within the food chain.

Typing of *Campylobacter* by species differentiation methods and by multi-locus sequence typing (MLST) has become key tools for diagnostics and source attribution. Specific gene targets have proven stable and were, therefore, chosen for this purpose. Two of commonly used species differentiation markers are mapA, a fitness factor in chicken colonization and ceuE playing a role in iron acquisition. For MLST, central enzymatic functions, which are conserved in the genome were defined and are commonly used for phylogenetic analysis.

It was shown that high level of interspecies transfer of genetic material can occur between *C. jejuni* and *C. coli*. Adaptation to hosts can modulate the gene pool and allele variants and was suggested to be of more relevance than geographical location.

Here we identified extensive interspecies gene transfer from *C. jejuni* to *C. coli*, impairing species differentiation and MLST analysis. Whole genome sequencing revealed that these hybrid strains shared *C. jejuni* gene variants, involved in stress response. Since the hybrids had predominantly been isolated from egg shells, we suggest that gene variations due to *C. jejuni* sequence introgression might have been a consequence of selection of survivors in a harsh environment.

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sequences but maintained cecum. These isolates apparently harboured an extended amount of *C. jejuni* exclusively from eggs, two others with ambiguous PCR result and were omitted from the table.

Further 1860 isolates from other sources did not result in an amplification at all (none, 3/31). A subsequent gel-based multiplex PCR, targeting the *ceuE* and *hipO* gene of *C. coli* with similar Ct values (*Cj/Cc mix, 28/31*) or by no amplification of both specific targets for *C. jejuni* and *C. coli* with ambiguous PCR results were defined by either amplification of both specific targets for *C. jejuni* and *C. coli* with similar Ct values (*Cj/Cc mix, 28/31*) or by no amplification at all (none, 3/31). A subsequent gel-based multiplex PCR, targeting the *hipO* gene of *C. jejuni* and the *glyA* gene of *C. coli*, indicated that all of these isolates belonged to the species *C. coli*.

The 31 strains with ambiguous real-time PCR signal had been isolated from poultry meat, turkey cecum or skin and eggs. The Federal State Laboratories either did not report any species (9/31), correctly identified *C. coli* (20/31) or in one case falsely reported *C. jejuni* (1/31). Surprisingly, when we compared the number of strains with ambiguous real-time PCR result with the total number of isolates analysed during the same time of collection, proportionally the highest percentage of strains with ambiguous qPCR results was derived from eggs (Table 1), although the total number of analysed eggs was low.

We characterized these isolates by whole genome sequence analysis. In addition, further *C. coli* isolates from previous years (*n* = 26, 2009–2015) were included. As the prevalence of strains with ambiguous qPCR was highest from eggs, we included those from eggs and additional isolates from laying hens, chicken meat and pig feces.

We performed a k-mer based analysis using the KmerFinder 3.1 (CGE, DTU, Denmark). For a typical *C. coli* it is expected that the k-mers match to different *C. coli* reference genomes. However, as expected from the real-time PCR results, the k-mers of the input *C. coli* sequences with ambiguous PCR results also exhibited *C. jejuni* genomic content. Different percentages of k-mers matching to *C. jejuni* reference genomes were observed, ranging from 0 (undetectable) to 15.5% (Fig. 1). Also correctly PCR-identified *C. coli* exhibited various amounts of *C. jejuni* content (Fig. 1, red squares). From the latter, those with the highest *C. jejuni* content (>10%) were exclusively from eggs, two others with *C. jejuni* content between 4.4–5.3% were from chicken meat and turkey cecum. These isolates apparently harboured an extended amount of *C. jejuni* sequences but maintained *C. coli* sequences at *mapA* and *ceuE*. In total, 29 *C. coli* isolates with a k-mer percentage of more than 10% *C. jejuni* content (Fig. 1, categorized *C. coli* with high *C. jejuni* content, named “hybrids”) were identified. “Half hybrid” strains were defined as harbouring <10% *C. jejuni* sequences but displaying an ambiguous qPCR result. According to MALDI-TOF analysis, all hybrid and half hybrid strains belonged to *C. coli* with a score of ≥ 2.000, which was

### Table 1. Distribution of *Campylobacter* isolation sources of isolates with ambiguous PCR results. *Cj*, *C. jejuni*; *Cc*, *C. coli*. *Isolates were obtained in the years 2016–2018; isolation source are displayed, from which isolates with ambiguous PCR results were obtained. Further 1860 isolates from other sources did not result in an ambiguous PCR result and were omitted from the table.

| Isolation source* | # total isolates investigated (*Cj* and *Cc*) | # isolates with ambiguous PCR | % of isolates with ambiguous PCR relative to *Cj* and *Cc* | # total *Cc* isolates | % of isolates with ambiguous PCR relative to *Cc* |
|-------------------|---------------------------------------------|--------------------------------|-------------------------------------------------|---------------------|-------------------------------------------------|
| eggs              | 39                                          | 5                              | 12.8                                           | 11                  | 45.5                                           |
| duck meat         | 63                                          | 1                              | 1.6                                            | 20                  | 5                                              |
| chicken meat      | 1245                                        | 7                              | 0.6                                            | 281                 | 2.5                                            |
| turkey meat       | 351                                         | 5                              | 1.4                                            | 95                  | 5.3                                            |
| turkey cecum/skin | 777                                         | 13                             | 1.7                                            | 414                 | 3.1                                            |

**Figure 1.** Percentage of *C. jejuni* genome content in *C. coli* isolates detected by k-mer analysis, categorized by qPCR result using *mapA*/*ceuE* as targets. *Cj/*Cc mix, both targets for *C. jejuni* and *C. coli* were amplified; *C. coli*, *C. coli* was correctly detected; false *jejuni*, *C. coli* was falsely detected as *C. jejuni*; none, none of the targets was amplified.

**Results**

Isolates had been collected from food and animal matrices during routine sampling or zoonosis monitoring by the Federal State Laboratories between January 2016 and December 2018 according to ISO 10272. The isolates were analysed by real-time PCR in the German National Reference Laboratory for *Campylobacter*. The target for *C. jejuni* is a fragment of *mapA*, coding for an outer membrane protein. The *C. coli* specific target *ceuE* encodes the enterochelin uptake substrate-binding protein, involved in iron acquisition. Out of 4,335 *C. jejuni* and *C. coli* isolates, 31 delivered ambiguous PCR results (0.72%). Ambiguous PCR results were defined by either amplification of both specific targets for *C. jejuni* and *C. coli* with similar Ct values (*Cj/Cc mix, 28/31*) or by no amplification at all (none, 3/31). A subsequent gel-based multiplex PCR, targeting the *hipO* gene of *C. jejuni* and the *glyA* gene of *C. coli*, indicated that all of these isolates belonged to the species *C. coli*.

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previously validated as indicative for correct species identification of Campylobacter spp.\textsuperscript{16–18}. In order to clarify genetic relationship of “hybrid” and “half hybrid” strains within the Campylobacter population we performed an average nucleotide identity (ANI) analysis using the tool FastANI\textsuperscript{19}. Population and ANI studies of recent years showed that organisms sharing at least 95% ANI among themselves are defined to be of the same species\textsuperscript{20,21}. Results of the ANI analysis were visualized in Fig. 2A and reveal that “hybrid” strains form a separate cluster but still share ~97% ANI with C. coli. Half hybrid isolates are spread across the C. coli population. (B) phylogeny of the Campylobacter core genomes based on Roary analysis. The branch length between C. coli, including hybrid and half hybrid strains and C. jejuni has been shortened for better visualization.

**Figure 2.** Relatedness of C. jejuni (turquoise), C. coli (pink), hybrid strains (purple) and half hybrid strains (mustard) according to ANI (A) and core genome analysis using Roary (B). (A) heatmap visualization of ANI values across all isolates. Hybrid strains form a separate cluster but still share ~97% ANI with C. coli. Half hybrid isolates are spread across the C. coli population. (B) phylogeny of the Campylobacter core genomes based on Roary analysis. The branch length between C. coli, including hybrid and half hybrid strains and C. jejuni has been shortened for better visualization.
We further asked whether the hybrid strains were of clonal origin and disseminated upon the/multiple horizonal *C. jejuni* gene transfer events had occurred. For this purpose, the MLST type was analysed based on the seven housekeeping genes (8, PubMLST.org). As visualized in Fig. 3 the isolates belonged to different sequence types, from which only two could be attributed to any known clonal complex (both ST-1150 complex), implying that none of the isolates belonged to the CC-828 complex, which is the most abundant clonal complex of *C. coli*. More specific sequence analysis showed that these housekeeping genes were also affected by *C. jejuni* sequence introgression (see below). Thus, MLST typing apparently has its limitations in those Campylobacter with substantial horizontal gene transfer activity.

Analysis via Ridom Seqsphere+ software using allele-based cgMLST22 of 1343 genes indicated that the 29 *C. coli* hybrids were in majority unrelated. The median number of allele differences between the nearest neighbour was 343 (¼ of all analysed genes, Fig. 3). Thus, most of the strains displayed a phylogenetically diverse origin. This was substantiated by different isolation dates, ranging from 2009 to 2018 and different isolation locations from six federal states of Germany. Taken together, these data indicate that horizontal gene transfer from *C. jejuni* independently occurred in the *C. coli* hybrids.

**Where did *C. jejuni* introgression take place?** An in-house k-mer analysis was performed. The *C. coli* sequences with >10% of *C. jejuni* introgression were split into 16-mers or 31-mers, which were compared against a set of 95 complete *C. coli* genomes obtained from the NCBI database and 18 further *C. coli* strains without PCR ambiguity, for which whole genome sequencing was performed in the laboratory (Supplementary Table S1). We excluded three genomes from the NCBI database, which were apparently *C. jejuni* strains, falsely annotated as *C. coli* (GCA_001292485.1, GCA_001292205.1 and GCA_001292265.1). K-mers with direct matching were subtracted from the k-mer pool and the residual k-mers were compared against 152 complete *C. jejuni* genomes from the NCBI database and 3 additional sequences from the BfR strain collection (Supplementary Table S1). Those k-mers, which matched sequences present in at least 95% of the *C. jejuni* and maximal 5% of the *C. coli* genomes, were mapped against the reference *C. jejuni* strain NCTC 11168. Example k-mer mappings of a “hybrid” (>10% *C. jejuni* introgression) and a “half hybrid” strain (<10% *C. jejuni* introgression but with ambiguous qPCR result) against the NCTC 11168 reference sequence are depicted in Fig. 4. The recombination events of *C. jejuni* sequences in *C. coli* appear to be distributed all over the chromosome.
An analysis of the recombination size was performed with a minimal assumed recombination event size of 100 bp and various maximal gaps between events of 100–500 bp between k-mer matchings. As expected, the recombination size was increased with increasing size of maximal gaps. However, the overall median size of recombination events ranged between 297 and 512 bp and the maximal event was between 11.4 and 11.8 kb detected in strain BfR-CA-08318. This might hint at the potential of \textit{C. coli} to incorporate large regions of more than 10 kb within one recombination event but that most of them were below 1 kb. The number of detected recombination events per strain ranged in median between 218 and 230 events. Note that our analysis might underestimate the number and the size due to the fact that only k-mers with exact and unique matches to the reference \textit{C. jejuni} NCTC 11168 and to 95% of all \textit{C. jejuni} strains included in the study were considered. A cumulative plot of recombination events (from gap size analysis of maximal 100 bp) in each strain sorted by the chromosomal location of the reference sequence is depicted in Fig. 4, indicating that common recombination events occurred in multiple \textit{C. coli} hybrid strains and that the overall \textit{C. jejuni} content in these strains was similar as analysed by k-mer analysis via the KmerFinder 3.1 of CGE.

What has happened at \textit{mapA} and \textit{ceuE} loci? In order to find out, why qPCR results led to ambiguous and even false results, k-mer mapping of these isolates to \textit{C. jejuni} NCTC 11168 was visualized with Geneious Prime. The k-mer analysis revealed different patterns of \textit{C. jejuni} introgression in \textit{C. coli} \textit{mapA} and \textit{ceuE} genes and their gene context (Fig. 5). In \textit{mapA} either several small or large recombination events, covering also the adjacent 3′ genes Cj1028c and \textit{gyrA} and the 5′ upstream located \textit{lepA}, were identified. In the false positive \textit{C. jejuni} isolates, the \textit{ceuE} locus exhibited a mosaic allele structure with the 5′ start of \textit{ceuE} displaying a typical \textit{C. coli} sequence and the 3′ end matching \textit{C. jejuni} sequences. In one strain with no amplification of any of both targets (“none”), the complete \textit{ceuE}, including adjacent \textit{ceuD} and \textit{ceuC} and 3′ downstream located tRNA and Cj1356c
Assuming that sequence introgression into C. coli C. jejuni Campylobacter homologues, summarized in functional categories (Supplementary Table S2).

Which genes/locations were exchanged by C. jejuni sequences? Considering the typing results with some genes overrepresented for C. jejuni introgression (e.g. cpn60 or asnA) and the observations of distinct recombination regions, the question arises, whether C. jejuni sequences were exchanged by C. jejuni k-mer match, which was not significant.

were exchanged by C. jejuni sequences. Thus, although introgression of C. jejuni sequences into C. coli mapA locus was more frequently observed, the ceuE locus can also partially or fully be introgressed by C. jejuni DNA in contrast to previous observations.

For a further analysis we looked at the annealing sites of the oligos used for mapA and ceuE amplification. A multiple alignment of the genes, displaying C. jejuni sequence content as identified by k-mer analysis, was done using MegAlign Pro 14 (Supplementary Fig. S1). As expected from the real-time PCR result, those strains from which both targets mapA and ceuE were amplified, exhibited a sequence at mapA, which is typical for C. jejuni but maintained a typical C. coli ceuE allele. Those four strains with no real-time PCR signal at all ("none" strains), had a C. coli allele at mapA but either a complete C. jejuni sequence of ceuE (BfR-CA-15489) or a mosaic gene as indicated in Fig. 5. Besides, this analysis corroborated the idea that independent recombination events led to similar outcome of the PCR result, since the sequence of the annealing sites of oligos and probes at the mapA locus was different in the strains with ambiguous PCR results and overall sequence of mapA and ceuE varied within PCR categories.

Are the strains with ambiguous qPCR result typable by other PCR assays? There are various other PCR assays published for species differentiation of C. jejuni and C. coli with and without detection of further Campylobacter spp. Thus, the whole-genome sequences of the strains leading to ambiguous species differentiation with mapA/ceuE targets and the correctly identified C. coli hybrids with high C. jejuni content were further assessed in silico (Supplementary Fig. S1). First, a second multiplex PCR targeting mapA/ceuE was evaluated, leading to 56% ambiguous or false results. The sequence data revealed that also cpn60 detection would lead to false species identification of all C. coli hybrid strains but would identify C. coli correctly in the “half hybrids”, in which mapA/ceuE were no reliable targets. Besides, strain BfR-CA-17110 harboured a C. jejuni sequence in the target cadF, leading to false C. jejuni identification of this C. coli strain. All other targets (hipO, glyA, lpxA and cooD) displayed either no or low C. jejuni introgression. If C. jejuni sequences were detected within the genes, the annealing sites of the PCR oligos were not affected, thus, a correct output of the PCR is expected.

As mentioned above, MLST typing based on the seven housekeeping genes aspA, glyA, gltA, glnA, tkt, uncA, pgm was impaired in the C. coli hybrid strains with high content of C. jejuni sequence introgression (Fig. 3 and Supplementary Table S1). In particular, aspA and tkt contained C. jejuni sequences in all hybrid strains for at least 38 or 26% gene coverage, respectively. pgm displayed C. jejuni sequences in 24 of the 29 hybrid strains between 13 and 29% of the gene length. Low amount of C. jejuni introgression up to 16% were found in gltA, glnA and uncA in 28 strains. glyA was the most “stable”, since all strains harboured a classical C. coli allele or displayed just one C. jejuni k-mer match, which was not significant.

Figure 5. Strains with ambiguous qPCR result display various C. jejuni recombination events at the mapA and ceuE locus. In blue, coverage of k-mers to the reference sequence of C. jejuni NCTC 11168 of example strains with ambiguous qPCR results. Cj/Cc mix, integration of C. jejuni sequences at the mapA locus; none, integration of C. jejuni sequences in the ceuE locus; false C. jejuni, integration of C. jejuni sequences in both targets.
hybrid strains (Supplementary Fig. S4). This supports our hypothesis of a selective process on the gene set affected by *C. jejuni* introgression in the hybrid strains.

Almost half of all identified genes encode proteins involved in oxidative stress response (*katA*, *Cj1386*, *mrsB*, *canB*, *Cj0833c* hydA, hydA2, *nadD*, *nuoA*, *nuoB*, *nuoC*, *Cj0081*), stress response in general (*clpA*, *htrB*, *htrA*, *cpn10*, *cpn60*), DNA metabolism and repair (*purF*, *pyrG*, *thyr*, *rarA*, *recJ*, *ung*, *ribA*, *guaB*, *dut*), chemotaxis and flagellar motor switch (*cheA*, *cheV*, *cheW*, *fliY*), signal transduction (*Cj1110c*, *Cj1227c*, *Cj1258*), membrane transporters (*crtB*, *Cj0832c*, *ktrA*, *ktrB*, *Cj1257c*, *Cj1687*), cell wall and capsule biosynthesis (*kpsS*, *kpsE*, *kpsF*, *kpsD*, *kpsT*, *murE*) and *metK* encoding a S-adenosylmethionine transferase, involved in providing the substrate for methylation reactions. This suggests that *C. jejuni* sequence recombination in the *C. coli* hybrid genes was not random but might modulate the fitness of the *C. coli* hybrid strains, selected for survival in a harsh environment. Intriguingly, an American isolate, *C. coli* RM4661, from turkey carcass origin (NZ_CP007181.1) was identified as a *Cc/Cj*-hybrid strain, sharing 106 of the 126 *C. jejuni* introgressed genes revealed in the majority of our hybrid strains (Supplementary Table S2). We propose that this strain underwent a similar selection procedure, which corroborates our hypothesis of independent functional adaptation upon selection in a harsh environment.

Figure 6. *Cc/Cj* hybrid strains share common genes with *C. jejuni* sequence content, which are distributed over the chromosome. Visualization of genes with at least 50% k-mer coverage of *C. jejuni* sequences identified in at least one of the 29 *Cc/Cj* hybrid strains (with >10% *C. jejuni* introgression). X-axis, strains; y-axis, genes. Left, heatmap sorted according to gene location in the reference *C. jejuni* sequence; right, heatmap sorted according to genes with high number of strains and high *C. jejuni* sequence exchange. Colours indicate coverage of gene length by *C. jejuni* sequence specific k-mer (16 bp) matches in % as detailed in the figure. Example genes with high coverage in the majority of strains are indicated. Below heatmaps, isolation source of the strains: c, chicken meat; tc, turkey cecum; tm, turkey meat and eggs.
Which *C. jejuni* sequence exchange leads to amino acid exchange in the protein and might represent a functional adaptation in *C. coli*? We checked whether the gene variants of the *C. coli* hybrid strains lead to protein variants different from *C. coli* proteins. Since *C. jejuni* and *C. coli* proteins differ in average by nearly 40 amino acids, it was expected that most of the newly found *C. jejuni* introgression covering at least 20% of the gene length leads to changes in protein sequence. BLAST analysis (https://blast.ncbi.nlm.nih.gov) was performed on a subset of the above-mentioned identified gene translations and in all of the cases amino acid exchanges were detected in the hybrid variants as compared to *C. coli* typical protein sequence. It remains to be investigated in future studies, how these variations impact protein function with respect to *C. coli* survival capacity under stress conditions.

**Discussion**

The German National Reference Laboratory for *Campylobacter* has access to a large collection of representative isolates from Germany. With this set of isolates in hand we were able to identify multiple strains with ambiguous species differentiation, in particular, isolated from eggs but also from poultry meat and turkey cecum. Further isolates from eggs showed that from this isolation source nearly half of all *C. coli* displayed an extended amount of *C. jejuni* sequences incorporated in the genome.

A study comparing *C. coli* clade 1 (ST-828 and ST-1150) from agriculture with nonagricultural unintrogressed *C. coli* clade 2 and 3 demonstrated the potential of incorporation of substantial *C. jejuni* sequences in clade 1 *C. coli*. The authors identified 26 *C. jejuni* genes present in *C. coli* clade 1 but absent in clade 2 and 3. Our analysis focussed on *C. coli* hybrid strains as a fraction of clade 1 and deciphers ongoing extended *C. jejuni* introgression in these strains. As expected, we only have an overlap of 2 genes (Cj0555 and htrB) out of the identified 26 with the study of Sheppard et al., since we compared our hybrid strains against 113 *C. coli* sequences (mostly clade 1), including sequences from the NCBI database. This supports the notion that the *C. jejuni* recombination events found in this study represent a further development of *C. coli* strains. Since the *C. coli* hybrids were predominantly isolated from eggs, this supports the notion that the identified *C. jejuni* sequence incorporations might be a consequence of functional adaptation to survival in a harsh environment. *Campylobacter* is transmitted on egg shells via fecal contamination. On the shell, the bacterium encounters oxidative stress but also dryness and, thus, osmotic stress as well as nutrient and cold stress. Usually after 5–6 days, *Campylobacter* are no longer cultivatable from faeces.

**Adaptation to harsh environment might explain shared *C. jejuni* recombinations in *C. coli* hybrids.** The hybrid strains carried gene variants of *C. jejuni* or mosaic genes involved in oxidative stress response, such as katalase (*katA*) and Cj1386, which was shown to encode an atypical hemin-binding protein, mediating the trafficking of hemin to katalase. Katalase is one of the key enzymes for protection against oxidative stress by cleaving peroxide to water and oxygen. *mrsB* (Cj1112c) encoding a methionine sulphoxide reductase, was shown to protect *C. jejuni* against oxidative and nitrosative stress. Furthermore, *canB* displayed *C. jejuni* sequences in the hybrid strains, encoding carbonic anhydrase, an enzyme important for growth at low CO2 concentrations. A further oxidoreductase (*Cj0833c*) and genes encoding for the NiFe hydrogenase small subunit (*hydA* (Cj1267c) and *hydA2* (Cj1399c) as well as *nadD* (Cj1404) involved in the synthesis of the redox cofactor NAD+ were found to harbour *C. jejuni* sequences. Furthermore, *nuoA*, *nuoB*, *nuoC* implicated in transfer of electrons in the respiration chain and *Cj0081*, encoding the cyanide-resistant CioAB, is proposed to lower oxygen levels and maintain microaerobic conditions, were identified to bear *C. jejuni* sequences in the hybrid strains. The *htrB* gene encoding a lipid A acyltransferase was proposed to play a role in regulation of cell responses to environmental harsh conditions, such as acid, heat, oxidative and osmotic stress. As mentioned above, also *htrA*, which encodes a protease and chaperone activity with roles in virulence and oxidative stress defence, was among the genes with *C. jejuni* sequence detected in all hybrid strains. Besides *cgb*, encoding a single-domain haemoglobin, was suggested to protect *Campylobacter* against nitric oxide and nitrosative stress.

Interestingly, also genes implicated in general stress response as the *clpA* ATPase and the chaperone genes *cnp10* and *cnp60* were affected by *C. jejuni* introgression. The latter *cnp60* (*groEL*) also serves as target for species differentiation, inevitably leading to false species identification of the hybrid strains. Among the genes with *C. jejuni* introgression in the hybrid strains were several with roles in DNA metabolism and repair, such as *purF*, *pyrG*, *thym*, *rareA*, *recF*, *ung*, *ribA*, *guaB* and *dut*. Moreover, motility-associated genes, like the chemotaxis genes *cheA*, *cheV*, *cheW* and *fliY*, encoding a flagellar motor switch protein, displayed *C. jejuni* sequences.

In addition, our list of genes with *C. jejuni* content in the hybrid strains, also contained genes implicated in cell wall (*murE*) and capsule biosynthesis (*kpsS*, *kpsE*, *kpsF*, *kpsD*, *kpsT*). Consistently, in *C. jejuni* strains enhanced biofilm formation capacity, which might also be associated with enhanced survival under oxidative stress, was attributed to genes implicated in oxidative stress defence, motility, cell wall and capsular biosynthesis.

We suggest that modification of genes by recombination of *C. jejuni* sequences in a common set of genes in most of the hybrid strains, might reflect selection of survivors from harsh environments.

**Practical implications for diagnostics.** The hybrid strains can elude molecular typing, such as species differentiation using the *mapA/ceuE* targets and MLST. It was previously found that *mapA/ceuE* targets might lead to ambiguous qPCR results in six identified strains out of a data collection of around 1700 sequences. In our study we identified in total 37 strains (21 “hybrid” strains and 16 “half hybrid” strains), which were not identifiable in the qPCR using *mapA/ceuE* targets, including two isolates, which were falsely identified as *C. jejuni*. All “hybrid” strains failed to be typed using the *cnp60* target and one “half-hybrid” would be incorrectly typed as *C. jejuni* using the *cadF* target.

*C. jejuni* sequence introgression into *aspa* and adjacent regions (including Cj0081) was previously detected in two *C. coli* strains from turkey. Our data showed that MLST as phylogenetic assay has limitations, since *C. jejuni* introgression covering at least 20% of the gene length leads to changes in protein sequence.
sequences were found in six of the seven housekeeping genes in the majority of “hybrid” strains. Hence, standardized typing methods should consider perturbations due to extended recombination activity in *Campylobacter*. Thus, it is recommendable to include multiple independent species differentiation methods as future molecular annex to ISO 10272-1/2:2017 and to be aware of phylogenetic bias in source attribution analysis.

**Conclusions and further aspects.** There are various studies dealing with the differential survival of *C. jejuni* and *C. coli* under different environmental and host conditions. It has to be noted, that stress survival of the microaerobic *Campylobacter* is one of the major and still enigmatic topics in order to explain the pathogens widespread dissemination. *C. jejuni* was shown to survive longer in liver juice\(^4\). Aerotolerant *C. jejuni* strains were identified\(^4\) but also aerotolerant *C. coli* isolates were highly prevalent in other studies\(^4\). Survival in harsh environments might be a result of various factors and also dependent on the specific genomic background. In aerotolerant *C. coli* point mutations were detected in other genes, not obviously implicated in oxidative stress response\(^4\). Thus, it remains elusive, how *Campylobacter* species modulate their gene pool in order to adapt to changing environments. However, the identification of hybrid strains, mainly selected from a harsh environment, exhibiting an extended amount of *C. jejuni* sequences in a common gene set, shows the enormous potential of *Campylobacter* for extensive genetic exchange for fitness enhancement.

**Materials and Methods**

**Strains and growth conditions.** *C. jejuni* and *C. coli* field strains were isolated from different food matrices and animal samples by the Federal State Laboratories according to ISO 10272\(^11\). At the National Reference Laboratory, isolates were cultured on Columbia agar (Oxoid, Germany) supplemented with 5% sheep blood (Oxoid, Germany) (ColbA) or passed through Bolton broth and subcultured on mCCDA in case strains still exhibited non-*Campylobacter* background flora. Incubation was performed for 48 h under microaerobic conditions (5% O\(_2\), 10% CO\(_2\), rest N\(_2\)) at 42 °C. Strains were stored at ~80 °C using the cryobank system (Mast Diagnostica GmbH, Germany). For DNA extraction strains from −80 °C stocks were grown on ColbA for 24 h under microaerobic conditions at 42 °C and once subcultured for another 24 ± 4 h prior to use.

**Species differentiation by PCR.** DNA of the strains was extracted by resuspension of the cell pellet in 5% Chelex 100 resin (Bio-Rad Laboratories GmbH, Germany), followed by incubation for 15 min at 95 °C and subsequent centrifugation. The supernatant was used for PCR analysis. For detection and species identification a real-time PCR method, targeting either a *C. jejuni* specific fragment of the *mapA* gene, a *C. coli* specific fragment of the *ceuE* gene or a *C. lari* specific fragment of the *glyA* gene was performed\(^4\),\(^5\). In case of ambiguity of the results, a second gel-based multiplex-PCR was applied, targeting specific fragments of the *hipO* gene for *C. jejuni*, the *glyA* gene for *C. coli* and *C. upsaliensis*, the *cpn60* for *C. lari*, the *sapB2* gene of *C. fetus* and a Campylobacterales specific fragment of the 23S rRNA gene\(^4\).

**Matrix-assisted laser desorption/ionization (MALDI-TOF) analysis.** Colony material of a 24 h ColbA plate was spotted onto the target plate (MSP 96 target polished steel (MicroScout Target) plate; Bruker Daltonik, Germany). After air drying the spots were overlaid with 1 μl of saturated α-cyano-4-hydroxy-cinnamic acid matrix solution (200 mg in 2.5% trifluoroacetic acid/50% acetonitrile) and dried completely. MALDI-TOF MS analysis was performed using MALDI-TOF Microflex LT (Bruker Daltonics, Germany) using a range of 2.000–20.000 m/z (mass to charge ratio) following the calibration with Bacterial Test Standard (Bruker Daltonics, Germany). For each spectrum 240 laser shots were summed up in 40 shot steps, but at least 80 shots per raster spot from different positions within the sample were acquired by the AutoXecute method using the software FlexAnalysis 3.4. The spectra were compared with the MBT Compass Library, Revision F (Bruker Daltonics, Germany). Each identification obtains a score value. The identification at the species level with a score > 2.000 was considered correct\(^4\),\(^5\).

**Whole genome sequence analysis.** *Campylobacter* strains grown on ColbA for 24 h under microaerobic atmosphere at 42 °C were harvested and DNA was extracted using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, USA). The quality of the DNA was evaluated by spectrophotometric analysis (NanoDrop Spectrophotometer, Thermo Fisher Scientific, USA) and the concentration was fluorimetrically quantified by Qubit 3.0 Fluorometer (dsDNA HS Assay Kit 0.2–100 ng; Thermo Fisher Scientific, USA). DNA libraries were prepared using the Nextera XT DNA Library Prep kit or the Nextera DNA Flex Library Prep Kit according to the manufacturer’s instructions (Illumina, San Diego, USA). Quality of the libraries was assessed by gel analysis or on a fragment analyser 3408 (Advanced Analytical Technologies Inc., USA). Paired-end sequencing was performed on the Illumina MiSeq (2 × 301 cycles) or the NextSeq (2 × 151 cycles) platform using the MiSeq v3 (600 cycles) reagent kit or the NextSeq 500/550 Mid Output kit v2.5 (300 cycles), respectively. The sequences were published within the BioProject No. PRJNA595957, BioSample No. SAMN13577876-SAMN13577920, SRA accession No. SRR10698060-SRR10698104 at NCBI sequence read archive (SRA). New MLST alleles and MLST-ST types were uploaded to PubMLST.

**Sequence analysis.** Sequences were analyzed by Ridom Seqsphere+ v. 6.0.0 (2019–04) (Ridom, Muenster, Germany) using the cgMLST scheme of 1343 gene targets previously proposed\(^2\), with 98% required identity and 98% required percentage of coverage to one of the known alleles (allele library status June 2018). Quality trimming was performed in a window of 20 bp with Phred score 30. The obtained average coverage (processed, unassembled) was >75-fold. Raw reads were de-novo assembled via SPAdes 3.11.1\(^4\) with careful option, which performs a mismatch correction. The number of assembled contigs was between 31 and 130, the total size of the assemblies ranged from 1.65 to 1.92 Mb. At least 95% “good targets” were found for cgMLST-based analysis using the previously proposed cgMLST scheme\(^2\). Average nucleotide identity (ANI) analysis was done using
the tool FastANIT9. Core genome phylogeny was calculated using Roary v.3.12.030 with a sequence identity of at least 80%. This resulted in 800 core genes that were used to build a phylogenetic tree with RAxML v.8.2.1031 (100 bootstraps). Finally, the phylogenetic tree was adjusted for recombination sites using ClonalFrameML v.1.1132.

For prescreening of sequences for C. jejuni introgressions, assembled contigs were analyzed on the web-based KmerFinder 3.1 (Center for Genomic Epidemiology, DTU, Denmark)13-15, which splits the assembly contigs into overlapping 16-mers and searches for homology matches in sequenced bacterial organisms, filtered on coding sequences (CDS; starting with ATG). The percentage of k-mers matching to distinct reference genomes was received as output data. An in-depth k-mer analysis was performed using an in-house pipeline. For this purpose the assemblies from Ridom Seqsphere+ after SPAdes assembly were used. A k-mer based databases from assembled and closed genomes from C. jejuni and C. coli (Supplementary Table S1) were built by kmc v.3 with a k-mer size of 16 bases or 31 bases. In order to identify C. jejuni specific genes, the database of C. jejuni includes k-mers present in at least 95% of the genomes whereas the C. coli database contains k-mers present in at least 5% of the genomes. K-mers from mixed isolates were subsequently counted and compared against the databases with kmc_tools. K-mers present in C. jejuni database and absent in C. coli genomes were mapped against a closed reference strain NCTC 11618 (NC_002163.1) of C. jejuni by bowtie234 and corresponding genes were identified with BEDTools v2.27.135. Since read mappers are optimized for longer sequences, k-mers of size 16bp were only considered for exact and unique matches for further downstream analysis. Since k-mers of size 31 bp are more specific, mapping results were not filtered at this step.

Genomes covered by k-mers in at least 20% or 50% of the gene length and in one of the high content C. coli hybrid genome were visualized as heatmap by the R package phemtap v.1.0.12. K-mers matched in C. coli hybrid genomes (as.bam files) were visualized in Geneious v.2019.2.1 using C. jejuni strain NCTC 11618 (NC_002163.1) as reference. Source code and scripts used to perform these steps are freely available at https://github.com/microbial_genomics/relative-kmer-project.

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Author contributions

J.G. performed experiments, analysed data and co-wrote the manuscript; L.E. designed the k-mer analysis, analysed data, co-wrote the manuscript; M.K., performed experiments; F.H., analysed data; M.B., C.D., B.M. and T.S. provided critical advice in experimental design and edited the paper; K.S. designed the study, analysed data and wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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