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Distribution of Colonization and Antimicrobial Resistance Genes in *Campylobacter jejuni* Isolated from Chicken

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Abstract

Campylobacter jejuni is an important worldwide foodborne pathogen commonly found as a commensal organism in poultry that can reach high numbers within the gut after colonization. Although information regarding some genes involved in colonization is available, little is known about their distribution in strains isolated specifically from chickens and whether there is a linkage between antimicrobial resistance (AMR) and colonization genes. To assess the distribution and relevance of genes associated with chicken colonization and AMR, a *C. jejuni* microarray was created to detect 254 genes of interest in colonization and AMR including variants. DNA derived from chicken-specific *Campylobacter* isolates collected in 2003 ($n=29$) and 2008 ($n=28$) was hybridized to the microarray and compared. Hybridization results showed variable colonization-associated gene presence. Acquired AMR genes were low in prevalence whereas chemotaxis receptors, arsenic resistance genes, as well as genes from the cell envelope and flagella functional groups were highly variable in their presence. Strains clustered into two groups, each linked to different control strains, 81116 and NCTC11168. Clustering was found to be independent of collection time. We also show that AMR weakly associated with the CJ0628 and *ars*R genes. Although other studies have implicated numerous genes associated with *C. jejuni* chicken colonization, our data on chicken-specific isolates suggest the opposite. The enormous variability in presumed colonization gene prevalence in our chicken isolates suggests that many are of lesser importance than previously thought. Alternatively, this also suggests that combinations of genes may be required for natural colonization of chicken intestines.

Introduction

Campylobacter jejuni is a major foodborne pathogen. Human campylobacteriosis has been linked to consumption and mishandling of contaminated poultry (Young *et al*., 2007). One way to reduce human exposure to *C. jejuni* would be to control pathogen levels at the farm (Nauta *et al*., 2009). In order to do so, a better understanding of the dynamic existing between *C. jejuni* and its chicken host is needed (Hermans *et al*., 2011).

Several studies have identified genes that may be associated with chicken colonization (Hermans *et al*., 2011): flagella/motility/chemotaxis (*fli*A, *pgl*E, *pfl*A, *mot*A, *che*Y, *rpo*N, *fli*A) (Wassenaar *et al*., 1993; Hendrixson and DiRita, 2004; Fernando *et al*., 2007), stress tolerance (*kat*A, *Ahp*C, *sod*B, *ahp*C PKK) (Klancnik *et al*., 2009; Palyada *et al*., 2009; Gangaiah *et al*., 2010), efflux pump (*cme*ABC) (Lin and Martinez, 2006; Quinn *et al*., 2007), nutrient acquisition and use (*cj1403*, *cj1198*, *cfr*A) (Palyada *et al*., 2004; Metris *et al*., 2011), secreted protein (*cia*) (Biswas *et al*., 2007), and adhesins (*cad*F, *dna*J, *pld*A, *por*A) (Ziprin *et al*., 2001; Hiett *et al*., 2008). The link existing between genes and chicken colonization is often validated using single strains and transposition mutants (Javed *et al*., 2010), without comparison of their presence in a large population of field strains. There are still knowledge gaps in their exact distribution and stability over time in chicken strains. Gaining more knowledge on their distribution would be helpful to better understand field strain genetic diversity in regard to chicken colonization and to establish which genes are more important in strains isolated from a commercial setting. Two separate reviews on *Campylobacter* chicken colonization (Dasti *et al*., 2010; Hermans *et al*., 2011) have stressed that more comprehensive studies are necessary in order to understand the relative importance of colonization factors, which could help in the design of suitable farm control strategies.

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Although virulence has been linked with antimicrobial resistance (AMR) (Pitout, 2012), such linkages are less clear in *C. jejuni* despite the fact that AMR is increasingly recognized as an important public health threat (Alfredson and Korolik, 2007). Monitoring of the association of *C. jejuni* colonization traits and AMR is important in order to anticipate the emergence of highly adapted strains due to the use of antimicrobial agents during rearing.

Given their inherent parallel processing power, microarrays are useful tools for assessing the genetic contents of a bacterial isolate. With regard to *Campylobacter*, they have been successfully used in comparative hybridization studies (Wilson *et al*., 2010), to measure differential gene expression levels (Malik-Kale *et al*., 2008), for typing (Rodin *et al*., 2008), for the detection of *Campylobacter* in various environmental matrices (Suo *et al*., 2010), and in epidemiological studies (Hepworth *et al*., 2011; Marotta *et al*., 2012). To our knowledge, no comprehensive microarray studies have focused on correlating the distribution of chicken colonization genes and their possible association with AMR in *C. jejuni* strictly isolated from chickens.

The aim of this study was to describe the relative distribution of genes related to chicken colonization and AMR, in strains isolated from cecal contents sampled at slaughterhouses, using a custom DNA microarray. This study also assessed the data for any association of the detected genes with the year of isolation as well as correlations between AMR and colonization genes.

Materials and Methods

Strains and media

Isolates collected in 2003 and 2008 were recovered from chickens at slaughter (average of 37 days of age) using the same method as described elsewhere (Arsenault *et al*., 2007). Thirty individual 1-g cecal samples were taken from different lots and then pooled in three 10-g samples. Samples were mixed 1:1 (wt/vol) in peptone buffered water (AES Laboratory, Montreal, Québec, Canada), directly streaked on mCCDA Agar (Oxoïd, Nepean, Ontario, Canada) and incubated at 42°C for 48 h in a microaerobic atmosphere using Oxoïd's Atmosphere Generation System with the *Campylobacter* gas generation kit. Typical colonies (ASPC MFLP-46) were purified on mCCDA Agar (Oxoïd) and plated on trypticase soy agar (TSA) 5% sheep blood agar (PML Microbiologicals [Québlab], Montreal, Quebec, Canada). Typical isolates (small Gram-negative spiral-shaped bacteria, motile) (Thibodeau *et al*., 2011) were further identified to the species level by polymerase chain reaction as previously described (Inglis and Kalischuk, 2003). A total of 57 *C. jejuni* confirmed isolates (29 from 16 lots in 2003 and 28 from 9 lots in 2008) were used in this study.

Control strains *C. jejuni* RM1221 (#BAA-1062, chicken carcass origin [Fouts *et al*., 2005]), *C. jejuni* NCTC11168 (#BAA-33291, human origin [Gundogdu *et al*., 2007]), and *Escherichia coli* ATCC 25922 were acquired from Cedarlane (Ontario, Canada). Strains *C. jejuni* 81–176 (human origin [Pearson *et al*., 2007]) and *C. jejuni* 81116 (human origin [Hofreuter *et al*., 2006]) were a kind gift from Dr. Shaun Cawthraw, Veterinary Laboratories Agency, United Kingdom. All strains were kept at −80°C in Brucella broth containing 0.1% agar and 25% glycerol. To minimize strain genetic variations, all experiments were started from an aliquot taken from a new −80°C stock, thus avoiding potential variations caused by the use of a single colony for starting inoculums. Strains were cultured on mCCDA, Mueller Hinton Agar (Oxoïd) or TSA sheep blood agar when needed.

Microarray gene selection and probe design

The *C. jejuni* microarray V1 was composed of 70-mer oligonucleotide probes specific for genes associated with envelope biosynthesis, pathogenesis, chemotaxis/mobility, detoxification, metabolism, transport and nutrient binding, global regulation, hypothetical or unclassified functions, and AMR genes. They were chosen based on a literature review of genes or functional groups involved to different degrees in chicken colonization.

Oligonucleotides were designed (see Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/fpd) as described previously with some modifications (Bruant *et al*., 2006). Some AMR probe sequences were derived from a published microarray (Hamelin *et al*., 2007). The rest of the probes were design using the YODA program (Nordberg, 2005). Probes were based on GenBank sequences including, whenever possible, sequences from the chicken strain RM1221 as a template. The specificity of each oligonucleotide sequence was individually verified through Blastn searches (nr/nt database, *Campylobacter jejuni* taxid 197, word size of 11) in GenBank. Details on probe specificity are given in supplementary Table S1. Probes were synthesized by Operon (Huntsville, AL) and printed at the National Research Council (Montreal, Quebec, Canada).

Microarray construction

Positive controls included *groEL (cpn60*, designed in this study*)* (Chaban *et al*., 2009) for the identification of *Campylobacter* genera as well as *map*A and *hip*O for the confirmation of *C. jejuni* species (Inglis and Kalischuk, 2003). A universal probe, EUB, able to hybridize to prokaryotic 16S rRNA genes was also added as a positive control (Maynard *et al*., 2005). The newly designed probe for *Arabidopsis thaliana* chlorophyll synthetase gene (*ara*) was included as a negative control. The complete array was composed of four subarrays, in which each oligonucleotide was printed in duplicate (except EUB, which was printed four times) on Corning Ultra GAPS^{tm} slides (Corning Canada, Whitby, Ontario) (Fig. 1). Three complete independent arrays were printed on the same slide. Some printing spots were left empty (printing buffer only) (Fig. 1).

DNA extraction, labeling, and hybridization

C. jejuni isolates were plated on mCCDA agar from a single −80°C storage aliquot. They were incubated 24 h at 42°C then streaked on Mueller-Hinton agar. DNA was extracted using the commercial Qiagen DNeasy Blood and Tissue Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's instructions. DNA labeling and hybridization was performed according to the protocol described in another study (Bruant *et al*., 2006). Complete details are provided in the Supplementary Materials and Methods.

Microarray analysis and validation

Arrays were read using a ScanArray Lite fluorescent scanner and the resulting images were analyzed with ScanArray Express version 1.1 (Canberra-Packard, Mississauga,

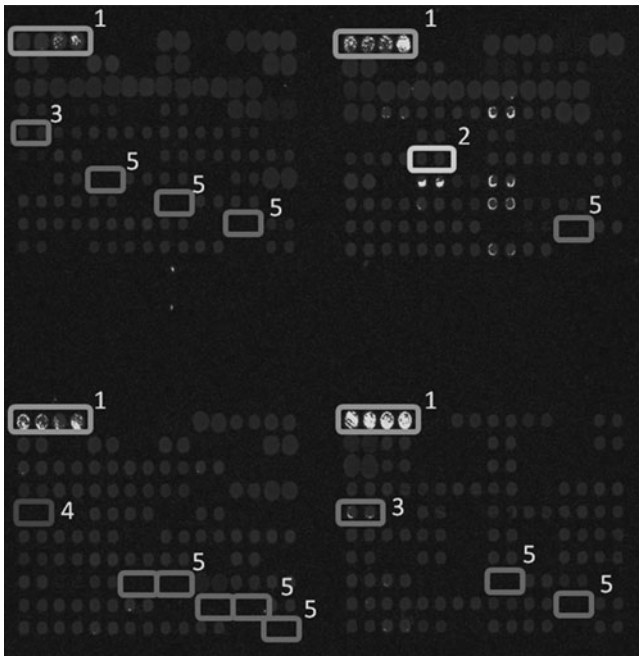

FIG. 1. Hybridization of the NCTC11168 strain to the custom microarray. 1, positive control (EUB); 2, *Campylobacter* sp. control (*GroEL*); 3, *C. jejuni* control (*mapA* and *hipO*); 4, negative control (ara); 5, printing buffer (empty). Gray and white spots represent a positive hybridization.

ON, Canada). After an initial visual inspection of the image was performed, a numerical analysis of the fluorescence obtained for each probe was carried out. For each hybridized strain, a probe was considered positive if the mean of the median probe fluorescence of the duplicates was above 1000 fluorescence units and four times higher than the background. The background was determined as the mean fluorescence of the negative control (*ara*) and empty spots (printing buffer). Microarray validation was done by multiple hybridizations of the four control *C. jejuni* strains. *C. coli* LSPQ 3655 (lab strain) and *E. coli* ATCC 25922 were used as negative controls. For each control strain, total DNA was extracted from three distinct fresh cultures, labeled, and hybridized to the microarray in three separate experiments, on three separate microarray slides.

Statistical analysis

Microarray results were analyzed with the TMeV 5 program (Hannon *et al*., 2009); a dendrogram was derived from the presence of all genes (genes and variants) with heterologous hybridization (Euclidian distance and single linkage for clustering). Cluster stability (percent) was defined by subjecting the chosen clusters to 500 bootstrapping iterations in TMeV 5. The Fisher's exact test (S-Plus, v8) and Bonferroni correction were used to assess the association of specific genes to strains clusters or to a recovery time. The relevance network analysis was also carried out using TMeV 5 with a minimal correlation value of 0.9. For all statistical analysis, α was fixed at 0.05.

Results

Gene distribution and correlation

All strains were positive for 94 genes (see Supplementary Table S1), which were distributed in the following fashion: 14 AMR, four envelope biosynthesis, seven pathogenesis, 11 chemotaxis/mobility, five detoxification, five metabolism, 32 transport and nutrient binding, three regulation, five hypothetical, and four unclassified genes including four controls. Although all probes on the microarray have been validated, a total of 13 genes were never detected in any of the strains in this study (see Supplementary Table S1) while most probes (147) demonstrated a variable presence, ranging from 2% to 98% (Table 1).

Relevance network analysis identified some co-linked genes (see Supplementary Tables S2–S4). Among the 2008 strains, the presence of an antibiotic resistance gene (*aph*A-3, coding for resistance to the aminoglycoside kanamycin [Gibreel *et al*., 2004]) correlated with a variant of a colonization gene (*CJ0628*, a lipoprotein, putative autotransporter, mediating adherence and invasion of epithelial cells [Ashgar *et al*., 2007]). In the 2003 strains, the presence of *CJE900*, a metallo-β-lactamase family protein, correlated with the presence of a variant of *ars*R, an arsenic resistance operon repressor.

Strains comparison

Dendrogram analysis, based on the heterogeneous presence of the genes, made it possible to divide the strains into two main groups (Fig. 2). A total of 24 strains were clustered in a group that included strain 81116 and 35 others with strain NCTC11168. These two groups were separated by a total of 37 genes (Table 2). Strain comparisons revealed that they mainly aggregated together according to their origin (lot; i.e., a group of chickens raised on the same farm and processed the same day) rather than the collection time period (Fig. 2), even though strains recovered from a specific sampling year were exclusively positive for some probes (Table 1). Control strains 81–176, RM1221, and *C. coli* were distant to all hybridized strains.

Discussion

Microarray

This novel study assessed the distribution of chicken colonization-associated genes and their correlation with antimicrobial resistance genes in a chicken-specific strain collection with the use of a newly customized DNA microarray. The microarray was found to be a reliable tool for the evaluation of 254 genes, including the detection of gene variants up to a 10% homology divergence. The designed probes were specific, as demonstrated by the *C. coli* and *E. coli* negative control hybridizations. The design of the microarray, as well the conservative cut-off criteria used to define positivity for a given probe, possibly resulted in an underestimation of the effective gene presence. However, this approach lowers the likelihood of reporting false-positive results and consequently increased the robustness of the hybridization data. As an example, more than 90% of the strains were positive for *cpr*R, a gene associated with regulatory functions. This was expected, considering the essential roles it plays for all bacterial functions (Raphael *et al*., 2005). The missing 10% is presumably due to sequence variants not covered by our new probes. It is important to note that our microarray may not be able to detect subtle genomic changes that may have an impact on the *C. jejuni* phenotype (Gaynor *et al*., 2004). Phenotypic properties of our strains will be determined in a future study.

Table 1. Variable Gene Occurrence of *Campylobacter jejuni* Poultry Strains Recovered from Quebec Flocks

		Occurrence			
Classification	*Gene*	*Total (%)*	*2003 (%)*	*2008 (%)*	*Gene description*
Antimicrobial resistance	*aad*E	2	0	4	Streptomycin resistance protein
	*aph*A-3	16	0	32	Aminoglycosidase
	*omp*50	17	30[a]	4[b]	50 kda outer membrane protein
	CJE0900v1	28	17	39	Metallo-β-lactamase family protein variant
	*tet*O	67	77	57	Tetracycline resistance
	CJE0900	74	87[a]	61[b]	Metallo-β-lactamase family protein
	CJE0175	84	79	90	Antibiotic transport protein, putative
	CJE1885	93	100[a]	86[b]	Acetyltransferase, GNAT family
	*bla-oxa*61	97	100	93	β-Lactamase
	*upp*P	97	100	93	Undecaprenyl-diphosphatase
	CJ1173	97	97	96	Multidrug resistance protein, SMR family
	*cme*E	98	100	96	Multidrug efflux system CmeDEF
	*mac*A	98	100	96	Macrolide-specific efflux protein macA
Cell envelope	Glf	2	3	0	UDP-galactopyranose mutase
	CJE1515	3	0	7	Formyltransferase putative
	*waa*Fv1	5	10	0	ADP-heptose–LPS heptosyltransferase II variant
	CJE1278	9	0	17	Lipooligosaccharide biosynthesis galactosyltransferase
	CJ1677nter	10	13	7	Putative lipoprotein autotransporter n-terminal
	CJ1677	10	13	7	Putative lipoprotein autotransporter
	*wcb*K v1	16	20	11	GDP-mannose 4,6-dehydratase, capsular biosynthesis
	CJ0628	26	40[a]	11[b]	Putative lipoprotein autotransporter
	CJ0628v2	29	26	32	Putative lipoprotein autotransporter variant 2
	C8J_1083	38	36	39	Hypothetical, lipooligosaccharide biosynthesis
	CJ0628v1	41	40	43	Putative lipoprotein autotransporter variant 1
	CJ0628nter	50	56	43	Putative lipoprotein autotransporter n-terminal
	CJE1277	52	53	50	Lipooligosaccharide biosynthesis glycosyltransferase
	*pgl*E(2)	69	80	57	UDP-4-keto-6-deoxy-GlcNAc C4 aminotransferase variant
	*ptm*C	81	97[a]	64[b]	*N*-Acetylneuraminic acid synthetase
	CJ1321	81	86	75	Probable transferase
	*pgl*F	90	96	82	UDP-GlcNAc C4,6 dehydratase
	CJE0821	93	90	96	Putative integral membrane protein
	*neu*C	95	100	89	DP-*N*-acetylglucosamine 2-epimerase
	*acp*P2v1	95	100	89	Putative Acyl carrier protein
	*waa*F	98	97	100	ADP-heptose–LPS heptosyltransferase II
	*waa*M	98	100	97	Lipid A biosynthesis lauroyl acyltransferase
Chemotaxis and motility	Maf1[c]	12	0[a]	25[b]	Motility accessory factor
	CJE0312	26	16	36	Methyl-accepting chemotaxis protein
	CJE0140	31	**53[a]**	**7[b]**	Methyl-accepting chemotaxis protein
	Maf3	33	40	25	Motility accessory factor
	CJE0314	34	43	23	Methyl-accepting chemotaxis protein
	CJE03112	43	60[a]	25[b]	Methyl-accepting chemotaxis protein
	*mot*A	59	60	57	Putative flagellar motor proton channel
	Maf5	71	80	61	Motility accessory factor
	*cet*A	81	87	75	Methyl-accepting chemotaxis protein
	Maf4	84	97[a]	71[b]	Motility accessory factor
	CJE1679	86	96[a]	75[b]	Methyl-accepting chemotaxis protein
	*doc*Bv1	90	100[a]	79[b]	Methyl-accepting chemotaxis protein variant 1
	CJ1564	91	87	96	Methyl-accepting chemotaxis protein
	Maf2	93	100[a]	86[b]	Motility accessory factor
	CJE1325	95	97	93	Methyl-accepting chemotaxis protein
	*fla*C	97	93	100	Flagellin C
	*fli*M	97	100	93	Flagellar motor switch protein M
	*che*R	98	100	96	Chemotaxis protein methyltransferase CheR
	*flg*K	98	100	96	Flagellar hook–associated protein
	*flg*E2	98	100	96	Flagellar hook–subunit protein
Detoxification	*ars*C	47	50	43	Arsenate reductase
	*ars*C2	47	50	43	Arsenate reductase probe 2

(*continued*)

Table 1. Continued

Classification	Gene	Occurrence			Gene description
		Total (%)	2003 (%)	2008 (%)	
	*ars*Rv1	71	80	61	Arsenical resistance operon repressor variant 1
	*ars*R	90	96	82	Arsenical resistance operon repressor
Hypothetical proteins	C8J_0065[c]	9	0[a]	18[b]	Hypothetical protein
	C8J_0035	9	10	7	Hypothetical protein
	CJJ81176_063	9	0[a]	18[b]	Conserved hypothetical protein
	C8J_0035v1	10	3	18	Hypothetical protein variant 1
	C8J_0140	11	7	15	Hypothetical protein
	C8J_0267	28	43[a]	11[b]	Hypothetical protein
	C8J_1456	43	43	43	Hypothetical protein
	C8J_0988	52	60	43	Hypothetical protein
	CJE1893	52	60	43	Hypothetical protein
	CJ1325	57	60	54	Putative methyltransferase
	CJ0223	67	76	57	Pseudogene putative IgA protease family protein
	CJ1322	71	80	61	Hypothetical protein
	CJ1323	74	87[a]	61[b]	Hypothetical protein
	CJ1324	83	87	79	Hypothetical protein
	CJE0147	95	97	93	Hypothetical protein
	CJ1417	97	100	93	Putative amidotransferase
	CJ1420	97	93	100	Putative methyltransferase
	*lol*A	98	97	100	Outer-membrane lipoprotein carrier protein precursor
	CJE0469	98	100	96	Putative periplasmic protein
Metabolism	*hsdsv*1	5	10	0	Hypothetical protein, Hsds gene variant 1
	CJEIv1	7	13	0	Type II restriction-modification enzyme variant 1
	Ggt[c]	9	0[a]	18[b]	γ-Glutamyltransferase
	Dns	16	7	23	Extracellular deoxyribonuclease
	CJEIv2	16	23	7	Type II restriction-modification enzyme variant 2
	*dms*A	43	43	43	Anaerobic dimethyl sulfoxide reductase chain A
	hsds	45	50	32	Hypothetical protein, Hsds gene
	*hsdsv*2	45	46	43	Hypothetical protein, Hsds gene variant 2
	Met	52	60	43	Homoserine *O*-acetyltransferase
	CJEI	74	63	86	Type II restriction-modification enzyme
	*trx*B	95	100	89	Thioredoxin-disulfide reductase
	*ccp*A-1	95	100	89	Cytochrome c551 peroxidase
	*ctp*A	98	100	96	Carboxyl-terminal protease
Pathogenesis	Flav2	5	0	11	Flagellin (A and B) 81176 and 81116
	*fla*Bv1	5	0	11	Flagellin B 81176 and 81116
	*fla*A	5	0	11	Flagellin A 81176 and 81116
	*vir*B11[c]	9	0[a]	18[b]	Putative type IV secretion system
	Flav1[c]	10	20[a]	0[b]	Flagellin (A and B) RM1221
	CJE1470	38	27	50	Toxin-antitoxin protein, putative
	*por*A	40	30	50	Major outer membrane protein
	*fsp*A	40	36	43	Hypothetical protein
	Fla	50	37	64	Flagellin (A and B) NCTC11168
	*por*Av1	52	73[a]	29[b]	Major outer membrane protein variant 1
	CJ1371	66	53	79	Putative lipoprotein vaCJ homologue
	*pfl*A	78	100	54	Paralysed flagellum protein
	*fla*Bv2	79	97[a]	61[b]	Flagellin B NCTC11168
	*ptm*B	90	100[a]	79[b]	Flagellin modification protein B
	Ptma	91	100	82	Flagellin modification protein A
	*fla*Av1	93	100[a]	86[b]	Flagellin A RM1221
	*fla*Av2	93	100[a]	86[b]	Flagellin A
	*fla*B	93	100[a]	86[b]	Flagellin B RM1221 and NCTC11168
	*cja*A	98	97	100	Surface antigen CJaA
Plasmid	pCJ419_p4[c]	9	0[a]	18[b]	Plasmid putative protein
Regulatory functions	*cpr*R	90	80[a]	100[b]	DNA-binding response regulator
	*rac*R	98	97	100	Two-component regulator
Transport and nutrient binding	*cfr*Av1	2	3	0	Ferric receptor CfrA variant 1
	CJE1754	10	17	4	Peptide ABC transporter, permease protein
	CJ1584v1	16	20	11	Putative peptide ABC-transport system periplasmic peptide-binding protein variant 1

(*continued*)

Table 1. Continued

Classification	Gene	Occurrence			Gene description
		Total (%)	2003 (%)	2008 (%)	
	CJE0534	38	36	39	Tartrate transporter, putative
	CJE1730	53	50	57	Permease, putative
	*lct*P	55	66	43	L-Lactate permease
	CJE0171	55	53	57	Putative TonB-dependent outer membrane receptor
	*cfr*A	57	57	57	Ferric receptor CfrA, erric enterobactin uptake receptor
	CJE1728	57	73[a]	39[b]	Permease putative
	CJE1820	65	73	54	ABC transporter, periplasmic substrate-binding protein, putative
	CJE1193	81	83	79	Transporter, LysE family
	CJE0830	84	87	82	ABC transporter, permease protein
	CJE0827	88	93	82	ABC transporter, periplasmic substrate-binding protein
	CJE0831	88	93	82	ABC transporter, permease protein
	CJ1584	90	100[a]	79[b]	Putative peptide ABC-transport system periplasmic peptide-binding protein
	CJE0937	91	100[a]	82[b]	Major facilitator family protein
	*cja*C	93	100	86	Histidine transporter
	*kgt*P	95	100	89	α-Ketoglutarate permease
	*dcu*B	97	93	100	Anaerobic C4-dicarboxylate membrane transporter
	CJE0138	98	100	97	Cation ABC transporter periplasmic cation-binding protein
	CJE0289	98	100	96	Mechanosensitive ion channel family protein
	CJE1833	98	97	100	ABC transporter, permease protein
	*dtp*T	98	97	100	Di-/tripeptide transporter
	CJE0980	98	100	96	Amino acid ABC transporter, His/Glu/Gln/Arg/family
Unknown/unclassified	*pse*D[c]	5	0	11	Hypothetical protein, pseD (81176)
	*rlo*A	45	47	43	Hypothetical protein rloA
	*rlo*B	45	47	36	Hypothetical protein rloB
	CJE1719	48	40	57	Putative NADP-dependent alcohol dehydrogenase
	CJE1719v1	53	63	43	Putative NADP-dependent alcohol dehydrogenase, variant 1
	CJE1538	83	93[a]	71[b]	Putative fibronectin/fibrinogen-binding protein
	CJE0464	98	97	100	Oxidoreductase, putative, putative GMC oxidoreductase subunit
	CJJ81176_024	98	100	96	Hypothetical protein

Total presence (%) of genes, following the microarray hybridizations, for all 57 strains: 2003 occurrence (%) was on 29 strains and 2008 occurrence (%) was on 28 strains. Genes that were present or absent in all strains are presented in Supplementary Table S1. Complete details on the genes and associated probes are given in Supplementary Table S1.

[a]Statistically different from [b]using Fisher's exact test ($p < 0.05$).

[c]Genes found only in isolates recovered in 2003 or in 2008. Statistically different genes between the collections periods, using Bonferroni correction, are highlighted in bold.

The *C. jejuni* genome contains 2427 gene families with an estimated 1295 gene families found in its core genome (Friis *et al*., 2010). It has been observed that minor nucleotide sequence variations in its core genome, such as mutations in flagella, lipooligosaccharide (LOS), or regulation genes, might account for differential *C. jejuni* pathogenicity in strains indistinguishable by comparative genomic hybridization or pulse-field gel electrophoresis (Duong and Konkel, 2009). Consequently, using probes able to detect gene variants became attractive in order to study *C. jejuni* colonization genes.

Distribution of genes with heterogeneous occurrence

Our data showed a large variation in the distribution of genes monitored by our microarray. As already observed, genes coding for the flagella and the cell envelope were the most variable (Taboada *et al*., 2004) while many genes (37), in the transport and nutrient binding groups, were more conserved. In an independent study (Marotta *et al*., 2012), the virulence gene content of different *Campylobacter* species isolated from different sources (i.e., humans and milk in addition to chicken [carcass and cecum]), was also found to be variable. Due to the different sources of isolation, parallels between the two studies are tenuous. However, some important differences were noted. For example, in their paper, *C. jejuni* strains originating from chicken feces showed variability in the presence of *cdt*A, B, and C, unlike our study where they were present in all isolates. Furthermore, *fla*B was never found in *C. jejuni*, while it was found in the majority of our isolates (93%). In contrast, *waa*M, *neu*C, *flg*K,

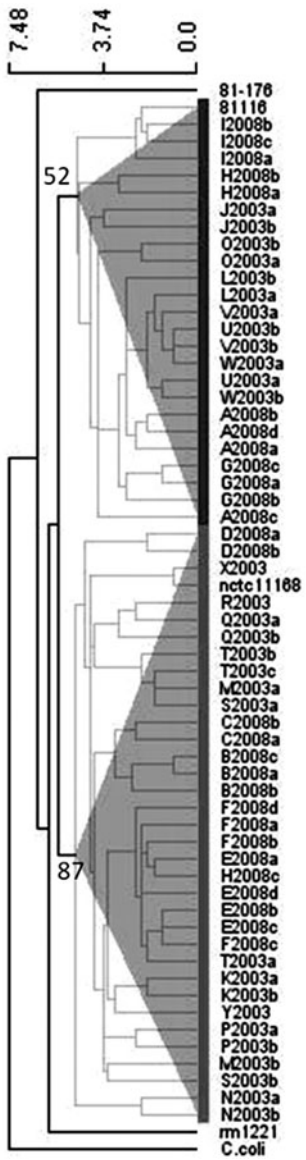

FIG. 2. Strain comparisons based on differential gene presence. Two clusters are resolved: cluster A associated with the 81116 reference strain (Bootstrapping value of 52%) while cluster B associated with the NCTC11168 strain (Bootstrapping value of 87%). Strains and Euclidean distance are presented horizontally. The last column lists the strains. Strain identification begins with a letter (example **H**2008b) representing the lot origin, followed by the year (example H**2008**b) of sampling and ends with an identification letter (example H2008**b**) to differentiate between strains isolated from the same lot.

*fli*M, *pgl*E, and *mot*A were conserved in all *C. jejuni* isolated from chicken feces while their presence was highly variable in our strain collection. Interestingly, both studies show that genes cj0903c and *cia*B were found in all tested *C. jejuni* strains. Finally, the strains in our study displayed more genetic diversity. These differences were presumably due to the incorporation of gene variants into our microarray design and possibly also to the different geographical origins of the strains used by both studies.

Table 2. Genes Differentiating Two Major Populations of *Campylobacter jejuni* Identified by Clustering

Cluster	*Cluster strain exclusive gene presence (%)*
81116 cluster	CJE0034(20)
NCTC11168 cluster	*aph*A-3(23), **CJE0171(88)**, **CJE0900v1(43)**, ***mot*A(94)**, **CJ0628v2(46)**, CJ1677(17), CJ1484v1(26), ***por*A(63)**, **CJE1277(83)**, **CJE0534(60)**, **CJE0140(51)**, **CJ0828(43)**, **CJ0828v3(80)**, ***met*(83)**, **CJE1730(86)**, ***cfr*A(91)**, **CJ1325(91)**, **CJE1893(83)**, *wcb*Kv1(26), **CJE1470(60)**, *fla*v1(17), **CJE1719(77)**, CJE1677v1(17)

Following hybridization, clustering showed that two main groups were present, each containing a different reference strain. Genes were found in one cluster but not in the other, using Fisher's exact test ($p < 0.05$). Genes statistically different using the Fisher's exact test with Bonferroni correction are highlighted in bold. Gene details may be found in Table 1 or in Supplementary Table S1.

In our study, the overall observed variable gene prevalence in our chicken-colonizing strains illustrates that genes previously thought important for chicken colonization may be of less relevance when characterizing field strains or alternatively, they may present more genetic diversity than expected. For example, the *ggt* gene, involved in persistence of *C. jejuni* in chicken flocks (Barnes *et al*., 2007), was only found in 16% of all our strains, in accordance with another study (Zautner *et al*., 2011) and only in the 2008 time period. This might be linked to the fact that *ggt* is not absolutely required for strain persistence or alternatively, that the chickens were only recently colonized. Therefore, the exact role of the observed variable genes and their relative importance in natural chicken colonization in many field strains remains to be elucidated.

It was previously reported that arsenic resistance genes differ from strains with different chicken colonization potentials (Ahmed *et al*., 2002) and that poultry isolates present variable levels of arsenic resistance (Sapkota *et al*., 2006; Wang *et al*., 2009). Arsenic compounds are commonly used in poultry for the control of coccidiosis (Li *et al*., 2011), and their impact on *C. jejuni* chicken colonization has yet to be assessed. In the present study, we noted that arsenic resistance genes had a variable presence among the isolates and that their presence in the 2008 strains correlated with the presence of other genes such as *dms*A, *fsp*A, and *rlo*A (see Supplementary Table S2). This suggests that arsenic resistance may only have an indirect role in chicken colonization.

Resistance to kanamycin can be mediated by the plasmid-based gene *aphA-3* in *C. jejuni.* (Gibreel *et al*., 2004). Kanamycin resistance is rarely monitored in *C. jejuni* as it is not the drug of choice for treatment of severe human campylobacteriosis (drugs of choice, in order of importance, are macrolide, quinolone/fluoroquinolone, and tetracycline [Young *et al*., 2007]). Monitoring this resistance is of interest since *C. jejuni* can act as a reservoir of the *aph*A-3 gene and could transfer this resistance to other bacteria. The *tet*O gene, mediating resistance to tetracycline by target protection, is also located on a plasmid. The *tet*O plasmid may be involved in *C. jejuni* episome plasticity and can be present in strains in

the absence of antibiotic pressure (Friis *et al*., 2007). In the current study, *aph*A-3 and *tet*O occurred in 16% and 67% of the isolates, respectively.

For some bacteria, it has been demonstrated that AMR genes may co-localize with virulence genes, making the co-selection of virulent AMR strains possible (Rosengren *et al*., 2009). For *C. jejuni*, involvement of AMR genes in chicken colonization remains to be clarified as the present study was not able to clearly confirm such a link even though some AMR genes weakly correlated with colonization genes.

Distribution of genes conserved in all strains

Among the 254 genes in this study, 94 genes were present in all strains regardless of the isolation year (see Supplementary Table S1). These gene products could be considered as a target of choice for control of *C. jejuni* (e.g., for either vaccination or a novel antimicrobial target). Interesting results were obtained for the chemotaxis gene group where the deletion of the chemotaxis protein *che*Y impaired chicken colonization (Hendrixson and DiRita, 2004), while modification of the regulatory proteins cheB and cheR (Kanungpean *et al*., 2011) or chemotactic receptors (Hendrixson and DiRita, 2004; Vegge *et al*., 2009) increased or lowered the *C. jejuni* virulence-associated phenotype. In our study, the presence of genes coding for most chemotaxis receptors was found to be variable as opposed to the consistent detection of *che* genes. This suggests that different chemoattractant recognition capacities existed in our test strains, potentially leading to different chemotaxis abilities.

Field strain comparison

Chicken colonization is a dynamic process driven by numerous genes. In some cases, strains indistinct by various typing methods presented different phenotypic properties (Malik-Kale *et al*., 2007). Consequently, the higher resolving power of a DNA microarray makes it attractive as a valuable method to compare closely related strains (Taboada *et al*., 2008).

In our study, test strains clustered around two different control strains, 81116 and NCTC11168. The strain 81116 was described as more efficient in chicken colonization than strain NCTC11168 (Ahmed *et al*., 2002). Previous studies found that virulence phenotypes may correlate with genotypes (On *et al*., 2006). The fact that these two reference strains were found in different clusters in our study suggests that different colonization potentials may exist between the two main *C. jejuni* groups. Confirming chicken colonization potential of strains present in both clusters remains to be done.

As expected, it was observed that strains did not cluster according to the time of sampling but rather to lot origin, suggesting that important genes required for chicken colonization may be stable over time. It was previously observed that *C. jejuni* is highly clonal within the same farm (Normand *et al*., 2008). In contrast, some strains from different lots in our study also clustered, which could reflect potential contamination by the same *C. jejuni* source.

Conclusions

Overall, our results illustrate the diversity of gene distribution in field strains over time by detailing the presence of chicken-associated colonization genes in a large chicken-derived *C. jejuni* collection. Our study also raised the possibility to revisit and refine the exact role of some chicken colonization determinants of *C. jejuni* in the context of natural colonization. Despite the genetic diversity of strains collected, clustering indicated the presence of two populations. We propose that this grouping is not associated with the collection time period but that it could correlate with the colonization potential of the chicken-specific *Campylobacter* isolates. Lastly, our results suggest a weak association between AMR and the studied colonization genes in our strain collection.

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Disclosure Statement

No competing financial interests exist.

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