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Fingerprinting of *Campylobacter jejuni* by Using Resolution-Optimized Binary Gene Targets Derived from Comparative Genome Hybridization Studies[▽]

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The aim of this investigation was to exploit the vast comparative data generated by comparative genome hybridization (CGH) studies of *Campylobacter jejuni* in developing a genotyping method. We examined genes in *C. jejuni* that exhibit binary status (present or absent between strains) within known plasticity regions, in order to identify a minimal subset of gene targets that provide high-resolution genetic fingerprints. Using CGH data from three studies as input, binary gene sets were identified with "Minimum SNPs" software. "Minimum SNPs" selects for the minimum number of targets required to obtain a predefined resolution, based on Simpson's index of diversity (*D*). After implementation of stringent criteria for gene presence/absence, eight binary genes were found that provided 100% resolution ($D = 1$) of 20 *C. jejuni* strains. A real-time PCR assay was developed and tested on 181 *C. jejuni* and *Campylobacter coli* isolates, a subset of which have previously been characterized by multilocus sequence typing, *flaA* short variable region sequencing, and pulsed-field gel electrophoresis. In addition to the binary gene real-time PCR assay, we refined the seven-member single nucleotide polymorphism (SNP) real-time PCR assay previously described for *C. jejuni* and *C. coli*. By normalizing the SNP assay with the respective *C. jejuni* and *C. coli* ubiquitous genes, *mapA* and *ceuE*, the polymorphisms at each SNP could be determined without separate reactions for every polymorphism. We have developed and refined a rapid, highly discriminatory genotyping method for *C. jejuni* and *C. coli* that uses generic technology and is amenable to high-throughput analyses.

Campylobacter jejuni and *Campylobacter coli* continue to persist as the most common etiological agents of human bacterial gastroenteritis worldwide. The sporadic nature of campylobacteriosis in humans, the ubiquitous distribution of *Campylobacter* spp. in the environment and in certain foodstuffs, and the lack of a well-understood relationship between genotype and pathogenicity render the utility of routine *Campylobacter* typing a matter of ongoing contention (33). However, typing has demonstrable value in monitoring small-scale *Campylobacter* outbreaks and for examining the genetic diversity and population biology of the species (5, 7, 14, 16, 19, 45).

While several phenotypic and molecular methods have been developed for characterizing *Campylobacter* spp., pulsed-field gel electrophoresis (PFGE) is considered the "gold standard" methodology due to the high degree of resolution obtained with this technique. Despite this, PFGE is limited to specialized applications as it is time-consuming and technically demanding and requires extensive normalization of protocols and genetic profiles to facilitate interlaboratory comparisons (6, 52). In recent years, multilocus sequence typing (MLST) has emerged as a practical approach for examining bacterial epidemiology and population genetics. MLST studies of *C. jejuni* and *C. coli* indicate a weakly clonal population structure generated as a result of high-frequency intraspecific recombination (7, 8, 9, 10, 29, 34). The lack of genetic linkage across the *Campylobacter* genome renders interrogation of multiple loci an effective means of gaining typing resolution, as demonstrated by the addition of the flagellin A short variable region (*flaA* SVR) locus to MLST (9, 12, 30). Consistent with this observation, MLST-*flaA* SVR yields resolution comparable to that of PFGE during examination of *Campylobacter* outbreaks (5, 31, 45).

We have previously pursued a distinctive approach to the development of DNA-based bacterial typing methods, which utilizes computerized analyses of known genetic diversity to identify highly informative sets of polymorphic targets. To date, the software package "Minimum SNPs" has been used to derive minimal sets of single nucleotide polymorphisms (SNPs) from MLST databases (22, 23, 43, 44, 47). "Minimum SNPs" uses Simpson's index of diversity (*D*) (21) to measure the resolving power of SNP sets, termed "high-*D* SNPs," with reference to the entire MLST database of a bacterium. It has been found that seven high-*D* SNPs can provide *D* values of between 0.95 and 0.99, depending on the bacterial species. Bacterial genotyping using real-time PCR has gained substantial popularity in recent years (2, 22, 23, 40, 46, 47), as it provides a flexible, inexpensive, single-step, and rapid means for characterizing an array of polymorphic targets on a single platform. We have previously developed allele-specific (AS) real-time PCR-based methods for interrogating the high-*D* SNPs of *Neisseria meningitidis*, *Staphylococcus aureus*, and *C. jejuni* (43, 44, 47). AS real-time PCR (also known as kinetic PCR) was chosen because this method places minimal con-

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TABLE 1. Primers used for detection of binary genes, *mapA*, and *ceuE* in *C. jejuni* and *C. coli*[a]

Gene	Primer name	Primer sequence (5′→3′)	Primer length (bp)	Amplicon size (bp)
Cj0629	Cj0629-For	CAAAACAATTCGGCAACTTGG	21	51
	Cj0629-Rev	ACTACCATTTTCGAGTTTTATACCAGC	27	
Cj0265c	Cj0265c-For	AAGCGAAAATAACAGGGTTTTGC	23	175
	Cj0265c-Rev	GCTTACCTTATCCCATTTGCCA	22	
Cj0178	Cj0178-For	GAGTGGTTTTGGGCGTGTTAATA	23	102
	Cj0178-Rev	GTTCCCGTTTGTGAATGAAATCTAG	25	
Cj0299	Cj0299-For	GAAAAACTTGGGCGAGTAACGA	22	51
	Cj0299-Rev	GAGAGAAAGTCTCCATAGCCCTTG	24	
Cj1319	Cj1319-For	CACTTTAAATATGCTCGAAGCAGCT	25	80
	Cj1319-Rev	TGCCATAAACTTCGCTTGTTGAG	23	
Cj1723c	Cj1723c-For	AAACCTCTGCAGTTGCGCC	19	56
	Cj1723c-Rev	ATATTGCGGATATACAGGATACGAAGT	27	
Cj0008	Cj0008-For	TGGAAAGTAAAAGATGAAAGCAAGACA	27	122
	Cj0008-Rev	GCATAAAAATCTTTATGGTTTGAGGTG	27	
Cj0486	Cj0486-For	ATTACTAAACAAGAAGAGGGTGCGA	25	56
	Cj0486-Rev	GCTACCAATGCAGCCTGGAT	20	
mapA	*mapA*-For	GCTAGAGGAATAGTTGTGCTTGACAA	26	67
	mapA-Rev	TTACTCACATAAGGTGAATTTTGATCG	27	
ceuE	*ceuE*-For	CAAGTACTGCAATAAAAACTAGCACTACG	29	72
	ceuE-Rev	AGCTATCACCCTCATCACTCATACTAATAG	30	

[a] All primers were designed with a T_m of 59.0°C ± 1.0°C.

straints on assay design and does not require labeled primers or probes.

The impetus for the current study was the recognition that "Minimum SNPs" could be used to derive informative sets of targets from databases other than DNA sequence alignments. The ability to compare multiple *C. jejuni* strains on a whole-genome level using comparative genome hybridization (CGH) DNA microarrays has facilitated identification of gene divergence and conservation patterns throughout the *C. jejuni* genome (3, 11, 25, 26, 41, 42, 51). Additionally, the determination of entire genome sequences for two *C. jejuni* strains (15, 39), coupled with similar undertakings for additional *Campylobacter* species (15), has substantially increased our understanding of these organisms. Genome sequencing and CGH of *C. jejuni* have revealed a largely colinear genome arrangement with few mobile elements or repeat sequences. Scattered within this stable genome "backbone" are regions consisting of multigene insertions and deletions, termed plasticity regions (PRs). The PRs contain genes involved in lipooligosaccharide, flagellin, and capsular biosynthesis; restriction-modification systems; and a large number of open reading frames with hypothetical or unknown functions (41, 51).

To date, whole-genome DNA arrays have been used to compare gene-to-gene differences between 226 *C. jejuni* strains. However, for routine genotyping of *C. jejuni*, genome sequencing and CGH approaches are not feasible. Therefore, the major aim of the current study was to use "Minimum SNPs" to derive from CGH data a minimal set of binary targets (i.e., genes present in some strains but not others, such as those within PRs) from CGH studies that are useful for genotyping.

The second aim of this investigation was to further refine a previously described SNP assay for *C. jejuni*/*C. coli* (43). Interrogation of seven highly discriminatory SNPs (identified using *D*) on the real-time PCR platform with SYBR green I detection, while a cost-effective technology, traditionally requires separate reactions targeting each polymorphism, unlike multiplex-amenable chemistries such as TaqMan (27), LUX (28, 36), or molecular beacons (32). Therefore, we examined a method for reducing the number of reactions required for kinetic PCR.

MATERIALS AND METHODS

***Campylobacter* isolates.** The OzFoodNet collection of 152 *C. jejuni* and two *C. coli* MLST- and *flaA* SVR-characterized isolates was used in this study (37, 43). A subset of these isolates ($n = 84$) have also been subjected to PFGE (37). A further 29 clinical *Campylobacter* isolates were obtained from the Princess Alexandra Hospital (PAH), Brisbane, Australia, collected between 1992 and 2004. All isolates were obtained from fecal samples of patients presenting with gastroenteric symptoms. Additionally, two well-characterized type culture strains, NCTC 11168 and NCTC 11351, were obtained from the Australian Collection of Microorganisms (Brisbane, Australia). Strains were grown and genomic DNA (gDNA) extracted as previously described (43).

Binary gene selection. Data generated from three CGH array studies of *C. jejuni* were used in the analysis (41, 42, 51). Eighteen *C. jejuni* strains were examined in the study by Pearson et al. (41). In addition, the recently genome-sequenced RM1221 strain (15) and CGH data from ATCC 43431 (42) were included in analyses. The presence or absence of CGH genes in RM1221 was tested in silico using the CampyDB BLAST server at *x*BASE (http://xbase.bham.ac.uk/ [4]). Binary genes for ATCC 43431 were assigned as "absent" when the array and PCR data were in complete agreement; otherwise, the genes were converted to "present" by default to minimize the effects of moderately divergent genes on the array signal (42).

The "Minimum SNPs" v2.042 software, described elsewhere (43, 44), was used to identify the minimal number of gene targets required for maximal resolution of the 20 *C. jejuni* isolates. As "Minimum SNPs" was designed to accept data in nucleotide format, the CGH data required conversion into concatenated nucleotide format. To achieve this, "A" was used to designate the absence of a gene within a strain, whereas "T" represented the presence of a gene. Once the data were converted to nucleotide format, the *D* function of "Minimum SNPs" (44) was used to find minimal gene sets.

Binary gene detection. Specific primers for each of the binary genes were identified using the *C. jejuni* NCTC 11168 genome and, where possible, the RM1221 genome. Gene-specific primers were designed using the Primer Express 2.0 (Applied Biosystems, Foster City, CA) and NetPrimer (http://www.premierbiosoft.com/netprimer/index.html/) facilities. All primers have a calculated melting temperature (T_m) of approximately 59.0°C and are listed in Table 1. The binary gene assay was tested on both the RotorGene 3000 (Corbett Robotics, Brisbane, Australia) and the ABI 7300 (Applied Biosystems) real-time

TABLE 2. Primers used for amplification and sequencing of binary genes[a]

Gene	Primer name	Primer sequence (5′→3′)	Primer length (bp)	Amplicon size (bp)
Cj0629	Cj0629gene-For	AAGGTGCAGGAGTAAATATATCTCAAGG	28	440
	Cj0629gene-Rev	AGCATTAGAACGGATAGATCCTGTG	25	
Cj0265c	Cj0265gene-For	TCTTTTGTTGTCCTAGCGACATGT	24	471
	Cj0265gene-Rev	CATAGAACGGAAAATTGCAGGC	22	
Cj0178	Cj0178gene-For	GCAACAAGCACAAGAGAGGGTATAG	25	487
	Cj0178gene-Rev	CCACATATCCATTATTTTCAAGTTCAGT	28	
Cj0299	Cj0299gene-For	AATTGGTTTAAAGAGTATAATACAAGCGG	29	481
	Cj0299gene-Rev	TCATTTCCTTAACACTATTCATTGCTTC	28	
Cj1319	Cj1319gene-For	TCGCTATCCCCTACTCCTACACA	23	489
	Cj1319gene-Rev	TGGAGTATTCCACTCCTGAGCCT	23	
Cj1723c	Cj1723gene-For	AAATTTATCCCTGTCATTTTAGCATGT	27	201
	Cj1723gene-Rev	TGGATAGAGATTTTGAATTTGACTGG	26	
Cj0008	Cj0008gene-For	TGGCAGGATTTCAATCACCAA	21	549
	Cj0008gene-Rev	AATACTGACACTTAAACCATTTTTGCTG	28	
Cj0486	Cj0486gene-For	AATGCGAGTTTTAGAATCAATGCTG	25	482
	Cj0486gene-Rev	GGAGTAGAAACAATGCGCCCTA	22	

[a] All primers were designed with a T_m of 59.0°C ± 1°C.

PCR platforms. The RotorGene 3000 PCR setup was as previously described (43), except that the total number of cycles was reduced from 40 to 30 cycles. For the ABI 7300 apparatus, amplifications were carried out in 96-well plates containing 5 pmol each primer (Sigma-Proligo, Lismore, Australia), 1 μl gDNA, 1× SYBR green I MasterMix (Applied Biosystems), and distilled water to a total volume of 20 μl. Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 30 cycles of 95°C for 15 s and combined annealing and extension at 59°C for 30 s. No-template controls were included in each real-time run for each primer set. For both real-time PCR platforms, dissociation curves spanning 61°C to 95°C were generated following amplification to detect primer dimer interference in no-template controls (seen as a distinct peak at or below 75°C) and to confirm melting profiles of amplicons.

***mapA* and *ceuE*.** To determine the status of each binary gene (present, absent, or intermediate) in the 154 OzFoodNet and 29 PAH isolates and to reduce well usage of a previously described seven-member SNP assay (43), a strategy to normalize the reactions to a standard gene was devised. Membrane-associated protein A (encoded by the 645-bp gene *mapA*) and a periplasmic enterochelin-binding protein (encoded by the 990-bp gene *ceuE*) have previously been described as genetic markers for differentiating *C. jejuni* and *C. coli* from other campylobacters (20, 49). Alignments of *mapA* and *ceuE* sequences from *C. jejuni*, *Campylobacter lari*, *C. coli*, and *Campylobacter upsaliensis* allowed species-specific primer design for *C. jejuni* and *C. coli*. Gene-specific primers for *mapA* and *ceuE* were designed with a T_m of 59.0°C. The *mapA* and *ceuE* primer sequences are listed in Table 1.

Nucleotide sequencing. Sequencing was performed on a subset of the OzFoodNet isolates to define cutoff values for present, absent, and intermediate genotypes of all eight binary genes (GenBank accession no. DQ983332 to DQ983360). Gene-specific sequencing primers (Table 2) were used for amplification and sequencing reactions and were designed to encompass the real-time PCR primer binding sites. MLST and *flaA* SVR sequencing were carried out as previously described (8, 30, 35), and alleles were assigned based on previous database submissions (http://pubmlst.org/campylobacter; http://hercules.medawar.ox.ac.uk/flaA/). Each sequencing mix contained 30 to 60 ng purified PCR product (QIAGEN) and 3.2 pmol of either forward or reverse primer, brought to a total volume of 12 μl with double-distilled water. Sequencing reaction mixtures were labeled using ABI PRISM BigDye Terminator 3.1 chemistry. Products were submitted to the Australian Genome Research Facility (University of Queensland, Brisbane, Australia) for processing.

RESULTS

Binary gene selection. "Minimum SNPs" was applied to the combined CGH data of 20 strains from diverse sources to identify a set of gene targets that provided maximal discrimination of these isolates. Only CGH genes classed as absent or highly divergent (HD) in one or more isolates were included in the "Minimum SNPs" analysis, with moderately divergent genes as designated by Taboada and coworkers (51) excluded. Selection of genes with highly negative log ratios minimizes inter- and intra-array variance and likely represents the loss or acquisition of an entire gene (50). Data from a meta-analysis CGH study of 97 *C. jejuni* strains (including data from references 25, 41, and 42) were used to assess appropriate cutoff values for the 277 genes identified as exhibiting binary variability in the 20 strains (51). Only those genes that were (i) divergent in more than one strain and (ii) considered HD or absent, based on a log ratio (tester signal/*C. jejuni* NCTC 11168 signal) of ≤3.3 for any strains in the data set (51), were assessed further. By these criteria, 87 HD or absent genes were included in the analysis.

Following reduction of the data set to 87 genes, the CGH data for all 20 strains were converted to a "pseudosequence alignment" format to facilitate analysis by "Minimum SNPs." Nonhybridizing genes (i.e., absent or HD) were coded as "A" and hybridizing genes coded as "T." Where the CGH data called a gene as present in a given isolate, this gene was converted to a "T." The resulting binary gene profiles of all 20 strains were subsequently analyzed using the *D* function of "Minimum SNPs." Of the 87 HD and absent genes analyzed, eight targets were identified by "Minimum SNPs" that completely resolved the 20 strains. The binary targets, with their respective cumulative resolution (*D*) and function, are shown in Table 3.

Of the eight binary genes, six were located within previously identified PRs. This was not surprising, as the PRs contain approximately half of the binary genes identified in *C. jejuni* (41). Four of the eight binary genes are absent in RM1221.

Real-time PCR interrogation of binary genes. Real-time PCR allows direct quantitative comparison of PCR amplifications in different templates when normalized to a ubiquitous gene, such as 16S rRNA and *recA* in bacteria (48) or 18S rRNA and β-actin in human tissues (36). In the current study, binary gene status cutoff values were determined by normalization of the binary gene cycle threshold (C_T) with the corresponding *mapA*/*ceuE* C_T of the gDNA preparation. Cutoff values for gene presence were determined using (i) sequence data and

TABLE 3. Binary genes identified in this study

Gene	Cumulative *D*	Presence in RM1221	Function	Qualifiers[a]	Location[b]	Gene length (bp)
Cj0629	0.491	No	Possible lipoprotein	588367–591303	PR8	2,937
Cj0265c	0.766	No	Putative cytochrome *c*-type heme-binding periplasmic protein	244448–245023	NA[c]	576
Cj0178	0.889	Yes	Putative outer membrane siderophore receptor	173764–176031	PR3	2,268
Cj0299	0.947	Yes	Putative periplasmic beta-lactamase	273321–274094	PR4 (PR1)	774
Cj1319	0.971	Yes	Putative nucleotide sugar dehydratase	1248624–1249595	PR12 (PR5)	972
Cj1723c	0.988	No	Putative periplasmic protein	1633895–1634119	PR16 (PR7)	225
Cj0008	0.994	No	Hypothetical protein	12644–14395	NA	1,752
Cj0486	1.0	Yes	Putative sugar transporter	453119–454375	PR6 (PR2)	1,257
mapA		Yes[d]	Outer membrane lipoprotein	960835–961479	NA	645
ceuE		Yes[e]	Periplasmic enterochelin-binding protein	1286672–1287664	NA	990

[a] In NCTC 11168.
[b] As assigned by Taboada et al. (51) and, in parentheses, by Pearson et al. (41).
[c] NA, not applicable.
[d] NCTC 11168 and RM1221 share 99% identity (643/645 bp) at *mapA*.
[e] Thirteen percent divergence between *C. jejuni* and *C. coli*; primers were designed to amplify only *C. coli*.

(ii) the C_T of *C. jejuni* NCTC 11168, which was used as a positive control in the binary gene assays. Genes were called as absent when the ΔC_T (difference in C_T) between the binary gene and *mapA*/*ceuE* C_T values exceeded 10 cycles. The binary gene was designated as "intermediate" if the ΔC_T relative to *mapA*/*ceuE* consistently fell between 3 and 10 cycles. Gene presence was ≤2 cycles of *mapA*/*ceuE*. These criteria were applicable using both the ABI 7300 and RG3000 apparatuses (Fig. 1). Of the eight binary targets, only Cj0629 was found to exhibit an "intermediate" genotype. DNA sequencing revealed that the intermediary genotype of Cj0629 was conferred by several 5′ polymorphisms at the Cj0629-Rev primer binding

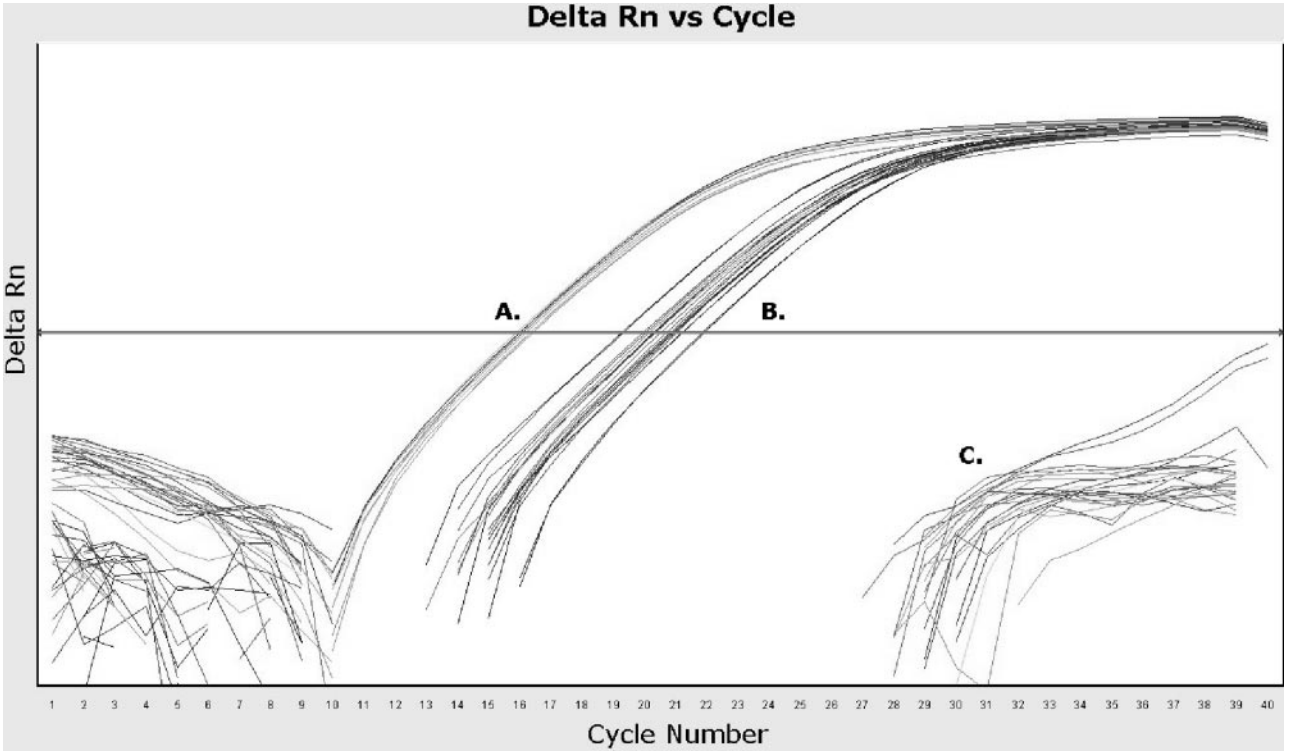

FIG. 1. Real-time PCR amplification plot of the *C. jejuni*/*C. coli* binary gene assay using an ABI 7300 apparatus. The horizontal line on the plot indicates the threshold. The *mapA* or *ceuE* gene indicates whether the isolate is *C. jejuni* or *C. coli*, respectively, and is used to quantitate the binary gene assay. The ΔC_T between *mapA*/*ceuE* and the binary gene is used to quantitatively determine whether the gene is present, absent, or intermediate within a given isolate. (A) *mapA*/*ceuE* and binary gene presence; (B) intermediate binary genotype; (C) binary gene absence and no-template control. Gene presence (binary gene C_T minus *mapA*/*ceuE* C_T), $\Delta C_T \leq 2$; gene absence, $\Delta C_T > 10$; intermediate, ΔC_T between 3 and 10.

site, at positions −16, −22, −23, −24, and −25. In some isolates, the Cj0629-Rev mismatches were coupled with another polymorphism 8 bp upstream in Cj0629-For.

The results of the binary gene typing on 181 out of the 183 isolates are displayed in Table 4. Two PAH isolates were negative for *ceuE* and *mapA* and gave uninterpretable results in the high-*D* SNP assay. Reextraction of gDNA from these isolates yielded a similar result. To confirm the quality of the DNA extractions, 01M28590 and 03M90835 were subjected to a thermophilic *Campylobacter* 23S rRNA gene PCR assay targeting *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* (13). Both isolates were positive for the Therm PCR, indicating that the gDNA extraction was successful. As 01M28590 and 03M90835 are not *C. jejuni* or *C. coli*, these isolates were not examined further.

As controls for the binary gene assay, two type cultures (NCTC 11351 and NCTC 11168) were tested at the eight binary genes. The NCTC 111351 isolate has been characterized by CGH (41), and NCTC 11168 has been used to construct and interrogate *C. jejuni* arrays (3, 11, 25, 26, 41, 42, 51). As expected, the NCTC 11168 strain was shown to possess all eight binary genes by the use of both real-time PCR platforms (Table 4). However, the CGH data and binary gene profiles of NCTC 11351 differed at seven of the eight markers, a result consistent between real-time PCR apparatuses. Although the exact reason for the incongruity could not be determined, MLST of NCTC 11351 confirmed that its genotype was nearly identical to a previous submission of this strain (http://pubmlst.org/campylobacter/).

The eighth binary gene, Cj0486, was the only gene that failed to resolve different strains of the same sequence type (ST). This was an unexpected finding, as Cj0486 is not in close proximity to any MLST locus and resides within PR6 (51). Additionally, RM1221 contains the insertion of a prophage element 3.6 kb downstream of PR6 (15), suggesting that this region is not genetically uniform between strains. It was investigated whether Cj0486 was redundant to the binary gene assay and could be removed from the data set. When Cj0486 was omitted from the OzFoodNet isolate profiles, binary types (BTs) 1 and 2, 17 and 29, 22 and 34, 10 and 33, 16 and 18, and 12 and 15 became indistinguishable from each other (Table 4). Although this removal did not decrease the ability to resolve unrelated isolates (i.e., the BTs still separated unrelated STs coupled with SNP groups), the resolution (*D*) of the binary typing method decreased from 0.934 to 0.897. It was concluded that a set of eight binary markers was identified that provides resolving power complementary to MLST and MLST-derived SNPs.

Performance of the binary markers with a larger CGH data set. Subsequent to development of the eight-member binary gene assay, Champion et al. (3) conducted a large CGH study examining 111 diverse *C. jejuni* strains from environmental, animal, and clinical sources. The performance of the eight binary genes identified in the current investigation was assessed by combining the CGH data for the 20 strains with the larger data set of Champion et al. (3). Binary gene data were obtained for the 111 strains from http://bugs.sgul.ac.uk/bugsbase/tabs/experiment.php (experiment accession: E-BUGS-22) and were assembled into the nucleotide format readable by "Minimum SNPs" as described in Materials and Methods. Four strains, 38857, 40209, 55703, and 59161, were removed from further analysis as they hybridized to all 1,654 NCTC 11168-derived open reading frames and were thus not able to be discriminated from each other or from NCTC 11168. In total, 127 strains were reexamined: 107 from the work of Champion et al. (3), 18 from the work of Pearson et al. (41), one from the work of Poly et al. (42), and RM1221 (15). Of the 696 genes that were binary within the 127-strain data set, 141 genes were found in a single isolate, and a further 149 genes have been classed as moderately divergent by Taboada et al. (51).

Taking these parameters into account, 406 genes were considered HD in the collection of 127 strains and were included for analysis using "Minimum SNPs." With unconstrained parameters, the first binary gene set identified by "Minimum SNPs" required only 12 targets to reach a *D* of 0.9975, and the targets identified are Cj0628 (0.5039), Cj0755 (0.7552), Cj1520 (0.8745), Cj0265c (0.9336), Cj0032 (0.9643), Cj1339c (0.9798), Cj0295 (0.9879), Cj0008 (0.9918), Cj1051c (0.9946), Cj0033 (0.9958), Cj0056c (0.9968), and Cj0618 (0.9975). The resolving power of the original binary gene set was recalculated using the larger CGH data set: Cj0629 (0.4927), Cj0265c (0.7377), Cj0178 (0.8161), Cj0299 (0.8448), Cj1319 (0.8761), Cj1723c (0.9198), Cj0008 (0.9528), Cj0486 (0.956), Cj1520 (0.9783), Cj0755 (0.9875), Cj0151c (0.9926), Cj0032 (0.9954), Cj1133 (0.9968), and Cj0416 (0.9975). It can be seen that two more targets were required to reach a *D* of 0.9975. Interestingly, six markers were shared between the two sets. These results indicate that effective sets of binary markers can be derived from small data sets and that these sets of markers can be progressively improved as additional data become available.

Reanalysis of SNP typing procedure. The utility of SNPs as an alternative to full MLST characterization of *C. jejuni* and *C. coli* has previously been detailed (1, 2, 43). In the study by Price et al. (43), SNPs were interrogated by AS real-time PCR combined with SYBR green I detection. The AS real-time PCR method coupled with SYBR green I chemistry provides a flexible, generic, and cost-effective means of genotyping SNPs, in comparison to labeled primer or probe methodologies (27, 32).

One substantial drawback of the AS real-time PCR procedure is that multiplexing is not feasible, as each AS reaction must be performed separately. Previous researchers (17, 38) have described a method of multiplexing SYBR green I AS real-time reactions by incorporation of GC-rich sequence onto the 5′ end of one AS primer. The addition of a GC clamp to one primer facilitates T_m discrimination of the resultant amplicons. However, incorporating two AS primers targeting the same SNP into a single tube substantially increases the frequency of misprimed PCR products (18), which subsequently amplify exponentially, potentially proving problematic for endpoint analyses such as T_m differentiation. To circumvent issues with mispriming, we investigated an AS real-time PCR method using SYBR green I that did not rely on multiplexing of primers but which reduced the well usage in a fashion similar to that of the GC clamp.

The method involved normalizing the AS reactions for seven SNPs, *asp*A174, *gly*267, *gln*A369, *glt*A12, *unc*A189, *pgm*348, and *tkt*297 (43), with the *mapA* or *ceuE* C_T of *C. jejuni* or *C. coli*, respectively. A similar strategy has been reported by Huygens et al. (23). Because of the high reproducibility of the AS

TABLE 4. Distribution of eight binary genes in 181 *C. jejuni* and *C. coli* isolates[a]

Isolate set and ST[b]	No. of isolates	*flaA* SVR(s)	Species	Clonal complex	Cj0629	Cj0265c	Cj0178	Cj0299	Cj1319	Cj1723	Cj0008	Cj0486	BT	BT with Cj0486 removed
OzFoodNet isolates														
5	3	11	*C. jejuni*	ST 353	I	A	P	A	P	A	A	A	1	1
527	2	11	*C. jejuni*	ST 353	I	A	P	A	P	A	A	A	1	1
21	2	8	*C. jejuni*	ST 21	P	A	P	P	P	P	P	P	8	8
53	1	1	*C. jejuni*	ST 21	I	A	P	P	P	P	P	P	3	3
190	1	1	*C. jejuni*	ST 21	A	P	P	P	P	A	P	P	13	13
569	1	1	*C. jejuni*	ST 21	A	P	P	P	P	A	P	P	13	13
43[c]	1	9	*C. jejuni*	ST 21	P	P	P	P	P	P	P	P	9	9
25	2	1	*C. jejuni*	ST 45	A	A	A	A	P	A	A	A	17	17
45	1	5	*C. jejuni*	ST 45	A	P	A	P	P	A	A	A	19	19
529	1	9	*C. jejuni*	ST 45	A	A	A	P	A	A	A	A	11	11
1616[d]	1	156	*C. jejuni*	ST 403	A	P	A	A	P	A	A	A	20	20
42	2	1, 9	*C. jejuni*	ST 42	A	A	A	A	A	A	A	A	24	24
42	2	9	*C. jejuni*	ST 42	A	A	A	P	A	A	A	A	11	11
48	3	1	*C. jejuni*	ST 48	A	A	P	P	P	A	P	P	22	22
48	11	1	*C. jejuni*	ST 48	I	A	P	P	P	A	P	P	7	7
48	9	1	*C. jejuni*	ST 48	P	A	P	P	P	A	P	P	10	10
50	4	1, 350	*C. jejuni*	ST 21	P	P	P	P	P	A	A	P	21	21
50	3	8, 10	*C. jejuni*	ST 21	P	P	P	P	P	P	A	P	25	25
451	2	1	*C. jejuni*	ST 21	A	P	P	P	P	A	P	P	13	13
536	1	10	*C. jejuni*	ST 21	P	A	P	P	P	A	A	P	14	14
51	1	2	*C. jejuni*	ST 443	A	A	P	P	P	A	A	P	16	16
52	5	4	*C. jejuni*	ST 52	P	A	P	P	P	A	A	P	14	14
161	4	2, 4, 10	*C. jejuni*	ST 52	A	A	P	P	P	A	A	P	16	16
70	1	4	*C. jejuni*	ST 52	P	A	P	P	P	A	A	P	14	14
61	1	14	*C. jejuni*	ST 61	A	A	A	P	A	A	A	A	11	11
227	4	1, 10	*C. jejuni*	ST 206	P	P	P	P	P	A	P	P	26	26
227	1	1	*C. jejuni*	ST 206	P	P	P	P	P	A	A	P	21	21
233	1	1	*C. jejuni*	ST 45	A	P	A	A	P	A	A	A	20	20
197	1	12	*C. jejuni*	ST 257	I	P	P	P	A	A	P	P	4	4
197	1	12	*C. jejuni*	ST 257	A	A	P	P	A	A	A	P	15	15
257	17	1, 2, 4, 8, 12, 20	*C. jejuni*	ST 257	A	A	P	P	A	A	A	P	15	15
532	2	12	*C. jejuni*	ST 257	A	A	P	P	A	A	A	P	15	15
312	1	1	*C. jejuni*	ST 658	A	A	P	P	P	A	P	A	34	22
354	3	18, 20, 37	*C. jejuni*	ST 354	A	A	P	P	P	A	A	P	16	16
528	18	1, 20	*C. jejuni*	ST 354	A	A	P	P	P	A	A	P	16	16
533	1	1	*C. jejuni*	ST 52	I	A	P	A	P	A	A	A	1	1
449	2	14, 33	*C. jejuni*	ST 61	A	A	P	P	P	A	A	A	18	16
531	9	1, 2, 5, 20	*C. jejuni*	NA	A	A	P	P	P	A	A	A	18	16
531	1	20	*C. jejuni*	NA	A	P	P	P	P	A	A	A	23	23
523	3	1, 71, 90	*C. jejuni*	ST 658	I	P	A	P	A	A	P	P	27	27
523	1	1	*C. jejuni*	ST 658	I	P	A	A	A	A	A	P	28	28
523	1	2	*C. jejuni*	ST 658	I	P	P	P	A	A	P	P	4	4
523	1	11	*C. jejuni*	ST 658	I	P	P	P	P	A	A	P	5	5
523	1	71	*C. jejuni*	ST 658	I	P	P	P	A	A	A	P	6	6
524	2	10	*C. jejuni*	ST 353	P	A	P	A	P	A	A	A	30	30
525	6	2	*C. jejuni*	ST 607	A	A	P	A	P	A	A	A	31	31

Continued on facing page

TABLE 4—*Continued*

Isolate set and ST[b]	No. of isolates	*flaA* SVR(s)	Species	Clonal complex	Cj0629	Cj0265c	Cj0178	Cj0299	Cj1319	Cj1723	Cj0008	Cj0486	BT	BT with Cj0486 removed
526	1	3	*C. jejuni*	NA	A	A	A	A	P	A	A	A	17	17
530	2	8	*C. jejuni*	NA	A	A	P	A	P	A	P	A	32	32
530	1	8	*C. jejuni*	NA	P	A	P	P	P	A	P	A	33	10
530	4	8	*C. jejuni*	NA	A	A	P	P	P	A	P	A	34	22
535	1	4	*C. jejuni*	ST 460	A	A	A	A	P	A	A	P	29	17
537	1	11	*C. jejuni*	ST 353	I	A	P	A	P	A	A	P	2	1
538	1	12	*C. jejuni*	ST 45	A	A	A	P	A	A	A	A	11	11
567	1	9	*C. jejuni*	ST 22	A	A	P	P	A	A	A	A	12	15
555	1	16	*C. coli*	NA	A	A	P	P	A	A	A	A	12	15
555	1	16	*C. coli*	NA	A	A	A	P	A	A	A	A	11	11
PAH isolates														
ND	3	17, 30	*C. coli*	ND	A	A	A	P	P	A	A	P	36	35
ND	1	16	*C. coli*	ND	A	A	A	A	P	A	A	A	17	17
ND	1	467	*C. coli*	ND	A	A	A	P	P	A	A	A	35	35
ND	3	16	*C. jejuni*	ND	A	A	P	P	A	A	A	P	15	15
ND	8	36	*C. jejuni*	ND	A	A	P	P	P	A	P	P	22	22
ND	1	36	*C. jejuni*	ND	P	A	P	P	P	A	P	P	10	10
ND	2	36	*C. jejuni*	ND	I	A	P	P	P	A	P	P	7	7
ND	1	222	*C. jejuni*	ND	A	A	P	P	P	A	A	A	18	16
ND	2	57	*C. jejuni*	ND	P	A	P	P	P	A	A	P	14	14
ND	1	18	*C. jejuni*	ND	A	A	P	P	P	A	A	P	16	16
227	1	9	*C. jejuni*	ST 206	P	A	P	P	P	A	P	P	10	10
ND	1	9	*C. jejuni*	ND	P	A	P	P	P	A	P	P	10	10
ND	1	9	*C. jejuni*	ND	P	A	P	P	P	A	A	P	14	14
583	1	239	*C. jejuni*	ST 45	A	A	A	P	A	A	A	A	11	11

[a] Abbreviations: ND, not determined; NA, not applicable; I, intermediate genotype; A, absent genotype; P, present genotype.
[b] Isolates are grouped according to their corresponding seven-member SNP profile (43).
[c] Genome-sequenced strain, NCTC 11168.
[d] NCTC 11351; used in CGH array study by Pearson et al. (41). The ST identity of this isolate was identified as part of this study.

method, it is superfluous to include all AS reactions for polymorphism determination. Using *mapA*/*ceuE* as a "universal control," the difference in cycles (ΔC_T) between the *mapA*/*ceuE* and the AS reactions could be quantitated, allowing polymorphisms to be determined without the need for performing all AS reactions.

Criteria for the reduced SNP interrogation method by normalization to *mapA*/*ceuE* are shown in Table 5. Using the normalization method, the number of reactions required to interrogate the seven-member *C. jejuni*/*C. coli* high-*D* SNP assay decreased from 15 to 9. The two-state polymorphisms at *glyA*267, *pgm*348, and *tkt*297 could be reduced to a single reaction, without affecting polymorphism determination. Similarly, for the three-state SNP *aspA*174, only two of the three polymorphisms require interrogation to confidently determine all polymorphisms. Using *glyA*267 as an example, if only the AS primer *glyA*267-G is tested, and the difference between the AS G reaction and the *mapA*/*ceuE* C_T is ≤ 8, the polymorphism present is A. However, if the difference between the *mapA*/*ceuE* C_T and the G reaction is ≥ 2, the G polymorphism is present in the sample. The criteria are mutually exclusive, thus removing any ambiguity in calling polymorphisms. By using the parameters for *aspA*174, *glyA*267, *pgm*348, and *tkt*297, all polymorphic variants were unambiguously determined.

*uncA*189, *glnA*369, and *gltA*12 differ from *glyA*267, *pgm*348, and *tkt*297 in that there are ΔC_T variations within a single polymorphism, allowing subdivision of polymorphisms into two allelic states (43). For *uncA*189, use of the *uncA*189-C primer in conjunction with *mapA*/*ceuE* permitted discrimination between the two T allelic states (T1 and T2), as well as efficiently called the matched C polymorphism. Similarly, the *glnA*369-T primer was capable of discriminating C, T1, and T2 polymorphic states at *glnA*369. At the *gltA*12 SNP, the A polymorphism could be further subdivided depending on whether it originated from *C. jejuni* (A1) or *C. coli* (A2). However, isolate NCTC 11351 did not fall into the assigned cutoffs for single

TABLE 5. Parameters for reduction of well usage in seven high-*D* *C. jejuni*/*C. coli* SNPs

High-*D* SNP	Reaction(s) required to determine polymorphism	Parameter (*mapA* C_T − AS reaction C_T)
*aspA*174 A/G/T	G+T	A: ≥7 at G; ≥13 at T G: ≤1 at G; ≥9 at T T: ≥9 at G; ≤0 at T
*glyA*267 A/G	G only	A: ≥9 at G G: ≤4 at G
*glnA*369 C/T	T only	C: ≥13 at T T1: ≤11 but ≥6 at T T2: ≤2 at T
*gltA*12 A/G	A+G[a]	
*uncA*189 C/T	C only	C: ≤2 at C T1: ≤9 but ≥4 at C T2: ≥12 at C
*pgm*348 A/G	A only	A: ≤0 at A G: ≥2 at A
*tkt*297 C/T	T only	C: ≥11 at T T: ≤5 at T

[a] Both reactions were required in order to call the genotype.

reaction interrogation of *gltA*12, despite readily calling the G polymorphism when both the *gltA*12-A and *gltA*12-G reactions were performed. To resolve this anomaly, the ST of NCTC 11351 was determined. This revealed that NCTC 11351 shares six out of seven loci with ST 403, except at the *gltA* locus, where it contains a unique allele harboring a single mismatch at position 13 of *gltA*, directly adjacent to the *gltA*12 SNP. The new allele (*gltA*156) and ST (ST 1616) for NCTC 11351 were deposited into the MLST database. MLST was also undertaken on two PAH isolates to confirm the fidelity of the refined SNP interrogation assay (Table 4).

Resolving powers of the different typing methods. Of the 181 *C. jejuni* and *C. coli* isolates used in the current investigation, 154 have previously been genotyped by MLST, *flaA* SVR sequencing, and SNP typing (37, 43). Within these 154 isolates, 84 have also been characterized by PFGE (37). The extensive data generated for these isolates facilitated performance comparisons of binary gene typing both independently and in conjunction with the other genotyping methods. In addition, single and combinatorial methods could be directly compared to PFGE.

As expected, PFGE was the most discriminatory independent method, resolving the greatest number of genotypes (n = 53) within the 84 isolates. MLST generated 32 genotypes corresponding to a D of 0.957 (37). SNP typing yielded fewer genotypes than MLST (n = 20), and *flaA* SVR alone provided the lowest resolution and the fewest genotypes of the three methods (43). Comparison of binary gene data generated in the current study identified 27 genotypes and a D of 0.950 in the 84 isolates, lower than those obtained with PFGE and MLST but higher than those obtained with SNP typing or *flaA* SVR (Table 6).

The combinatorial power of the genotyping methods was also compared to PFGE. MLST in combination with the binary genes provided resolution comparable to that of PFGE and higher than that of MLST-*flaA* SVR in the collection of 84 isolates, despite resolving fewer genotypes. The SNP-binary assay equalled SNP-*flaA* SVR in discriminating the 84 isolates, a trend very similar in the larger collection of 154 isolates. Binary-*flaA* SVR was the most discriminatory of the double genotyping methods and outperformed MLST-*flaA* SVR in both collections of isolates. By utilizing any two genotyping methods in combination, a degree of resolution similar to that of PFGE within the 84 isolates is obtained. When three methods are used in combination (SNP-binary-*flaA* SVR or MLST-binary-*flaA* SVR), the degree of resolution surpasses that of PFGE (Table 6).

It has previously been demonstrated that SNP typing can resolve isolates unrelated by MLST when used in combination with *flaA* SVR sequencing (43). Of the 154 isolates, SNP-*flaA* SVR was unable to resolve only two unrelated STs, STs 533 and 528. In all other cases, unrelated STs sharing SNP profiles could be differentiated when *flaA* SVR was included as an additional locus. It was investigated whether the binary gene assay could replace *flaA* SVR when used in combination with SNP typing. The binary genes performed comparably to *flaA* SVR in differentiating between unrelated isolates sharing the same SNP profile and yielded a D comparable to that for SNP-*flaA* SVR (Table 4). Similarly to SNP-*flaA* SVR, a single circumstance arose (STs 449 and 531) in which unrelated isolates were unable to be differentiated with the SNP-binary gene approach, with the remaining unrelated isolates resolved following the addition of the eight binary genes. The binary gene markers efficiently provide typing resolution that is complementary to the SNP-based genotyping and can be conveniently interrogated on the same platforms as the SNPs.

DISCUSSION

This investigation describes the development and refinement of rapid real-time PCR-based genotyping assays for the food-borne pathogens *C. jejuni* and *C. coli*. Polymorphic targets were identified using a systematic computerized approach that enabled the selection of a small, resolution-optimized set of binary targets from CGH data. We have previously described the derivation of high-resolution SNP sets from MLST databases of *C. jejuni*/*C. coli*, *S. aureus*, and *N. meningitidis* (43, 44). Using "Minimum SNPs," seven high-*D* SNPs were identified from the *C. jejuni*/*C. coli* MLST database that in combination provide a D of 0.98 relative to the MLST database. While the SNP typing method was unable to resolve some isolates belonging to different MLST complexes, the addition of the *flaA* SVR locus to the seven-member SNP profile enabled resolution comparable to that of MLST-*flaA* SVR and clustered isolates in a similar fashion (43).

The advantage of the SNP/*flaA* SVR approach over MLST/*flaA* SVR is the substantial reduction of DNA sequencing performed. However, *flaA* SVR remains sequence based and is therefore not adaptable to real-time PCR or low-density array platforms. In contrast, the binary gene interrogation is inherently simple and can be performed on the same platform as SNP interrogation. A major rationale for this study was to determine whether the binary gene assay could replace *flaA* SVR when used in combination with SNP typing or MLST. It has been shown that the binary genes are appropriate for this purpose. As the binary targets were efficient at adding resolving power to the MLST and SNP profiles, it can be speculated that these markers represent regions of the genome that un-

TABLE 6. Comparative resolution of genotyping methods for *C. jejuni*

Typing method(s)	Value with isolate group:					
	84 isolates[a]		154 isolates[b]		PAH isolates	
	No. of genotypes	*D*	No. of genotypes	*D*	No. of genotypes	*D*
Binary	27	0.950	33	0.934	11	0.883
MLST	32	0.957	40	0.94		
flaA SVR	15	0.877	19	0.858	10	0.812
SNPs	20	0.935	24	0.919	9	0.801
PFGE	53	0.972				
SNPs and binary	37	0.963	47	0.947	13	0.895
SNPs and *flaA* SVR	39	0.963	53	0.952	12	0.826
Binary and *flaA* SVR	45	0.976	64	0.969	14	0.900
MLST and binary	41	0.973	55	0.958		
MLST and *flaA* SVR	45	0.969	63	0.959		
SNPs, binary, and *flaA* SVR	47	0.978	69	0.970	14	0.900
MLST, binary, and *flaA* SVR	51	0.981	75	0.975		

[a] Isolates previously typed by PFGE, MLST, and *flaA* SVR sequencing (37).
[b] Comprising 84 isolates from the work of O'Reilly et al. (37). Isolates were previously typed by SNP typing, *flaA* SVR, and MLST (43).

dergo rapid evolution, similarly to *flaA* SVR. However, the specific mechanisms driving this apparent selective pressure remain to be elucidated.

A very recent *C. jejuni* CGH study (3) used a phylogenomics approach to reveal genetic markers characteristic of isolates from different sources. Two clusters of genetically distinct isolates were identified; one cluster, termed the "livestock" clade, was associated predominantly with poultry, cattle, and sheep, whereas the "nonlivestock" clade comprised isolates primarily from environmental sources. Human campylobacteriosis isolates were distributed between the clades in approximately equal numbers, suggesting transmission to humans from both nonlivestock and livestock origins. The CGH data, in conjunction with phylogenetic analysis, identified a genetic island from Cj1321 to Cj1326 within the O-linked flagellin glycosylation locus whose presence was strongly correlated with the livestock clade but which was predominantly absent in the nonlivestock clade (3). Investigations that are focused on *Campylobacter* transmission from chicken or livestock sources could be readily tailored to include one or more binary genes from the Cj1321-to-Cj1326 flagellin glycosylation locus in the current binary gene assay. An advantage of the computerized approach adopted in this investigation is the ability to directly select particular polymorphic targets of interest or to exclude unsuitable targets according to the requirements of the end user. As an example of this utility of "Minimum SNPs," the Cj0486 gene, which completely correlated with ST identity in the OzFoodNet isolate collection in the current study, may be readily replaced by one of the genes residing in the Cj1321-to-Cj1326 genetic island and the resolution of the new binary gene set assessed. Alternatively, one or more of the genes in the flagellin glycosylation locus may be added to the existing eight-target binary gene assay, or the "include" function of "Minimum SNPs" (43) could be applied to derive a new resolution-optimized set of binary markers that includes the Cj1321-to-Cj1326 locus.

A comparison was made between the original eight binary genes identified using 20 strains (15, 41, 42) and 127 strains, which included the 107 strains from the work of Champion et al. (3). The performance of the eight-binary gene set was compared with that of an unconstrained binary gene set generated from the 127 strains. Unsurprisingly, the original eight binary markers were not as discriminatory when the extra 107 strains were incorporated into the data set, yielding a *D* of 0.956 versus a *D* of 0.9918 for an unconstrained eight-member gene set. However, when "Minimum SNPs" was used to determine resolution of the 127 strains using either (i) the eight binary markers identified in this study or (ii) unconstrained parameters, only two additional binary markers were required to achieve the same resolution between the two pathways. Six of the binary markers were common to both data sets. This finding suggests that the original binary gene set identified from comparative genomics of 20 strains is powerful in resolving much larger data sets. In support of this, 181 isolates were empirically tested at the eight binary genes, and high-resolution fingerprints within and between clonal complexes were observed.

The resolving powers of the genotyping methods for 84 isolates (37) were compared. While the SNP/binary assay performed well against SNP/*flaA* SVR, it was unable to reach the resolution obtained with PFGE. Other real-time PCR-based methods, such as high-resolution melting temperature analysis of hypervariable genetic loci, may be useful additions to the SNP/binary gene assay to augment their resolving power. We are currently developing T_m-based assays targeting the "clustered regularly interspaced short palindromic repeat" region of *C. jejuni* (24) suitable for this purpose. In addition, assessing the performance of the SNP and binary gene typing methods with outbreak isolates will provide valuable information on the utility of these methods for tracing and epidemiology.

In addition to the binary gene assay, we have refined a seven-member SNP typing method for *C. jejuni* and *C. coli* that enables well usage comparable to that of the multiplex TaqMan or molecular beacon systems. The benefits of the refined SNP typing assay are its flexibility and cost. Using the generic SYBR green I dye allows a simple and cost-effective setup in comparison to the multiplexed fluorescent resonance energy transfer-based systems. By normalizing the SNP assay to *mapA*/*ceuE*, each polymorphism at the seven SNPs can be

unambiguously determined and the number of reactions can be reduced from 15 to 9. Only one SNP, *gltA*12, required both reactions to determine whether the A or G polymorphism was present in the gDNA, due to a penultimate mismatch between the *gltA*12-A primer and the NCTC 11351 template.

We have shown that the eight-member binary gene assay, in concert with the refined SNP typing assay, can provide high-resolution bacterial fingerprints of *C. jejuni* and *C. coli* comparable to those for MLST-*flaA* SVR or SNP-*flaA* SVR by using a unified real-time PCR approach. Further advantages of this combinatorial method over sequence-based methods such as MLST and *flaA* SVR include its simplicity, flexibility, robustness, cost-effectiveness, short turnaround time, and high-throughput potential. This approach to the derivation of resolution-optimized sets of binary markers from CGH is straightforward, novel, and applicable to any species for which such data are available.

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