Genetic diversity in Campylobacter jejuni is associated with differential colonization of broiler chickens and C57BL/6J IL10-deficient mice

Wilson, D L ; Rathinam, V A K ; Qi, W ; Wick, L M ; Landgraf, Jeff ; Bell, J A ; Plovanich-Jones, A ; Parrish, J ; Finley, R L ; Mansfield, L S ; Linz, J E

DOI: https://doi.org/10.1099/mic.0.035717-0

Microbiology Papers in Press. Published April 1, 2010 as doi:10.1099/mic.0.035717-0

3-24-10

Genetic Diversity in *Campylobacter jejuni* Is Associated with Differential Colonization of Broiler Chickens and C57BL/6J IL10 Deficient Mice.

Running Title: Genetic diversity and host colonization by Campylobacter

Contents Category: Environmental and Evolutionary Microbiology

David L. Wilson[1], Vijay K. Rathinam[1], Weihong Qi[1], Lukas M. Wick[1], Jeff Landgraf [2], Julia A. Bell[1], Anne Plovanich-Jones[1], Jodi Parrish[3], Russell L. Finley[3], Linda S. Mansfield [1,4,5] and John E. Linz [1,4,6]

1. National Food Safety and Toxicology Center, Michigan State University, East Lansing Michigan, 48823.

2. Research Technology and Support Facility, Michigan State University, East Lansing Michigan, 48823.

3. Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, Michigan 48201

4. Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing Michigan, 48823.

5. Department of Large Animal Clinical Science, Michigan State University, East Lansing Michigan, 48823.

6. Department of Food Science and Human Nutrition, Michigan State University, East Lansing Michigan, 48823.

Corresponding Author: John E. Linz, 234B GM Trout Building, Michigan State University, East Lansing Michigan 48823. Phone, 517-355-8474 x 126; E-mail, jlinz@msu.edu; FAX, 517-353-8963.

Summary

Previous studies demonstrate that C*ampylobacter jejuni*, the leading causative agent of bacterial food borne disease in the US, experiences high frequency genetic variation that is associated with changes in cell surface antigens and ability to colonize chickens. To expand our understanding of the role of genetic diversity in the disease process, we analyzed the ability of three *C. jejuni* human disease isolates (strains 11168, 33292, 81-176) and genetically marked derivatives to colonize Ross 308 broilers and C57BL/6J IL-10 deficient mice. *C. jejuni* colonized broilers at much higher efficiency (all three strains, 23 of 24 broilers) than mice (11168 only, 8 of 24 mice). *C. jejuni* 11168 genetically marked strains colonized mice at very low efficiency (2 of 42 mice); however, *C. jejuni* re-isolated from mice colonized both mice and broilers at high efficiency suggesting that this pathogen can adapt genetically in the mouse. We compared the genome composition in the three wild type *C. jejuni* strains and derivatives by microarray DNA/DNA hybridization analysis; the data demonstrated a high degree of genetic diversity in three gene clusters associated with synthesis and modification of the cell surface structures capsule, flagella, and lipo-oligosaccharide. Finally, we analyzed the frequency of mutation in homopolymeric tracts associated with the contingency genes *wlaN* (GC tract) and *flgR* (AT tracts) in culture and after passage through broilers and mice. *C. jejuni* adapted genetically in culture at high frequency and the degree of genetic diversity was increased by passage through broilers but was nearly eliminated in the gastrointestinal (GI) tract of mice. The data suggest that the broiler GI tract provides an environment which promotes outgrowth and genetic variation in *C. jejuni*; the enhancement of genetic diversity at this location may contribute to its importance as a human disease reservoir.

Introduction

Campylobacter jejuni is a small, motile, ubiquitous, Gram (-) bacterium with a small (1.7 Mbp), AT rich genome (Parkhill *et al*., 2000; Fouts *et al*., 2005). This important foodborne disease agent is associated with 2.4 to 3 million human disease cases in the US each year at an estimated cost of $1.6 to $6.2 billion (reviewed in Snelling et al., 2005; Humphrey et al., 2007; Young et al., 2007). In immuno-competent individuals, *C. jeuni* infection results in a self-limiting enteritis with an average duration of 3 to 5 days. Auto-immune disorders such as Guillain-Barre (GBS) and Miller-Fisher syndromes occur more rarely subsequent to *C. jejuni* infection (1/1000 infections) (Humphrey et al., 2007; Yuki, 2010) and these disorders have been linked to certain serotypes that carry epitopes similar to human gangliosides; this process is known as molecular mimicry (Linton et al., 2000; Guerry et al., 2002; Prendergast et al., 2004; Godschalk *et al*., 2004; van Doorn et al., 2008; Vucic et al., 2009).

Campylobacter establishes a commensal relationship in the GI tract of migratory waterfowl and commercial broiler flocks where it can persist in these animal reservoirs; the pathogen then gains access to food supplies predominantly through the slaughter and processing of broiler chickens (Lee and Newell, 2006; Knudsen *et al*., 2006). Poultry and poultry products therefore represent a major reservoir for human disease (reviewed in Suzuki and Yamamoto, 2009; Horrocks et al., 2009). The prevalence of *C. jejuni* in

commercial broiler flocks has been well documented in Europe, Australia and in the United States (reviewed in Humphrey et al., 2007; Horrocks et al., 2009; Suzuki and Yamamoto 2009).

Despite its high-profile as the leading causative agent of foodborne bacterial gastroenteritis, the mechanisms by which *C. jejuni* causes disease have proven difficult to define and relatively few virulence factors (eg. flagella, cytolethal distending toxin, FspA (Poly *et al*., 2007) have been identified. However, recent studies demonstrate that homopolymeric tracts associated with several loci in the *C. jejuni* genome (Parkhill et al., 2000) are subject to high frequency mutation (as high as 1 in every 2 cells in a population) and that these genetic changes likely arise by slip strand mutation (Bernatchez et al., 2007; Linton et al., 2000; Guerry et al., 2002; Prendergast et al., 2004; Hendrixson, 2006). Many of these loci are associated with the synthesis and modification of key surface antigens including capsule, flagella, and lipo-oligosaccharide (LOS); their expression appears to be regulated by phase variation prompting their characterization as "contingency genes" (Guerry et al., 2002. Prendergast et al., 2004; Moxon, 2006; Hendrixson, 2008; van Alphen, 2008). The ability to control the conformation and antigenicity of surface structures via mutation strongly suggests that this genetic diversity could affect host colonization and disease. In support of this idea, others observed that mutations in specific contingency genes affect the ability of *C. jejuni* to invade epithelial cells (*in vitro*) (Guerry et al., 2002), affect the antigenicity of specific cell surface molecules (glycoproteins) associated with molecular mimicry both *in vitro* and in experimental human infection (Bernatchez et al., 2007; Linton et al., 2000; Guerry et al., 2002; Prendergast et al., 2004), and affect the colonization of chickens (Ashgar et al., 2007; Hendrixson, 2008). Several groups also demonstrated that *C. jejuni* genotype can impact colonization of the GI tract of poultry (Ahmed et al., 2002; Hook et al., 2005; Coward et al., 2008; Ridley et al., 2008) and that passage through poultry can impact both genotype and colonization of poultry (Cawthraw et al., 1996; Wassenaar et al., 1998; Ringoir and Korolik., 2003; Jones et al., 2004; Kakuda and DiRita, 2006).

In order to understand the ability of *C. jejuni* to cause disease in a human host, we believe that it is important to understand the mechanisms by which *C. jejuni* establishes a reservoir in poultry, how the organism is transferred from the reservoir to the human host, and how growth in the reservoir affects subsequent ability to colonize and cause disease in the host. Based on the discussion above, we hypothesized that rapid mutation in contingency genes generates genetic diversity in *C. jejuni* sub-populations that enhances the ability to colonize poultry and to subsequently colonize and cause disease in humans. To begin to test this hypothesis, we analyzed the ability of 3 *C. jejuni* human disease isolates (11168, 33292, 81-176) and derivatives to colonize broiler chickens (Ross 308) and our mouse model for human disease (C57Bl/6J IL-10 deficient mice) (Mansfield et al., 2007). We then compared the genetic diversity in the genomes of these three isolates and derivatives (microarray DNA/DNA hybridization analysis) and analyzed genetic changes that specifically occurred in the homopolymeric tracts in two contingency genes (DNA sequence analysis) in culture, in the poultry reservoir, and after passage through mice.

Methods

***C. jejuni* Culture and Growth Conditions.** Glycerol stocks of *C. jejuni* 11168, 81-176, 33292, and genetically marked derivatives were streaked onto Trypticase Soy Blood Agar (TSBA) and incubated at 37ºC under microaerophilic conditions (10% CO_2, 10% H_2, and 80% N_2) in BBL anaerobic jars (Becton, Dickinson and Company, Franklin Lakes, NJ) . The cultures were incubated for 16 to 24 hours, harvested with a sterile dacron or cotton swab (Fisher Scientific), and resuspended to a concentration of 1 x 10^9 CFU/ml in Trypticase Soy Broth (TSB). 100 µl aliquots were plated onto TSBA and the plates incubated at 37ºC under a microaerophilic atmosphere of 10% CO_2, 10% H_2, and 80% N_2. The culture was incubated for approximately 12 hours, harvested with a sterile Falcon cell scraper (Becton, Dickinson and Company) and suspended in TSB as either a single culture or a co-culture of two strains to a concentration of 5 x10^{10} cfu ml^{-1} for mouse inoculation or 5 x10^8 cfu ml^{-1} for chicken inoculation. DNase I (Roche) was added to a concentration of 1U μl^{-1} in co-cultures to prevent DNA transfer prior to inoculation. This inoculum culture was immediately placed on ice prior to oral gavage.

At day of necropsy, cecal tissue was excised, placed in sterile PBS, and its contents gently removed. Cecal tissue was weighed and separated into halves. One half of the tissue was dried at 75º C for two days to determine tissue dry weight. The second half of the tissue was homogenized in 150 to 300 µl of TSB, serially diluted, and plated onto TSBA supplemented with 10 µg ml^{-1} cefoperazone, 20 µg ml^{-1} vancomycin, and 2 µg ml^{-1} amphotericin B (CVA). Chloramphenicol (20 µg ml^{-1}) or kanamycin (30 µg ml^{-1}) was added to TSBA-CVA when appropriate. Culturing for *C. jejuni* colonization prior to necropsy of mice was performed by collecting fecal pellets directly from mice. Fecal pellets were homogenized in TSB, a portion dried as above, and a sample serially diluted and plated onto TSBA-CVA. Cultures recovered from animals were incubated at 37ºC under standard microaerophilic conditions as described above for 72 hours.

Genetically Marked *C. jejuni* Strains. Genetically marked *C. jejuni* strains were derived from 11168 through homologous recombination with transposed chromosomal DNA or with a pUC- based suicide vector. DNA was introduced into competent *C. jejuni* by electroporation as described in Wilson *et al*., 2003. Recombination occurred by double-crossover of a kanamycin or chloramphenicol marker (Wilson *et al*., 2003) with one integration per mutant as demonstrated by Southern blot (data not shown).

Integration of the kanamycin marker into the *C. jejuni* 11168 chromosome was accomplished via the pJB23SK suicide vector described in Wilson *et al*. (2003). The suicide vector pDW*hip*OK, produced by methods similar to those utilized by de Boer *et al*. (2002), was used to introduce the kanamycin marker into the *C. jejuni* 11168 *hip*O chromosomal locus. Briefly, primers JL1100 (5'-AGAGCTAATGATACCCTACC-3') and JL1101 (5'-ATGCTATTGTCTGGAGGAGC-3') were used to amplify the 11168 *hip*O locus (1.9 kb), which was subsequently cloned into pCR2.1-Topo (Invitrogen) and electroporated into EC TOP10 (Invitrogen) according to manufacturer specifications. An 1.9 kb *EcoRI* fragment containing the *hip*O gene was excised from the above engineered plasmid, ligated into the *EcoRI* site of pBluescript II SK(+) (Stratagene), and transformed into *E. coli* DH5α (Life Technologies) to produce plasmid pDW*hip*O. The *Campylobacter aphA*-3 kanamycin marker (Trieu-Cuot *et al*., 1985) was excised from

pILL600 by *SmaI* and blunt-end ligated into the *SphI* site of pDW*hip*O (polished with T4 DNA polymerase), generating pDW*hip*OK. Both pDW*hip*OK and pJB23SK were amplified in *E. coli* DH5α before electroporation into *C. jejuni*.

Mice and Chickens. C57BL/6J IL10 deficient (IL10 $^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and a breeding colony established in a *Campylobacter*/*Helicobacter* free facility with autoclaved food, bedding, and water at Michigan State University (MSU) (Mansfield *et al*., 2007).

Ross 308 broiler eggs were acquired from a commercial hatchery that supplies fresh poultry to the Chicago and Detroit markets via regional grow-out farms and a centralized processing plant. Eggs were incubated for 18 days at the MSU Poultry Farm before transfer to a portable Brower Incubator (Houghton, IA) at the University Research Containment Facility (URCF). Eggs were sanitized in a dilute bleach solution prior to setting at URCF. Hatch occurred at about 3 days post-setting and chicks were housed in a single isolator for about 1 day before oral gavage. Chickens were provided a commercial high-protein chicken feed (Broiler Starter and Grower W/Maxiban 72 – medicated for prevention of coccidiosis), and sterile water *ad libitum*. Chicken feed was irradiated at a target dose of 1 mrad. Chickens, like mice, were housed in separate cages to maintain isolation of each individual *C. jejuni* population.

Animals were inoculated with 200 µl of culture through a 5.0 French feeding tube/catheter (Sovereign, Mansfield, MA) and a one ml Luer-Lok syringe (Becton, Dickinson, and Co.). All animal protocols were reviewed and approved by the MSU All University Committee on Animal Use and Care.

Microarray DNA-DNA Hybridization Analysis. Gene specific primers were designed using Primer 3 software and used to amplify 1,685 predicted ORFs in the *C. jejuni* 11168 sequence (ftp://ftp.sanger.ac.uk/pub/pathogens/cj/) carried in the Sanger Center and TIGR database. *C. jejuni* 11168 chromosomal DNA was used as template for PCR to produce a clone library which was subsequently re-amplified; the PCR products were ethanol precipitated and resuspended in 3x SSC print buffer. DNA representing each clone was spotted in triplicate on UltraGAPS slides (Corning) using a GeneMachines Omnigrid 100 robot (San Carlos CA) and Telechem Chipmaker (Sunnyvale CA). Test and reference sample DNA was sonically sheared to produce a majority of fragments between 500 bp and 2,000 bp and labeled with Cy3 and Cy5 fluorescent dyes (Amersham, Louisville CO).

Each sample consisted of a combined statistical analysis of four chips that included two chips for biological replicates and two chips for dye reversals of test and reference chromosomal DNA. Each chip contained triplicate representation of the *C. jejuni* chromosome. Competitive hybridizations were performed in a SlideHyb (Ambion, Austin, TX) at 54°C followed by post hybridization washes at 42°C. Chips were scanned for fluorescent signal with an Affymetrix 428 scanner (Santa Clara, CA). Hybridization data were analyzed as the distribution of the two-color signal ratios by using GACK (Genomotyping Analysis by Charlie Kim) (Kim *et al*., 2002). The GACK program uses the shape of the $\log_2$ fluorescent signal distributions to assign estimated probability of presence (EPP) values to genes. For each array, analysis of the $\log_2$ (test strain/reference strain signals) distribution of good probes was performed.

DNA Sequence Analysis. The chromosomal DNA of *C. jejuni* cultures recovered from broilers and mice was extracted using Easy-DNA (Invitrogen, Carlsbad, CA) according to manufacturer recommendations. DNA concentration was determined with a Nanodrop ND-1000 spectrophotometer (Wilmington, DE), and 200 - 300 ng amplified with the high-fidelity *Pfx50* DNA Polymerase (Invitrogen). Primers JL 1273 (5'TGC TGG GTA TAC AAA GGT TGT G 3') and JL 1274 (5' GTG CTA AAG TAG CAA CTT CAC C 3') were used to amplify a 415 bp fragment of *wlaN*. Primers JL 1225 (5' GAG CGT TTA GAA TGG GTG TG 3') and JL 1226 (5' GCC AGG AAT TGA TGG CAT AG 3') were used to amplify a 390 bp fragment of *flgR*. PCR concentrations were as follows: 1x *Pfx50* PCR buffer (Invitrogen), 0.2 mM (each) dNTP, 0.5 pmol of each primer/µl, 0.1 unit polymerase/µl. The PCR thermocycling included an initial denaturation at 94°C for 2 min, followed by 35 cycles that included DNA annealing at 60° C for 30 s, polymerization at 68°C for 30 s, and denaturation at 94°C for 30 s. A final polymerization at 68°C for 5 min was performed. In preparation for TA cloning, each PCR reaction was incubated with 0.5 units of Taq DNA Polymerase (Invitrogen) for an additional 10 min after thermocycling, and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA).

PCR products were cloned into the pCR 4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen) and transformed into One Shot TOP10 Competent *E. coli*. For each PCR and cloning reaction, plasmids from 20 clones were prepared using standard alkaline lysis (Ausubel *et al*., 2009) and purified with Wizard Plus SV Miniprep Columns (Promega, Madison, WI). Plasmid insert DNA was sequenced in both directions using T3 and T7 primers and either an ABI 3730 Genetic Analyzer or an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) through the Research Technology Support Facility at Michigan State University.

Results

***C. jejuni* Colonization of C57BL/6J IL10 Deficient (IL10$^{-/-}$) Mice.** In initial pilot experiments, *C. jejuni* failed to colonize C57BL/6J IL10 deficient mice on a consistent basis. We hypothesized that single colony isolation during preparation of the mouse inoculum may have introduced a bottleneck in the genetic diversity of the *C. jejuni* population thereby reducing colonization potential and generating inconsistencies in the colonization data. We eliminated the single colony isolation step during inoculum preparation for all strains and achieved consistent colonization data with strain 11168; this change in protocol did not result in colonization by strain 33292 or 81-176.

Three sets of eight C57BL/6J IL10 deficient mice were inoculated with *C. jejuni* strains 11168, 33292, and 81-176. Each set (4 male and 4 female) was inoculated with one strain and each mouse received an inoculum of 10^{10} CFU. Two control mice received TSB only. Colonization was monitored by fecal culture on Days 1, 4, 7, 10, and 13. Mice were necropsied on Day 14 and colonization assayed by cecal culture. Control mice were culture negative at all time points. At Day 1 (24 h), all 24 mice inoculated with *C. jejuni* were culture positive. On average, mice carried 11168, 33292, and 81-176 at 29,000 $\pm$ 8,400, 1,300 $\pm$ 540, and 1,500 $\pm$ 790 CFU mg^{-1} dry fecal mass, respectively (mean $\pm$ standard error). Mice inoculated with *C. jejuni* 33292 and 81-176 were culture negative on Days 4, 7, 10, 13, and 14, with the exception of a single mouse inoculated

with 33292 that carried 4 CFU mg^{-1} of dry fecal mass on Day 7. The limit of detection during culture ranged from 160 to 400 CFU $gram^{-1}$ of dry fecal or cecal mass based on the negative culture data from control mice.

All eight mice inoculated with *C. jejuni* 11168 were culture positive at each time point analyzed (Fig. 1). Differences in colonization level between individual mice inoculated with 11168 fluctuated over the 14 Day period. At Day 1, colonization level in individual mice ranged from 8.3×10^2 to 7.1×10^4 CFU mg^{-1} of dry fecal mass, a difference of approximately 100 fold. The difference in colonization level peaked at Day 7, with a range from 4.7×10^0 to 1.3×10^5 CFU mg^{-1} of dry fecal mass (a difference of approximately 10,000 fold). The diversity of colonization levels between individual mice inoculated with 11168 was again demonstrated during examination of cecal tissue at necropsy with a range of colonization from 8.9×10^1 to 3.5×10^5 CFU mg^{-1} of dry cecal mass. *C. jejuni* isolates 11168-728 and 1168-732 were obtained from individual colonized mice and used for DNA analysis because of their extreme differences in colonization level. On Day 1, 11168-728 and 11168-732 each carried 2.0×10^4 CFU $mg^{-1}$of dry fecal mass (*C. jejuni*). On Day 14, 11168-728 (cecal sample) carried 3.1×10^5 CFU mg^{-1} dry cecal mass (*C. jejuni*) while 11168-732 (cecal sample) carried 8.9×10^1 CFU mg^{-1} of dry cecal mass, a greater than 3,000 fold difference in population level.

***C. jejuni* Colonization of Ross 308 Broilers.** The lack of colonization of mice by *C. jejuni* 33292 and 81-176 prompted us to determine if these strains were able to efficiently colonize broiler chickens, an important human disease reservoir. Three sets of eight Ross 308 broiler chickens were inoculated with *C. jejuni* strains 11168, 33292, and 81-176. Each set was inoculated with one strain and each chicken received an inoculum of 10^8 CFU. Chicken experiments were performed subsequent to mouse studies, and chicken doses were reduced to 10^8 CFU after it was demonstrated by Mansfield *et al.*, (2007) that there was not a significant difference in colonization between C57BL/6J IL10 deficient mice inoculated with 10^8 and 10^{10} CFU of *C. jejuni*. Six control chickens received TSB only. Colonization was monitored by cloacal swab on Days 1, 4, 7, and 10. Chickens were necropsied on Day 14 and colonization assayed by cecal culture. Chickens, unlike mice, produce a watery feces which is difficult to collect from the bird, thus cloacal swabs were used to assay colonization. Cloacal swab data were reported as colonization positive or negative and they were not normalized to fecal mass.

Control chickens were culture negative at all time points. Ross 308 broilers inoculated with *C. jejuni* were all culture positive on Day 1 post-inoculation. Twenty one of twenty four broilers inoculated with *C. jejuni* were culture positive at all time points analyzed. A single Ross 308 broiler (17) inoculated with strain 81-176 was culture negative (cloacal swab) at Days 7 and 10, however, this bird carried a low concentration of 81-176 (5×10^{-1} CFU mg^{-1} dry cecal tissue) on Day 14. Two chickens (24 and 25) inoculated with strain 33292 were culture negative on Day 4, and a single chicken (27) inoculated with this strain was culture negative on Days 7 and 10, and 33292 was not detected in the cecum sample at Day 14. Chickens 24 and 25 on Day 14 carried *C. jejuni* at concentrations of 1.4×10^3 and 3.5×10^3 CFU mg^{-1} dry cecal tissue, respectively. The limit of detection for cecal culture of chickens was similar to that of mice.

At Day 14 post-inoculation all birds inoculated with *C. jejuni* strain 11168 were culture positive (cecal samples; Fig. 2). The range of 11168 colonization levels in

chickens was 5.2 x 10^4 to 1.6 x 10^6 CFU mg^{-1} dry cecal tissue, while the 11168 colonzation levels in mice varied by approximately 1,000 fold (see above); the range in colonization levels in chickens was 100 fold greater than in mice. While strains 81-176 and 33292 failed to colonize mice they successfully colonized chickens; colonization ranged from 6.7 x 10^2 to 9.2 x 10^3 CFU mg^{-1} dry cecal tissue in 14 of 16 chickens.

Colonization of Ross 308 Broilers and C57BL/6J IL10 Deficient Mice by Genetically Marked *C. jejuni* 11168. A long-term goal of our research is to understand the process of natural transformation and its role in the generation of genetic diversity in *C. jejuni* strains. To this end, we examined the ability of populations of genetically marked progeny derived from the same parent to colonize chickens and mice. Genetically marked strains (chloramphenicol resistant and kanamycin resistant populations) derived from *C. jejuni* 11168 were co-inoculated into 42, C57BL/6J IL10 deficient mice (Table 1). At approximately one day post-inoculation all mice were culture positive for C*ampylobacter*, however, by day four 39 of 42 (93%) mice were culture negative, and by day seven 40 of 42 (95%) mice were culture negative for *Campylobacter*. Two mice remained culture positive on Day 14 (necropsy). Mouse 712 carried a chloramphenicol resistant population (MATn5CamR6) at a concentration of 4.9 x 10^5 CFU mg^{-1} dry cecal tissue and Mouse 682 maintained a kanamycin resistant population (MA*23S*KanR4) at a concentration of 2.4 x 10^5 CFU mg^{-1} dry cecal tissue.

In a subsequent experiment, the MATn5CamR6 and MA*23S*KanR4 cultures recovered from mouse cecal tissue were co-inoculated into eight C57BL/6J IL10deficient mice (Table 1). All mice inoculated with these mouse-adapted *C. jejuni* cultures were *Campylobacter* positive at every time point examined and carried 2.1 x 10^1 to 1.9 x 10^5 CFU *C. jejuni* mg^{-1} dry cecal tissue (necropsy), a range of approximately 10,000 fold (Fig. 3). Kanamycin resistant *C. jejuni* cultures were not recovered from cecal tissue in these mice, and serial dilutions of recovered cultures gave similar *C. jejuni* densities on TSBA-CVA and TSBA-CVA plus chloramphenicol, indicating that a majority if not all of the population recovered was chloramphenicol resistant. Six control mice (TSB only) were culture negative for *C. jejuni* at all time points. MATn5CamR6, MA*23S*KanR4, and 11168-Tn5CamR2 were also inoculated as individual cultures into four C57BL/6J IL10 deficient mice per strain. At Day 14 post-inoculation, all eight mice inoculated with the mouse-adapted cultures were culture positive for *C. jejuni* and carried 1.5 x 10^2 to 1.5 x 10^6 CFU mg^{-1} dry cecal tissue (Fig. 3). Three of the four 11168-Tn5CamR2 inoculated mice were culture negative for *Campylobacter* on Day 14 (cecal culture); the fourth mouse carried 3.0 x 10^4 CFU/mg dry cecal tissue (Fig. 3). Two control mice (TSB only) were culture negative for *C. jejuni* at all time points.

C. jejuni 11168-Tn5CamR2 and 11168-*23S*Kan4 were inoculated as individual cultures into seven Ross 308 broilers each. Broilers were assayed for *C. jejuni* by cloacal swab culture on Days 1, 4, 7, and 10, and by cecal culture on Day 14. The chickens were culture positive on all days examined. Each chicken carried between 9.7 x 10^0 to 3.2 x 10^5 CFU mg^{-1} dry cecal tissue (Fig. 3). Five control broilers (TSB only) were culture negative for *C. jejuni* at all time points.

Microarray DNA-DNA Hybridization Analysis. We conducted microarray analysis to explore whether genetic diversity might at least in part explain the observed differences

in colonization levels between different *C. jejuni* strains inoculated into mice (comparison of 11168, 81-176, and 33292 for example) and between high and low level colonizing isolates of the same strain (11168-728 and 11168-732) obtained from mice. Chromosomal DNAs isolated from "test" strains were fluorescently labeled and used as probes in a competitive hybridization against a labeled chromosomal DNA probe from 11168, the "reference" strain. PCR products from 1681 ORFs representing the entire 11168 genome were printed in replicate on the microarray slide and these served as a reporter target for hybridization. Three adjacent gene clusters demonstrated a high degree of genetic diversity between 11168, 33292, and 81-176 (Fig. 4). The first of these clusters, a protein glycosylation (PG) gene cluster, lies adjacent to the second cluster, a LOS biosynthesis cluster. Together they form one "locus" called PGLOS. This "locus" comprises approximately 33,600 bp in the 11168 chromosome; the ends of the locus are delineated by the genes *wlaM*/*pglG* (gene position 1086) and *waaD*/*hldD* (gene position 1116). The capsule biosynthesis locus (CAP) is approximately 42,600 bp in length whose ends are defined by the gene *kpsS* (gene position 1373) at one end and *kpsM* (gene position 1408) at the other. The PGLOS locus has a GC content of 29%, compared to 30% to 31% for the equivalent size regions flanking PGLOS and the CAP locus has a GC content of 28% compared with 30% in the flanking sequences. The GC content of the entire 11168 chromosome is 31% (Parkhill *et al*., 2000).

In contrast, over 99% of the 1,681 ORFs analyzed in the isolate of the high efficiency mouse colonizer 11168-728 (Fig. 1) exhibited high sequence similarity ($\geq$ 99.5% EPP) to ORFs in the 11168 reference strain (original inoculum). Similarly, over 97% of ORFs in the isolate of the low efficiency colonizer 11168-732 demonstrated high sequence similarity ($\geq$ 99.5% EPP) to the 11168 reference strain. Of particular significance, very few significant genetic differences were observed between 11168-728 and 11168-732 within the 3 gene clusters discussed above.

Analysis of Homopolymeric Tracts in Contingency Genes. To further explore the potential role of genetic diversity in the ability of *C. jejuni* to colonize broilers and mice, we analyzed the frequency of slip-strand mutations in two genes, *wlaN* and *flgR*, shown to carry homopolymeric tracts (Parkhill *et al*., 2000), as indicators of genetic diversity.

The *C. jejuni* gene *wlaN* encodes a 1'3 -linked galactosyltransferase involved in LOS biosynthesis (Linton *et al*., 2000). If *wlaN* expression or enzyme activity is blocked, the LOS structure lacks a terminal galactose residue. Wild type *wlaN* generates a LOS structure that carries a terminal galactose and mimics human ganglioside GM_1 resulting in an increased risk for GBS in infected patients. Linton *et al.* (2000) demonstrated that WlaN enzyme activity is subject to phase variation (high frequency genetic event that alters gene expression or protein activity) by slip-strand mutations (see below) that occur at very high frequency within a homopolymeric (poly G) tract in the *wlaN* open reading frame. We measured mutation frequency in *wlaN* as an indicator (sentinel) of the degree of genetic diversity in CJ populations; this is particularly relevant because this genetic change appears to have a direct impact on human health. *wlaN* carries a homopolymeric tract of 8 G residues.

flgR encodes one protein in a two component regulatory pathway that controls flagellar synthesis and modification (Hendrixson, 2006). The homopolymeric sequence in *flgR* consists of 4, A/T rich stretches (7 to 8 residues each) within a 50 bp fragment. Slip-

strand mutations in either gene eliminate protein activity. We purified genomic DNA from *C. jeuni* populations isolated from: 1) wild type 11168 broiler inoculum (CI); 2) wild type 11168 mouse inoculum (MI); 3) wild type 11168 colonized broilers (4 birds, C4, 5, 7, and 11); and 4) wild type 11168 colonized mice (4 mice, M51, 719, 728, and 732). DNA (400 bp) surrounding the *wlaN* homopolymeric tract was amplified (PCR), the PCR fragment ligated into plasmid PCR4-TOPO, and the recombinant plasmid was transformed into *E. coli*. The *wlaN* region carried in twenty independent plasmids derived from each of the 10 PCR fragments (200 plasmids total) were subjected to DNA sequence analysis (Fig. 5). In "wild type" *C. jejuni* 11168 animal inocula, 30 to 40% of the *wlaN* clones carried a single base insertion in the *wlaN* homopolymeric tract (+1) while 60 to 70% of the clones carried wild type *wlaN*. These data suggest that nearly 1 in every 2 bacterial cells in the so-called "wild type" inocula carried a mutation in the *wlaN* homopolymeric tract. In contrast, in *wlaN* clones obtained from 4 different broilers (80 plasmids total), nearly 80% of the clones carried a single base insertion (+1), 5% carried two base insertions (+2), 2% carried single base deletions (-1), and 13% were wild type in *wlaN*; the (+2) and (-1) mutant categories were not observed in the original broiler *C. jejuni* inoculum (CI) suggesting that they either originated or became enriched in the broiler GI tract. Of particular interest, in *wlaN* clones obtained from 4 different colonized mice (80 plasmids total), the vast majority (>90%) carried the wild type *wlaN* homopolymeric tract. Only a small number of mouse *wlaN* clones (<10%) carried single base (+1) insertions and these occurred in only 1 of 4 mice.

In *wlaN* clones prepared from genomic DNA from *C. jejuni* 11168 Tn5CamR2 animal inocula (that colonized only 1 in 24 mice), the vast majority of *C. jejuni* isolates (93%) carried the wild type *wlaN* hompolymeric tract. Since the identification of marked strains during strain construction (such as Tn5CamR2) requires single colony isolation, these data provide strong supporting evidence that single colony isolation of *C. jejuni* reduces the overall genetic diversity in the population and reduces ability of *C. jejuni* to colonize mice.

A similar analysis was conducted on the *flgR* homopolymeric sequence in genomic DNA prepared individually from mouse inoculum (MI), chicken inoculum (CI), 2 mice (M728 and M732) and 1 broiler (C4). Genetic variation in *flgR* was observed at lower frequency overall than in *wlaN*. A single base deletion (-1) was observed in 5% of the *flgR* clones in MI and C4. No mutations were observed among 40 *flgR* clones prepared from *C. jejuni* populations in the 2 mice or in the animal inoculum. The data generated from this second contingency gene tend to support our observations based on *wlaN*. Controls demonstrated that genetic variation in *wlaN* and *flgR* occurred within *C. jejuni* and not during cloning in *E. coli*.

Discussion

Genetic Diversity and Host Colonization. In the current work, we generated several independent lines of evidence that support our research hypothesis; that is that genetic diversity in sub-populations of *C. jejuni* enhances the ability to colonize the host animal. Previous studies demonstrated a high mutation frequency in specific contingency genes and support a role for the resulting genetic diversity in the ability to invade INT407 cells, to modify the host immune response (molecular mimicry) in experimental human

infection (Guerry et al., 2002; Prendergast et al., 2004), and to colonize chickens (Ashgar et al., 2007; Hendrixson, 2008). Our work expands on these previous studies by demonstrating a strong correlation between the degree of genetic diversity in *C. jejuni* populations and the differential ability to colonize broilers and mice.

1) Genetic Diversity within Gene Clusters Required for Synthesis/Modification of Cell Surface Structures. The three *C. jejuni* human disease isolates chosen for these analyses (strains 11168, 33292, 81-176) colonized Ross 308 broilers at higher frequency (all birds) and efficiency (higher cfu/g cecal material) than our mouse model for disease (C57 BL/6J IL-10 deficient mice). In general, 11168 colonized all mice and broilers, whereas 33292 and 81-176 colonized all broilers, but failed to colonize mice. Microarray DNA-DNA hybridization analysis demonstrated genetic diversity between each of the 3 *C. jejuni* strains within gene clusters associated with the synthesis and modification of the cell surface structures, flagella, capsule, and lipo-oligosaccharide. Although these analyses did not tell us the exact nature of the genetic changes that occurred within these gene clusters at the DNA sequence level, the data do strongly suggest that; 1) the degree of DNA sequence divergence between these 3 strains within these 3 gene clusters is large as evidenced by the fact that the genetic differences can be detected reproducibly by a relatively insensitive tool (the probability of presence of a particular gene sequence as measured by DNA/DNA hybridization); and 2) the high degree of genetic divergence occurred predominantly within these three gene clusters. Because a high level of DNA sequence divergence can be demonstrated over a span of several genes within each of the 3 gene clusters, the data also suggest that the observed genetic diversity likely accumulated over a relatively long time span (years as opposed to hours). Presumably, genetic diversity within the gene clusters generated changes in flagella, LOS, and capsule that modulated interaction between the pathogen and the host via effects on host cell attachment and invasion, and on the ability of the host to mount an effective immune response. Future work will analyze surface structure changes that accompany accumulation of genetic diversity and the specific mechanisms by which these changes impact colonization and disease.

2) Genetic Diversity within Contingency Genes. We examined genetic variation in two well-characterized genes that carry homopolymeric tracts susceptible to slip-strand mutation (Linton et al., 2000; Hendrixson, 2006). The *wlaN* gene in *C. jejuni* 11168 expresses a β-1,3-galactosyltransferase; the presence or absence of a terminal galactose on LOS impacts attachment of sialic acid residues via the activity of sialic acid transferases. Sialic acid modification of LOS contributes to its antigenic specificity and to the ability of specific LOS structures to mimic GM1a, GD3, GD1a, GT1a, and/or GD3 gangliosides associated with GBS (Linton *et al*., 2000; Gilbert *et al*., 2002; Godschalk *et al*., 2004). An eight nucleotide poly G homopolymeric tract in the coding sequence of *wlaN* generates a full length active enzyme (wild type); one or two base insertions or deletions in this tract generate frameshift mutations that terminate translation and eliminate enzyme activity.

The homopolymeric tract in *wlaN* exhibited a higher frequency (>80%) and diversity (one and two base insertions, one base deletions) of mutations in *C. jejuni* populations isolated from broilers as compared to the *C. jejuni* animal inoculum (40%; predominantly one base insertions). In contrast, *C. jejuni* populations after passage in mice carried predominantly wild type homopolymeric tract (>80%). We observed a

similar frequency of mutation as detected previously by others in *C. jejuni* populations (Parkhill *et al*., 2000; Linton *et al*., 2000; Gilbert et al., 2002; Karlyshev *et al*., 2005). In contrast to these studies, Wassenaar *et al*., (2002) observed that the *wlaN* homopolymeric tract in *C. jejuni* 11168 remained stable under all conditions examined, including chicken colonization. Because Wassenaar *et al*. detected high mutation frequencies in other contingency genes, the absences of mutation in *wlaN* in their study may reflect differences in selective pressure experienced by *C. jejuni* during growth *in vivo*.

We also analyzed *flgR* which carries a series of four polyadenosine tracts, each seven or more nucleotides in length, within the 11168 coding region. FlgR is a σ^{54}-dependent NtrC-like protein that regulates flagellar expression in *C. jejuni*. It is the reponse regulator in a two-component regulatory system but is also subject to phase-variation through slip-strand mutagenesis in its poly A/T tracts (Hendrixson 2006; Joslin and Hendrixson 2008). Our data suggest that mutations may occur more frequently in *flgR* in the chicken host than in the mouse host implying that motility is essential in the mouse GI tract but is less so in chickens. However, more extensive experiments must be conducted to expand on these observations.

DNA sequence analysis of homopolymeric tracts embedded in *wlaN* and *flgR* demonstrated that subtle genetic differences (one or two base insertions or deletions) can arise at extremely high frequency within populations derived from a single strain during growth in culture and this mutation process is enhanced during colonization of broilers. Although high mutation frequencies have been observed previously in specific *C. jejuni* contingency genes (including *wlaN*) (Linton et al., 2000; Guerry et al., 2002; Prendergast et al., 2004) we demonstrated a strong correlation between the degree of genetic diversity observed in the homopolymeric tract in *wlaN* and the differential ability to colonize mice and broilers. This is an extremely important observation because it suggests that *C. jejuni* can control the chemical composition and conformation of cell surface structures via phase variation at high frequency and in real time.

It is important to note that *wlaN*, *flgR,* and several other contingency genes are located within the 3 adjacent gene clusters that display a high degree of genetic diversity among the 3 three *C. jejuni* human disease strains. This observation reinforces the notion that genetic diversity is important in colonization but also implies that this particular region of the genome not only is subject to high rates of mutation over very short time periods, but also to more stable accumulation of a large number of mutations that occurs over a much longer time interval. The data also demonstrate a strong ability for *C. jejuni* to adapt genetically in chickens. Future work will focus on the role of genetic adaptation in the subsequent ability to colonize and to cause disease in the mouse model.

3) Reduced Genetic Diversity (a Genetic Bottleneck) Reduces Colonization. In initial studies, we constructed genetically marked strains of *C. jejuni* 11168 to trace the frequency of genetic exchange by natural transformation in the GI tract of mice. These marked strains were inoculated into C57BL/6J IL10 deficient mice and they either failed to colonize or colonized at low frequency (2 in 42 mice); a similar result was observed during initial attempts to colonize mice with wild type 11168 that had been single colony isolated during inoculum preparation. We hypothesized that single colony isolation reduced genetic variation within the culture resulting in reduced colonization efficiency. This supposition was supported by three observations: 1) when we eliminated single colony isolation during inoculum preparation of wild type 11168, mouse colonization

increased markedly; 2) when we recovered a *C. jejuni* 11168 genetically marked derivative from a mouse and re-inoculated it into a new set of mice, all mice were colonized; and 3) we detected only a low level of genetic diversity (>10%) in *wlaN* in 11168 Tn5CamR2 (subjected to single colony isolation during strain construction) that was used as an animal inoculum. Our *C. jejuni* culture protocol now avoids the bottleneck that results from single colony isolation whenever practical.

These data also illustrate an extremely important principal; that is, that genetic adaptation occurs in the GI tract of mice and this directly impacts subsequent ability to colonize mice (although the mechanism and nature of this adaptation process are not completely understood). As discussed above, we also observed genetic adaptation in broilers. Of particular interest, the adaptation process in both host animals results in large fluctuations in genetic diversity within contingency genes. However, in chickens adaptation enhances genetic diversity while mouse adaptation severely limits diversity. Our current work shows that mouse adaptation has a major impact on subsequent ability to colonize mice. Preliminary data generated in our laboratory also suggest that chicken adaptation enhances subsequent mouse colonization (manuscript in preparation). Future work will focus on understanding the mechanisms that underlie genetic adaptation in chickens and mice and the role of adaptation in regulating colonization and disease in the host animal.

Proposed Role of the Chicken Reservoir in Human Disease. We now propose that the poultry reservoir plays at least two important roles in human disease caused by *C. jejuni*. The broiler GI tract provides a favorable environment for expansion of *C. jejuni* populations. This increases the potential for human exposure to this foodborne pathogen in improperly prepared poultry products. One novel contribution of the current study is that *C. jejuni* populations also experience a major expansion in genetic diversity during outgrowth in the broiler GI tract that appears to enhance colonization of our mouse model for human disease. Future work will explore the association between genetic diversity and mouse colonization in more detail.

The Host Immune Response and Genetic Diversity. Based on these analyses, we propose a model that we believe, at least in part, explains the role of the host immune system in shaping genetic diversity in *C. jejuni*. According to this model, the host immune system provides strong selective pressure that acts as a filter for genetic diversity in *C. jejuni* populations. We hypothesize that the broiler immune system is much more tolerant in its ability to recognize and eliminate *C. jejuni* from the GI tract. As a result, most *C. jejuni* can colonize broilers at high population levels. In contrast, we hypothesize that the mouse immune system is more stringent. It recognizes *C. jejuni* cells that carry LOS lacking a terminal galactose (due to mutation and inactivation of *wlaN*) and successfully eliminates these from the GI tract. Cells expressing wild type *wlaN* escape immune surveillance; this population becomes predominant in mice. We also propose that the host immune system plays an important role in the observed accumulation of a high level of genetic diversity within the PGLOS and CAP gene clusters (loci). Genetic variation within these clusters can generate subpopulations that carry cell surface antigens that escape immune surveillance and these successful subpopulations expand. These subpopulations then can predominate in future rounds of

colonization resulting in a high level of diversity being "fixed" over a long period of time. This analysis implies that selective pressure shapes the predominant genotypes observed in human clinical isolates of *C. jejuni*.

This analysis prompts us to hypothesize that the host immune system generates subpopulations with specific patterns of mutations within contingency genes and that understanding this pattern may allow us to predict the potential virulence of specific genotypes. Theoretically, one could analyze patterns of mutations in contingency genes to help understand the relative importance of individual genes and gene combinations in colonization and disease.

References

Ahmed, I.H., Manning, G., Wassenaar, T.M., Cawthraw, S., Newell, D.G. (2002) Identification of genetic differences between two *Campylobacter jejuni* strains with different colonization potentials. *Microbiology* **148**, 1203-1212.

Ashgar, S.S.A., Oldfield, N., Woldridge, K.G., Jones, M.A. Irving, G.J., Turner, D.P.J., Ala'Aldeen, D.A.A. (2007). CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. *J Bacteriol* **189**:1856-1865.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (2009). Current Protocols in Molecular Biology. John Wiley and Sons, New York.

Bernatchez, S., Gilbert, M., Blanchard, M.C., Karwaski, M.F., Defrees, S., Wakarchuk, W.W. (2007). Variants of the (beta)-1,3-galactosyltransferase CgtB from the bacterium *Campylobacter jejuni* have distinct acceptor specificities. *Glycobiology* **17,** 1333-1343.

Cawthraw, S.A., Wassenaar, T.M., Ayling, R., Newell, D.G. (1996). Increased colonization potential of *Campylobacter jejuni* strain 81176 after passage through chickens and its implication on the rate of transmission within flocks. *Epidemiol Infect* **117**, 213-215.

Coward, C., van Dierman, P.M., Conlan, A.J., Stevens, M.P., Jones, M.A., Maskell, D.J. (2008). Competing isogenic Campylobacter strains exhibit variable population structures in vivo. *Appl Environ Microbiol* **74**, 3857-3867.

de Boer, P., Wagenaar, J.A., Achterberg, R.P., van Putten, J.P., Schouls, L.M., Dulm, B. (2002). Generation of *Campylobacter jejuni* genetic diversity *in vivo*. *Mol Microbiol* **44**, 351-359.

Fouts, D.E., Monogodin, E.F., Mandrell, R.E., Miller, W.G., Rasko, D.A., Ravel, J., Brinkac, L.M., DeBoy, R.T., Parker, C.T., and other authors. (2005). Major structural differences and novel potential virulence mechanisms from the genomes of multiple campylobacter species. *PLoS Biol* Epub Jan. 4.

Gilbert, M., Karwaski, M.F., Bernatchez, S., Young, N.M., Taboada, E., Michniewicz., J., Cunningham, A.M., Wakarchuk, W.W. (2002). The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. *J Biol Chem* **277**, 327-337.

Godschalk, P.C., Heikema, A.P., Gilbert, M., Komagamine, T., Ang, C.W., Glerum, J., Brochu, D., Li, J., Yuki, N., and other authors. (2004). The crucial role of *Campyloabacter jejuni* genes in anti-ganglioside antibody induction in Guillain Barre syndrome. *J Clin Invest* **114**, 1659-1665.

Guerry, P., Szymanski, C.M., Prendergast, M.M., Hickey, T.E., Ewing, C.P., Pattarini, D.L., Moran, A.P. (2002). Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects gangliocide mimicry and invasiveness in vitro. *Infect Immun* **70**, 787-793.

Hendrixson, D.R. (2006). A phase-variable mechanism controlling the *Campylobacter jejuni* FlgR response regulator influences commensalism. *Mol Microbiol* **61**, 1646-1659.

Hendrixson, D.R. (2008). Restoration of flagellar biosynthesis by varied mutational events in *Campylobacter jejuni*. *Mol Microbiol* **70**:519-536.

Hook, H., Fattah, M.A., Ericsson, H., Vagsholm, I., Danielsson-Tham, M.L. (2005). Genotype dynamics of *Campylobacter jejuni* in a broiler flock. *Vet Microbiol* **106**, 109-117.

Horrocks, S.M., Anderson, R.C., Nisbert, D.J., Ricke, S.C. (2009). Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. *Anaerobe* **15,** 18-25.

Humphrey, T., Obrien, S., Madsen, M. (2007). Campylobacters as zoonotic pathogens: a food production perspective. *J. Food Microbiol*. **117,** 237-257.

Jones, M.A., Marston, K.L., Woodall, C.A., Maskell, D.J., Linton, D., Karlyshev, A.V., Dorrell, N., Wren, B.W., Barrow, P.A. (2004). Adaptation of *Campylobacter jejuni* NCTC 11168 to high level colonization of the avian gastrointestinal tract. *Infect Immun* **72,** 3769-3776.

Joslin, S.N., Henrixson, D.R. (2008). Analysis of the *Campylobacter jejuni* FlgR response regulator suggests integration of diverse mechanisms to activate an NtrC-like protein. *J Bacteriol* **190,** 2422-2433.

Kakuda, T., DiRita, V.J. (2006). Cj 1496c encodes a *Campylobacter jejuni* glycoprotein that influences invasion of human epithelial cells and colonization of the chick gastrointestinal tract. *Infect Immun* **74**, 4715-4723.

Karlyshev, A.V., Champion, O.L., Churcher, C., Brisson, J.R., Jarrell, H.C., Gilbert, M., Brochu, D., St.Michael, F., Li, J., and other authors. (2005). Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. *Mol Microbiol* **55**, 90-103.

Kim, C.C., Joyce, E.A., Chan, K., Falkow, S. (2002). Improved analytical methods for microarray-based genome-composition analysis. *Genome Biol* **29**, Epub.

Knudson, K.N., Bang, D.D., Anderson, L.O., Madsen, M. (2006). *Campylobacter jejuni* strains of human and chicken origin are invasive in chickens after oral challenge. *Avian Dis* **50**, 10-14.

Lee, M.D., Newell, D.G. (2006). Campylobacter in poultry; filling an ecological niche. *Avian Dis* **50**, 1-9.

Linton, D., Gilbert, M., Hitchen, P.G., Dell, A., Morris, H.R., Wakarchuk, W.W., Gregson, N.A., Wren, B.W. (2000). Phase variation of beta-1,3, galactosyl transferase involved in generation of the ganglioside GM1-like oligosaccharide of *Campylobacter jejuni*. *Mol Microbiol* **37**, 501-514.

Mansfield, L.S., Bell, J.A., Wilson, D.L., Murphy, A.J., Eishikha, H.M., Rathinam, V.A., Fierro, B.R., Linz, J.E., Young, V.B. (2007). C57BL/6 and congenic interleukin 10-deficient mice can serve as models of *Campylobacter jejuni* colonization and enteritis. *Infect Immun* **75,** 1099-1115.

Moxon, R., Bayliss, C., Hood, D. (2006). Bacterial contingency loci: the role of simple sequence DNA repeats in bacterial adaptation. *Ann Rev Genet* **40**, 307-333.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T. and other authors. (2000). The genome sequence of the foodborne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**, 665-668.

Poly, F., Ewing, C., Goon, S., Hickey, T.E., Rockabrand, D., Majam, G. Lee, L., Phan, J., Savarine, N.J., Guerry, P. (2007). Heterogeneity of a *Campylobacter jejuni* protein that is secreted through the flagellar filament. *Infect Immun* **75**, 3859-3867.

Prendergast, M.M., Tribble, D.R., Baqar, S., Scott, D.A., Ferris, J.A., Walker, R.L. Moran, A.P. (2004). In vivo phase variation and serologic response to lipo-oligosaccharide of *Campylobacter jejuni* in experimental human infection. *Infect Immun* **72**, 916-922.

Ridley, A.M.. Toszeghy, M.J., Cawthraw, S.A., Wassenaar, T.M., Newell, D.G. (2008). Genetic instability is associated with changes in the colonization potential of *Campylobacter jejuni* in the avian intestine. *J Appl Microbiol* **105**, 95-104.

Ringoir, D.D., Korolik, V. (2003). Colonization phenotype and colonization potential differences in *Campylobacter jejuni* strains in chickens before and after passage in vitro. *Vet Microbiol* **92,** 225-235.

Snelling, W.J., Matsudea, M., Moore, J.E., Dooley, J.S. (2005). *Campylobacter jejuni*. *Lett Appl Microbiol* **41**, 297-302.

Suzuki, H., Yamamato, S. (2009). Campylobacter contamination in retail poultry meats and by-products in the world: a literature survey. *J Vet Med Sci* **71** 255-261.

Trieu-Cuot, P. Gerbaud, G., Lambert, T., Curvalin, P. (1985). In vivo transfer of genetic information between gram-positivie and gram-negative bacteria. *EMBO J* **16**:3583-3587

Van Alphen, L.B., Wuhrer, M., Bleumink, N.M.C., Hensberger, P.J., Deelder, A.M., vanPutten, J.P.M. (2008) A functional *Campylobacter jejun*i *maf4* gene results in novel glycoforms on flagellin and altered agglutination behavior. *Microbiol* **154**:3385-3397.

van Doorn, P.A., Ruts, L., Jacobs, B. (2008). Clinical features, pathogenesis, and treatment of Guillain-Barre syndrome. *Lancet Neurol* **7,** 939-950.

Vucic, S., Kiernan, M., Cornblath, D.R. (2009). Guillain-Barre syndrome; an update. *J Clin Neuroscience*. **16,** 733-741

Wassenaar, T.M., Geihausen, B., Newell, D.G. (1998). Evidence of genome instability in *Campylobacter jejuni* isolated from poultry. *Appl Environ Microbiol* **64**, 1816-1821.

Wassenaar, T.M, Wagenaar, J.A., Rigter, A., Fearnley, C., Newell, D.G., Dulm, B. (2002). Homonucleotide stretches in chromosomal DNA of *Campylobacter jejuni* display high frequency polymorphism as detected by direct PCR analysis. *FEMS Microbiol Lett* **18**, 77-85.

Wilson, D.L., Bell, J.A., Young, V.B., Wilder, S.R., Mansfield, L.S., Linz, J.E. (2003). Variation of the natural transformation frequency of *Campylobacter jej*uni in liquid shake culture. *Microbiol* **149**, 3603-3615,

Young, K., Davis., L.M., and DiRita, V.J. (2007). *Campylobacterjejuni:* molecular biology and pathogenesis. *Nat Rev Microbiol*. **5,** 665-679.

Yuki, N. (2010). Human gangliocides and bacterial lipo-oligosaccharides in the development of autoimmune neuropathies. *Methods Mol Biol* **600,** 51-65

Table 1: Co-Inoculation and Colonization of C57BL/6J IL10 Deficient Mice in Cecal Tissue 14 or 16 Day Post-Inoculation by Genetically Marked *C. jejuni* 11168. Mouse cecal samples were homogenized in TSB, serially diluted, plated onto TSBA-CVA medium and TSBA-CVA supplemented with chloramphenicol and/or kanamycin, and incubated at 37°C under under microaerophilic conditions for 72 hr (see Methods). Culture positive mice carried *Campylobacter* at necropsy. *, represents a mouse-adapted strain.

Table 1

***Cj* 11168 Mutant Progeny Co-inoculum**	**Mice Inoculated**	**Culture Positive Mice, Day 14 -16 Post-Inoculation**
Tn5CamR2 + *23S*KanR4	5 male, 5 female	Detected in 1 of 10 mice (**Cj* 11168 Kan^R) 2.4×10^5 CFU mg^{-1} cecal tissue
Tn5CamR2 + *hipO*KanR1	5 male, 5 female	Not Detected
Tn5CamR6 + *23S*KanR4	5 male, 5 female	Detected in 1 of 10 mice (**Cj* 11168 Cam^R) 4.9×10^5 CFU mg^{-1} cecal tissue
Tn5CamR6 + *hipO*KanR1	5 male, 5 female	Not Detected
*hipO*CamR1 + *23S*KanR4	1 male, 1 female	Not Detected
*Tn5CamR6 + **23S*KanR4	4 male, 4 female	Detected in 8 of 8 mice

Figure Legends

Figure 1: Time-course of *C. jejuni* 11168 Colonization of Individual C57BL/6J IL10 Deficient Mice. Mice were orally gavaged with 10^{10} CFU of *C. jejuni* 11168 at day zero. Fecal samples were taken at Day 1, 4, 7, 10 and 13 and plated onto TSBA-CVA selective medium. Mice were sacrificed on Day 14 and cecal tissue homogenized and plated onto TSBA-CVA. (a) Plot represents the mean and standard error of colonization data for eight *C. jejuni* 11168 inoculated mice. (b) Each plot represents colonization data from a single, separately caged mouse. The chromosomal DNA from 11168-728 and 11168-732 was harvested for DNA-DNA microarray analysis to determine the stability of the *C. jejuni* chromosome during colonization.

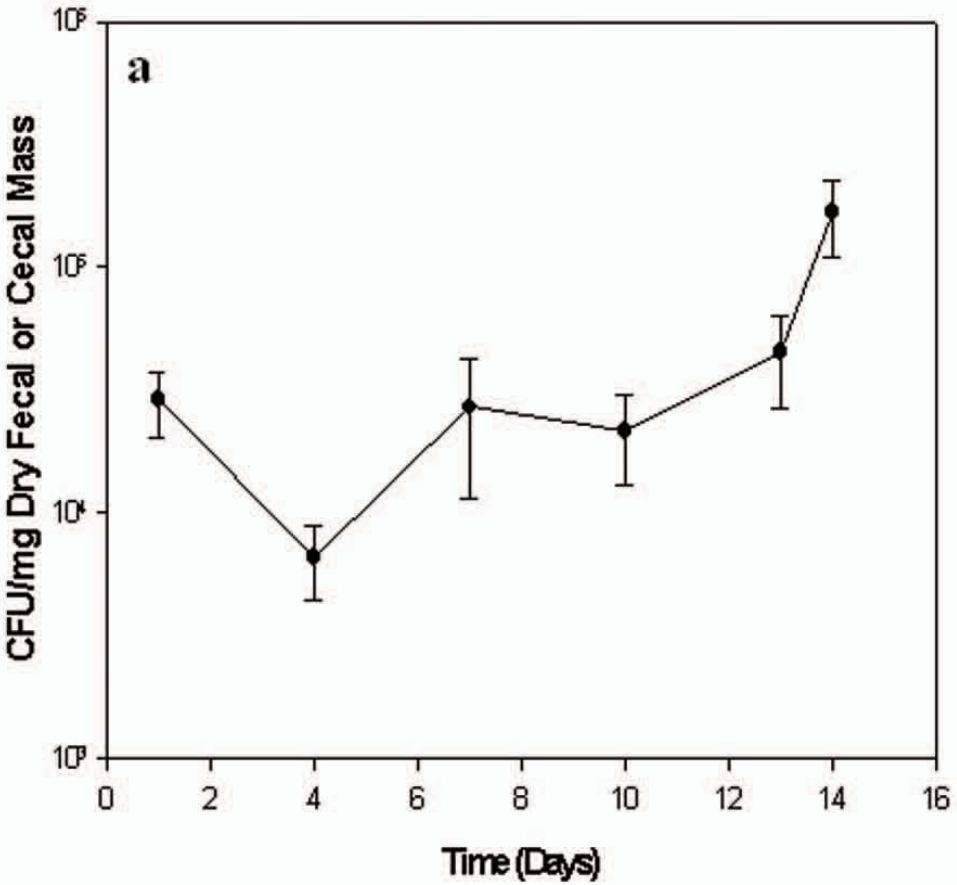
Figure 2: *C. jejuni* Colonization of C57BL/6J IL10 Deficient Mice and Ross 308 Broiler Cecal Tissue 14 Day Post-Inoculation. *C. jejuni* strains 11168, 33292 and 81-176 are represented on the x-axis. Eight mice and eight chickens were sacrificed on Day 14 and cecal tissue homogenized, serially diluted, and plated onto TSBA-CVA. Mice inoculated with strains 33292 and 81-176 were culture negative after 14 Days post-inoculation, with the limit of detection in these experiments ranging from 150 to 300 CFU $gram^{-1}$ of dry cecal tissue. Chicken #17 produced a relatively low *C. jejuni* concentration of 5×10^{-1} CFU mg^{-1} dry cecal tissue while chicken #27 did not produce a detectable level of *C. jejuni*. Each animal was housed separately in an individual cage.

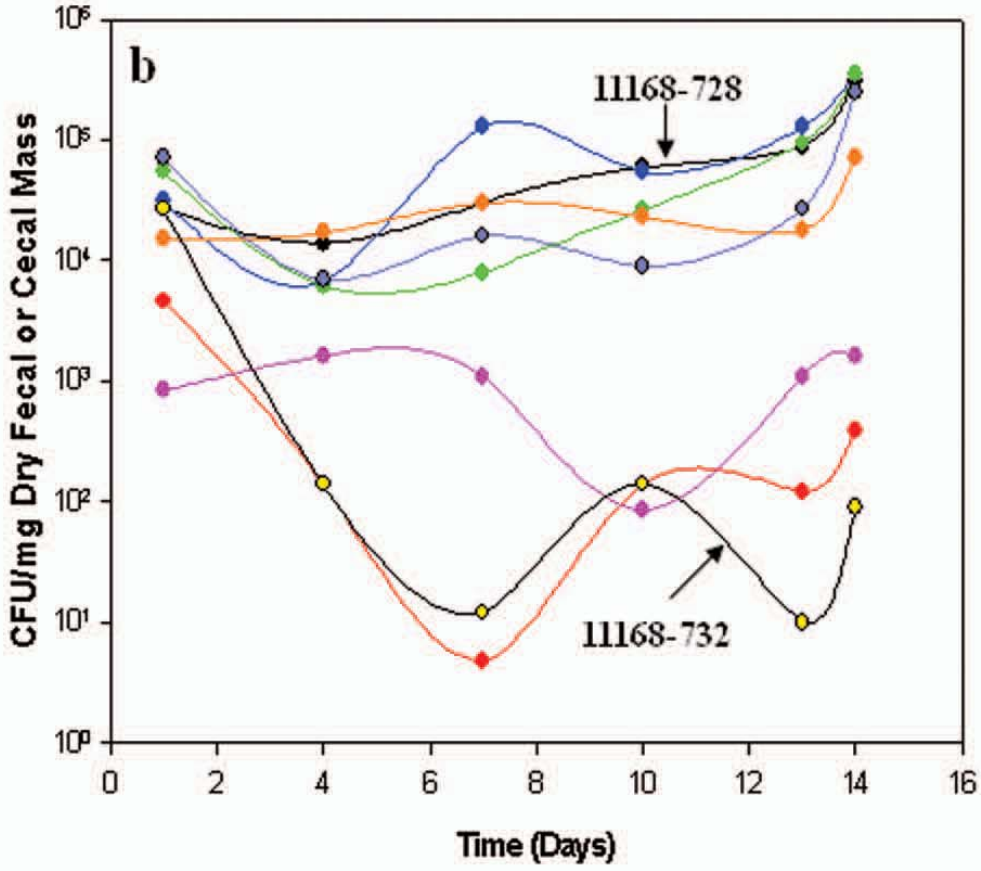
Figure 3: Colonization of C57BL/6J IL10 Deficient Mice and Ross 308 Broiler Cecal Tissue 14 or 16 Day Post-Inoculation by Genetically Marked *C. jejuni* 11168. Colonization data for wildtype *C. jejuni* 11168 in mouse and chicken cecal tissue are presented (as in Figure 2), for comparison with 11168 mutant progeny. For inoculation of mice, the majority of genetically marked 11678 strains were inoculated as co-cultures as defined in Table 1. 43 of 46 mice inoculated with non-adapted mutant progeny were culture negative for *C. jejuni* two weeks post-inoculation, with 42 of the mice receiving co-inocula. The mouse-adapted cultures were 11168-Tn5CamR6 (indicated by a circle in the Adapted column) and 11168-*23S*KanR4 (indicated by a triangle in the Adapted column) recovered from the cecal tissue on Day 14 post-inoculation of mouse 712 and 682 respectively. The limit of detection in these experiments ranged from 150 to 900 CFU $gram^{-1}$ of dry cecal tissue. Each animal was housed separately in an individual cage.

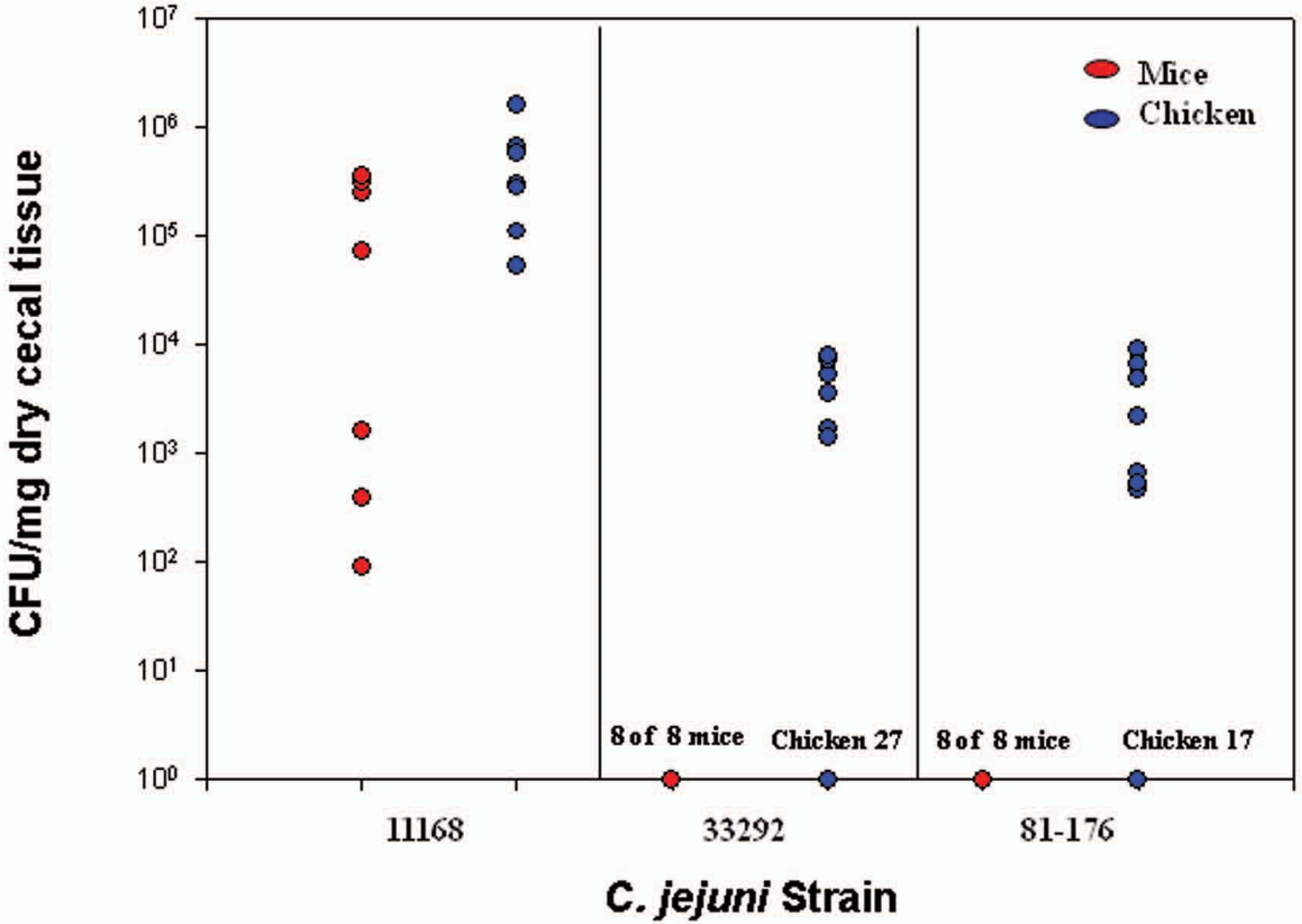
Figure 4: Microarray DNA-DNA Hybridization of *C. jejuni* Chromosomal DNAs. A glass slide array of *C. jejuni* 11168 gene-specific PCR ORFs served as a microarray reporter. *C. jejuni* strains D0121 and D2600 are distantly related human isolates as demonstrated by MLST analysis (personal communication, Erin Stanley). These strains were not used in colonization studies but are included here to provide more robust analysis between chromosomes. Samples consisted of two fluorescently labeled chromosomal preps (test and reference conditions) in a competitive hybridization against the reporter DNA. *C. jejuni* 11168 chromosomal DNA served as reference in all hybridizations. A GACK value of –0.5 represents a 100% chance of sequence

divergence and an Estimated Probability of Presence (EPP) $\leq$ 0.5%. A GACK score of 0.5 represents a 100% chance of identical sequence and an EPP $\geq$ 99.5%.

Figure 5. DNA Sequence Analysis of the Homopolymeric G/C Tract of *wlaN* in *C. jejuni* 11168 Cultures Recovered from the Cecum of C57BL/6J IL10 Deficient Mice and Ross 308 Broilers. We prepared chromosomal DNA extracts for *C. jejuni* 11168 used to inoculate C57BL/6J IL10 deficient mice (MI) and Ross 308 Broilers (CI) as well as from *C. jejuni* isolates recovered from cecal contents of four mice and four chickens. DNA extracts were amplified with Pfx50 high fidelity DNA polymerase and *wlaN* primers. A specific 415 bp amplification product containing the *wlaN* homopolymeric G/C tract was purified, ligated and transformed into *E. coli* using the PCR4-TOPO cloning system. Twenty individual *wlaN* clones were selected from each chromosomal DNA amplification and sequenced with either an ABI 3730 Genetic Analyzer or an ABI Prism 3700 DNA Analyzer using T3 or T7 primers. The relative *wlaN* DNA sequence populations from each culture are reported above (C – Ross 308 Broiler, M = C57BL/6J IL10 deficient mouse). Total sample size for each group is also given.


a
CFU/mg Dry Fecal or Cecal Mass
Time (Days)


b
11168-728
11168-732
CFU/mg Dry Fecal or Cecal Mass
Time (Days)


CFU/mg dry cecal tissue
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Mice
Chicken
8 of 8 mice
Chicken 27
8 of 8 mice
Chicken 17
11168
33292
81-176
C. jejuni Strain

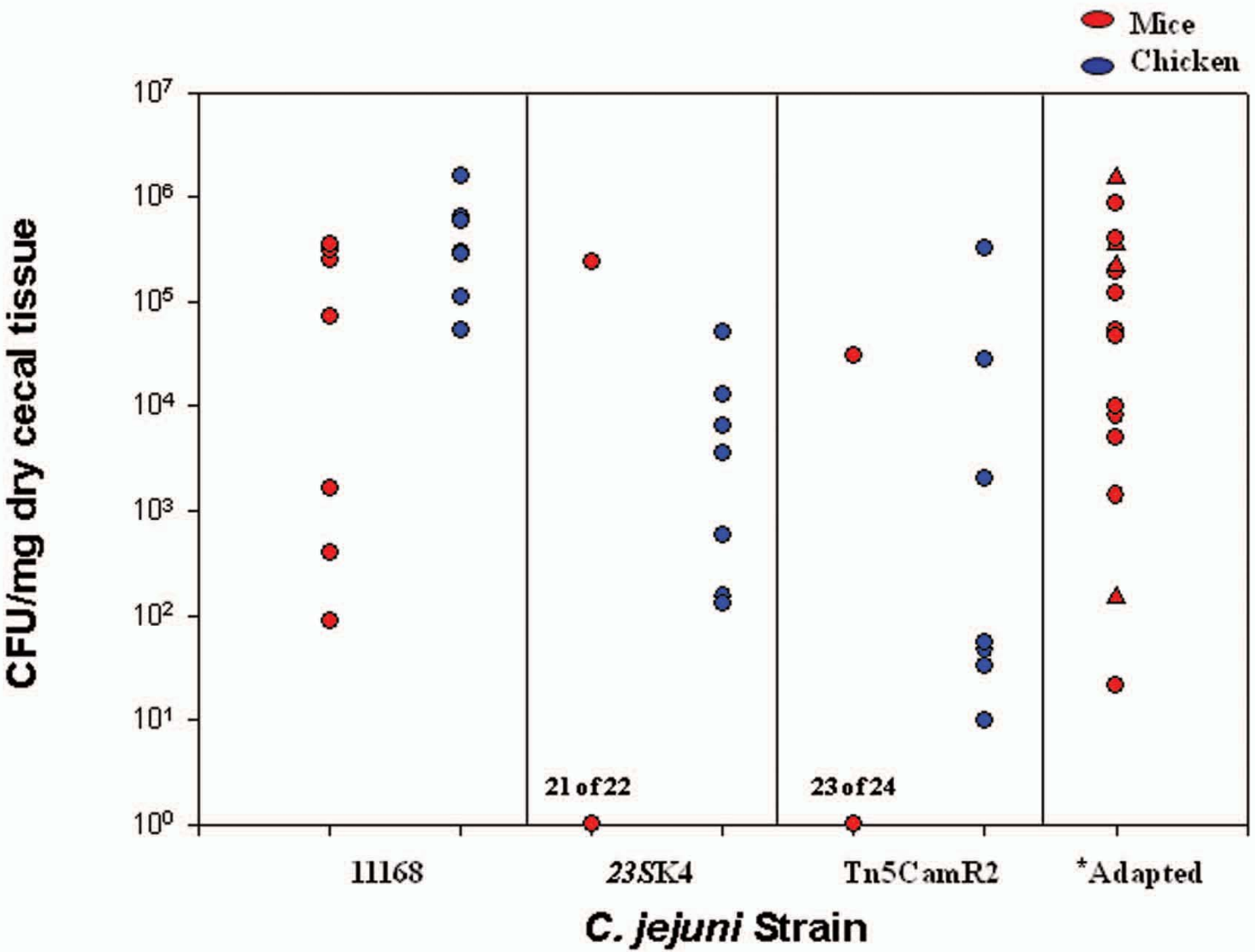
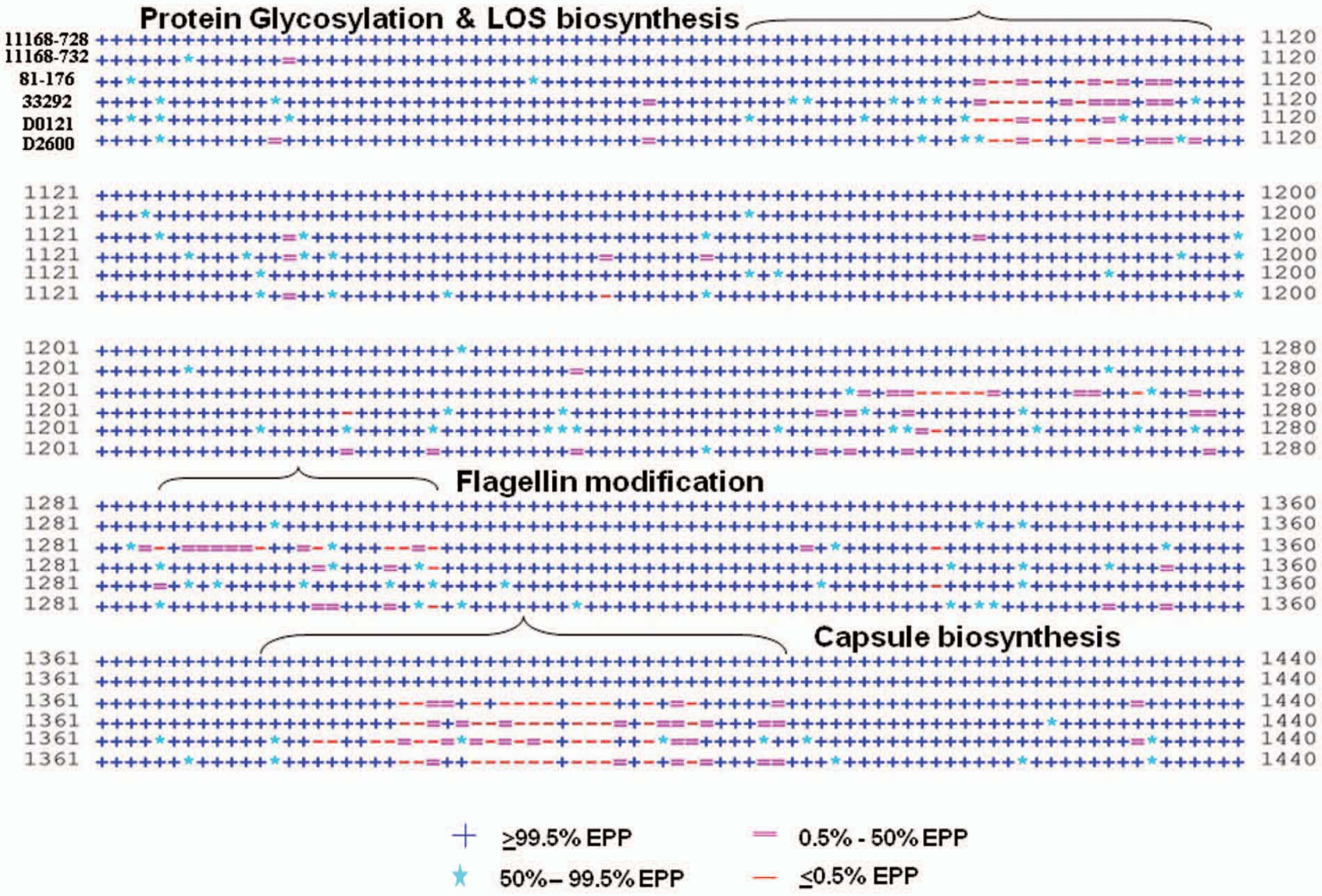
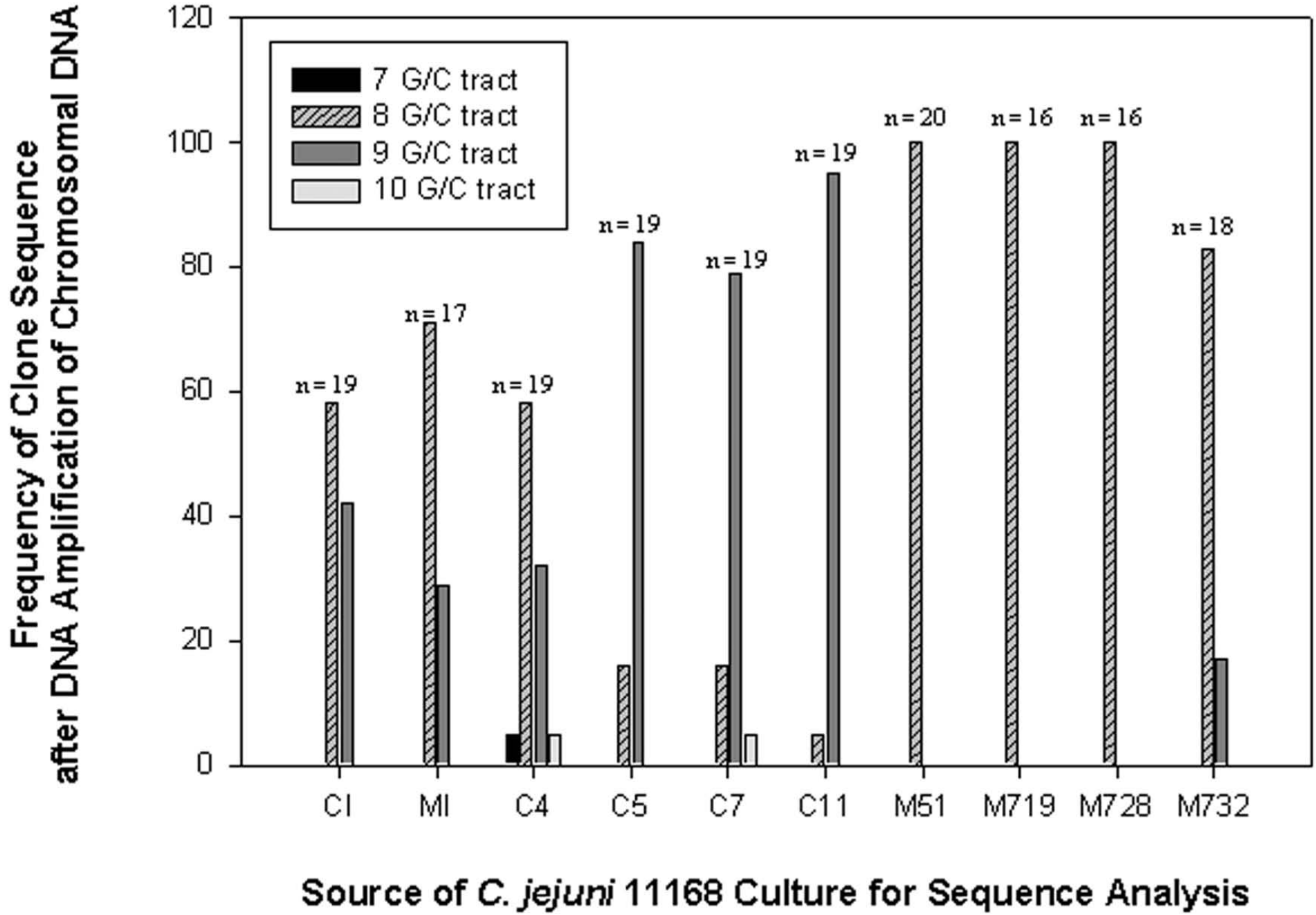

Mice
Chicken
CFU/mg dry cecal tissue
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
21 of 22
23 of 24
11168
23SK4
Tn5CamR2
*Adapted
C. jejuni Strain

Figure 4


Protein Glycosylation & LOS biosynthesis
11168-728
11168-732
81-176
33292
D0121
D2600
1120
1121
1200
1201
1280
Flagellin modification
1281
1360
Capsule biosynthesis
1361
1440
≥99.5% EPP
0.5% - 50% EPP
50%– 99.5% EPP
≤0.5% EPP


Frequency of Clone Sequence
after DNA Amplification of Chromosomal DNA
120
100
80
60
40
20
0
7 G/C tract
8 G/C tract
9 G/C tract
10 G/C tract
n = 19
n = 17
n = 19
n = 19
n = 19
n = 19
n = 20
n = 16
n = 16
n = 18
CI
MI
C4
C5
C7
C11
M51
M719
M728
M732
Source of C. jejuni 11168 Culture for Sequence Analysis