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High-Throughput Sequencing of *Campylobacter jejuni* Insertion Mutant Libraries Reveals *mapA* as a Fitness Factor for Chicken Colonization

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***Campylobacter jejuni* is a leading cause of gastrointestinal infections worldwide, due primarily to its ability to asymptomatically colonize the gastrointestinal tracts of agriculturally relevant animals, including chickens. Infection often occurs following consumption of meat that was contaminated by *C. jejuni* during harvest. Because of this, much interest lies in understanding the mechanisms that allow *C. jejuni* to colonize the chicken gastrointestinal tract. To address this, we generated a *C. jejuni* transposon mutant library that is amenable to insertion sequencing and introduced this mutant pool into day-of-hatch chicks. Following deep sequencing of *C. jejuni* mutants in the cecal outputs, several novel factors required for efficient colonization of the chicken gastrointestinal tract were identified, including the predicted outer membrane protein MapA. A mutant strain lacking *mapA* was constructed and found to be significantly reduced for chicken colonization in both competitive infections and monoinfections. Further, we found that *mapA* is required for *in vitro* competition with wild-type *C. jejuni* but is dispensable for growth in monoculture.**

Campylobacter jejuni is a leading cause of bacterially derived gastroenteritis in the United States, with 13.6 illnesses/100,000 persons annually. The incidence of infection is similar to and often surpasses the annual incidence of similar food-borne infection caused by *Salmonella* (1). It is reported that *C. jejuni* costs the United States approximately $1.7 billion annually (1, 2). The high incidence of *C. jejuni* infections is largely due to its ability to persistently colonize the gastrointestinal tracts of poultry and other agriculturally relevant animals (3). During processing of meat products, bacteria are released from the gastrointestinal tracts of these animals, contaminating the meat. Following consumption of either undercooked poultry or contaminated food, human infection often occurs from inocula as low as 360 organisms and results in moderate to severe and bloody diarrhea (4).

Due to the importance of chicken colonization to the infection cycle of *C. jejuni*, much emphasis has been placed on identifying and characterizing factors that promote colonization of the chicken gastrointestinal tract. To date, many different classifications of genes have been found to play a role in colonization of the chicken gastrointestinal tract, including those involved in chemotaxis, invasion, iron regulation, motility, and oxidative and nitrosative stress responses (5). Many of these studies identified chicken colonization phenotypes as a consequence of characterizing a factor of interest and often using monoinfections. Only one previous study attempted to identify colonization factors of *C. jejuni* in the chicken using a genome-wide approach (6). Employing a signature-tagged mutagenesis (STM) screen, this study identified factors involved in motility, *N*-linked protein glycosylation, and chemotaxis. While these factors have been further confirmed to be involved in chicken colonization, the STM approach identified only 22 new loci and was plagued by a high rate of false-negative identification, presumably due to technical limitations of STM. Not surprisingly, based on the cumulative work in the field, it is postulated that the earlier STM study did not identify a majority of the factors involved in chicken colonization (5).

Recently, development of various methodologies that take advantage of advances in next-generation sequencing technologies has allowed more comprehensive identification of bacterial factors of interest. One such protocol, insertion sequencing (INSeq), relies on a Mariner transposon system with an MmeI site introduced into the Himar1 inverted repeats of the transposon (7). Following construction of a transposon mutant library, extracted genomic DNA can be prepared and digested with MmeI, leaving a 16-bp fragment of flanking genomic DNA attached to the transposon. After ligating sequencing adapters to these fragments, the 16-bp region can be sequenced and mapped to a reference genome, allowing identification and quantification of mutants within a pool. Fortunately, the Mariner transposon system has been developed for use in *Campylobacter*, making the use of INSeq an attractive approach for identifying determinants of chicken colonization.

Here we describe using INSeq to identify novel colonization determinants of *C. jejuni* in the chicken ceca (7). We constructed an INSeq-compatible *C. jejuni* transposon mutant library of several thousand mutants and used it to inoculate day-of-hatch chicks. Analysis of INSeq data from inocula and from *C. jejuni* recovered from the ceca of infected chickens enabled us to identify mutants that were either lost or enriched during infection. One mutant underrepresented in the ceca contained a transposon insertion in *mapA*, which encodes a putative outer membrane lipo-

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TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Description or sequence (5′–3′)[a]	Reference or source
Strains		
C. jejuni DRH212	Streptomycin-resistant *C. jejuni* 81-176 (Sm^r)	10
C. jejuni Δ*rpoN*	*rpoN* deletion mutant of *C. jejuni* DRH212 (Sm^r)	10
C. jejuni kpsM::*kan*	*kpsM* insertion mutant of *C. jejuni* 81-176	15
C. jejuni mapA::*kan*	*mapA* insertion mutant of *C. jejuni* DRH212 (Kn^r Sm^r)	This study
E. coli DH5α	*E. coli* strain used for cloning	Invitrogen
Plasmids		
pRY109	*Campylobacter coli cat* cassette plasmid (Cm^r Ap^r)	26
pGEM-T Easy	Subcloning vector (Ap^r)	Promega
pKinnick	Plasmid containing *hawkeye* transposon (Cm^r Ap^r)	This study
Oligonucleotides		
5′cat_INSeq	ACGCGTCCTAACAGGTTGGATGATAAGTCCCCGGTCTTCGTATGCCGTCTTCTGCTTG GCGCGCCCTCGAGCAATTGTGCTCGGCGGTGTTCCTTTCCAA	
3′cat_INSeq	ACGCGTCCTAACAGGTTGGATGATAAGTCCCCGGTCTTCGTATGCCGTCTTCTGCTTGGCG CGCCCTGCAGTCTAGTGCGCCCTTTAGTTCCTAAAGGGT	
JJ17	CTAGCGAGCTTCCTCCTGTT	
JJ18	CATCTCTTGCGTCAGGCAAA	
JJ19	GGATCCCCGCATTAAAATTCACATCAAC	
JJ20	GGATCCGCTAGAGGAATAGTTGTGCTT	

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance.

protein previously shown to be a species-specific determinant that is immunogenic in both chickens and humans (8, 9). Analysis of a *mapA* mutant confirmed its importance to *C. jejuni* fitness in the chicken gastrointestinal tract both in competition with wild-type *C. jejuni* and during monoinfections. Similarly, *mapA* was found to be required for *in vitro* competition with wild-type *C. jejuni* but was dispensable when the mutant was grown in rich media alone.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. *Escherichia coli* strains used for subcloning were routinely grown using LB media at 37°C under aerobic conditions. *C. jejuni* strains were routinely grown under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) using either Mueller-Hinton (MH) agar containing 10% sheep's blood or MH broth. Cultures were grown at either 37°C or 42°C as noted specifically below. The following antibiotics were used at the given concentrations: carbenicillin at 50 μg/ml, chloramphenicol at 30 μg/ml, kanamycin at 150 μg/ml, and trimethoprim at 10 μg/ml.

DNA manipulations were performed using commercially available kits (Qiagen, Valencia, CA) and enzymes (NEB, Ipswich, MA) per the directions of the manufacturers.

Analysis of pseudoread mapping. To determine whether the 16-bp genomic fragments flanking the transposons are sufficient to identify the points of insertion, a pseudoread analysis was performed, as previously described (7). Briefly, every TA dinucleotide—the insertion site of mariner transposons—in the *C. jejuni* 81-176 genome (including all three replicons, namely, chromosome, pTet, and pVir) was identified and reads of various lengths (12 to 20 bp) were generated on each side of the predicted transposon insertion site. These reads were analyzed using our pipeline, and the percentage of those reads that were successfully mapped back to the correct "insertion" locus was determined.

Construction of *C. jejuni* INSeq library. A *C. jejuni* INSeq transposon, termed *hawkeye*, was generated by amplifying the *cat* cassette from plasmid pRY109 using oligonucleotides 5′cat_INSeq and 3′cat_INSeq. These primers were adapted from those used by Goodman et al. (7) but included regions complementary to the *Campylobacter coli cat* gene present in pRY109. This approximately 900-bp amplicon was cloned into the pGEM-T Easy vector (Promega, Madison, WI), resulting in pKinnick, and was sequenced to confirm conservation of required INSeq features, including the Himar1 insertion sequences, the introduced MmeI restriction sites, and the P7 priming sites. Following sequencing, *in vitro* transposition using purified *C. jejuni* genomic DNA was performed as previously described (10). Briefly, transposition was performed using 2 μg of purified DRH212 genomic DNA, approximately 500 ng of pKinnick, and 250 ng of purified maltose binding protein-tagged MarC9 transposase (MBP-MarC9) in transposition reaction buffer (10% glycerol, 25 mM HEPES [pH 7.9], 100 mM NaCl, 5 mM $MgCl_2$, 0.25 mg bovine serum albumin [BSA], and 2 mM dithiothreitol [DTT]) at 30°C for 4 h. After repair of the transposon junctions, genomic DNA was purified and introduced into *C. jejuni* DRH212 via natural competence (11). Successful recombinants were selected under microaerobic conditions on MH agar containing chloramphenicol for approximately 48 h at 37°C.

Prior to *en masse* collection of the INSeq library, random and single insertion of *hawkeye* into *C. jejuni* genomic DNA was determined by Southern blotting. Genomic DNA was isolated from 17 individual mutants and digested with RsaI (Promega). Digested DNA was separated on a 1% Tris-acetate-EDTA agarose gel and transferred to an Amersham Hybond-XL membrane (GE Healthcare, Little Chalfont, United Kingdom). Bound DNA was probed using ^{32}P-labeled *hawkeye* fragments generated by random priming and visualized by autoradiography.

Mutants were picked to individual wells of 96-well plates con-

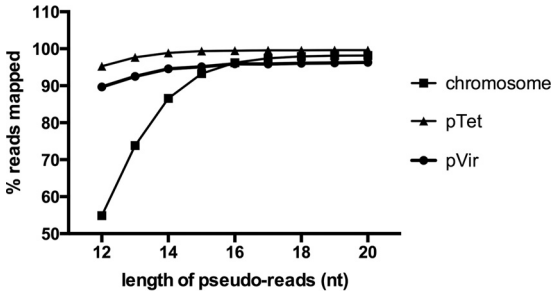

FIG 1 Analysis of pseudoreads flanking TA dinucleotides in the *C. jejuni* 81-176 genome. Reads of various lengths are plotted against the percentage of those reads that were successfully mapped to the correct location in each replicon.

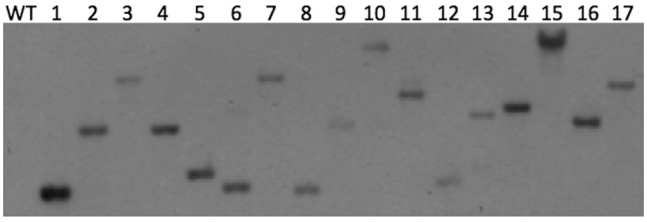

FIG 2 Southern blot analysis of *C. jejuni* INSeq mutants. Genomic DNA of wild-type *C. jejuni* (WT) and 17 INSeq mutants was digested with RsaI and separated on a 1% agarose gel. Transposon-containing fragments were detected using ^{32}P-labeled *hawkeye* sequence.

taining MH broth and grown an additional 48 h at 37°C microaerobically before being stored at −80°C. To collect the individually stored mutants into a single pool, each 96-well plate was replicated using a 96-pin replicator in a 150-mm-diameter MH agar plate. The plates were grown microaerobically for approximately 48 h at 37°C before the growth from all plates was harvested into a single MH broth–20% glycerol suspension and stored at −80°C.

Infection of day-of-hatch chickens with the INSeq library. The pooled *C. jejuni* INSeq mutant library was thawed on ice, and then 100 μl of the stock suspension was placed onto MH agar and grown microaerobically for 4 h at 37°C. Cells were harvested and used to make a suspension of approximately 10^7 CFU/ml in sterile phosphate-buffered saline (PBS). This suspension was used to inoculate white leghorn chicks (Charles River, Wilmington, MA) by oral gavage with approximately 10^6 CFU (100 μl of suspension). To profile the input, INSeq libraries were prepared using two different methods. In the first, approximately 10^9 cells were prepared following 4 h, as previously described (12). In the second, a sample of 10^6 bacteria that was used to inoculate the day-of-hatch chicks was grown overnight and the input library was generated from that growth. The mutant library was allowed to colonize for 7 days before cecal contents were harvested, and *C. jejuni* was enumerated following 48 h of microaerobic growth at 37°C on *Campylobacter* selective media (MH agar with 10% sheep blood containing 40 μg/ml cefoperazone, 100 μg/ml cyclohexamide, 30 μg/ml chloramphenicol, 10 μg/ml trimethoprim, and 100 μg/ml vancomycin).

Preparation of INSeq libraries from chickens. Following harvest of cecal contents, a *Campylobacter* enrichment step was performed immediately, as previously described (13). Briefly, an equal volume of PBS containing a 1:100 volume of phenol (Life Technologies, Grand Island, NY) was added to PBS suspensions of cecal contents. These suspensions were subjected to low-speed centrifugation at 800 × *g* for 3 min at 4°C to remove large insoluble particles. The supernatant from this step was removed and subjected to further centrifugation at 9,260 × *g* for 5 min at 4°C, resulting in a pellet consisting primarily of bacterial cells and small cecal particles (13). Genomic DNA was extracted from these pellets and subjected to the protocol of Goodman et al. (12). Generally, the amount of sample or bacteria from one chick was insufficient to use for this protocol. As a result, genomic DNA from bacteria enriched from the cecal contents of two to five birds was pooled prior to generating INSeq libraries.

Analysis of INSeq results. To identify mutants present in both input and output pools, DNA fragments were sequenced using an Illumina HiSeq 2000 sequencing system (San Diego, CA) available through the University of Michigan DNA Sequencing Core. Sequence data were analyzed using the following pipeline. Reads were trimmed and aligned to the *C. jejuni* 81-176 genome (accession no. NC_008787) using the Burrows-Wheeler Aligner (BWA) (14). Reads that (i) did not have a complementary read within 4 bp on the opposite strand, (ii) had a right-left read ratio > 10, or (iii) occurred within the last 10% of the reading frame were eliminated. The reads from each sample were normalized per million total reads. To determine the effect of mutations on colonization, fitness ratios were calculated for each locus by dividing the normalized reads in the output by the number of normalized reads for the same locus in the input.

Construction and characterization of a *mapA* mutant. Regions of homology flanking the *mapA* locus were amplified from DRH212 genomic DNA. The upstream region was amplified using primers JJ17 and JJ19, while the downstream region was amplified using primers JJ18 and JJ20. These fragments were subcloned into pGEM-T Easy and subsequently cloned together, introducing an internal BamHI restriction site into *mapA*. The *Campylobacter* kanamycin resistance cassette was excised from pILL600 using BamHI and ligated into *mapA* in the construct described above. This plasmid was introduced into DRH212 by electroporation and grown on MH agar overnight at 37°C under microaerobic conditions. Cells were plated on MH agar containing kanamycin, and successful integration of *mapA*::*kan* into the chromosome was confirmed by PCR.

To examine *mapA in vivo*, equal numbers of the wild-type DRH212 and *mapA* mutant strains were used in competition assays. For these assays, an inoculum of approximately 5×10^4 CFU of each strain was given to day-of-hatch chicks by oral gavage. Strains were allowed to colonize for 7 days when cecal contents were isolated, and bacterial counts were determined for each strain using the *Campylobacter* selective media mentioned above. Additionally, the wild-type and the *mapA* mutant strains were individually introduced into day-of-hatch chicks using the same inocula of approximately 5×10^4. Similarly, at 7 days postinoculation, cecal contents were harvested and *C. jejuni* numbers were determined.

To examine whether reductions in colonization were due to a general growth defect, competitive and individual growth curves were determined for both wild-type *C. jejuni* and the *mapA* mutant. Briefly, each strain was grown overnight on MH agar containing trimethoprim and subsequently passed for a second night on the same media at 37°C. These cultures were used to inoculate

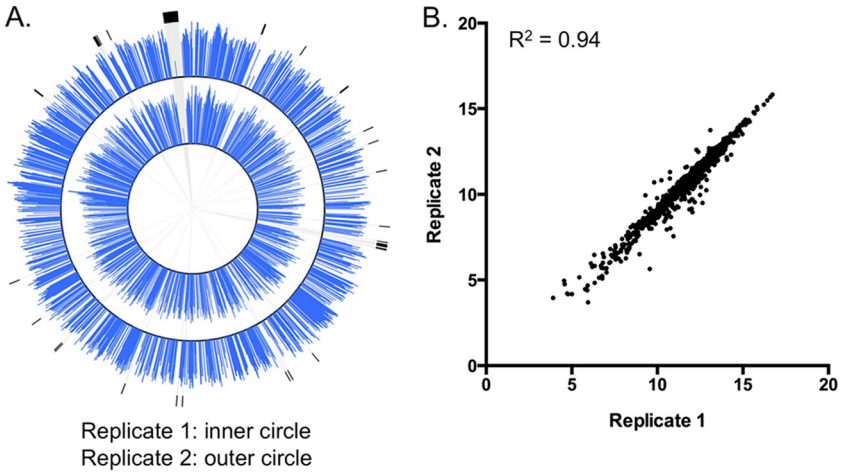

FIG 3 Analysis of *C. jejuni* INSeq mutant library. (A) Plots of insertion frequencies (log2 of total reads per ORF) in the *C. jejuni* chromosome generated using Circos (27). The inner and outer plots correspond to 2 independent cultures of ~10^9 INSeq mutants. Regions highlighted in black correspond to ORFs encoding ribosomal proteins. (B) The correlation of the locus abundances of the two technical replicates was determined using a Pearson correlation coefficient (R^2 = 0.94).

50 ml of MH broth to an A_{600} optical density (ODA_{600}) of 0.025 and grown under microaerobic conditions at 42°C. At each indicated time point, the ODA_{600} was recorded (for all growth curves) and the numbers of viable *C. jejuni* and *mapA* mutant bacteria were determined using selective media (for *in vitro* competition).

Motility was examined following growth of wild-type *C. jejuni*, the *mapA* mutant, and a nonmotile control, the Δ*rpoN* mutant, at 37°C under microaerobic conditions. Cells from these plates were inoculated into MH swim agar (0.4% agarose) and incubated overnight at 42°C. This assay was repeated in triplicate, and motility was visualized and measured. Capsule expression was determined by growing wild-type *C. jejuni*, the *mapA* mutant, and a nonencapsulated *kpsM* mutant control at 37°C under microaerobic conditions (15). These strains were passed again onto MH media, as described above, and grown overnight before cell extracts were assayed for capsular material as previously described (16). Briefly, cells were pelleted in 100 μl of lysis buffer (31.25 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 0.025% bromophenol blue, and 20% glycerol) and boiled for 5 min. After 20 μl was transferred to a new tube, extracts were treated with 1 μl of a 20 mg/ml proteinase K stock and incubated for 1 h at 50°C. Extracts were separated on a 15% Tris–polyacrylamide gel and stained with alcian blue (0.1% alcian blue, 40% ethanol, 5% acetic acid) prior to visualization.

RESULTS AND DISCUSSION

The genomic fragments flanking the INSeq transposon are sufficient for identifying insertions sites. To provide a proof of concept that the use of INSeq was possible in *C. jejuni*, we first determined whether the 16-nucleotide (nt) reads produced by the INSeq protocol were sufficient to identify the locations of insertions in the genome. Pseudoreads of various lengths were generated from every TA dinucleotide in the genome and mapped back to the *C. jejuni* 81-176 genome. Of all 16-nt pseudoreads generated, 97.21% ± 1.97% were mapped to the correct location in the genome, an efficiency similar to that reported by Goodman et al. for both *Bacteroides thetaiotaomicron* and *Saccharomyces cerevisiae* (Fig. 1) (7). While shorter reads could be accurately mapped

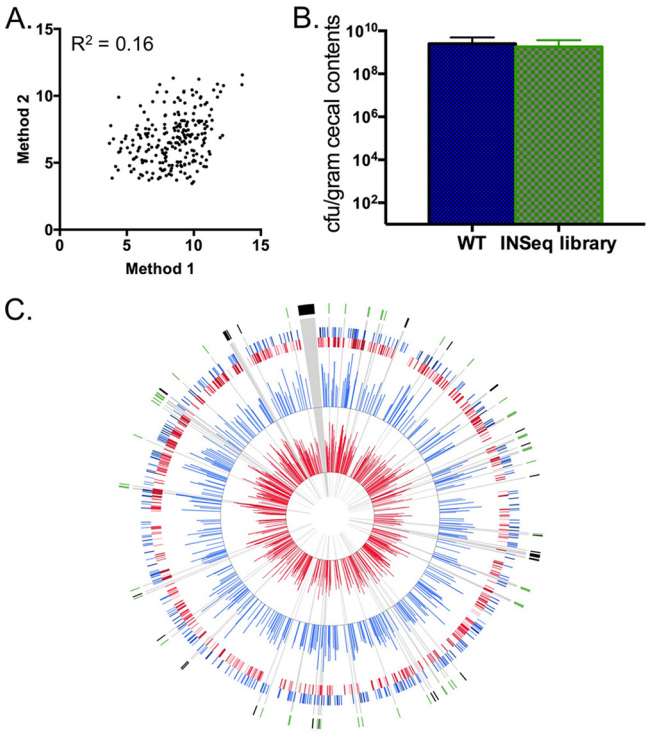

FIG 4 Identification of mutants affected for colonization of the chicken gastrointestinal tract. (A) Correlation between mutant library inputs prepared using two different methods; data were determined using a Pearson correlation coefficient (R^2 = 0.16). (B) Cecal colonization of day-of-hatch chicks by either wild-type *C. jejuni* or mutants from the *C. jejuni* INSeq mutant library. (C) Plots of insertion frequencies in the *C. jejuni* chromosome generated using Circos (27). The inner 2 plots correspond to the insertion frequencies (log2 of total reads per ORF) of the input (red) and output (blue) samples, respectively. The outer 2 plots show a heatmap of the inner 2 plots in which the 4 quartiles of insertion frequencies are plotted with increasingly darker shades of blue or red. Regions highlighted in black and green correspond to ORFs encoding ribosomal proteins and motility-associated gene products, respectively.

TABLE 2 Additional factors exhibiting at least a 2-log decrease in abundance from chicken cecal samples relative to the input

Locus	Annotation/predicted function[a]	Fitness ratio[b]
CJJ81176_1449	Hypothetical protein	0.00027
CJJ81176_0310	*cheA*	0.00028
CJJ81176_1411	Hypothetical protein	0.00034
CJJ81176_0025	*flgE* flagellar hook protein	0.00037
CJJ81176_0311	*cheV*	0.00037
CJJ81176_1452	Hypothetical protein	0.00053
CJJ81176_0283	Sulfatase, putative	0.00056
CJJ81176_0305	*carB* carbamoyl phosphate synthase large subunit	0.00056
CJJ81176_0246	Pathogenicity domain-containing protein	0.00072
CJJ81176_0573	*fliD* flagellar capping protein	0.00095
CJJ81176_0343	*dxs* 1-deoxy-D-xylulose-5-phosphate synthase	0.00096
CJJ81176_0038	*rbr* rubrerythrin	0.00119
CJJ81176_1567	Peptide ABC transporter, permease protein	0.00133
CJJ81176_0290	*zupT* zinc transporter	0.00144
CJJ81176_0764	Putative outer membrane protein	0.00148
CJJ81176_0098	*fliM* flagellar motor switch protein	0.00155
CJJ81176_0356	Antioxidant AhpCTSA family protein	0.00157
CJJ81176_0918	Hypothetical protein	0.00168
CJJ81176_1062	Hypothetical protein	0.00168
CJJ81176_1087	Hypothetical protein	0.00175
CJJ81176_1242	*htrA* protease DO	0.00178
CJJ81176_0389	*cmeB* RND efflux system, inner membrane transporter CmeB	0.00184
CJJ81176_1214	2OG-Fe(II) oxygenase family oxidoreductase	0.00188
CJJ81176_1457	Hypothetical protein	0.00237
CJJ81176_1341	Hypothetical protein	0.00238
CJJ81176_0671	*cbrR* response regulator/GGDEF domain-containing protein	0.00246
CJJ81176_0444	Hypothetical protein	0.00248
CJJ81176_0079	Hypothetical protein	0.00265
CJJ81176_0083	Hypothetical protein	0.00272
CJJ81176_0357	*flhB* flagellar biosynthesis protein	0.00276
CJJ81176_1598	*rpsT* 30S ribosomal protein S20	0.00277
CJJ81176_1061	Putative transmembrane transport protein	0.00296
CJJ81176_0813	Hypothetical protein	0.00298
CJJ81176_1206	Methyl-accepting chemotaxis protein	0.00302
CJJ81176_1548	Methyl-accepting chemotaxis protein	0.00303
CJJ81176_0508	*rplL* 50S ribosomal protein L7/L12	0.00305
CJJ81176_1433	Putative sugar transferase	0.00307
CJJ81176_0040	Inner membrane protein YagU	0.00311
CJJ81176_0215	Ser/Thr protein phosphatase family protein	0.00313
CJJ81176_1438	*kpsD* capsular polysaccharide ABC transporter, periplasmic polysaccharide-binding protein	0.00316
CJJ81176_1675	*gltA* citrate synthase	0.00324
CJJ81176_0414	Hypothetical protein	0.00346
CJJ81176_0080	*flgD* flagellar basal body rod modification protein	0.00347
CJJ81176_0109	Hypothetical protein	0.00352
CJJ81176_0574	*fliS* flagellar protein	0.00356
CJJ81176_0294	Hypothetical protein	0.00365
CJJ81176_0906	*pheS* phenylalanyl-tRNA synthetase subunit alpha	0.00367
CJJ81176_1415	Putative sugar-1-phosphate nucleotidyltransferase	0.00367
CJJ81176_0589	Hypothetical protein	0.00374
CJJ81176_0236	OPT family oligopeptide transporter	0.00379
CJJ81176_0359	*motA* flagellar motor protein	0.00390
CJJ81176_1462	Hypothetical protein	0.00391
CJJ81176_1602	Hemin ABC transporter, permease protein, putative	0.00399
CJJ81176_1541	Hypothetical protein	0.00400
CJJ81176_1050	Hypothetical protein	0.00402
CJJ81176_1203	*gidA* tRNA uridine 5-carboxymethylaminomethyl modification enzyme	0.00416
CJJ81176_0307	*tal* transaldolase	0.00435
CJJ81176_1413	*kpsC* capsule polysaccharide export protein	0.00451
CJJ81176_0299	Hypothetical protein	0.00454
CJJ81176_0273	MCP-domain-containing signal transduction protein	0.00459
CJJ81176_1335	Motility accessory factor	0.00462
CJJ81176_1529	Integral membrane protein	0.00462
CJJ81176_0602	*ilvB* acetolactate synthase 3 catalytic subunit	0.00463
CJJ81176_1455	*flgI* flagellar basal body P-ring protein	0.00468
CJJ81176_1459	*flgK* flagellar hook-associated protein	0.00468
CJJ81176_0032	Na+/H+ antiporter family protein	0.00479
CJJ81176_0641	*ftn* nonheme iron-containing ferritin	0.00493
CJJ81176_1337	*pseE*	0.00493
CJJ81176_1012	*argF* ornithine carbamoyltransferase	0.00495
CJJ81176_0219	Hypothetical protein	0.00519
CJJ81176_1416	Class I glutamine amidotransferase, putative	0.00525
CJJ81176_0590	Hypothetical protein	0.00537
CJJ81176_0892	*rpsO* 30S ribosomal protein S15	0.00542
CJJ81176_1652	ABC transporter, permease protein	0.00550

(Continued on following page)

TABLE 2 (Continued)

Locus	Annotation/predicted function[a]	Fitness ratio[b]
CJJ81176_0251	*argB* acetylglutamate kinase	0.00559
CJJ81176_0296	*ilvE* branched-chain amino acid aminotransferase	0.00564
CJJ81176_0674	*rlpA* rare lipoprotein A	0.00566
CJJ81176_0005	*ksgA* dimethyladenosine transferase	0.00571
CJJ81176_1238	Hypothetical protein	0.00576
CJJ81176_1606	*kgtP* alpha-ketoglutarate permease	0.00587
CJJ81176_1143	*pglB* general glycosylation pathway protein	0.00588
CJJ81176_1679	Major facilitator superfamily protein	0.00590
CJJ81176_1436	Putative glycosyl transferase	0.00593
CJJ81176_0245	Hypothetical protein	0.00596
CJJ81176_1136	*cheY* chemotaxis protein	0.00603
CJJ81176_1147	*wlaB* ABC transporter, permease/ATP-binding protein	0.00668
CJJ81176_1605	Hypothetical protein	0.00668
CJJ81176_0366	*uvrA* excinuclease ABC subunit A	0.00674
CJJ81176_0585	MATE efflux family protein, authentic frameshift	0.00677
CJJ81176_0801	*napA* nitrate reductase catalytic subunit	0.00702
CJJ81176_0386	Coproporphyrinogen III oxidase	0.00713
CJJ81176_1654	ABC transporter, ATP-binding protein	0.00720
CJJ81176_0185	*hom* homoserine dehydrogenase	0.00724
CJJ81176_0274	Hypothetical protein	0.00728
CJJ81176_0730	*waaA* 3-deoxy-D-manno-octulosonic-acid transferase	0.00728
CJJ81176_0119	*cydB* cytochrome *d* ubiquinol oxidase, subunit II	0.00736
CJJ81176_1417	Hypothetical protein	0.00737
CJJ81176_1392	*metC* cystathionine beta-lyase	0.00752
CJJ81176_1547	MerR family transcriptional regulator	0.00752
CJJ81176_1154	Beta-1,4-N-acetylgalactosaminyltransferase	0.00756
CJJ81176_1405	Hypothetical protein	0.00760
CJJ81176_0252	*argD* acetylornithine aminotransferase	0.00764
CJJ81176_1550	*pflA* paralyzed flagellar protein	0.00766
CJJ81176_0543	Hypothetical protein	0.00769
CJJ81176_0572	*flaG* flagellar protein	0.00777
CJJ81176_1458	Hypothetical protein	0.00786
CJJ81176_0223	*clpP* ATP-dependent Clp protease proteolytic subunit	0.00791
CJJ81176_0295	SPFH domain-containing protein	0.00795
CJJ81176_0076	Hypothetical protein	0.00800
CJJ81176_0107	Hypothetical protein	0.00805
CJJ81176_1421	Putative glycosyltransferase	0.00814
CJJ81176_1336	*pseD*	0.00816
CJJ81176_0534	*alaS* alanyl-tRNA synthetase	0.00829
CJJ81176_1519	Bacterioferritin, putative	0.00829
CJJ81176_0856	Hypothetical protein	0.00839
CJJ81176_0802	*napG* quinol dehydrogenase periplasmic component	0.00865
CJJ81176_0661	Hypothetical protein	0.00882
CJJ81176_1431	Putative sugar transferase	0.00882
CJJ81176_0202	Hypothetical protein	0.00893
CJJ81176_0893	Cell division protein FtsK, putative	0.00936
CJJ81176_0110	Hypothetical protein	0.00955
CJJ81176_1352	*ceuC* enterochelin ABC transporter, permease protein	0.00955
CJJ81176_1387	*katA* catalase	0.00962
CJJ81176_1530	Flavodoxin-like fold domain-containing protein	0.00962
CJJ81176_0024	Hypothetical protein	0.00969
CJJ81176_0339	*hisC* histidinol-phosphate aminotransferase	0.00969
CJJ81176_0483	*miaB* (dimethylallyl)adenosine tRNA methylthiotransferase	0.00969
CJJ81176_0210	Iron ABC transporter, permease protein	0.00983
CJJ81176_1569	Peptide ABC transporter, periplasmic peptide-binding protein	0.00983
CJJ81176_1063	AraC family transcriptional regulator	0.00997

[a] MATE, multiantimicrobial extrusion protein; OPT, oligopeptide transporter; RND, resistance-nodulation-cell division.

[b] Fitness ratio data were calculated by dividing normalized sequence abundance in output by normalized input abundance. All genes listed are significantly decreased in abundance ($P < 0.05$).

within the smaller replicons (pTet and pVir), the mapping accuracy of chromosomal reads < 15 nt in length was below 90%. These results demonstrate that, despite the relatively low sequence complexity of an AT-rich organism such as *C. jejuni* (30% GC content), the 16-mer sequences produced by the INSeq protocol are sufficient for accurate, unambiguous mapping of transposon insertions.

The INSeq-compatible *hawkeye* transposon inserts randomly throughout the *C. jejuni* genome. By the use of *in vitro* transposition reactions, *hawkeye* was transposed throughout the genome of DRH212, typically yielding 400 to 500 mutants per transposition reaction. Though the randomness of Mariner insertion into the *C. jejuni* genome had already been demonstrated, adapting the transposon for insertion sequencing made it necessary to confirm that this had not been altered. To determine whether the transposon inserted singly and randomly, RsaI-digested genomic DNA was subjected to Southern blot analysis using radiolabeled probes that hybridized to the *hawkeye* transposon. This analysis indicated that the transposition conditions that were used resulted in single insertions of *hawkeye* into the

genomic DNA of DRH212, as only a single fragment within each of several isolates was able to hybridize to the probe (Fig. 2). Additionally, *hawkeye* inserted randomly, as those single fragments were of discrete sizes among the different mutant isolates. To ensure heterogeneity of the library, only 100 to 150 transposon mutants were selected from each individual transposition reaction until approximately 8,500 mutants were collected. At this size, based on the approximately 1,700 genes in the *C. jejuni* genome, we calculated that the mutant library should represent approximately 5× coverage.

Preparation of 10^9 cells from the pooled *C. jejuni* INSeq mutant library and analysis by our pipeline determined that the library consisted of 3,994 independent insertions into the chromosome, which inserted into 1,002 open reading frames (ORFs) (58.8% of chromosomal ORFs). Many of the ORFs that were missed included those genes encoding ribosomal components or replication factors and other essential functions (Fig. 3A). That *C. jejuni* can tolerate insertions into only ~60% of its ORFs is not surprising; *C. jejuni* likely has to dedicate a relatively large portion of its small genome (1.7 Mbp) to growth. Insertions were mapped to every ORF within the pVir plasmid (52 genes) but only infrequently into pTet (5 genes). The latter observation is likely due to the frequency with which the pTet plasmid is lost from *C. jejuni*—presumably, pTet would have been mostly absent in genomic preparations for *hawkeye* mutagenesis. Overall, 1,059 of the 1,758 predicted *C. jejuni* ORFs were mutagenized, or 60.2% of those in the genome. Additionally, these results were reproducible using separate samples of the library, as a significant correlation between two independently prepared samples was observed ($R^2 = 0.94$) (Fig. 3A and B). This indicates not only that the library is consistent but also that the INSeq protocol provides reproducible results.

Insertion sequencing identified several determinants that are required for efficient colonization of the chicken gastrointestinal tract. As stated above, two different methods for characterizing inputs were used. In the first method, an INSeq library was prepared from 10^9 cells after 4 h of growth at −80°C. In the second method, 10^6 cells from the inoculum were grown overnight before a sample of 10^9 cells was used to generate an INSeq library (Fig. 4A). The log2 abundances of reads mapping to each gene from these preparations were plotted against one another and found to be significantly different ($R^2 = 0.16$). This is likely due to both viable and nonviable cells being present in inputs generated using the first method and the sampling error introduced by using only 10^6 cells in the second method. While the complexity of the input diminishes using the second method, libraries are more likely to resemble the mutant populations that were in the inocula, because the cells were derived directly from those. Using this method, the inoculum was determined to contain a mutant population representing 1,155 independent insertions into 545 chromosomal genes.

Following inoculation with 6×10^5 CFU of the INSeq mutant library, day-of-hatch chicks were fully colonized at 7 days postinoculation. Cecal loads of the mutant library had a mean of 1.79×10^9 CFU/gram of cecal content, while the wild-type strain yielded a mean cecal load of 2.52×10^9 CFU/gram (Fig. 4B). These results were found to be statistically indistinguishable (P value = 0.43), indicating that the *C. jejuni* INSeq library, as a whole, is proficient at colonizing the chicken gastrointestinal tract. Libraries were generated from six genomic samples representing 22 birds in total.

TABLE 3 Factors exhibiting at least a 3-log increase in abundance from chicken cecal samples relative to the input

Locus	Annotation/predicted function[a]	Fitness ratio[b]
CJJ81176_0636	*macB* macrolide-specific efflux protein	137,828.30
CJJ81176_1116	*pyrB* aspartate carbamoyltransferase catalytic subunit	113,410.73
CJJ81176_0372	*trpB* tryptophan synthase subunit beta	87,386.48
CJJ81176_0159	Hypothetical protein	47,536.15
CJJ81176_1393	PurB-2 adenylosuccinate lyase	43,154.53
CJJ81176_0043	*exsB* protein ExsB	34,172.52
CJJ81176_1608	Hypothetical protein	30,149.72
CJJ81176_1344	Hypothetical protein	27,510.78
CJJ81176_0041	Membrane protein, putative, degenerate	27,237.86
CJJ81176_0015	*leuC* isopropylmalate isomerase large subunit	22,670.49
CJJ81176_0190	Tetrapyrrole methylase family protein	15,887.70
CJJ81176_0626	Hypothetical protein	6,533.57
CJJ81176_1668	*eno* phosphopyruvate hydratase	6,111.82
CJJ81176_1655	Thiredoxin-like protein	5,811.00
CJJ81176_1164	*waaF* ADP-heptose LPS heptosyltransferase II	5,388.21
CJJ81176_1008	Hypothetical protein	5,142.55
CJJ81176_0437	Hypothetical protein	4,427.12
CJJ81176_1325	Formyl transferase domain-containing protein	4,020.13
CJJ81176_0738	Transthyretin-like protein	3,743.10
CJJ81176_0863	*psd* phosphatidylserine decarboxylase	3,261.48
CJJ81176_1527	Hypothetical protein	3,023.00
CJJ81176_0973	Peptidyl-arginine deiminase family protein	2,437.90
CJJ81176_0503	*nusG* transcription antitermination protein	2,430.53
CJJ81176_0402	Hypothetical protein	1,963.88
CJJ81176_0363	*ydfJ* inner membrane metabolite transport protein	1,635.86
CJJ81176_1552	*nuoM* NADH-quinone oxidoreductase, M subunit	1,633.78
CJJ81176_1553	*nuoL* NADH dehydrogenase subunit L	1,633.78
CJJ81176_0588	Hypothetical protein	1,313.33
CJJ81176_1447	*yliG* putative tRNA modifying protein	1,309.45
CJJ81176_1188	SMR family multidrug efflux pump	1,244.10
CJJ81176_0815	Hypothetical protein	1,242.03
CJJ81176_0072	Hypothetical protein	1,216.87
CJJ81176_1270	Hypothetical protein	1,142.36
CJJ81176_1521	*galU* UTP-glucose-1-phosphate uridylyltransferase	1,119.73
CJJ81176_1311	Aminotransferase	1,098.35
CJJ81176_0163	Inositol monophosphatase family protein	1,032.00
CJJ81176_1700	*rpsS* 30S ribosomal protein S19	1,016.91
CJJ81176_0576	*efp* elongation factor P	1,012.63

[a] LPS, lipopolysaccharide; SMR, small multidrug resistance.
[b] Fitness ratio data were calculated by dividing normalized sequence abundance in output by normalized input abundance

Illumina HiSeq 2000 sequencing typically yielded between 500 and 1,500 Mb per sample. The input and each of the individual outputs were analyzed using parameters described in Materials and Methods.

Outputs were pooled and found to contain mutants that represented 565 independent insertions in 329 chromosomal genes. This represents a loss of a slight majority of those mu-

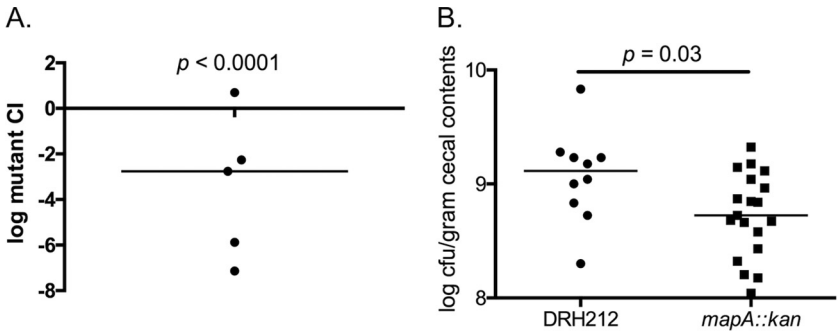

FIG 5 Cecal colonization of a *mapA* mutant. (A) Competitive index (CI) calculated by determining ratio of wild-type *C. jejuni* to *mapA* mutant in cecal samples from chicks infected with equivalent amounts of each strain. Significance was determined using a Wilcoxon signed-rank test ($P < 0.0001$). (B) Colonization loads in the cecum following monoinfections with either wild-type *C. jejuni* or the *mapA* mutant. Significance was determined using a Mann-Whitney test ($P = 0.03$).

tants present in the input, with 51% of the independent insertions and 40% of the marked genes being lost, which was evidenced by the relative abundance of sequenced loci in the input and the lack of those same loci in the output (Fig. 4C). We reasoned that this was likely due both to a loss of mutants following passage through the chick due to their reduced fitness *in vivo* and to a large number of the mutants being present at numbers too low to allow efficient colonization of the cecum, regardless of phenotype.

To address the likelihood that some mutants were present at numbers too low to allow colonization, we determined a fitness ratio for each locus that takes into account the abundance of a mutant in the output relative to its abundance in the input. We set a 2-log decrease in abundance within output pools as a standard for those that warranted further investigation. At this level of effect, a mutant would have been present at infectious levels within the inoculum and would have often been uniformly absent in all cecal outputs. Many of the loci identified with this level of stringency were shown to be required for colonization of the chicken gastrointestinal tract in the STM work mentioned previously, including those involved in flagellar motility, N-linked protein glycosylation, and chemotaxis (Table 2) (6). In all, 130 genes exhibited fitness ratios < 0.01, with approximately 15% involved in flagellar motility (*flgE*, *motA*, *fliS*, etc.) and chemotaxis (*cheA*, *cheY*, etc.), which have been highlighted (Fig. 4C).

These results also confirm several other processes required for colonization or survival within the chicken gastrointestinal tract. For example, INSeq mutants of the GGDEF domain-containing protein CbrR were uniformly absent from cecal samples (17). This determinant has previously been shown to be required for resistance of *C. jejuni* to the bile acid sodium deoxycholate, making it required for survival within the chicken gastrointestinal tract (17). Additionally, insertions in genes required for capsule biosynthesis were absent from cecal samples, including mutations in genes required for capsule transport, *kpsC* and *kpsD* (18). Insertions in genes required for stress response—catalase (*katA*), for example—or energy production and metabolism (such as cytochrome *d* ubiquinol oxidase subunit II [*cydB*]) were also absent from cecal samples. Based on transcriptome sequencing (RNA-Seq) analysis, transcripts from genes involved in these processes are increased in abundance during infection of the chicken ceca compared to their abundance in *in vitro* cultures (19).

In addition to those mutants with decreased abundance in the output pools, insertion mutants of several genes were enriched following passage through the chicken (Table 3). Of these, more than 25% encode hypothetical proteins, making it difficult to interpret their biological significance. Several might be predicted to be at a disadvantage during colonization of the chicken gastrointestinal tract, rather than being enriched, including mutations within *trpB*, encoding a product required for tryptophan biosynthesis, and *waaF*, encoding lipopolysaccharide heptosyltransferase, a component of the *C. jejuni* lipooligosaccharide (LOS). Previous work demonstrated that hyperosmotic stress caused elevated *trpB* expression, which may be required for osmotic stress resistance and efficient colonization of the chicken gastrointestinal tract (20). Mutation of *waaF* resulted in a truncated core oligosaccharide, which resulted in increased sensitivity to serum killing and decreased binding to

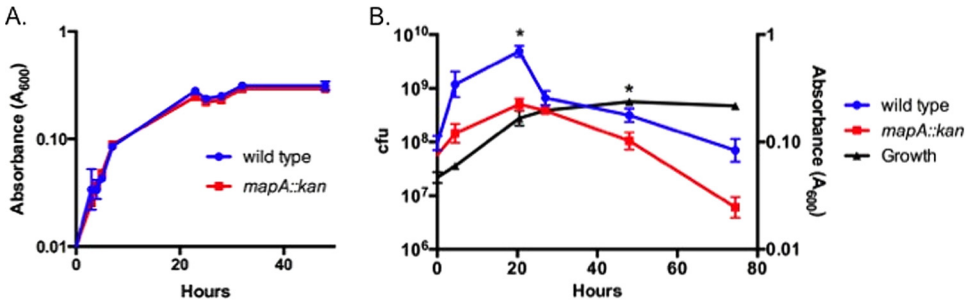

FIG 6 Growth phenotypes of the *mapA* mutant. (A) Growth curves for wild-type *C. jejuni* and the *mapA* mutant in monocultures. (B) Competitive growth assay of wild-type *C. jejuni* and the *mapA* mutant. CFU counts for each strain are plotted against the growth curve for the combined culture. Student's *t* test was used to determine significance ($P < 0.05$).

cellular ligands (21). Notwithstanding the presumed effects of mutations in these genes, neither has been previously shown to be required for chicken colonization, underscoring the need to repeat colonization studies with isogenic mutants.

A *C. jejuni mapA* mutant exhibits decreased colonization of the chicken gastrointestinal tract. We chose one representative locus, *mapA*, which exhibited a >3-log decrease in its fitness ratio, for further analysis. It encodes a species-specific outer membrane lipoprotein that is immunogenic in both chickens and humans but has not been previously shown to play a role in colonization of the chicken gastrointestinal tract (8, 9, 22). We reconstructed a *mapA* mutation using allelic exchange mutagenesis and carried out two separate colonization studies. In one, a *mapA* mutant was introduced at a 1:1 ratio with wild-type *C. jejuni* DRH212 (4.1×10^4 and 5.3×10^4, respectively) into day-of-hatch white leghorn chicks by oral gavage, and the mixture was allowed to colonize for 7 days before bacterial loads from the ceca were determined. In these infections, the *mapA* mutant was at a significant competitive disadvantage, with a median competitive index of 2.2×10^{-3} (P value < 0.0001) (Fig. 5A). We also carried out an infection with the *mapA* mutant alone (i.e., not in competition with the wild type). For this, the mutant and wild-type DRH212 strains were individually introduced into day-of-hatch chicks. In these infections, the *mapA* mutant was recovered with a median colonization level of 5.3×10^8 CFU/gram of cecal contents, while DRH212 colonized at a median of 1.3×10^9 CFU/gram of cecal contents, a decrease of approximately 0.5-log (P value = 0.03) (Fig. 5B). Thus, while it is required for competing against wild-type *C. jejuni* in the chicken gastrointestinal tract, the *mapA* gene product is less critical during monoinfections. Further, the difference in colonization proficiency following assays of competitive infection and monoinfection demonstrated the value of genome-wide approaches such as INSeq because protocols focusing on monoinfection phenotypes likely would have been unable to identify MapA as a fitness determinant.

To examine whether the competitive colonization defect of the *mapA* mutant is due to a general growth defect, growth curves were determined for individually and competitively grown wild-type DRH212 and *mapA* mutant strains (Fig. 6A and B, respectively). During competitive growth with wild-type *C. jejuni*, the *mapA* mutant exhibited decreased growth potential, with a peak concentration of 5×10^8 CFU/ml at 20 h of growth compared to 4.9×10^9 CFU/ml for the wild type (Fig. 6B). Also, the *mapA* mutant exhibited a more rapid decrease in viability as cultures continued into stationary phase. The *mapA* mutant did not exhibit any growth defect as a monoculture, consistent with the observation that *mapA* is more critical during competition with the wild-type strain in the chicken gastrointestinal tract than in monoinfections. We tried without success to complement this phenotype, as a plasmid encoding *mapA* appears to alter the viability of *C. jejuni* (data not shown); this may be due to gene dosage effects. We examined transcript levels of downstream genes in both wild-type and *mapA* mutant backgrounds and observed that transcription was not significantly affected by the insertion mutation (data not shown).

As motility and capsule production have previously been shown to be required for colonization of the chicken gastrointestinal tract, the ability of the *mapA* mutant to swim and produce capsule was determined (6, 18). The mutant exhibited motility in semisolid agar similar to that of the wild type (see Fig. S1A and B in the supplemental material). Additionally, the *mapA* mutant produced capsule at levels similar to wild-type levels, and both strains produced much more than a strain lacking KpsM, encoding a capsular polysaccharide transporter (see Fig. S1C).

Analysis of MapA amino acid sequence using the protein structure and activity predictor I-TASSER provided few clues about its function, although weak homologies to various zinc-containing endopeptidases, e.g., endothelin-converting enzyme I, were detected (23, 24). Thus, it is possible that MapA functions as an endopeptidase, but it is currently unknown what the consequence of this activity might be for *in vivo* or *in vitro* competition. Whatever its precise role in chicken colonization, *mapA* sequences are highly conserved among several sequenced strains of *C. jejuni*, most with different origins. Despite the relatively high rate of genomic sequence variation in *C. jejuni* strains, *mapA* typically contains few nucleotide changes in the sequences of 100 strains, representing greater than 97% identity. This may indicate that MapA serves an important role in *C. jejuni*, preventing significant variation in the coding sequences.

This study demonstrated the value of deep sequencing analysis with transposon mutagenesis for identifying fitness determinants encoded by *C. jejuni*. Of the 130 mutations with relative abundances of at least 0.01, 37 are in genes annotated as hypothetical proteins, though some hypothetical determinants have been shown to encode colonization factors. These include Cjj81176_0083 and Cjj81176_0414, which encode a new class of flagellar coexpressed determinants (Feds) required for full colonization of the chicken gastrointestinal tract (25). Thus, approximately 28% of those genes identified as required for colonization of the chicken gastrointestinal tract exhibit no significant primary homology to known protein domains, signaling that an immense amount of information still remains to be uncovered about *C. jejuni* colonization factors.

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