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Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract

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Summary

Campylobacter jejuni **is the leading cause of bacterial gastroenteritis in humans in developed countries throughout the world. This bacterium frequently promotes a commensal lifestyle in the gastrointestinal tracts of many animals including birds and consumption or handling of poultry meats is a prevalent source of *C. jejuni* for infection in humans. To understand how the bacterium promotes commensalism, we used signature-tagged transposon mutagenesis and identified 29 mutants representing 22 different genes of *C. jejuni* strain 81–176 involved in colonization of the chick gastrointestinal tract. Among the determinants identified were two adjacent genes, one encoding a methyl-accepting chemotaxis protein (MCP), presumably required for proper chemotaxis to a specific environmental component, and another gene encoding a putative cytochrome *c* peroxidase that may function to reduce periplasmic hydrogen peroxide stress during *in vivo* growth. Deletion of either gene resulted in attenuation for growth throughout the gastrointestinal tract. Further examination of 10 other putative MCPs or MCP-domain containing proteins of *C. jejuni* revealed one other required for wild-type levels of caecal colonization. This study represents one of the first genetic screens focusing on the bacterial requirements necessary for promoting commensalism in a vertebrate host.**

Introduction

The interactions between microorganisms and their respective hosts may range on a continuum from a pathogenic relationship in which the microorganism profits by being able to replicate or perform other beneficial biological processes to the detriment of the host, to a commensal relationship in which one partner benefits biologically with no harm to the other, and, finally, to a symbiotic relationship in which both partners benefit biologically. Regardless of the outcome of these interactions, specific requirements are needed by both the microbe and the host to evolve these relationships, ultimately affecting the lifestyles of one or both of the organisms.

Numerous studies have focused on the requirements of many pathogenic organisms to infect and cause disease in their respective host. Fewer studies have sought to clarify the requirements of a microorganism or the host to successfully produce a commensal or symbiotic relationship. Cases where significant progress has been made to elucidate bacterial–host interactions to initiate and maintain symbiosis include the programmed development of intestinal mucosa of germ-free mice by interactions with *Bacteriodes thetaiotaomicron* (Bry *et al*., 1996; Hooper *et al*., 1999; 2001; 2003; Stappenbeck *et al*., 2002); nodulation of roots of certain legumes by nitrogen-fixing rhizobia (Perret *et al*., 2000); and formation of a functional light organ harbouring *Vibrio fischeri* in the squid *Euprymna scolopes* (Ruby, 1996; Visick and McFall-Ngai, 2000). Recent analysis of another symbiotic interaction has revealed bacterial genes of *Xenorhabdus nematophila* required for colonization of the intestinal vesicle of the nematode *Steinernema carpocapsae* (Heungens *et al*., 2002).

The bacterium *Campylobacter jejuni* naturally colonizes the gastrointestinal tract of many birds and animals, resulting in a harmless commensal relationship, but this microbe can also promote pathogenesis in humans, resulting in a productive gastroenteritis leading to a mild to bloody diarrhoeal syndrome. *Campylobacter jejuni* has been suggested to be the leading cause of bacterial gastroenteritis in humans in many developed countries, with estimates in the United States and Great Britain indicating that one out of 100 individuals becomes ill from *C. jejuni* infection each year (Kendall and Tanner, 1982; Altekruse *et al*., 1999; Friedman *et al*., 2000). The tropism of *C. jejuni* for the avian gastrointestinal tract – particularly to that of the chicken – involves mainly the lower gastrointes-

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tinal tract, including the caeca and large intestines and, to a lesser extent, the upper gastrointestinal tract and other organs (Beery *et al*., 1988). Whereas multiple surveys have had variable outcomes, it is generally believed that colonization of the chicken gastrointestinal tract may occur at a young age and can last for several weeks or months up to the time of slaughter (Lindblom *et al*., 1986; Pokamunski *et al*., 1986). During the slaughtering process, the gastrointestinal contents may contaminate the meat products; analysis of retail meats has demonstrated that as much as 67–98% of chicken meats may contain viable *C. jejuni* (Wempe *et al*., 1983; Stern and Line, 1992). Ingestion or handling of contaminated poultry meats are important causes of sporadic cases of *C. jejuni* disease (Altekruse *et al*., 1999; Friedman *et al*., 2000). Understanding the genetic requirements of *C. jejuni* for colonization of the chicken gastrointestinal tract may help elucidate mechanisms used by the bacterium to initiate and maintain this commensal relationship.

We used signature-tagged transposon mutagenesis of *C. jejuni* to identify genes involved in caecal colonization of chicks in a 1-day old chick model of commensalism. We screened 1550 *C. jejuni* mutants and identified 29 mutants representing 22 different genes required for wild-type levels of caecal colonization. Two genes demonstrating the most significant reductions in colonization include a gene encoding a putative methyl-accepting chemotaxis protein (MCP) and a gene encoding a putative cytochrome *c* peroxidase. Mutation of genes encoding 10 other MCPs or MCP-domain-containing proteins of *C. jejuni* revealed requirement of only one other MCP for wild-type caecal colonization. This study provides identification of the requirements of a bacterium in a natural host–microbe commensal relationship.

Results

Chick gastrointestinal colonization capacity of C. jejuni *strain 81–176*

Campylobacter jejuni strain 81–176 was isolated during an outbreak of *C. jejuni* disease associated with consumption of raw milk or hand milking of cows (Korlath *et al*., 1985). This strain elicits gastroenteritis in humans (Black *et al*., 1988) and reproduces the diarrhoeal syndrome in a ferret model of disease (Doig *et al*., 1996), but its colonization capacity in chicks has not been studied. We determined whether this strain could naturally colonize the gastrointestinal tracts of chickens, resulting in commensalism using a 1-day-old chick model of infection (Beery *et al*., 1988; Stern *et al*., 1988).

Approximately 12–36 h after hatching, chicks were orally infected with a range of doses of *C. jejuni* strain 81–176 and at 7 days post infection *C. jejuni* in the caeca were enumerated. With inocula of approximately 900 bacteria and above, all chicks were colonized and the bacterial loads in the caeca ranged from 3×10^8 to 8×10^9 colony-forming units (cfu) per gram of caecal content (Fig. 1). Caecal colonization was observed after inoculation with as low as approximately eight, 20 and 48 organisms (Fig. 1). Four of five chicks inoculated with eight organisms were colonized and three of four colonized chicks contained bacterial loads above 2×10^8 cfu per gram of caecal content after 7 days, similar to the caecal bacterial loads of chicks infected with inocula of higher orders of magnitude (Fig. 1). Colonized chicks appeared healthy and showed no signs of disease, as reported for chicks infected with other *C. jejuni* strains (Beery *et al*., 1988; Stern *et al*., 1988). Chicks gavaged with phosphate-buffered saline (PBS) alone as a nega-

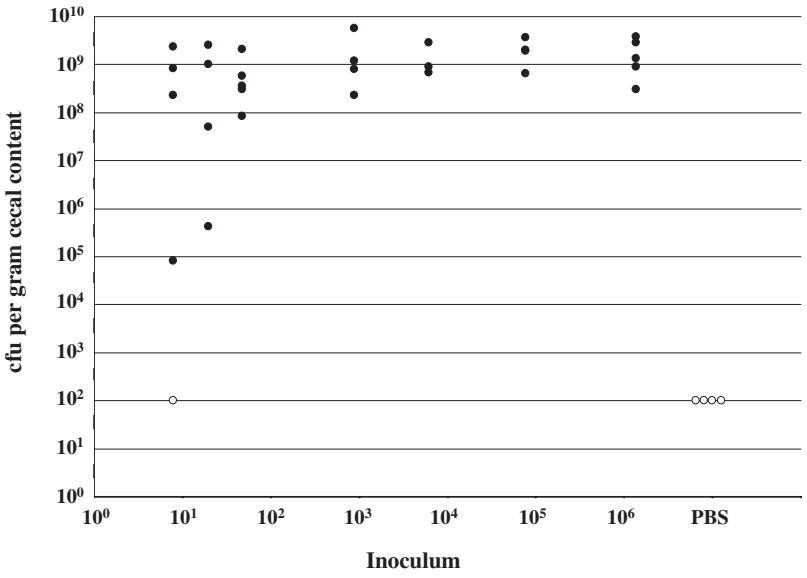

Fig. 1. Chick caecal colonization capacity of *C. jejuni* 81–176 at different inoculum. Chicks were orally infected with PBS alone or an inoculum ranging from eight to 1.37×10^6 cfu of *C. jejuni* 81–176. The levels of caecal colonization seven days post infection are reported as the number of cfu per gram caecal content. Each closed circle represents the number of bacteria obtained from an individual chick. Open circles represent chicks containing bacterial loads below the limit of detection (<100 cfu per gram caecal content). The actual inoculum doses were as follows: 8, 20, 48, 896, 6.2×10^3, 7.7×10^4 and 1.37×10^6 cfu.

tive control did not have *C. jejuni* in their caeca 7 days post infection.

We next studied the dynamics of colonization by *C. jejuni* strain 81–176 throughout the chick gastrointestinal tract by giving three different doses of the bacterium orally (~10^6, ~10^4 and ~10^1) and determining the number of bacteria in the upper gastrointestinal tract (the proximal and distal small intestines) and the lower gastrointestinal tract (the caeca and large intestines) over time. At day 1 post infection, chicks infected with 3×10^6 bacteria harboured the majority of *C. jejuni* in the lower gastrointestinal tract, with levels in the caeca and large intestines greater than 3×10^7 and 3×10^6 cfu per gram content respectively (Fig. 2A); the number of *C. jejuni* in the upper gastrointestinal tract (the proximal and distal small intestines) was at least 100-fold lower. By day 4, the levels of bacteria in all organs increased to an apparent maximal load and somewhat sustained this level of colonization at day 7 (Fig. 2A). At the end of the assay, the caeca of each chick still contained the most *C. jejuni*, with the large intestines containing a significant amount of bacteria. As with earlier time points, the colonization levels in the upper gastrointestinal tract at day 7 were lower than in the caeca or large intestines (Fig. 2A). Chicks inoculated with lower doses of *C. jejuni* (1.73×10^4 and 37 bacteria) displayed the same general trend: colonization of primarily the caeca early during the infection period with increasing colonization in all organs over the course of infection (Fig. 2B and C). Compared with infections with a higher level of 81–176 (~10^6; Fig. 2A), the time required to reach the maximal bacterial load in each organ was delayed and achieved between days 4 and 7 with an inoculum of 1.73×10^4 bacteria and around day 7 with an inoculum of 37 bacteria (Fig. 2B and C). Overall, the caeca contained the most bacteria at all time-points in each infection; of the 23 colonized chicks in these experiments (Fig. 2A–C), over 90% of the entire bacterial load of *C. jejuni* 81–176 per chick were in the caeca in 18 chicks (and over 75% in 21 chicks). These results demonstrate that the pathogenic *C. jejuni* strain 81–176 initiates commensal colonization of the chick intestinal tract, with tropism for the lower gastrointestinal tract – particularly the caeca – similar to what was found with another *C. jejuni* strain (Beery *et al.*, 1988).

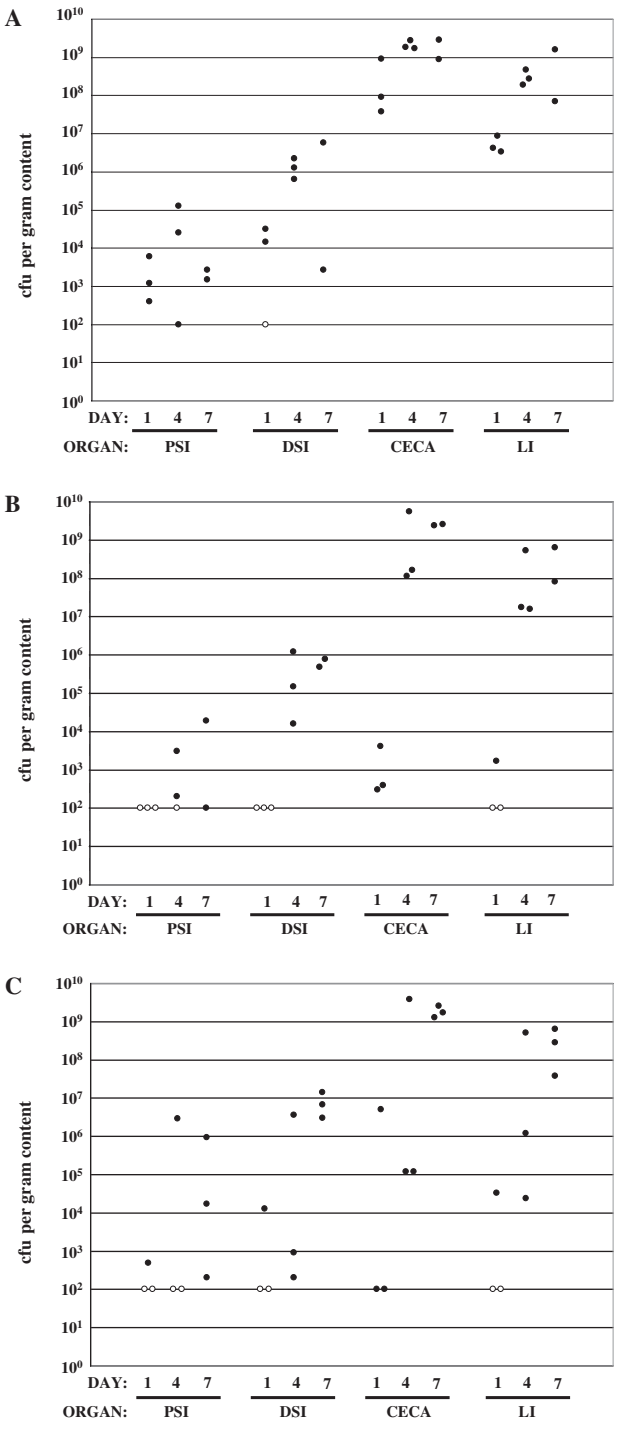

Fig. 2. Gastrointestinal colonization dynamics of *C. jejuni* 81–176. Chicks were orally infected with *C. jejuni* 81–176 and sacrificed at day 1, 4 or 7 post infection. The number of bacteria in the proximal small intestines (PSI), distal small intestines (DSI), caeca, and large intestines (LI) are reported as the number of cfu per gram organ content. Closed circles represent the number of bacteria obtained from an individual chick. Open circles represent chicks containing bacterial loads below the limit of detection (<100 cfu per gram organ content). Chicks were orally infected with (A) 3×10^6 cfu; (B) 1.73×10^4 cfu; (C) 37 cfu.

Identification of C. jejuni *genes involved in colonization of chick caeca by signature-tagged transposon mutagenesis*

In order to identify genes of *C. jejuni* 81–176 involved in colonization of the chick caeca, we employed signature-tagged transposon mutagenesis (STM; Hensel *et al.,* 1995; Shea *et al.*, 2000). We adapted the *Himar1*-based *solo* transposon (Hendrixson *et al.*, 2001) with 82 different

DNA signature tags, created 82 different transposon mutant libraries, and constructed pools containing 74–82 different transposon mutants of *C. jejuni* 81–176, with one mutant coming from a different transposon mutant library. We orally infected 1-day-old chicks with different pools and recovered the bacteria from the caeca 7 days post infection, as the caeca contained a high majority of bacteria in the chicks at this time during the infection period (Fig. 2A–C). We tested 1550 transposon mutants and obtained 187 putative mutants attenuated for chick caecal colonization.

To test the colonization capacity of the putative mutants, we attempted to perform a competition assay by mixing each transposon mutant with streptomycin-resistant 81–176 Sm^R (DRH212; Hendrixson *et al.,* 2001) in a 1:1 ratio and determining the levels of each strain in the caeca 7 days post infection. Control experiments in which 81–176 was competed with 81–176 Sm^R demonstrated that the number of bacteria of each strain obtained from the chick caeca was highly variable. This finding suggested that results from competition experiments were not reliable, and thus we switched to using infections with only one *C. jejuni* strain per chick. As an alternative method for determining the caecal colonization capacity of the putative mutants, we orally gavaged three to five chicks with approximately 10^4 cfu of each mutant and determined the number of bacteria 7 days post infection. This inoculum was chosen because it is the lowest that we have found routinely gives about $1–2 \times 10^9$ cfu per gram caecal content at day 7 post infection for strain 81–176. A transposon mutant was considered attenuated for caecal colonization if it colonized the caeca of all infected chicks at least 10-fold lower than wild-type 81–176 ($\sim < 2 \times 10^8$ cfu of the transposon mutant per gram caecal content). Of the 187 putative mutants from our primary screen, 29 mutants were attenuated for caecal colonization by this measure (Table 1 and Fig. 3A–C); many of the remaining mutants had significantly less colonization defects ranging from two- to fivefold and were not characterized further. These 29 mutants represent 22 different genes required for wild-type colonization of the chick caeca.

Seventeen of the 29 mutants exhibited a non-motile or altered flagellar motility phenotype in Mueller–Hinton (MH) motility medium (Table 1; Fig. 3A and B; data not shown). Previous studies found that motility of *C. jejuni* is required for wild-type levels of caecal colonization

Table 1. Location of *solo* in *C. jejuni* mutants attenuated for colonization of the chick caeca.

Gene[a,b]	Name[a]	Identification/proposed function[a]	Motility phenotype[c]
Cj0019c (2)	*docB*[d]	Probable methyl-accepting chemotaxis domain singal transduction protein	Motile
Cj0020c	*docA*[d]	Probable cytochrome *c* peroxidase	Motile
Cj0061c (2)	*fliA*	RNA polymerase σ^{28} subunit	Non-motile
Cj0248 (2)		Unknown/no identity	Altered
Cj0336c	*motB*	Possible flagellar motor protein	Non-motile
Cj0337c (3)	*motA*[e]	Probable flagellar motor proton channel	Non-motile
Cj0454c		Possible membrane protein	Reduced
Cj0456c		Unknown/no identity	Motile
Cj0618[f]		Unknown/similarity to C-terminus of Cj1305c, Cj1306c, Cj1310c, Cj1342c	Non-motile
Cj0670	*rpoN*	RNA polymerase σ^{54} subunit	Non-motile
Cj0688	*pta*	Probable phosphate acetyltransferase	Motile
Cj0883c[g]		Unknown/no identity	Non-motile
Cj0903c		Probable amino acid transport protein	Motile
Cj0938c	*aas*	Probable 2-acylglycerophosphoethanolamine acyltransferase/acyl-acyl carrier protein synthetase	Motile
Cj1019c	*livJ*	Probable branched-chain amino-acid transport ABC transport system periplasmic binding protein	Motile
Cj1118c	*cheY*	chemotaxis regulatory protein	Non-motile
Cj1120c	*pglF*	Protein glycosylation; possible sugar epimerase/dehydratase	Motile
Cj1121c (2)	*pglE*	Protein glycosylation; possible aminotransferase	Motile
Cj1129c	*pglH*	Probable glycosyltransferase	Motile
Cj1179c (2)	*fliR*	Probable flagellar biosynthesis protein	Non-motile
Cj1466	*flgK*	Possible flagellar hook associated protein	Non-motile
Cj1565c	*pflA*	Paralyzed flagellum protein	Non-motile

a. Gene designation, name, and proposed function are based on the annotated genome sequence from *C. jejuni* NCTC 11168 (Parkhill *et al*., 2000).
b. Number indicates number of mutants identified with different *solo* insertions in the respective gene.
c. Motile, motility similar to wild-type *C. jejuni* 81–176; non-motile, no migration; reduced, less migration with typical motility ring pattern; altered, less migration with atypical motility ring pattern.
d. Based on our data presented, we propose to designate *Cj0020c* and *Cj0019c* as *docA* and *docB*, respectively, for *d*eterminant *o*f chick *c*olonization.
e. One mutant has a *solo* insertion 5 base pairs upstream of the coding sequence of *motA*.
f. This mutant was originally derived from a putative signature-tagged transposon mutant that contained two *solo* insertions. The DNA from this original mutant was purified and transformed into *C. jejuni* 81–176. Transformants recovered contained either a *solo* insertion in *Cj0618*, which was attenuated in caecal colonization or the other unknown *solo* mutation which was not defective for colonization (data not shown).
g. *solo* Insertion is in the coding sequence of *Cj0883c*. *Cj0883c* is immediately upstream of *flhA* (Parkhill *et al*., 2000); the motility and efficient caecal colonization defect by the *solo* insertion in this mutant may result from a polar effect on the transcription of *flhA*.

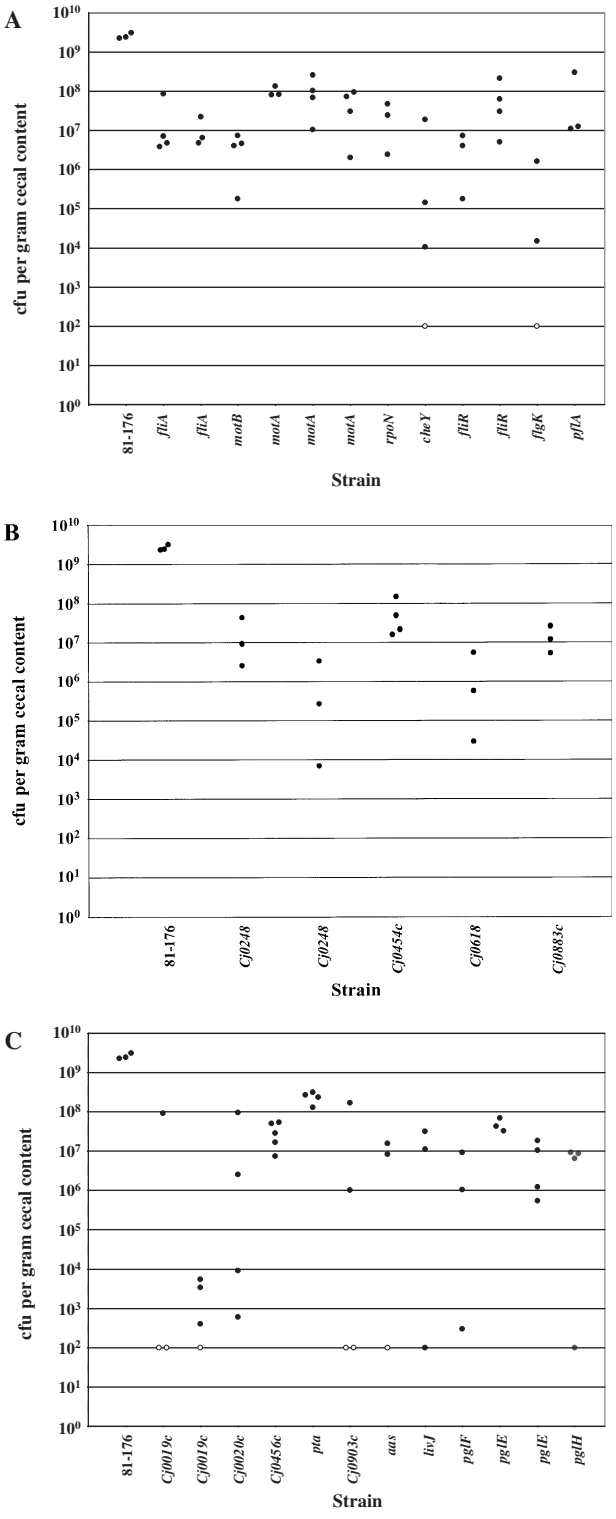

Fig. 3. Caecal colonization capacity of *C. jejuni* 81–176 signature-tagged transposon mutants. Chicks were orally infected with *C. jejuni* 81–176 or with a 81–176 transposon mutant. The number of bacteria present in the caeca 7 days post infection is reported as the number of cfu per gram caecal content. Each closed circle represents the number of bacteria obtained from an individual chick. Open circles represent chicks containing bacterial loads below the limit of detection (<100 cfu per gram caecal content). The same colonization assay for wild-type *C. jejuni* 81–176 is shown in all graphs.
A. Colonization capacity of non-motile mutants with transposon insertions in known genes required for motility. Inocula ranged from 3.9×10^3 to 2.3×10^4 cfu.
B. Colonization capacity of non-motile mutants with transposon insertions in genes with unknown functions. Inocula ranged from 1.63×10^4 to 3.4×10^4 cfu.
C. Colonization capacity of mutants with wild-type motility phenotype. Inocula ranged from 6.4×10^3 to 5.4×10^4 cfu.

(Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993), validating our screening procedure for identifying mutants of *C. jejuni* attenuated for colonization. Included among these genes involved in motility and chick colonization are genes encoding transcription factors, σ^{28} and σ^{54}, required for transcription of specific flagellar genes (Hendrixson *et al.*, 2001; Hendrixson and DiRita, 2003); flagellar secretory or structural components (*fliR* and *flgK*); flagellar motor proteins (*motA* and *motB*); a chemotaxis regulatory protein (*cheY*); a protein required for proper flagellar function (*pflA*; Yao *et al.*, 1994); and four proteins with no significant homologies to proteins with known functions (*Cj0248*, *Cj0454c*, *Cj0618* and *Cj0883c*; Parkhill *et al.,* 2000). *Cj0248* and *Cj0883c* have previously been identified as being required for motility (Hendrixson *et al.*, 2001). Mutation of many of these genes result in 10- to 1000-fold reductions in the bacterial loads in the chick caeca (Fig. 3A and B). The *cheY* and *flgK* mutants displayed a more dramatic defect in colonization; recovery of these mutants after infection ranged from 100-fold less than wild type to below the limit of our detection ability (<100 cfu per gram caecal content; Fig. 3A).

Twelve colonization mutants displayed wild-type motility in MH motility medium (Table 1 and data not shown); these mutants represent 10 different genes required for wild-type levels of caecal colonization. Four mutants contained transposon insertions in three different genes, *pglE*, *pglF* and *pglH*, which are part of a multigene locus encoding a general protein glycosylation system responsible for adding α-linked *N*-acetylgalactosamine residues to many different proteins in *C. jejuni* including flagellin (Szymanksi *et al.*, 1999; Linton *et al.*, 2002). These mutants typically displayed a 100- to 1000-fold reduction in colonization, but in a few chicks displayed over a 10^6-fold reduction in colonization (Fig. 3C). We also identified transposon insertions in attenuated mutants in *Cj0456c*, encoding a protein of unknown function, and two genes encoding proteins that probably function in amino acid transport, *livJ* and *Cj0903c* (Parkhill *et al.*, 2000). Mutation of *Cj0456c* reduced caecal colonization capacity of 81–176 approximately 100-fold, whereas mutation of *livJ* or *Cj0903* caused equivalent to more dramatic defects in colonization (Fig. 3C).

Transposon insertions in two adjacent genes *Cj0019c* and *Cj0020c* resulted in generally large decreases in caecal colonization (Fig. 3C). *Cj0019c* encodes a putative MCP; homologues of these proteins in other bacteria detect specific environmental components and transduce signals to the flagellar motor to alter the direction of motility (Blair, 1995; Falke and Hazelbauer, 2001). Transposon insertions in *Cj0019c* did not affect *in vitro* motility however (data not shown), and resulted in caecal colonization levels in six of seven chicks approximately 10^6-fold lower than wild-type 81–176 and below the limit of detection in three other chicks (Fig. 3C). *Cj0020c*, which is upstream of *Cj0019c* and may be co-transcribed with this gene, encodes a putative periplasmic cytochrome *c* peroxidase. A transposon insertion in this gene resulted in 20-fold to more than a 10^6-fold reduction in caecal colonization (Fig. 3C).

To ensure that the caecal colonization defects of the above mutants were specific for *in vivo* growth conditions, we analysed the growth curve of each mutant in MH biphasic medium. The growth curves of all the mutants over a 48 h period were similar to that of wild-type *C. jejuni* 81–176 (data not shown), suggesting that the colonization defects were due specifically to *in vivo* growth and not the result of a general growth defect.

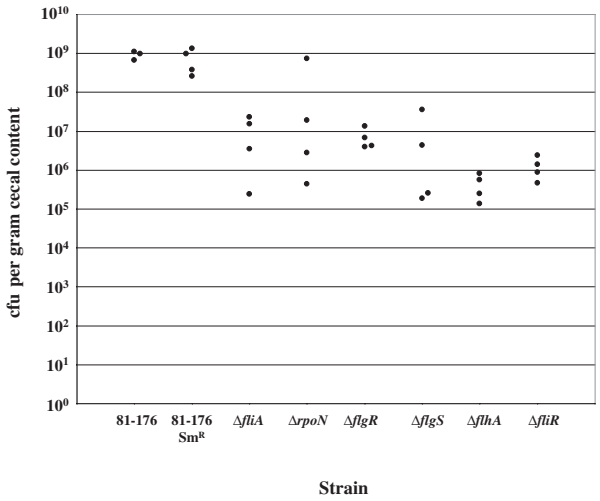

Fig. 4. Caecal colonization capacity of defined *C. jejuni* flagellar mutants. Chicks were orally infected with *C. jejuni* 81–176, 81–176 Sm^R (DRH212), or mutants containing defined deletions of certain genes required for motility. The number of bacteria present in the caeca seven days post infection is reported as the number of cfu per gram caecal content. Each closed circle represents the number of bacteria obtained from an individual chick. Open circles represent chicks containing bacterial loads below the limit of detection (<100 cfu per gram caecal content). Inocula ranged from 1.02×10^4–3.48×10^4 cfu.

Analysis of colonization defect by defined motility mutants

Some of the chick colonization mutants defective for motility had transposon insertions in genes encoding σ-factors required for transcription of flagellar genes including *rpoN (*encoding σ^{54}), which is required for transcription of *flgDE2* (encoding putative flagellar hook-associated proteins) and *flaB* (encoding a minor flagellin), and *fliA* (encoding σ^{28}), which is involved in transcription of *flaA* (encoding the major flagellin; Hendrixson *et al*., 2001; Hendrixson and DiRita, 2003). In addition, we found transposon insertions in *fliR* and *Cj0883c*, which is directly upstream of *flhA* (Parkhill *et al*., 2000); a transposon insertion in *Cj0883c* may have polar effects on the transcription of *flhA*. Both *fliR* and *flhA* encode putative components of the flagellar secretory apparatus, which is presumably required to secrete flagellar proteins, and, in conjunction with the FlgRS two-component regulatory system, is required for σ^{54}-dependent transcription of flagellar genes (Hendrixson and DiRita, 2003).

To determine the colonization capacity of *C. jejuni* mutants with defined deletions of flagellar genes, we infected chicks with derivatives of *C. jejuni* 81–176 Sm^R (DRH212) containing in-frame deletions of *fliA*, *rpoN*, *flgR*, *flgS*, *flhA* or *fliR* (Hendrixson *et al*., 2001; Hendrixson and DiRita, 2003). All flagellar regulatory mutants demonstrated colonization capacities approximately 100- to 10,000-fold lower than wild-type strains (81–176 or 81–176 Sm^R) in four separate chicks (the Δ*rpoN* mutant colonized to wild-type levels in one of four chicks; Fig. 4). The phenotypes of the *fliA*, *rpoN* and *fliR* deletion mutants verify those of the corresponding transposon mutants identified in our screen. Additionaly, because *flhA* is downstream of *Cj0883c* and the Δ*flhA* mutant was defective for colonization, the transposon insertion originally isolated in the *Cj0883c* mutant may have polar effects on the expression of *flhA*. These data also indicate that the FlgRS two-component regulatory system governing σ^{54}-dependent transcription of flagellar genes is involved in commensal colonization, and along with the additional characterization of the above flagellar gene deletion mutants, emphasize the importance of flagellar biosynthesis and motility in efficiently promoting commensal colonization of the chick caeca.

Characterization of the dynamics of colonization of Cj0019c *and* Cj0020c *deletion mutants in the chick gastrointestinal tract*

Two genes identified in our screen, *Cj0019c* and *Cj0020c*, are adjacent to each other in the *C. jejuni* genome (Parkhill *et al*., 2000). As mentioned above, *Cj0019c* displays homology to numerous bacterial MCP proteins that are responsible for detecting environmental cues and transducing signals to the flagellar motor to change the direction of motility towards chemoattractants and away

from chemorepellants. *Cj0020c* shows significant homology to bacterial periplasmic cytochrome *c* peroxidases, which bind two c-type haeme compounds to convert hydrogen peroxide to water (Rönnberg and Ellfolk, 1979; Ellfolk *et al*., 1983; Goodhew *et al*., 1990). Transcription of these two genes may be linked as the last four codons of *Cj0020c* overlap the beginning of the *Cj0019c* coding sequence.

To verify the colonization defect originally observed with the *Cj0019c* and *Cj0020c* transposon mutants, we deleted each gene from *C. jejuni* strain 81–176 Sm^R (DRH212; Hendrixson *et al.* 2001). For *Cj0019c*, we fused in frame codon four to the stop codon, deleting the intervening 588 codons. For *Cj0020c*, we fused in frame the start codon to the last 12 codons, deleting the intervening 292 codons.

The mutants were given orally to chicks at two different doses and caecal bacterial loads were determined 7 days post infection. At an inoculum of approximately 10^4, colonization of the Δ*Cj0019c* mutant was undetectable in four of five chicks and the colonization level in the remaining chick was slightly lower than 81–176 Sm^R (Fig. 5). Although increasing the inoculum to approximately 5×10^5 bacteria resulted in increased number of chicks colonized, the levels of colonization were 100-fold lower than what was seen with 81–176 Sm^R given at a similar inoculum (Fig. 5). The caecal colonization capacity for the Δ*Cj0020c* mutant was more dramatically altered relative to wild type. With an inoculum of 10^4 bacteria, two chicks were colonized with the Δ*Cj0020c* mutant at levels 10- to 10,000-fold lower than wild-type and three chicks appeared not to be colonized at all; raising the inoculum by approximately 50-fold resulted in four of five chicks being colonized, but the bacterial loads in the caeca did not significantly increase. These deletion mutants confirmed results obtained with the respective transposon mutants isolated in the STM random screening procedure.

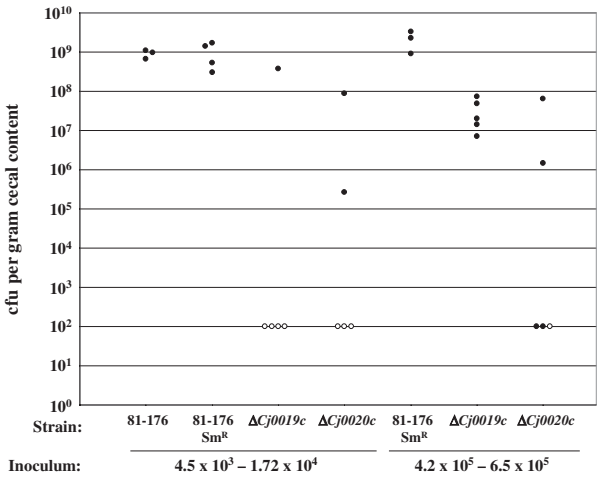

Fig. 5. Caecal colonization capacity of *C. jejuni* Δ*Cj0019c* and Δ*Cj0020c* mutants. Chicks were orally infected with 81–176, 81–176 Sm^R (DRH212), 81–176 Sm^R Δ*Cj0019c*, and 81–176 Sm^R Δ*Cj0020c* at two different inocula. The number of bacteria present in the caeca seven days post infection is reported as the number of cfu per gram caecal content. Each closed circle represents the number of bacteria obtained from an individual chick. Open circles represent chicks containing bacterial loads below the limit of detection (<100 cfu per gram caecal content).

To determinine whether *Cj0019c* and *Cj0020c* are required for specific tropism for the caeca or for general gastrointestinal colonization of chicks, we further characterized their colonization capacity by analysing colonization dynamics of each in the chick gastrointestinal tract over time. When inoculated at a dose of 5.6×10^5, the Δ*Cj0019c* mutant was detected in all intestinal organs of each chick at day 1 (Fig. 6A). However, colonization decreased over time to undetectable levels in the proximal small intestines and 10- to 100-fold in the large intestines (compare Fig. 6A with the colonization dynamics of wild-type 81–176 in Fig. 2A). The colonization levels in the caeca and distal small intestines remained relatively constant. Again, the lower gastrointestinal tract – the caeca and the large intestines – contained the highest amounts of the Δ*Cj0019c* mutant. Compared with the chicks infected with similar numbers of wild type 81–176 (Fig. 2A), the colonization level of the Δ*Cj0019c* mutant was generally 100-fold lower in all organs. When the inoculum of the Δ*Cj0019c* mutant was decreased to 10^4, significant colonization was not detected in the organs until day 4 and colonization increased in all organs through day 7, but remained at least 100-fold lower in all organs compared with wild-type 81–176 (data not shown).

Dynamics of colonization by the Δ*Cj0020c* mutant were different from those of the Δ*Cj0019c* mutant. When inoculated at a dose of 8.2×10^5, this mutant colonized all intestinal organs of chicks at day 1 and the amount recovered increased until day 4 (Fig. 6B). However, after day 4 the level of colonization in all organs stalled or began to decrease; the colonization levels in all organs at day 7 were 10- to 100-fold lower than wild-type 81–176 used at the same inoculum (compare Fig. 6B to Fig. 2A). When the inoculum of the Δ*Cj0020c* mutant was decreased to 10^4, colonization was not consistently detected in all organs at days 1 and 4, and by day 7 only one chick (out of three) showed detectable caecal colonization (data not shown); no other organs harboured detectable *C. jejuni*, suggesting that the infection was almost cleared. These results suggest that *Cj0019c* and *Cj0020c* are not required for specific tissue tropism and are instead required for wild-type colonization of the entire gastrointestinal tract. Because of these results, we propose to annotate *Cj0020c* and *Cj0019c* as *docA* and *docB*, respectively, for *d*eterminant *o*f chick *c*olonization.

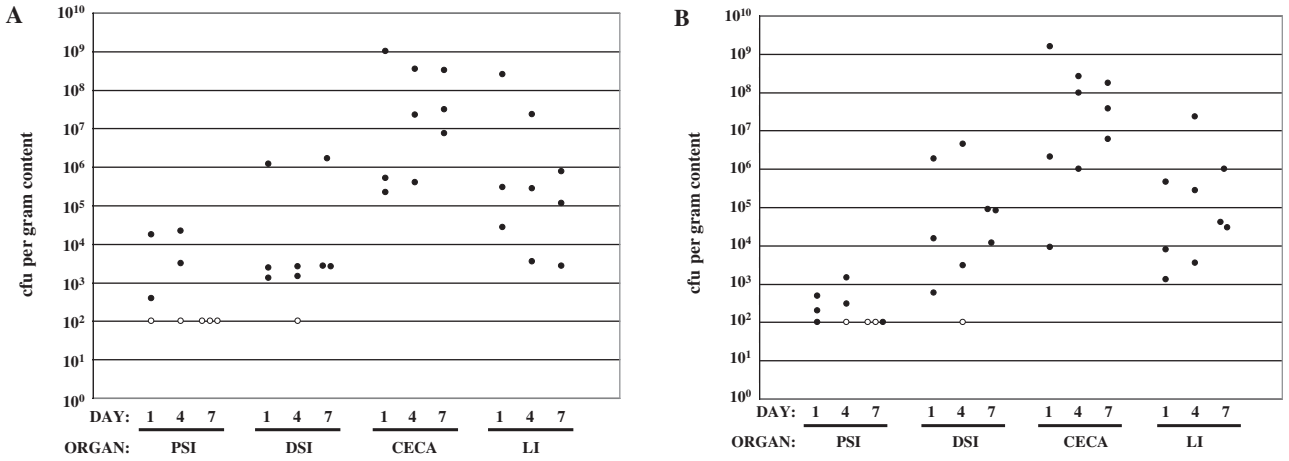

Fig. 6. Gastrointestinal colonization dynamics of *C. jejuni* Δ*Cj0019c* and Δ*Cj0020c* mutants. Chicks were orally infected with bacteria and sacrificed at day 1, 4 or 7 post infection. The number of bacteria in the proximal small intestines (PSI), distal small intestines (DSI), caeca, and large intestines (LI) is reported as the number of cfu per gram organ content. Closed circles represent the number of bacteria obtained from an individual chick. Open circles represent chicks containing bacterial loads below the limit of detection (<100 cfu per gram organ content). Chicks were orally infected with (A) 5.6×10^5 *C. jejuni* Δ*Cj0019c* or (B) 8.2×10^5 *C. jejuni* Δ*Cj0020c*.

Analysis of other MCP and MCP-domain containing proteins for chick caecal colonization

Because *docB* encodes a putative MCP involved in establishing colonization of the chick gastrointestinal tract, we thought it relevant to determine whether other MCP-like proteins in *C. jejuni* were required for this process. Twelve genes (including *docB*) are proposed to encode MCPs or proteins containing MCP domains in the *C. jejuni* NCTC11168 genome (Parkhill *et al.*, 2000; Marchant *et al.*, 2002). Three of these proteins in *C. jejuni* include CetA and CetB, which are required for energy taxis (specific migration towards the energy-generating substrates fumarate and sodium pyruvate; Hendrixson *et al.*, 2001), and Cj1191c, which shows high homology to CetB but is not required for energy taxis (Hendrixson *et al.*, 2001). The other eight MCP or MCP-domain-containing proteins have so far remained uncharacterized.

We inactivated each gene encoding an MCP or MCP-domain-containing protein (except for *Cj0246c*, which we were unable to inactivate) either by inserting a *cat-rpsL* or a *kan-rpsL* cassette or by deletion mutagenesis in *C. jejuni* 81–176 Sm^R (DRH212). All mutants displayed wild-type motility in MH motility medium (data not shown). The mutants were used to orally infect 1-day-old chicks at inocula of approximately 10^4 and the bacteria in the caeca were enumerated 7 days post infection. Only one other MCP, encoded by *Cj0262c*, appeared to be required for wild-type colonization (Fig. 7). Of the eight chicks infected with one of two isolated *Cj0262c* mutants, only one chick became colonized close to the level at which chicks infected with wild-type were. Four chicks had bacterial loads in the caeca 10- to 100-fold lower than wild type and three chicks contained bacterial loads at or below the limit of detection in the assay (<100 cfu per gram caecal content). Mutation of the remaining 10 genes encoding an MCP or MCP-domain-containing protein did not significantly alter the colonization capacity of *C. jejuni*. Of note, the genome sequence of *C. jejuni* NCTC11168 contains a stop codon in *Cj0951c* that causes a premature stop codon. In *C. jejuni* strain 81–176, this mutation is not present and the coding sequence therefore extends downstream, resulting in an open reading frame of ~1600 nucleotides encoding an MCP with domains similar to those of other bacteria. Nevertheless, this protein in *C. jejuni* 81–176 does not function in chick colonization (Fig. 7). These experiments also demonstrate that Cj1191c and the energy taxis system mediated by CetA and CetB are not required for colonization, as mutation of the these genes did not alter the colonization capacity of *C. jejuni*. These results indicate that only two MCPs, DocB and Cj0262c (which we term DocC), are required for wild-type level of colonization of the chick gastrointestinal tract.

Discussion

Our study provides one of the first whole-genome screening procedures for identifying genes of a bacterium involved in establishing commensalism with a natural host. Using signature-tagged transposon mutagenesis, we identified 22 different genes of *C. jejuni* required for wild-type levels of colonization of the chick gastrointestinal tract. Genes previously identified as recognized for chick colonization by other *C. jejuni* strains, such as *cadF* (Bras *et al.*, 1999) and *racRS* (Ziprin *et al.*, 1999), were not identified in our screen, indicating that our screen was not exhaustive. In addition, until detailed analysis of each

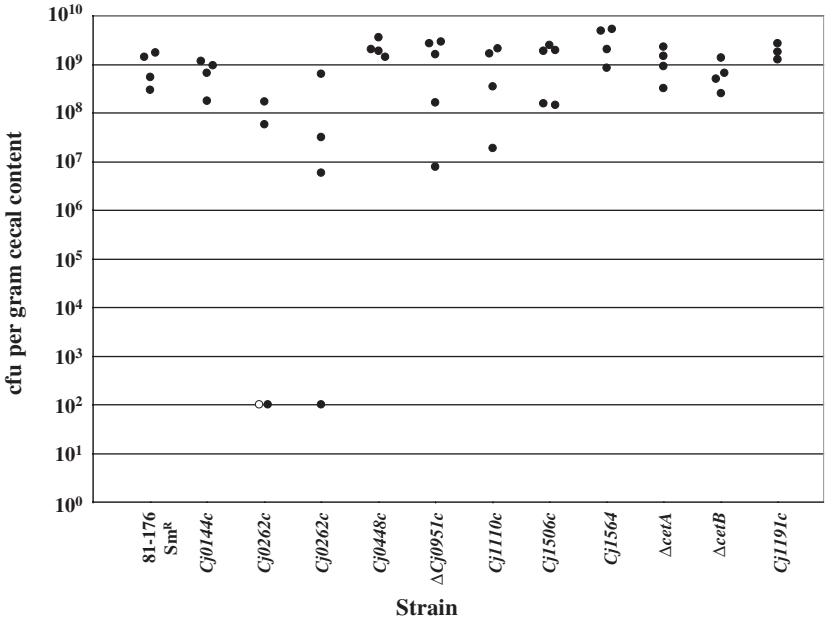

Fig. 7. Chick caecal colonization capacity of *C. jejuni* MCP mutants. Chicks were orally infected with *C. jejuni* 81–176 Sm^{R} (DRH212) or mutants lacking genes encoding MCP or MCP-containing domain proteins. The number of bacteria present in the caeca seven days post infection is reported as the number of cfu per gram caecal content. Each closed circle represents the number of bacteria obtained from an individual chick. Open circles represent chicks containing bacterial loads below the limit of detection (<100 cfu per gram caecal content). Inocula ranged from 5×10^3 to 3.7×10^4 cfu. All mutants contained genes insertionally inactivated with a *cat-rpsL* or *kan-rpsL* cassette except for the *Cj0951c*, *cetA*, and *cetB* mutants which contained in frame deletions of the respective genes.

identified locus has been carried out, we cannot rule out polarity effects on transcription of genes downstream of the transposon insertions.

Our screen confirmed an earlier observation that flagellar mutants can colonize chicks, albeit with greatly reduced ability (Wassenaar *et al*., 1993). Currently we do not know the role of motility in colonization. Whereas our study suggests that non-motile mutants can promote short-term colonization (up to 7 days post infection) with lower bacterial loads in the caeca, we have not determined whether motility is required for persistence; chickens in the field can be colonized with *C. jejuni* for many weeks to months (Lindblom *et al*., 1986; Pokamunski *et al*., 1986). One hypothesis for the role of motility in colonization is that it may be required to reach a specific niche in the intestinal tract *C. jejuni* normally occupies; non-motile mutants may be unable to migrate to that niche and establish prolonged colonization like that seen with wild-type *C. jejuni*. Additionally, because *C. jejuni* apparently resides in an extracellular environment in the chick caeca – the mucus layer of the caecal crypts (Beery *et al*., 1988) – motility may be required to resist gut peristalsis that may otherwise expel the organisms from the gastrointestinal tract. Non-motile mutants may be able to colonize the chick gastrointestinal tract but eventually be shed from the host with time.

We are aware of only one other study in which a large-scale genetic approach was taken to identify bacterial genes involved in commensal colonization of a natural host. In this study, Heungens *et al*. (2002) applied signature-tagged transposon mutagenesis to identify genes of the bacterium *X. nematophila* involved in symbiotic colonization of the intestinal vesicle of the nematode host, *S. carpocapsae*. By comparing the results of our study with those found in the analysis of the *X. nematophila–S. carpocapsae* model, and then comparing these two studies with many previous studies using signature-tagged transposon mutangenesis to identify genes of a bacterial pathogen required for virulence, common themes may be emerging regarding the differing bacterial requirements for a commensal versus a pathogenic bacteria for colonization of their respective hosts. For instance, in pathogenesis studies bacterial genes encoding proteins involved in LPS/LOS, amino acid and purine biosynthesis are commonly found (Perry, 1999; Shea *et al*., 2000), but in our system we did not find any of these types of genes and instead found only two genes predicted to encode proteins involved in amino acid transport. In the *X. nematophila–S. carpocapsae* model, no apparent genes involved in LPS biosynthesis and only two genes encoding proteins involved in amino acid biosynthesis were found (Heungens *et al*., 2002). These differences may in part be caused by a reflection of the extracellular location of the commensal organisms in their host in comparison to the invasive nature of many pathogens where the eventual sites inhabited by the pathogens, such as intracellular vacuoles, may be quite nutrient limiting. Another reason for these differences may be that the host actually provides many nutrients for the commensal organism to allow for colonization, unlike in bacterial pathogen–host relationships. This point is highlighted in the bacterial symbiont–host model involving *V. fischeri* colonizing the light organ of the squid *E. scolopes*, where it has been shown that amino acid auxotrophs of *V. fischeri* are able to colonize the squid, albeit at reduced efficiency, but this defect in auxotrophy can be overcome because the squid appar-

ently freely provides amino acids in various forms for the bacterium (Graf and Ruby, 1998). By comparing commensal and pathogenesis model systems, we may begin to obtain a picture of the different requirements of commensal bacteria and pathogenic bacteria in colonizing their respective host.

One of the genes identified with the most severely attenuated colonization phenotype when mutated is *Cj0019c (docB*), predicted to encode an MCP. MCPs are bacterial signalling proteins that sense certain environmental components and transduce signals to the chemotaxis regulatory proteins, thereby altering the direction of motility towards chemoattractants or away from chemorepellants (for more extensive description, see reviews by Blair, 1995; Falke *et al*., 1997; Falke and Hazelbauer, 2001). An MCP is usually arranged with two N-terminal transmembrane regions separated by a periplasmic domain that functions to sense signals in the environment, often amino acids or sugars. Once the periplasmic domain has sensed the component, the MCP transduces signals to the cytoplasmic domain. The cytoplasmic domain is highly conserved amongst the MCPs and interacts with the chemotaxis regulatory proteins to transmit signals to the flagellar motor.

Through a process termed 'adaptation' mediated by methylation of its cytoplasmic domain, an MCP can keep a memory of the concentration of a chemoattractant or chemorepellant encountered previously. The degree of methylation of a particular MCP (which is controlled by two chemotaxis regulatory proteins) allows the bacterium to record the concentration of a particular substance in recent environments. Adaptation allows the bacterium to fine-tune its movement towards or away from a particular substance.

The role of methylation in *C. jejuni* MCPs has not been studied. Whereas DocB (Cj0019c) and DocC (Cj0262c) are predicted to contain periplasmic, transmembrane and highly conserved cytoplasmic domains typical of other bacterial MCPs (Marchant *et al*., 2002), the methylation sites in these proteins contain differences. Some MCPs in *C. jejuni* such as DocC (Cj0262c) have putative methylation sites similar to the typical methylation site found in well-characterized MCPs of other bacteria (Falke *et al*., 1997; Marchant *et al*., 2002). In contrast, DocB (Cj0019c) and others have no obvious methylation sites similar to canonical MCP methylation sites. These observations suggest that the site and process of methylation of MCPs in *C. jejuni* may be different from typical bacterial MCPs.

One of two hypotheses may account for the mechanism of DocB function. The simplest explanation is that DocB is methlyated at one or more specific sites that are divergent from the more canonical methylation site of other bacterial MCPs. Alternatively, DocB may be able to promote proper movement towards its particular chemotactic cue in conjunction with another MCP, which has been observed with a mutant form of the Trg MCP in *Escherichia coli* (Hazelbauer *et al*., 1989). MCPs appear to be arranged in bacteria as homodimers clustering with other MCP homodimers to form a complex that has been described as 'trimers of dimers', which allows signal recognition by one MCP and a signal trasduction to the chemotaxis regulatory proteins by another MCP in the mixed cluster (Kim *et al*., 1999; Ames *et al*., 2002). In this hypothesis, DocB may associate with a methylation-competent MCP; an obvious candidate is DocC (Cj0262c), which we also identified in our analysis of other MCPs that may be required for chick colonization. DocC has a putative methylation site that is more similar – but not identical – to the typical methylation sites of well-studied MCPs.

Another mutation that severely affected the chick colonization capacity of *C. jejuni* occurred in the gene immediately upstream of *docB*. This gene, *Cj0020c (docA*), is predicted to encode a periplasmic cytochrome *c* peroxidase that binds two c-type haeme groups to accept electrons from a periplasmic c cytochrome to convert hydrogen peroxide to water (Rönnberg and Ellfolk, 1979; Ellfolk *et al*., 1983; Goodhew *et al*., 1990). *Campylobacter jejuni* also encodes another periplasmic cytochrome *c* peroxidase in its genome in the gene *Cj0358* (Parkhill *et al*., 2000). Currently we do not know what the specific roles of these two cytochrome *c* peroxidases are in the biology of *C. jejuni*. One possibility is that a specific type of metabolism may occur in the avian gut resulting in periplasmic hydrogen peroxide formation that DocA detoxifies. We have been unable to isolate a mutant deficient for *Cj0358*, indicating that this gene may be essential for *in vitro* growth whereas *docA* may be required for *in vivo* growth.

We have identified genes required for wild-type caecal colonization leading to commensalism in the chick gastrointestinal tract. It is as yet unclear whether these same genes are required for pathogenesis in a disease model of *C. jejuni* gastroenteritis, such as the ferret, which produces an inflammatory response to the invading *C. jejuni* (Fox *et al*., 1987). One of the genes we identified that is involved in chick colonization, *cheY*, is also required for promoting gastroenteritis in ferrets (Yao *et al*., 1997), suggesting that at least some genes we identified as involved in commensalism are required for pathogenesis. By analysing both commensal and pathogenic models of *C. jejuni* infection, we hope to provide new insights into interactions between the bacterium and each host that result in two such different outcomes.

Experimental procedures

Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are

located in Table S1 (see *Supplementary materials*). For details regarding the construction of plasmids and strains for producing specific *C. jejuni* mutants, see *Supplementary materials. Campylobacter jejuni* was grown in microaerophilic conditions at 37°C on Mueller–Hinton (MH) agar as previously described (Hendrixson et al., 2001). For *C. jejuni*, antibiotics were used at the following concentrations: trimethoprim, 10 μg ml^{-1}; cefoperazone 30 μl ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 15 μg ml^{-1}; and streptomycin, 0.5, 1 or 2 μg ml^{-1}. *E. coli* DH5α and DH5αλ*pir* were grown in Luria–Bertani (LB) agar or broth. For *E. coli*, antibiotics were used in the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and chloramphenicol, 15 μg ml^{-1}.

Construction of signature-tagged solo transposon mutants and mutant pools

The *solo* transposon was amplified from pFalcon (Hendrixson *et al*., 2001) by PCR using primers that added 5′ *Pme*I restriction sites. The amplified fragment contained 98 bp of sequence 5′ and 217 bp of sequence 3′ to *solo*. The *Pme*I-digested PCR fragment was ligated into pUC19 that had been digested with *Eco*RI and *Hin*dIII and blunt-ended with T4 DNA polymerase (Invitrogen) to generate pFalcon2.

Eighty-two unique DNA signature tags, each contained in a separate pUTmini-Tn*5*Km2 derivative (Martindale *et al*., 2000) were individually amplified by PCR with primers P3 and P5 (Hensel *et al*., 1995), digested with *Kpn*I, and ligated into *Kpn*I-digested pFalcon2. After cloning each signature tag, a collection of 82 pFalcon2 derivatives each containing a different signature tag in the *solo* transposon was obtained. Each plasmid was purified and used in *in vitro* transposition reactions. Transposition reactions were performed as previously described (Hendrixson *et al*., 2001; Hendrixson and DiRita, 2003) using 500 ng *Himar1* C9 transposase purified from DH5α/pMALC9 (Akerley and Lampe, 2002), 2 μg of purified *C. jejuni* strain 81–176 chromosomal DNA, and 1 μg of each pFalcon2 derivative containing a signature-tagged *solo* transposon. After transposition, the transposed DNA was repaired and transformed into *C. jejuni* 81–176 as previously described (Hendrixson *et al*., 2001). Transformants were recovered on MH agar containing 50 μg ml^{-1} kanamycin. As a result, 82 *C. jejuni* 81–176 signature-tagged *solo* mutant libraries were created in which each library contained mutants with a unique signature-tagged transposon. Combined, the mutant libraries totalled over 59 000 transposon mutants.

For construction of signature-tagged pools of *C. jejuni* 81–176 mutants, each of the 82 signature-tagged *solo* mutant libraries was streaked on MH agar containing 50 μg ml^{-1} kanamycin and grown for 48 h at 37°C under microaerophilic conditions. To construct a mutant pool, a mutant from each library was patched onto two identically sectored MH agar plates and grown for 48 h at 37°C under micraerophilic conditions. All 82 signature-tagged 81–176 *solo* mutants of one pool from one plate were recovered, mixed together, and stored at −80°C in MH broth containing 15% glycerol. The mutants on the other plate were each arrayed in a 96-well plate in MH broth containing 15% glycerol and stored at −80°C. This procedure was repeated to generate 20 different pools of signature-tagged 81–176 *solo* mutants.

Chick colonization assays

White leghorn strain Δ chicken eggs were acquired from a local farm and incubated in an egg incubator (Sportsman Incubator Model 1202; Georgia Quail Farms) for 21 days at 37.8°C with the appropriate humidity and rotation of eggs according to manufacture's instructions until the chicks hatched from the eggs.

For testing the caecal colonization capacity of *C. jejuni* 81–176 and derivatives, each strain was streaked on MH agar and grown at 37°C under microaerophilic conditions for 48 h. Strains were streaked heavily onto three MH agar plates and grown at 37°C under microaerophilic conditions for 16 h. Each strain was resuspended, diluted in MH broth to an OD_{600} = 0.4, and then diluted in PBS appropriately to obtain the proper inoculum. Twelve to 36 hours after hatching, chicks were divided into groups of three to 10 and infected orally with 100 μl of each inoculum for each strain. Dilutions of each inoculum were plated on MH agar to determine the number of bacteria in each inoculum. Each group of chicks was housed separately in brooders and given water and food *ad libitum*. Chicks were sacrificed at day 1, 4 or 7 post infection and the appropriate organs were removed. The contents of each particular organ were collected, weighed, and resuspended in PBS to a final concentration of 0.1 g of organ content per ml. Ten-fold serial dilutions of each sample were made and plated on MH agar containing 10 μg ml^{-1} trimethoprim and 30 μg ml^{-1} cefoperazone to select for growth of *C. jejuni*. Plates were incubated for 48 h at 37°C under microaerophilic conditions and the colonies recovered were counted. The colonization capacity of *C. jejuni* in an organ from each chick was reported as the number of cfu per gram of organ contents.

Screening of signature-tagged transposon mutants for mutants attenuated in caecal colonization

Hybridization blots for screening of mutants present in the input and output pools from infections with signature-tagged mutant pools were prepared by amplifying each of the 82 tags individually from the respective pFalcon2-containing signature-tagged *solo* derivative by PCR with P3 and P5 primers (Hensel *et al*., 1995). Each 40-bp unique signature tag was purified after *Hin*dIII-digestion and eluted as previously described (Merrell *et al*., 2002) and specifically arrayed onto Duralon nitrocellulose membranes (Stratagene). Membrane-bound DNA was denatured with 0.4 M NaOH for 8 min, rinsed with 0.5 M Tris-HCl (pH 7.0) for 5 min, rinsed twice in 2× SSC for 5 min, and cross-linked to the membranes in a UV Stratalinker (Stratagene). Membranes were stored in 2× SSC at 4°C.

To screen each pool for mutants attenuated for colonization of the chick caeca, each pool was streaked onto MH agar and grown for 48 h at 37°C under microaerophilic conditions. Each pool was then streaked heavily onto five MH agar plates and grown for 16 h at 37°C under microaero-

philic conditions and then resuspended in PBS, pelleted, and diluted in PBS to $OD_{600} = 1$. Bacteria were concentrated fivefold and 100 μl was used to orally infect each of three chicks (approximately 10^9 bacteria). The remaining inoculum suspension was used to purify chromosomal DNA from each pool (input pool). Seven days post infection, chicks were sacrificed and the contents of both caeca from each chick were collected, combined, weighed and resuspended in PBS to a final concentration of 0.1 g caecal content per ml. Dilutions of bacteria were plated on MH agar containing 10 μg ml^{-1} trimethoprim and 30 μg ml^{-1} cefoperazone to recover approximately 10 000 bacteria per chick. The output pool bacteria obtained from chicks infected with identical pools were combined and chromosomal DNA was purified.

For identification of putative mutants attenuated in caecal colonization, the signature tags from the input and output pool chromosomal DNAs from each infection were amplified with P2 and P4 primers (Hensel *et al*., 1995), Pfu polymerase (Stratagene), and DIG DNA labelling mix (Roche) to generate dioxygenin (DIG)-dUTP labelled probes. The probes were then used in dot blot hybridizations with the signature tag-arrayed membranes. Hybridization and development of dot blots were performed using the DIG DNA Labeling and Detection Kit (Roche) according to manufacture's instructions. Tags present in the input pool but absent in the output pool represent putative mutants deficient for efficient caecal colonization. Occasionally, the primary screening of an individual pool revealed between 20 and 50 putative mutants absent from the output pools, suggesting a great loss of many mutants, presumably the result of a bottleneck in the colonization process. These putative mutants from an individual pool were combined into a second pool consisting of 20–50 mutants and screened again for caecal colonization in chicks as described above. The second screening procedure often reduced the number of putative mutants in a pool to below 20 individual mutants.

Each putative mutant was recovered from the frozen arrayed pool and individually tested for caecal colonization in chicks at an inoculum of approximately 10^4. The number of bacteria per gram of caecal colonization 7 days post infection was determined as described above. A putative mutant was considered attenuated for colonization if the caecal bacterial loads in all chicks infected with the mutant were at least 10-fold lower than those of chicks infected with wild-type *C. jejuni* 81–176 (caecal colonization capacity $<2 \times 10^8$ cfu of mutant per gram caecal content compared with $\sim 2 \times 10^9$ cfu of wild type per gram caecal content). Identification of the site of the transposon insertion in each attenuated mutant was determined by DNA sequencing. The generated DNA sequences were compared with the genomic sequence of *C. jejuni* NCTC 11168 (Parkhill *et al*., 2000) to determine the site of the transposon insertion in *C. jejuni* 81–176.

Motility assays

Motility phenotypes of strains were tested in MH motility media containing 0.4% agar as previously described (Hendrixson *et al*., 2001).

In vitro *growth curve determination*

To determine the *in vitro* growth rates of *C. jejuni* mutants, strains were streaked on MH agar and grown for 48 h at 37°C under microaerophilic conditions. Each strain was streaked heavily onto three MH agar plates and the plates were incubated for 16 h at 37°C under microaerophilic conditions. Growth from each strain was resuspended in MH broth and diluted to a final concentration of approximately 2×10^5 cfu per ml. For each strain, 20 mls of MH agar were placed in sterile T75 tissue culture flasks. After the agar had solidified, the inoculated MH broth was placed in the tissue culture flasks to create a biphasic medium. Growth was monitored both spectrophotometrically by OD_{600} readings and by determining the number bacteria per ml by plating dilutions of the biphasic media on MH agar at 8, 24, 32 and 48 h.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3988/mmi3988sm.htm

Appendix 1: Construction of *C. jejuni* 81–176 mutants.

Table S1. Bacterial strains and plasmids.

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