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Use of Genome-Wide Expression Profiling and Mutagenesis To Study the Intestinal Lifestyle of *Campylobacter jejuni*

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Campylobacter jejuni **is the most common bacterial cause of diarrhea worldwide. To colonize the gut and cause infection, *C. jejuni* must successfully compete with endogenous microbes for nutrients, resist host defenses, persist in the intestine, and ultimately infect the host. These challenges require the expression of a battery of colonization and virulence determinants. In this study, the intestinal lifestyle of *C. jejuni* was studied using whole-genome microarray, mutagenesis, and a rabbit ileal loop model. Genes associated with a wide range of metabolic, morphological, and pathological processes were expressed in vivo. The in vivo transcriptome of *C. jejuni* reflected its oxygen-limited, nutrient-poor, and hyperosmotic environment. Strikingly, the expression of several *C. jejuni* genes was found to be highly variable between individual rabbits. In particular, differential gene expression suggested that *C. jejuni* extensively remodels its envelope in vivo by differentially expressing its membrane proteins and by modifying its peptidoglycan and glycosylation composition. Furthermore, mutational analysis of seven genes, *hspR*, *hrcA*, *spoT*, Cj0571, Cj0178, Cj0341, and *fliD*, revealed an important role for the stringent and heat shock response in gut colonization. Overall, this study provides new insights on the mechanisms of gut colonization, as well as possible strategies employed by *Campylobacter* to resist or evade the host immune responses.**

Campylobacter is the most common nonviral etiological agent of infectious enteritis in humans and has been implicated in 14.2% of the 76 million food-borne illnesses reported annually in the United States (21). *Campylobacter* infections vary from mild diarrhea to severe abdominal pain (49). Rarely, they result in the development of Guillain-Barré, syndrome, which is the primary cause of acute neuromuscular paralysis in the United States (49). Because of the extremely high number of cases of food-borne infections reported yearly worldwide, the development of new strategies to fight these infections is urgently needed and will depend on developing an understanding of host-pathogen interactions. The complete genomic sequence of *Campylobacter jejuni* NCTC 11168 was released in 2000 (27), providing new opportunities for the investigation of *Campylobacter* pathogenesis.

Despite the high incidence of *Campylobacter*-mediated diarrhea, the microbial factors that govern gut colonization and pathogenesis are poorly understood in comparison with other enteric pathogens. In a complete infection cycle, *Campylobacter* cells are transferred from contaminated foods to the stomach, the intestinal tract and, finally, to the feces, allowing their transmission to a new host (6, 13). During this stressful journey through the gastrointestinal tract, *Campylobacter* encounters and must adapt to life-threatening environmental conditions, such as the acidic pH of the stomach, the high osmolarity of the gastrointestinal tract, intestinal gases, reactive oxygen and nitrogen compounds, changes in nutrient availability, and low inorganic ion concentrations (13). For successful colonization, *Campylobacter* cells must survive in the intestinal tract, either as free-living microorganisms in the mucus layer, attached to the epithelium, or intracellularly in epithelial cells (6). *Campylobacter* determinants involved in colonization and pathogenesis include flagella, host cell adherence and invasion, and toxin production (6). Nonmotile and aflagellated *Campylobacter* were shown to be affected in their ability to colonize the gastrointestinal tract as well as to invade the epithelial cells (25, 56). Many suspected adhesins have been identified, such as lipo-oligosaccharide (20), flagella (56), or surface-exposed proteins (CadF and PEB1) (23, 29). Host cell invasion has been extensively studied and is thought to be an important step in *Campylobacter* infection (6). Indeed, biopsies of humans diagnosed with *C. jejuni* enteritis revealed the presence of intracellular *Campylobacter* cells (47). Interestingly, toxin production has recently been proposed to modulate the host immune response, allowing the bacterium to escape the immune surveillance (9).

To identify new potential virulence factors, we analyzed *C. jejuni* lifestyle in the gut using microarray technology. The *C. jejuni* NCTC 11168 genome-wide expression profile was assessed during host colonization and pathogenic development, using a mammalian model of gastroenteritis, the rabbit ileal loop (RIL) model. In addition, mutants were constructed by deleting genes of interest identified by our microarray analysis, and they were assessed for their ability to survive in the gastrointestinal tract of rabbits.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and preparation of inocula. The bacterial strains and plasmids used in this work are listed in Table 1. The *C. jejuni* NCTC 11168

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
E. coli strain		
DH5α	*endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA rel A1 Δ(lacZYAargF U169deoR [f80dlacΔ(lacZ0 M15]*	Invitrogen
C. jejuni strains		
NCTC 11168	*C. jejuni* NCTC 11168	NCTC
AS283	NCTC 11168ΔCj0571	This study
AS287	NCTC 11168Δ*spoT*	This study
AS272	NCTC 11168Δ*hspR*	This study
AS277	NCTC 11168Δ*hrcA*	This study
AS211	NCTC 11168ΔCj0178	26
AS317	NCTC 11168ΔCj0341	This study
AS318	NCTC 11168Δ*fliD*	This study
Plasmids		
pUC19	Cloning and suicide vector, Amp^r	Biolabs
pRY111	*cam* resistance gene	55
pAS282	pUC19 carrying ΔCj0571::*cam*	This study
pAS271	pUC19 carrying Δ*hspR*::*cam*	This study
pAS315	pUC19 carrying Δ*hrcA*::*cam*	This study
pAS286	pUC19 carrying Δ*spoT*::*cam*	This study

[a] *cam,* chloramphenicol resistance gene; Amp^r, ampicillin resistant.

strain was acquired from the National Collection of Type Culture (NCTC) in Spring 2000. *Campylobacter* strains were cultured in Mueller-Hinton (MH) medium or on MH agar plates at 37°C in a microaerophilic chamber (Don Whiteley, West Yorkshire, England). Chloramphenicol-resistant mutants were maintained on MH medium supplemented with 20 μg of chloramphenicol/ml.

C. jejuni inocula were prepared by microaerobic culture (84% N_2, 5% O_2, and 11% CO_2) in MH medium at 37°C with agitation using a stirrer. The bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}). At early-mid-log phase (OD_{600} of approximately 0.3) the bacterial culture was split in two, and one half was used to produce purified total RNA from *C. jejuni* grown in vitro, while the other half was used to inoculate the RILs. Bacteria were collected by centrifugation (10 min, 6,000 × *g*), washed once with sterile phosphate-buffered saline (PBS) buffer, and resuspended in PBS buffer at a concentration of approximately 6.6×10^{10} CFU/ml.

For the in vitro growth experiments, the *C. jejuni* wild-type and mutant strains were grown in MH biphasic medium at 37°C under microaerophilic conditions (83% N_2, 4% H_2, 8% O_2, and 5% CO_2).

RIL and isolation of *C. jejuni* total RNA. Rabbits were checked upon arrival to see if they carried *Campylobacter* by taking cloacal swabs for culture. Ileal loops were prepared according to published methods (3, 8). Briefly, New Zealand White rabbits (<2 kg; females) were anesthetized, a laparotomy was performed, and two 20-cm sections of ileum with intact mesenteric blood supply were ligated per animal. Each loop was inoculated with approximately 10^{11} mid-log-phase *C. jejuni* in 1.5 ml of PBS buffer. The size of the inoculum was confirmed by bacterial enumeration on MH agar plates. Loops of two rabbits were injected with sterile PBS buffer and served as control animals. After replacing the intestinal loops in their appropriate position in the abdominal cavity, the abdominal wall and skin were closed in standard fashion and the rabbits were allowed to recover from anesthesia. The rabbits were anesthetized again 24 or 48 h after the inoculation, the intestinal loops were excised intact, and the animals were then euthanized.

RNA turnover in the samples was quickly stopped by submerging the entire loops into 10 ml of RNAlater solution (Ambion, Austin, Tex.). The loops were first weighed in order to evaluate fluid accumulation. The contents of each loop as well as the mucus layer were recovered into 20 ml of a 50% solution of RNAlater in PBS buffer and centrifuged at low speed to remove epithelial cells (5 min at 1,000 × *g*). Thereafter, *Campylobacter* bacteria were separated from the intestinal microflora by filtration through 0.8-μm-pore-size filters. *Campylobacter* cells were pelleted by centrifugation, and total RNA was isolated using a hot phenol-chloroform protocol, as previously described (40). Traces of genomic DNA were removed by two or three consecutive treatments with DNase I Amp-grade enzyme (Invitrogen, Carlsbad, Calif.). The absence of contaminating genomic DNA was confirmed by PCR. RNA was further purified two to five times using a QIAGEN RNeasy mini kit (QIAGEN, Valencia, Calif.), and the concentration of RNA was determined using the RiboGreen RNA quantitation reagent (Molecular Probes, Eugene, Oreg.).

Microarray construction and hybridizations. DNA microarrays were prepared using PCR-amplified fragments of each annotated open reading frame from *C. jejuni* NCTC 11168, as previously described (40, 41). Twenty micrograms of total RNA from each growth condition (in vitro and in vivo) was converted to cDNA using 2 pmol of *C. jejuni* 3′-specific primers (set of 1654 3′ primers used for the PCR amplification of *C. jejuni* open reading frames) and coupled to monoreactive fluors (Cy3 and Cy5), according to previously described procedures (40).

Data collection and analysis. Microarray slides were scanned at 532-nm (Cy3) and 635-nm (Cy5) wavelengths with a laser-activated confocal scanner (ScanArray 3000) at 10-μm resolution, generating two TIFF images. Fluorescence intensities of each spot were collected using the GenePix Pro 3.0.5 software (Axon Instruments, Foster City, Calif.) after manual optimization of spot registration and exported to OriginPro 7 spreadsheets (OriginLab Corporation, Northampton, Mass.). The analysis of the fluorescence data was conducted as follows: (i) the spots were filtered and excluded based on slide abnormalities or low signal (corresponding to spots flagged bad or not found); (ii) after background subtraction, all spots with fluorescent mean intensities below three times the standard deviation of the background in both channels were removed from the final data analysis; and (iii) the fluorescence intensity in each wavelength was $\log_2$ transformed and normalized using locally weighted linear regression (lowess) performed by the MIDAS software (available from The Institute for Genomic Research; http://www.tigr.org/software/).

For the microarray analysis, *Campylobacter* RNA was isolated 48 h postinfection from five RIL rabbits. Each cDNA sample was individually cohybridized with cDNA obtained from in vitro growth (mid-log-phase bacteria) on microarray slides. The microarray hybridization was repeated up to three times depending on the amount of RNA purified from each rabbit, yielding between two and six measurements per gene per rabbit (each gene was spotted in duplicate on each slide). The microarray data were statistically analyzed using the significant analysis of microarray (SAM) algorithm, which was specifically developed for genomic expression data mining (the Microsoft Excel add-in software is available at http://www-stat.stanford.edu/~tibs/SAM/) (46). Briefly, SAM uses the standard deviation of repeated gene expression measurements to assign a score to each gene. It then estimates, for a particular score, a false discovery rate by permutations of the data. This SAM analysis ascertains that genes identified as differentially expressed do not arise from a random fluctuation of the large quantity of data generated (46). To identify genes whose expression differed significantly between in vivo and in vitro growth, we performed a one-class response analysis by considering the five rabbits as one class. We applied a false discovery rate of 0.11% and a delta value of 0.9. To identify genes with variable expression between rabbits, we performed a multiple-classes analysis by treating each rabbit as one class. We applied a false discovery rate threshold of 1.64% and a delta of 0.19. The microarray data of SAM-positive genes were extracted into a text output file using the Samster software (available at http://falkow.stanford.edu/whatwedo/software/software.html). Finally, the ratios of the fluorescence intensities of all replicate spots from the hybridization of RNA derived from each rabbit were averaged and used for further analysis. The data generated by this study are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab.

***C. jejuni* mutant construction.** Knockout mutants of *C. jejuni* NCTC 11168 were constructed by independently mutating six genes: *hrcA*, Cj0571, *spoT*, *hspR*, Cj0341, and *fliD*. The same inactivation strategy was used for the *hrcA*, Cj0571, *spoT*, and *hspR* mutants. Briefly, the gene to be mutated was amplified by PCR from *C. jejuni* NCTC 11168 chromosomal DNA, which was extracted using a standard protocol (35). The PCR product was cloned into pUC19, using a unique restriction site (Table 2), and deletions of 42, 600, 627, and 72 bp were made by inverse PCR in *hrcA*, Cj0571, *spoT*, and *hspR*, respectively. The chloramphenicol resistance cassette (Cam^r) was PCR amplified from pRY111 (55) using primers with appropriate restriction sites and cloned into the deletion site. Specific primers used for the first PCR amplification and the following inverse PCR are listed in Table 2. Recombinant plasmids carrying the Cam^r gene in the same orientation as the genes of interest were selected by DNA sequencing and transformed into *C. jejuni* NCTC 11168 using standard protocols (55). Transformants were identified on MH agar plates containing 20 μg of chloramphenicol/ml. The identity of the mutants was confirmed by PCR analysis using a combination of primer sets annealing within the mutated gene and the Cam^r gene.

The Cj0341 and *fliD* mutants were isolated from a library of random mutants generated using the EZ::TN pMOD-3<R6Kγori/MCS> transposon (Epicen-

TABLE 2. Primers used in this study

Primer	DNA sequence from 5′ to 3′ (restriction site)[a]
For gene cloning	
spoT-01	ATGC**GAATTC**GAGATTTTAACCTATCCTTGACAC (EcoRI)
spoT-02	ATGC**CTGCAG**CCATGATGCCATTCTTGAAA (PstI)
Cj0571-01	ATGC**GAATTC**ATGCAAGAAAATTTCATACGC (EcoRI)
Cj0571-02	ATGC**CTGCAGT**CCCGTTGTAGCATCTTTTG (PstI)
hrcA-01	ACGT**GGTACC**AATAGAGTGCTAGATATGAAGGAA (KpnI)
hrcA-02	ACGT**GGTACC**GATGGTTTTCACTATCTACATGAA (KpnI)
hspR-01	ACGT**GGTACC**GGAAAACAAAGCAGAAATGGAGC (KpnI)
hspR-02	ACGT**GGTACC**GCACCACTGCGGTTGAAGAAA (KpnI)
For inverse PCR	
spoT-03	ATGC**GGATCC**AAAACAGGAAAAGCAAAAGCA (BamHI)
spoT-04	ATGC**GGATCC**AATGCCTTTTCTTTGCAT (BamHI)
Cj0571-03	ATGC**GGATCC**GCTTAATTTTCCCAAAGCAAA (BamHI)
Cj0571-04	ATGC**GGATCC**AGAACTGAAAATACGGCTAGAAGA (BamHI)
hrcA-03	ACGT**CCATGG**ATTGCTTCTATAGCAAATCAA (NcoI)
hrcA-04	ACGT**GCGGCCGC**AAGCTCTTAGATTGTCAAATTCAC (NotI)
hspR-03	ACGT**CCATGG**TTGCCATCGGTTCTACTTGG (NcoI)
hspR-04	ACGT**GCGGCCGC**GAATCAATCTTGCTGGAGTA (NotI)

[a] The restriction sites used for cloning are highlighted in bold and indicated in parentheses.

tre), in which the Cam^r cassette from pRY111 has been cloned (A. Stintzi, unpublished data). The mutant library was constructed following the manufacturer's recommendation, and the Cj0341 and *fliD* mutants were identified during the first trial to assess the randomness of the library. The insertion site of the transposon in the Cj0341 and *fliD* mutants was identified using a single-primer PCR procedure and DNA sequencing of the resulting amplification product, as described by others (16). The Cj0341 and *fliD* mutations were confirmed by PCR amplification using a primer that anneals within the mutated gene and another primer that anneals within the Cam^r gene.

In vivo survival assays using a pool of mutants. The ability of the *C. jejuni* mutants to survive within the RIL was assessed. Each mutant was grown individually in MH broth to mid-log phase, harvested by centrifugation (10 min, 6,000 × *g*), washed, and resuspended in PBS buffer. The mutants were mixed to constitute the input pool by combining 5×10^9 CFU of each mutant with 7.5×10^{10} CFU of the wild-type strain, *C. jejuni* NCTC 11168, in 5 ml of PBS buffer. One milliliter of this suspension was used to prepare genomic DNA following standard protocols. The remaining bacterial suspension was used to equally inoculate four ileal loops from two rabbits (1 ml per loop). At 48 h postinfection, loop contents were harvested in PBS buffer and centrifuged at low speed to remove debris and epithelial cells. The supernatant was immediately processed to prepare genomic DNA, constituting the recovered pool of genomic DNA. Each loop was processed individually.

The difference in relative abundance of each mutant between the input and recovered pools was evaluated by quantitative PCR, which was performed using an ABI Prism 7700 DNA analyzer (Applied Biosystems, Foster City, Calif.) and the QuantiTect SYBR Green PCR kit (QIAGEN), according to the following protocol: 500 ng of genomic DNA was added to 25 μl of 2× QuantiTect SYBR Green PCR solution and a 0.3 μM concentration of each specific primer in a 50-μl final volume. The HotStar *Taq* DNA polymerase was activated by heating the reaction mixture at 95°C for 15 min. PCR amplification was performed by 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s. The specificity of the PCR was confirmed by melting curve analysis of the PCR product following the manufacturer's recommendations (Applied Biosystems). Genomic DNA of each mutant was specifically amplified from both pools by using a combination of two primers, with one of them annealing within the mutated gene and the other within the chloramphenicol resistance cassette. The sequences of the specific primer sets are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab. The relative abundance of each mutant was then normalized to the DNA pool by using the *cydA* or *argD* gene. The competitive ratio of the relative abundance of each mutant between the input and recovered pools was obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method, as recommended by Applied Biosystems. The abundance of each mutant was assessed twice per loop, and the mean C_T value for each ileal loop was used for further analysis. The C_T value corresponds to the PCR threshold cycle at which the fluorescence detected is significantly higher than the baseline value. The ratio of the mutant in the input to that in the recovered pool was calculated as follows: ratio input/recovered = $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T,\ \text{recovered}} - \Delta C_{T,\ \text{input}}$, and $\Delta C_{T,\ \text{recovered or input}}$ is obtained by subtracting the mean C_T value of the specific gene from the mean C_T value of the reference gene (*cydA* or *argD*) in the genomic DNA from the input or recovered pool. Given that the wild-type strain was inoculated at a higher level than the mutants, the population as a whole (the mutants plus the wild-type strain) should have a growth similar to the wild-type strain alone. The same assumption is usually made for the analysis of data generated by signature tag mutagenesis (10). Consequently, the normalized competitive ratio of input versus recovered *C. jejuni* NCTC 11168 should be approximately equal to 1. A competitive input/recovered ratio above 1 indicated that the mutant was attenuated in vivo, while a competitive ratio below 1 indicated that the mutant survived better in vivo than the wild type. The data were statistically analyzed using the Student *t* test, and a *P* value below 0.01 was considered significant.

In vitro and in vivo competition experiments. *C. jejuni* wild-type and mutant strains were grown in biphasic MH medium to mid-log phase, centrifuged, and resuspended in PBS buffer to an OD_{600} of ÷1.8 (approximately 10^{10} CFU/ml). Two milliliters (each) of the wild-type and the mutant strains was mixed at a 1:1 ratio. Four ileal loops (from two rabbits) were injected (each) with 1 ml of this suspension. The initial 1:1 mixture of mutant and wild-type strains was confirmed by plating serial dilutions of this mixed culture on MH agar with and without chloramphenicol (20 μg/ml). At 48 h postinfection, the loops were recovered as described above. Their content as well as the mucus layer were collected and homogenized in 10 ml of PBS buffer. Serial dilutions of bacteria recovered from each rabbit's loop were plated on karmali-agar plates (*Campylobacter* agar base [Oxoid CM935] supplemented with the *Campylobacter*-selective karmali supplements [Oxoid SR167E]) and karmali-agar plates containing chloramphenicol (20 μg/ml). Plates were incubated at 37°C for 3 days before the colonies were counted. The titer of the mutant was obtained from the CFU recovered on karmali-agar plates containing chloramphenicol, and the titer of the wild-type bacteria was calculated by subtracting the number of mutants from the total number of bacteria recovered on karmali-agar plates without antibiotic. Finally, the in vivo competitive index was calculated for each loop and corresponded to the ratio of the mutant to the wild-type strain.

For the in vitro competition assays, overnight cultures of the wild-type and mutant strains were mixed in a 1:1 suspension into fresh MH medium. This suspension was used to inoculate three replicate biphasic MH cultures. The bacterial growth was monitored by measuring the OD_{600} over time. The titer of each strain was determined in the inoculum and at early stationary phase, by plating on MH agar and MH agar containing chloramphenicol (20 μg/ml). The in vitro competitive index was calculated for three independent growth experiments and is defined as the ratio of mutant to wild-type.

Student's *t* test was used to statistically analyze the data from the in vivo and in vitro competition assays.

Real-time quantitative RT-PCR analysis. The relative expression of nine genes (*flgE2*, Cj0178, *katA*, *spoT*, *ahpC*, *fliD*, Cj0571, *cydA*, and Cj0366) was confirmed by real-time quantitative reverse transcription-PCR (RT-PCR), as previously described (40), using the QuantiTect SYBR Green RT-PCR kit (QIA-

GEN) according to the manufacturer's recommendations. The relative expression of each gene was normalized to either the 16S or 23S RNA, and the extent of its induction was obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method, as described above. The primers used are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab.

Necropsy and histopathology. Four rabbits were used to evaluate the pathological changes in the RIL model caused by *C. jejuni* NCTC 11168. The loops were prepared as described above for the transcriptional profiling experiments. The loops from two rabbits were inoculated with 10^{11} CFU of mid-log-phase *C. jejuni* NCTC 11168, while the loops of the two other rabbits were injected with sterile PBS buffer. At 48 h postinfection, the rabbits were anesthetized, the loops were recovered, and the animals were then euthanized with an overdose of Beuthanasia D (>0.25 ml/kg of body weight). A 0.5-cm middle section of each loop was immediately excised, flushed with formalin, linearly opened, placed flat on a card, fixed in buffered 10% formalin, embedded in paraffin, section at 5 μm, and stained with hematoxylin and eosin. Specimens were examined for evidence of inflammation, villus epithelial cell attenuation, and crypt dilatation or hyperplasia.

RESULTS AND DISCUSSION

RIL model for campylobacteriosis. The RIL model was initially chosen because of its documented ability to accurately model the histopathological lesions associated with human *Campylobacter* gastroenteritis (8). In addition, *Campylobacter* cells can be collected in a number sufficient for the investigation of in vivo genome-wide transcript abundance. The model was created by surgical ligation of 20-cm sections of the ileum, resulting in the cessation of the normal peristaltic flux, thereby facilitating *Campylobacter* gut colonization. The strain of *C. jejuni* NCTC 11168 used in our study is helically shaped, fully motile, and colonizes the gastrointestinal tract of chicks (26). Therefore, this strain is phenotypically different from the sequenced *C. jejuni* NCTC 11168 strain recently described by Gaynor et al. (11), which was described to be straight rod-shaped, nonmotile, and a poor colonizer of chicks. To explore the feasibility of this model to study *Campylobacter* lifestyle in the gut by transcriptome profiling, we undertook a pilot study involving four rabbits: RIL were sampled at 24 or 48 h postinoculation (using two rabbits per time point). To note, the *C. jejuni* strain was passaged three times in vitro before its inoculation in the ileal loops. *C. jejuni* NCTC 11168 colonized the rabbit gut at bacterial concentrations of 10^5 CFU/loop and 10^7 to 10^9 CFU/loop, at 24 and 48 h postinoculation, respectively. The initial decrease in bacterial population from 10^{11} to 10^5 CFU per loop during the first 24 h after inoculation reflects the challenges of surviving in a hostile environment, while the subsequent bacterial growth from 10^5 to 10^9 CFU/loop at 48 h suggests the successful adaptation and colonization of *C. jejuni* in the rabbit intestinal tract. Considering that 10^5 cells would not generate sufficient amounts of RNA to perform microarray hybridization, we decided to harvest the bacterial cells at 48 h postinfection in the present study.

Intestinal distension resulting from accumulation of gas and fluid (the first signs of diarrhea) was qualitatively observed at 48 h postinfection in all infected rabbits but not in the control animals injected with the PBS buffer. Two infected rabbits and two control animals were used to evaluate pathological changes. Fluid accumulation was quantitatively estimated by weighing the intestinal content of the eight loops, which indicated an increase of 0.09 ± 0.05 g of content/g of ileal tissue in the infected loops compared with control loops. This difference was found to be statistically significant ($P < 0.05$ using a paired *t* test). In contrast to the observations of Everest et al. (8), histopathological analysis of the ileal tissues did not reveal any severe pathology. This lack of damages likely reflects the inability of *C. jejuni* NCTC 11168 to invade epithelial cells or to exert morphologically evident cytotoxic effects on intestinal epithelial cells. In fact, the strain of *C. jejuni* NCTC 11168 used in our study is poorly invasive into human epithelial INT407 cells (30). Consequently, the transcriptome profile presented in this work reflects noninvasive *Campylobacter* lifestyle in the intestine during survival, colonization, and the initial stages of pathogenesis.

In vivo expression profiling validation. While microarrays provide a powerful approach for the investigation of gene expression, the performance of these expression studies in vivo is technically challenging. To date, expression profiling experiments have been limited mainly to in vitro environments. In the present study, in vivo colonization of the intestinal tract by *C. jejuni* was investigated by conducting transcriptional expression profiling experiments during growth and survival within the natural gut environment. We utilized microarrays containing spotted PCR products representing approximately 98% of the annotated open reading frames of *C. jejuni* NCTC 11168 (40). The challenge of recovering intact *C. jejuni* mRNA from the intestine to ensure acquisition of an accurate and specific transcriptome profile was addressed by excising the entire intestinal loops and immediately submerging them in RNA stabilization solution to block RNA turnover. In order to minimize RNA degradation and/or changes in the gene expression level, loops were immediately processed for RNA extraction and quantitative histopathological traits were not recorded. The content of each loop, including the mucus layer, was recovered in RNA stabilization solution, and *C. jejuni* was purified by filtration through 0.8-μm filters. This physical separation removed most of the endogenous microflora; more than 80% of the bacterial population was estimated to be constituted of *C. jejuni*. The yield of RNA recovered was between 12 and 55 μg per loop. The total RNA extracted from each rabbit's two loops were combined. Twenty micrograms of RNA was reverse transcribed using *C. jejuni*-specific 3′-end primers and fluorescently labeled with the Cy5 dye, which fluoresces red. The relative abundance of transcripts was monitored by competitive hybridization with RNA extracted from bacteria grown in vitro to mid-log phase and labeled with the green fluorescent Cy3 dye. To address any potential cross-hybridization with RNA extracted from the remaining natural intestinal microflora, RNA was also purified from ileal loops of rabbits which had been injected with PBS buffer only. The yield of total RNA purified from the uninfected loops was between 1 and 3 μg per loop. The total RNA harvested from two uninfected loops was combined, reversed transcribed, labeled with Cy5, and hybridized to the *C. jejuni* microarray. As shown in Fig. 1, this RNA did not cross-hybridize with genes from *C. jejuni*. In addition to physical enrichment of *C. jejuni* by filtration, the use of 3′-specific primers to synthesize cDNA further enhanced the specificity of the assay. A similar approach was employed by Talaalt and coworkers to amplify mycobacterial RNA from a mixture containing mammalian RNA (43).

Global gene expression analysis and validations. *C. jejuni* NCTC 11168 was inoculated into five rabbits and colonized the loops of these rabbits at a bacterial concentration of 3.10^8

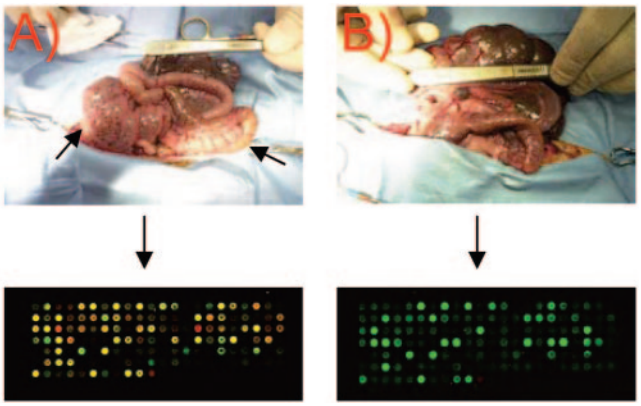

FIG. 1. Detection of *C. jejuni* transcriptome in vivo. (A and B) The RIL 48 h postinoculation with *C. jejuni* or PBS buffer, respectively. The arrows indicate intestine distended with gas and fluid accumulation. Total RNA was extracted from the intestinal contents, reverse transcribed using *C. jejuni*-specific 3′ primer, and labeled with the Cy5 dye. This labeled cDNA was cohybridized to the microarray with Cy3-labeled cDNA, obtained from in vitro-grown bacterial RNA.

CFU/loop (rabbit 1), 2.10^8 CFU/loop (rabbit 2), 5.10^8 CFU/loop (rabbit 3), 4.10^7 CFU (rabbit 4), and 4.10^8 CFU/loop (rabbit 5). *Campylobacter* RNA samples were extracted from each rabbit 48 h postinfection and individually hybridized to the microarray slides up to three times, depending on the amount of RNA purified from each loop. Specifically, rabbits 1, 4, and 5 yielded two measurements per gene, rabbit 2 yielded six measurements per gene, and rabbit 3 yielded four measurements per gene. The data were quantified, normalized, and reported as the ratio of gene expression of *C. jejuni* grown in the rabbits to that of *C. jejuni* grown in vitro. To limit the number of genes falsely identified as differentially expressed, we performed a statistical procedure. This test consisted in applying the SAM algorithm to our microarray data. This statistical method has been shown to be more reliable than a standard *t* test or the use of a fold change threshold and is relatively conservative in declaring a significant change in gene expression (46). A one-class-response SAM analysis, using the five rabbits as one group, identified 348 genes as being differentially expressed between in vivo and in vitro growth with a false discovery rate of 0.11%. All SAM-selected genes exhibited expression ratios greater than 1.5. As demonstrated in our previous study using the same microarray platform, a 1.5-fold differential expression is technically and biologically significant (40). This *Campylobacter* microarray platform has previously been shown to generate data with a high level of concordance with quantitative RT-PCR (40). However, in order to address the reliability of the microarray data generated in this study, the change in transcript abundance in rabbit 4 between in vitro and in vivo growth was confirmed for nine genes (*flgE2*, Cj0178, *katA*, *spoT*, *ahpC*, *fliD*, Cj0571, *cydA*, and Cj0366) by real-time quantitative RT-PCR. The *flgE2* gene was found to be 4-fold up-regulated, Cj0178 was 200-fold up-regulated, *katA* was 130-fold up-regulated, *spoT* was 61-fold up-regulated, *ahpC* was 4-fold up-regulated, *fliD* was 5-fold down-regulated, Cj0571 was 2.5-fold up-regulated, *cydA* was 350-fold up-regulated, and Cj0366 was 300-fold up-regulated. Similarly to our previous study, while the quantitative RT-PCR confirmed the trend in differential gene expression observed with the microarray analysis, a quantitative difference in the fold change was observed between these two technologies. This difference reflects a lower dynamic range for the microarray experiments compared to that of quantitative real-time RT-PCR, as previously reported by others (53). Notably, very few genes were found to be differentially regulated more than 20-fold by the microarray analysis, while the real-time RT-PCR found several genes up-regulated more than 100-fold. This observation highlights the semiquantitative nature of microarray experiments and the low dynamic range of this technology (4). Furthermore, this technical limitation appears to be amplified in situations where a gene exhibits a very low expression level under only one of the growth condition, which is the case of in vivo genome-wide expression analysis. As a consequence, the fold change in gene expression presented in this study should be significantly underestimated. Nevertheless, differentially expressed genes were readily identified by statistical analysis. Overall, 185 genes were found to be induced in vivo in all five rabbits. Among them, 177 exhibited more than twofold differential expression, with 91 of them showing more than fourfold differential expression. Of the 199 genes found to be repressed in vivo, the expression level of 153 genes was reduced twofold and the expression level of 32 genes was reduced more than fourfold.

Multiple-class response SAM analysis (considering each rabbit as one group) as well as two-class unpaired data SAM analysis (considering each rabbit as one group and comparing each rabbit with each of the others) revealed some gene expression variability between rabbits. Multiple-class response SAM analysis identified 170 genes differentially expressed between rabbits with a false discovery rate of 1.64%. Importantly, very few genes were found to be antagonistically expressed between rabbits. Indeed, the trend of differential expression remained essentially the same, while only the amplitude of change in transcript abundance varied. To confirm the variability of gene expression between rabbits and to rule out the possibility of intrinsic noise, we compared the expression measurements of these 170 genes within each rabbit and between rabbits (using the microarray data from rabbits 2 and 3). A high level of concordance with a correlation coefficient higher than 0.9 was obtained between replicate microarray hybridizations of RNA isolated from the same rabbit (Fig. 2A and B), whereas a very weak correlation was observed between hybridizations of RNA samples originating from two different rabbits (Fig. 2C). The variability of gene expression between rabbits was further confirmed by quantitative real-time RT-PCR for *flgE2* and Cj0178, which encode the flagellar hook subunit protein and a putative outer membrane ferric-siderophore receptor, respectively. These were found to be differentially expressed between rabbits 3 and 4. The microarray analysis indicated that the *flgE2* gene was overexpressed in rabbit 4 and down-regulated in rabbit 3, while the expression of Cj0178 was essentially unaffected in rabbit 3 and up-regulated in rabbit 4. By using the same RNA preparation as the one used for the microarray hybridization, quantitative RT-PCR confirmed the differential expression of both genes. The expression of *flgE2* was found to be down-regulated approximately 70-fold in rabbit 3 and up-regulated 4-fold in rabbit 4, compared with in vitro growth. Cj0178 was found to be equally expressed in rabbit 3

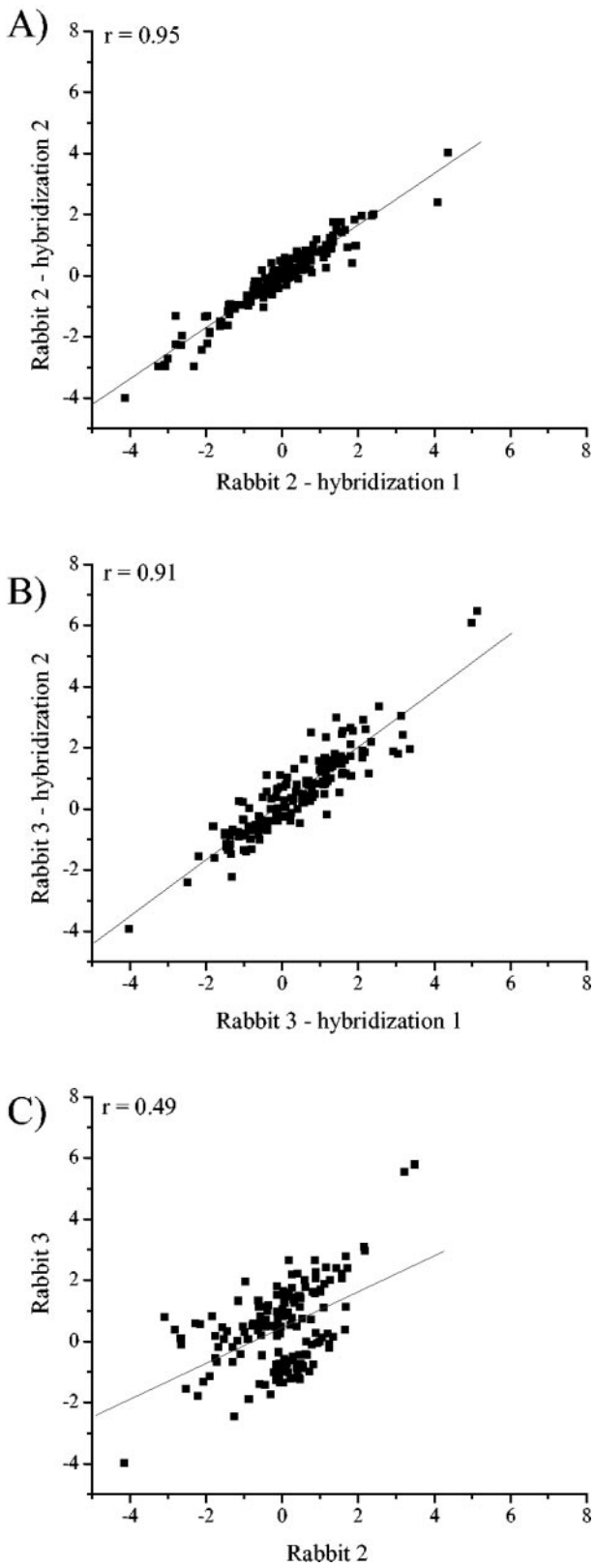

FIG. 2. Scatter plots showing the relationship between the $\log_2$ value of the gene expression ratio obtained from hybridization experiments with bacterial cDNA derived from the same rabbit (A and B) or from two different rabbits (C). The solid lines represent the linear regression fit of the data.

and overexpressed 200-fold in rabbit 4 relative to in vitro growth.

The observed variability in gene expression patterns is unclear but likely reflects both physiological and intrinsic variations in the rabbits. This hypothesis is in agreement with the observed difference in colonization level (1 log) and the variation in the amount of fluid accumulation (±55%) between rabbits. Obviously, the gastrointestinal environment cannot be controlled and is likely to vary from one rabbit to another, leading to variations in *C. jejuni* colonization and gene expression profiles. In addition, it is unknown if the rabbits used in this study had previously encountered *C. jejuni*. If it was the case, an immune response would likely take effect by 48 h postinfection and might also result in the observed gene expression variability. Recently, Boyce et al. reported the genome-wide expression profile of *Pasteurella multocida* recovered from blood of infected chickens 20 h after inoculation (2). Although blood has questionable pathological relevance because it is not the site of infection of *P. multocida*, similarly to our study those authors observed a variable bacterial gene expression profile between infected hosts (2). More recently, Xu et al. characterized the transcriptome of *Vibrio cholerae* during intestinal growth 8 h postinfection using the RIL model (54). In contrast to our study, *V. cholerae* gene expression was similar in the three rabbits tested. All together, these data highlight the complexity of studying genome-wide gene expression in vivo.

***Campylobacter* lifestyle in the gut.** Overall, the expression of 482 genes was found to be significantly altered in vivo. Based on their expression profiles, genes can be grouped into two major categories: (i) genes exhibiting similar differential expression in all five rabbits tested (348 genes) (Fig. 3), and (ii) genes with variable expression between rabbits (170 genes) (Fig. 4). It should be noticed that 36 genes belong to both categories. These genes exhibit similar expression alteration in vivo in all five rabbits but different change amplitudes between rabbits.

To elucidate further the intestinal lifestyle of *Campylobacter*, we grouped genes by functional annotations and mapped their expression profiles to all known biological processes, thus allowing the investigation of the overall physiological status of *C. jejuni* grown in vivo. This approach revealed the involvement of a wide range of metabolic, morphological, and pathological processes (Fig. 3 and 4). Figures 3 and 4 list only the genes found to be significantly differentially expressed between in vivo and in vitro growth by SAM analysis. However, a biological process was considered to play a role in *Campylobacter* physiology in the intestine when the constituting genes were found to be either up-regulated or equally expressed in vivo compared to in vitro growth.

Energy and central intermediary metabolism. The in vivo transcriptome pattern of *C. jejuni* was consistent with the oxygen-limited environment found in the intestine. The expression of genes encoding the key enzymes in the oxidative phosphorylation pathway was decreased dramatically in all five rabbits. These genes encode NADH dehydrogenase (*nuoG*, *nuoL*, and *nuoH*) and succinate dehydrogenase (*sdhABC*). Recently, fumarate, nitrate, nitrite, and N- or O-oxides have been shown to constitute alternative terminal electron acceptors, allowing *C. jejuni* to carry out respiration under oxygen-re-

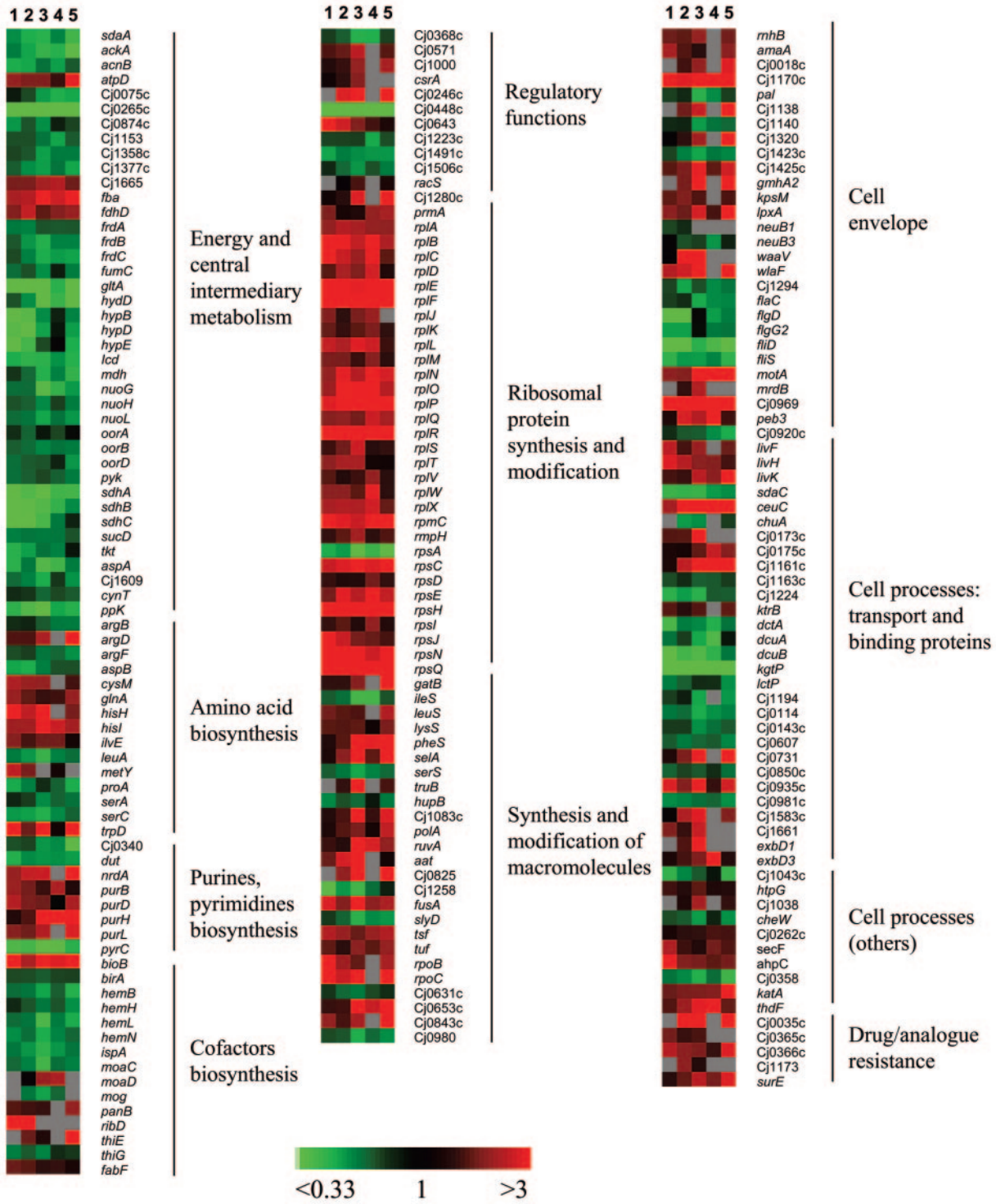

FIG. 3. Global view of genes with similar expression patterns between rabbits grouped by functional categories according to the Sanger Center *C. jejuni* genome database. Each row represents one gene, and each column represents the expression profile in one rabbit (the mean fold change in the expression ratio of the technical replicates). The column label corresponds to the rabbit numbering. An increasing red intensity denotes a gene for which expression was significantly increased in vivo compared to in vitro growth, and an increasing green intensity indicates a gene for which expression was significantly decreased in vivo compared to in vitro growth. A gray color indicates missing data. Genes with unknown functions are not represented.

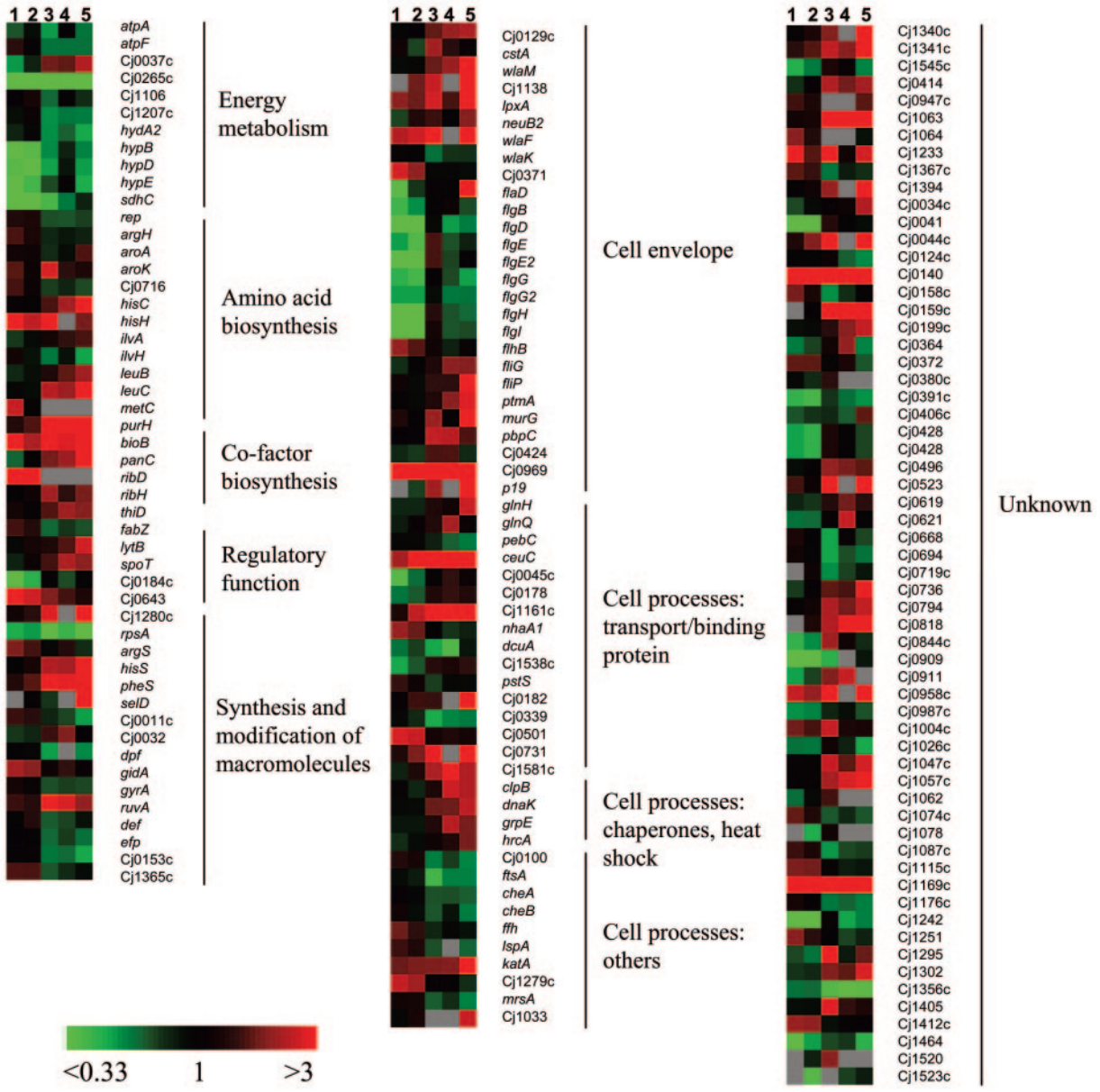

FIG. 4. Global view of genes with a variable expression pattern between rabbits. Each row represents one gene. Columns 1, 2, 3, 4, and 5 represent the expression profiles in rabbits 1, 2, 3, 4, and 5, respectively. For each rabbit, the microarray data correspond to the mean fold change in the expression ratio of the technical replicates. Red and green denote transcripts for which abundance was increased or decreased in vivo compared to in vitro growth, respectively. The red and green intensities are proportional to the increase or decrease, with maximal fold changes in transcript abundance of 3 and 0.33, respectively. A gray color denotes missing data.

stricted conditions in vitro (36). However, the genes encoding the reductases involved in this alternative respiratory pathway were all down-regulated in vivo. In contrast, the genes encoding for the cytochrome *bd* oxidase (*cydAB*) were expressed in vivo but were not or were only slightly expressed in vitro. The differential expression of the *cydA* gene was confirmed by quantitative real-time PCR. The expression of *cydA* was found to be 350-fold higher in vivo compared to in vitro growth. Although the CydAB oxidase catalyzes the oxidation of menaquinone using oxygen as an electron acceptor, this enzymatic complex has been shown in *Escherichia coli* to possess a high affinity for oxygen, allowing the bacterium to carry out respiration under limited oxygen tension (5). In addition, the expression of *E. coli cydAB* is known to be induced under limiting oxygen conditions (5). Similarly, the CydAB complex could facilitate *C. jejuni* respiration in the oxygen-limited environment of the intestine. Interestingly, formate dehydrogenase (encoded by *fdhABCD*) was the only enzyme identified by the microarray analysis to be overexpressed or equally expressed in vivo relative to that in vitro and capable of transferring electrons to the menaquinone pool. Other genes encoding enzymes with similar activity were found to be down-regulated in vivo. The FdhABCD enzyme participates in the respiratory chain of many bacterial species, enabling these organisms to respire

using formate as an alternative terminal electron donor under anaerobic conditions (33). In *C. jejuni*, the formate dehydrogenase, together with the CydAB complex, could allow the bacterium to carry on oxygen respiration even under extreme oxygen-limited conditions.

The expression of the genes encoding enzymes involved in gluconeogenesis, the citric acid cycle, and the pentose phosphate pathway, were all down-regulated in vivo, except for fructose biphosphate aldolase (*fba*). Down-regulation of these genes is consistent with the oxygen-deprived intestinal environment and the up-regulation of the carbon storage regulator, *csrA*. In *E. coli*, CsrA has been shown to repress the expression of genes involved in glycogen catabolism, gluconeogenesis, glycolysis, and motility (34). This enzyme likely performs a similar function in *C. jejuni*.

Macromolecular synthesis and processing. Genes encoding proteins involved in the synthesis and modification of macromolecules, in particular the ribosomal proteins (with the exception of the *rpsA* gene) and aminoacyl tRNA synthetases, were among the most highly up-regulated in vivo. The significance of this contradictory expression of *rpsA* (which encodes the ribosomal protein S1) and other genes from the same functional group is puzzling and requires confirmation by an alternative method and further investigation. In *E. coli*, the ribosomal protein S1 has been shown to be essential for cell viability, to promote the efficiency of translation, and to act as a repressor for its own synthesis (37). Depletion of the protein S1 resulted in a stringent response consistent with amino acid starvation and an increased production of ppGpp (37). Therefore, the down-regulation of the *rpsA* expression would suggest the induction of a stringent response in *C. jejuni* during intestinal colonization.

Biosynthesis of cofactors. Another group of genes expressed in vivo encodes proteins involved in the biosynthesis of the cofactors, biotin (*bioABCD*), riboflavin (*ribADFH*), thiamine (*thiCDEGHJL*), pantothenate (*panBC*), coenzyme A (*accB* and *acs*), and folic acid (*folCD*). These genes were found to be either up-regulated or equally expressed in vivo relative to in vitro growth (with the exception of two genes, *thiG* and *D*, from the thiamine biosynthetic pathway, which were found to be down-regulated). The expression of these genes suggests that these cofactors are unavailable in the intestine. As a consequence, and because biotin, riboflavin, thiamine, and pantothenate are produced only by microbes and higher plants, these biosynthetic pathways could constitute an ideal target for drug development. This evidence that biotin is unavailable in the intestine is corroborated by the up-regulation of *V. cholerae* biotin biosynthetic genes during intraintestinal growth (54) and by the inability of a *V. cholerae* biotin biosynthesis mutant to colonize the gastrointestinal tract of mice (22).

Virulence and colonization determinants. Suspected virulence and colonization factors of *Campylobacter* include motility and chemotaxis, host cell adherence and invasion, toxin production, lipo-oligosaccharide and surface structure biosynthesis, oxidative stress defense, iron acquisition, and heat shock response (49). In contrast to genes encoding proteins involved in general metabolism or bacterial physiology, the expression levels of many genes related to virulence and/or colonization factors were highly variable among infected rabbits. The most notable among genes with flexible expression were those coding for proteins involved in flagellum biosynthesis. Motility is known to be an essential requirement for *C. jejuni* to colonize the host gut and ultimately cause disease (49). Considering that the flagellin subunit is the immunodominant antigen recognized during human or animal infection, it is assumed that the gene encoding this protein is expressed in vivo (28). However, our microarray data suggest that there is considerable interanimal expression variability among genes belonging to the flagellum locus. Most of the genes belonging to the flagellum locus were found to be down-regulated in four rabbits (and at a different level), while they were slightly up-regulated in one rabbit. This variability may allow the bacterium to evade the host immune system by shutting down flagellum production once colonization is accomplished. In support of this hypothesis, *C. jejuni* flagella have recently been proposed to be necessary for passage through the gastrointestinal tract of chickens, but not for persistence in the chickens' cecum (51). A similar effect on the expression of flagellar genes was also recently demonstrated in *Salmonella enterica* during macrophage intracellular growth (7).

Another functional category of genes expressed in vivo relates to iron-responsive genes which encode proteins involved in iron metabolism and oxidative stress defense. Several of the genes encoding iron acquisition systems were found to be either up-regulated or equally expressed between in vivo and in vitro growth. These genes code for a putative ferric-siderophore transporter system (Cj0178 and Cj0173c-Cj0175c), a putative iron transporter (p19 and Cj1658), and the three TonB-ExbB-ExbD energy-transducing complexes. The genes encoding a putative heme outer membrane transporter (ChuABCD) were found to be only slightly expressed in vivo, suggesting that heme does not constitute the main iron source in the gut. The genes encoding the components of the ferric-enterobactin uptake permease (*ceuBCDE*) were found to be up-regulated in vivo; however, the *cfrA* gene encoding the ferric-enterobactin receptor appeared to not be expressed. As a microaerophilic bacterium, *C. jejuni* must deal with free oxygen radicals and other reactive molecules generated by normal aerobic metabolism and host defenses against microbial attack (49). The expression of most genes known to be associated with the *C. jejuni* oxidative stress response was found to be increased during gastrointestinal growth (49). To note, the up-regulation of these genes is in agreement with an iron-limited environment. Theses genes include *sodB* (superoxide dismutase), *ahpC* (alkyl hydroperoxide reductase), *tpx* (probable thiol peroxidase), and *katA* (catalase). Consistent with the expression of these genes in vivo, a mutation of *C. coli sodB* has been reported to impede colonization of chick gut (31). Furthermore, a mutation in *katA* sensitizes *C. jejuni* to hydrogen peroxide and reduces its intracellular survival in macrophages (49). In *Helicobacter pylori*, the catalase KatA has been shown to be required for persistent colonization in the mouse model (14). These results highlight the iron-restricted conditions in the rabbit intestine. The importance of iron metabolism for successful host colonization has been established for most pathogens (32) and should also be an essential factor for *C. jejuni* colonization in the intestine. Indeed, a *fur* mutant of *C. jejuni*, as well as *cfrA*, *ceuE*, and Cj0178 mutants, was recently shown to be significantly affected in the ability to colonize the gastrointestinal tract of chicks (26).

Interestingly, *C. jejuni* possesses a system of general protein glycosylation which has been proposed recently to play an important role in *C. jejuni* pathogenesis (42). Indeed, mutation of genes encoding the glycosyltransferases, *pglB* (also named *wlaF*) and *pglE* (also named *wlaK*), affected their ability to adhere to and invade human intestinal cells, as well as to colonize the gastrointestinal tract of mice (42). In our study, the expression of three genes, *pglB*, *pglE*, and *pglG*, which belong to this functional category, was found to vary between growth conditions. The expression of *pglB* and *pglG* was highly induced in vivo (in four rabbits), while *pglE* expression was either similar or repressed in vivo compared with that under in vitro growth. The expression of the other genes from the glycosylation cluster (*pglH*, *pglA*, *wlaJ*, and *pglF*) was found to be similar under both growth conditions. It is not clear why expression of the genes belonging to the same biological pathway varied. However, a similar difference in expression between the *pgl* genes was observed previously in *Campylobacter* in response to temperature up-shift (40) and iron starvation (26). Considering the absence of data on the functional role of each *pgl* gene in protein glycosylation, the significance of their expression profiles is difficult to assess.

The genes encoding the recently discovered multidrug efflux pump in *C. jejuni*, *cmeABC*, were found to be highly up-regulated in vivo. Expression of *cmeB* was confirmed by real-time RT-PCR to be up-regulated by approximately 300-fold in vivo compared with that under in vitro growth. This tripartite multidrug efflux transporter is composed of an outer membrane protein, CmeC (Cj0367c), a periplasmic fusion protein, CmeA (Cj0365c), and an inner membrane efflux transporter, CmeB (Cj0366c) (18). Interestingly, this efflux system has been shown to contribute greatly to bile resistance and to be required for the colonization of the chick's gastrointestinal tract (18, 19). Given the presence of a high concentration of bile salts in the gut, the up-regulation of these genes could contribute significantly to the survival of *Campylobacter* in the host by allowing the bacterium to resist the harmful effects of these salts.

Another important functional set of genes with variable expression between rabbits encodes proteins involved in peptidoglycan biosynthesis. Specifically, *murB* (a putative UDP-*N*-acetylenolpyruvoyl glucosamine reductase), *murC (*UDP-*N*-acetylmuramate-alanine ligase), and *pbpC (*penicillin-binding protein) were among the genes from this category that were the most differentially regulated. The differential expression of this category of genes suggests that there may be a modification of the murein sacculus in vivo, probably in response to the high osmolarity of the intestinal environment. Likewise, *Staphylococcus aureus* modifies its peptidoglycan layer under conditions of high osmolarity (50). While the activation of genes involved in peptidoglycan synthesis may constitute a repair mechanism necessary for the bacterial adaptation to environmental stress, the modification of the peptidoglycan structure may have broad implications for the stiffness and elasticity of the cell surface, thereby conditioning the bacterium to its ecological niche.

Among the other genes annotated or previously characterized as virulence- or colonization-associated factors, Cj1279c (putative fibronectin domain-containing lipoprotein) and several genes involved in the heat shock response were found to be significantly up-regulated in vivo. Heat shock proteins are induced in response to various stresses and act by repairing and preventing damage caused by the accumulation of unfolded proteins. The importance of the heat shock response for *Campylobacter* intestinal tract colonization has previously been demonstrated (49). Genes identified as heat shock proteins (40) and induced in vivo include *clpB* (ATP-dependent CLP protease ATP-binding subunit), *dnaK* (heat shock protein), *grpE* (heat shock protein), *hrcA* (putative heat shock regulator), and *htpG* (hsp90 family heat shock protein). The up-regulation of these genes, together with the previous identification of ClpB as a B-cell antigen in human disease (45), suggests that these proteins play an important role in bacterial growth within the gastrointestinal tract.

Mutational analysis. In order to study further the *C. jejuni* lifestyle in the intestinal tract of rabbits, we constructed knockout mutations of genes identified by our microarray analysis and investigated the ability of these mutants to survive in the RIL by comparison with the parent strain, *C. jejuni* NCTC 11168. Seven genes were selected and individually mutated. The main goal of this mutational analysis was to disrupt physiological functions that appeared to be important for the colonization of the ileal loop. In particular, the microarray data suggested an important role for the genes involved in the heat shock response, the stringent response, iron metabolism, and the biogenesis of the flagellum in the intestinal lifestyle of *C. jejuni*. Therefore, the heat shock response was disrupted by mutagenesis of its two transcriptional regulators, *hrcA* and *hspR*. The stringent response was disrupted by mutagenesis of the *spoT* gene, which encodes the guanosine-3′,5′-bis(diphosphate) 3′-pyrophosphohydrolase. The iron metabolism was disrupted by mutagenesis of the Cj0178 gene, which encodes a ferric-siderophore outer membrane receptor. This gene has been previously shown to be induced under iron restriction and is highly up-regulated in vivo. The flagellum biogenesis was disrupted by mutagenesis of the *fliD* gene, which encodes the flagellar hook-associated protein. A mutant in the *fliD* gene has been shown to be nonmotile (12). The *fliD* gene was found to be down-regulated in all five rabbits. Interestingly, among the genes encoding transcriptional regulators, Cj0571 was the only one from this category found to be significantly up-regulated in all five rabbits by the microarray analysis, suggesting an important role for this protein in vivo. Consequently, this gene was also mutated. Finally, a mutant in Cj0341 was chosen to be tested in the ileal model as an experimental control. The expression of this gene was found to be off under in vivo and in vitro growth conditions. Therefore, this mutant should not be affected in its ability to colonize the ileal loop.

The seven mutants and the parent strain were pooled together and inoculated into four ileal loops constructed in two different rabbits. After 48 h postinoculation, the ileal contents were recovered and directly processed for chromosomal DNA purification. Then, the relative amount of each mutant was evaluated by quantitative real-time PCR, as described in Materials and Methods. The competitive ratio of the number of cells at the time of the inoculation (in the input pool) to the number of cells recovered 48 h postinoculation was normalized to the entire bacterial population in the pool for each mutant (Fig. 5) so that the competitive ratio of the whole population was equal to 1. Considering that the wild-type strain was present in excess, compared with each mutant in the input

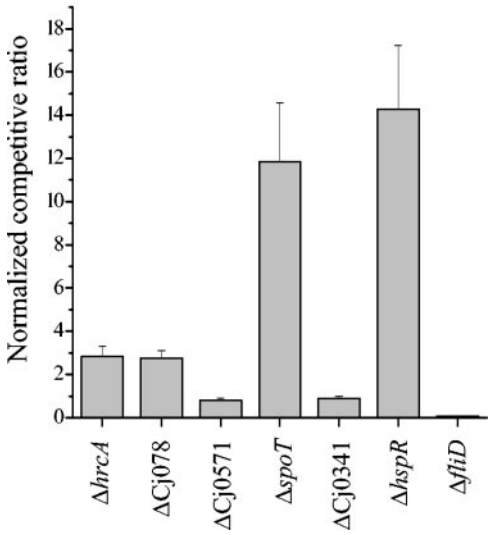

FIG. 5. Competitive colonization ability of seven mutants (*hrcA*, Cj0178, Cj0571, *spoT*, Cj0341, *hspR*, and *fliD*). The strains were pooled with the parent strain *C. jejuni* NCTC 11168 (constituting the input pool) and inoculated into four RIL. Forty-eight hours postinoculation, the intestinal contents were recovered and processed for chromosomal DNA extraction. The number of bacteria was estimated by quantitative real-time PCR for each mutant as described in Materials and Methods. The normalized competitive ratio corresponds to the ratio of the number of mutant cells to the total number of bacterial cells in the input pool divided by the ratio of the number of mutants to the total number of bacteria in the recovered pool. The data are the mean of eight determinations (four biological replicates with two technical replicates each), and the error bars represent the standard deviations.

pool, and assuming that it represents the major proportion of the population in the recovered pool, it should have a competitive input/recovered ratio equal to approximately 1. Therefore, any deviation of the competitive ratio from a value of 1 would indicate an effect of the mutation on the survival ability of *C. jejuni* in the RIL. As expected, the competitive ratio of the Cj0341 mutant was 0.9, indicating that this strain colonizes the ileal loop as well as the wild type. Of the six other mutants, one was unaffected (Cj0571), one had an advantage over the others with respect to survival in and colonization of the RIL (*fliD*; $P < 0.003$), and four were significantly attenuated (*hrcA*, Cj0178, *spoT* and *hspR*; $P < 0.002$). The *spoT* and *hspR* mutated strains were the most affected mutants, while the *hrcA* and Cj0178 mutants were only slightly attenuated. Because mutants are out-competed by many other strains during a mixed infection, the in vivo phenotype of the affected mutants was confirmed in a one-to-one competition assay. Each mutant was independently mixed with the wild-type strain in equal numbers and injected into four ileal loops (constructed in two rabbits). The one-to-one ratio of the inocula was confirmed by CFU determination. Forty-eight hours postinfection, the loop contents were plated on selective medium for bacterial enumeration. Then, the competitive index was calculated as the ratio of the mutant to the wild-type strain recovered from each ileal loop. As shown in Fig. 6A, three out of the five mutants were confirmed to be statistically affected in their colonization ability (with a P value of $\leq 10^{-4}$). In order to determine whether the colonization phenotype of these mutants was specific for in vivo growth, an in vitro competition assay was performed. An equal amount of each mutant and wild-type strain was mixed in MH broth. The cultures were incubated at 37°C until late log phase (~30 h), after which serial dilutions were plated on MH agar with or without chloramphenicol. These experiments were performed in triplicate. The in vitro competitive index was determined as described for the in vivo competition assay (Fig. 6B). Four of the five mutants were found to be statistically affected in their ability to out-compete the wild-type strain during in vitro growth. Finally, in order to determine whether the in vitro growth defect was caused by the competition with the wild-type strain, the growth kinetic of each mutant was independently determined (Fig. 7). All five mutants were found to have a growth defect in vitro, with the *spoT*, *hspR*, and *fliD* mutants being the most affected.

The competitive index of the *hspR* mutant in vitro and in vivo was 1.5×10^{-2} and 7×10^{-5}, respectively. While this mutant is affected in vitro, the 200-fold difference between the in vitro and in vivo competitive indices indicates a significant in vivo-specific growth defect. The attenuation of the *hspR* mutant in vivo suggests a role for the heat shock regulatory net-

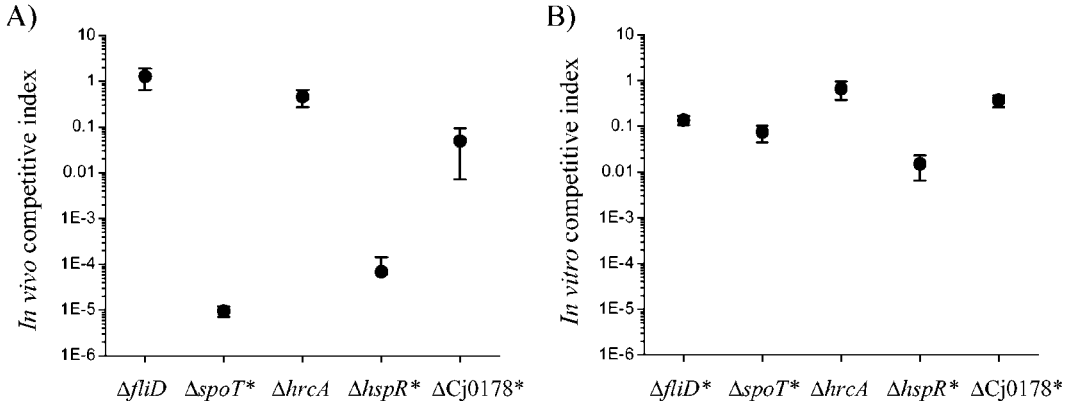

FIG. 6. In vivo (A) and in vitro (B) competition assays. The in vivo competitive index is the ratio of the mutant to the wild-type strain recovered in the ileal loop 48 h postinfection. Four loops were infected with a mixture of each mutant and the wild-type strain at a ratio of 1:1. The in vitro competitive index is the ratio of the mutant to the wild-type strain in MH broth at late log phase. The in vitro competition assay was performed in triplicate. The error bars indicate the standard deviations. *, statistical significance of $P < 0.001$.

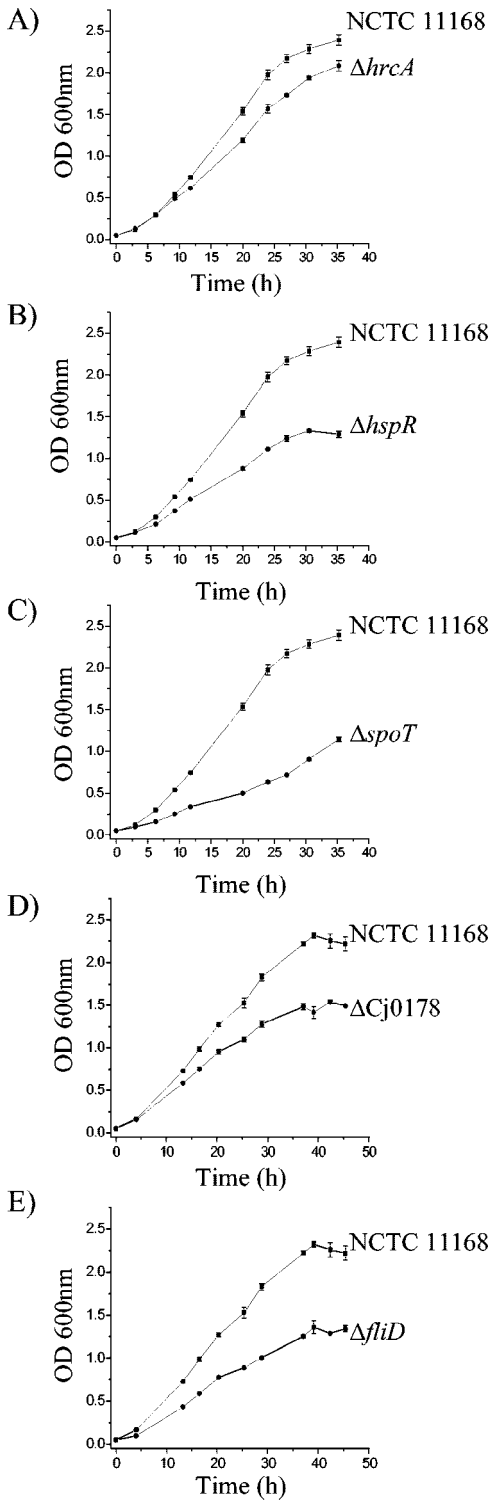

FIG. 7. Growth kinetics of *C. jejuni* NCTC 11168 and five mutants, Δ*hrcA* (A), Δ*hspR* (B), Δ*spoT* (C), ΔCj0178 (D), and Δ*fliD* (E). Biphasic MH cultures were incubated at 37°C under microaerophilic conditions. The growth kinetics were performed in triplicate, and the error bars represent the standard deviations.

work in *Campylobacter* gut colonization. In contrast, the *hrcA* mutant was not significantly affected in its in vitro growth nor in its ability to colonize the ileal loop. While the function of *hspR* and *hrcA* in *C. jejuni* is essentially unknown, the products of these genes were recently demonstrated to repress the transcription of the major heat shock proteins in *H. pylori* (*groESL*, *hrcA*, *grpE*, and *dnaK*) (38, 39). In addition, the HrcA-mediated repression was shown to be dependent on the binding of HspR to the promoter region (38). Interestingly, the transcription of the *cbpA-hspR-orf* operon was found to be exclusively regulated by HspR (38). Consequently, it is tempting to propose that the loss of HspR induces an increase in the abundance of heat shock proteins. Given that heat shock proteins are major immunodominant antigens, the overexpression of these proteins would likely contribute to host resistance (52). As a result, the *hspR* mutants should be less capable of colonization and survival in the host. In addition, given the absence of a colonization defect for the *hrcA* mutant, the amount of these proteins would not be increased at a sufficient level in this mutant to induce host resistance. In support of this hypothesis, *H. pylori hspR* mutants and *hspR-hrcA* double mutants were reported to have attenuated colonization efficiency in wild-type mice, while they were unaffected in interleukin-12-deficient mice (15). Furthermore, *C. jejuni*-infected patients have been shown to develop a humoral response against the heat shock protein DnaK (44).

In *E. coli*, *spoT* codes for a bifunctional enzyme able to catalyze the biosynthesis and the degradation of hyperphosphorylated guanine [(p)ppGpp] (1). In most eubacteria, (p)ppGpp has been shown to accumulate in response to stringent conditions, such as amino acid starvation, triggering the down-regulation of genes encoding the transcription and translation apparatus (1). As shown in Fig. 6, the *spoT* mutant exhibited a competitive index of 9×10^{-6} in vivo. In contrast to this in vivo result, the in vitro competitive index was only 0.06, suggesting a significant in vivo-specific defect. The decreased ability of the *C. jejuni spoT* mutant to colonize the host gut suggests an important role for the stringent response in vivo, likely allowing *Campylobacter* to deal with periods of nutrient starvation or other environmental stresses in the intestinal tract.

While the function of Cj0178 has not been characterized, this protein exhibits high homology with ferric-siderophore outer membrane receptors. In addition, the expression of Cj0178 is Fur regulated and induced in response to iron starvation (26). Consequently, Cj0178 is probably required for the acquisition of iron from an uncharacterized siderophore. The Cj0178 mutant exhibits a competitive index of 0.05 in vivo and 0.37 in vitro. Given that *C. jejuni* NCTC 11168 does not seem to produce any siderophore (48), the growth defect of this mutant in vitro is unclear. The significant attenuation of the Cj0178 mutant in vivo is in agreement with its overexpression in our microarray experiment and might suggest an important role for this iron acquisition system in gut colonization.

Interestingly, while the *fliD* mutant had a significant growth defect in vitro (exhibiting a competitive index of 0.1), it colonized the ileal loop as well as the wild-type strain (exhibiting a competitive index of 1.3). The *fliD* gene encodes a putative flagellar hook-associated protein. In *H. pylori*, *fliD* is an essential component in the assembly of a functional flagellum and is

required for colonization of the gastric mucosa of mice (17). Similarly to *H. pylori*, the *C. jejuni fliD* mutant is nonmotile and aflagellated (12). While the survival of the *fliD* mutant in the gut is in disagreement with the essential role of the flagellum in the colonization of the gastrointestinal tract, it is consistent with the characteristics of the RIL animal model. Indeed, the physical ligation of the rabbit intestinal tract likely favors the survival of mutants affected in their ability to adhere to the mucus or the intestinal epithelial cells. In addition, considering that flagellin is the major immunodominant antigen during infection (24), the loss of the flagellum structure should promote evasion of the immune system. Consequently, a *fliD* mutant will have an advantage over the wild type in vivo and thus compete better during in vivo than in vitro growth.

Concluding remarks. This genome-wide expression profiling study revealed important elements of the *Campylobacter* lifestyle during host intestinal tract colonization. In addition to the genes discussed above that have known or potential functions, many other genes of unknown function were also found to be differentially expressed between in vivo and in vitro growth and therefore constitute many new directions for future investigations. The transcriptome pattern of *C. jejuni* in vivo was consistent with that expected in an environment that is oxygen limited, hyperosmotic, nutrient restricted, and containing reactive oxygen compounds. Interestingly, the comparison of the *C. jejuni* transcriptomes between different rabbits revealed gene expression variability during the course of an infection. This flexibility in gene expression is probably essential for *Campylobacter* to adapt to the changing environment of the gut. Furthermore, the genes encoding proteins involved in flagellum biogenesis were found to be differentially expressed between rabbits. They were up-regulated in one rabbit and down-regulated (at a different level) in all others. While the up-regulation of these genes is in agreement with the role of the flagellum in gut colonization, the decreased expression of these genes probably reflects a bacterial strategy to evade the host response. Finally, regulation of both the heat shock response and the stringent response were found to be necessary for efficient colonization of the host gastrointestinal tract.

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