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# Metabolic Diversity in *Campylobacter jejuni* Enhances Specific Tissue Colonization

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## SUMMARY

***Campylobacter jejuni* is a leading cause of foodborne illness in industrialized countries. This pathogen exhibits significant strain-to-strain variability, which results in differences in virulence potential and clinical presentations. Here, we report that acquisition of the capacity to utilize specific nutrients enhanced the ability of a highly pathogenic strain of *C. jejuni* to colonize specific tissues. The acquisition of a gene encoding a γ-glutamyltranspeptidase enabled this strain to utilize glutamine and glutathione and enhanced its ability to colonize the intestine. Furthermore, the acquisition of a DNA segment, which added a *sec*-dependent secretion signal to an otherwise cytoplasmic asparaginase, allowed this pathogen to utilize asparagine and to more efficiently colonize the liver. Our results reveal that subtle genetic changes in a bacterial pathogen result in significant changes in its ability to colonize specific tissues. In addition, these studies revealed remarkably specific nutritional requirements for a pathogen to effectively colonize different tissues.**

## INTRODUCTION

*Campylobacter jejuni*, a representative of the diverse group of ε-proteobacteria, is the leading cause of foodborne diarrhea in industrialized nations (Allos, 2001). Although enteritis resulting from *C. jejuni* infections is usually self-limiting, more than 10% of untreated patients may suffer prolonged severe illness (Blaser, 1997). Furthermore, a number of convalescent patients develop a neurological complication known as Guillain-Barré Syndrome (Nachamkin, 2002). Despite its public health importance, the molecular mechanisms of *C. jejuni* pathogenesis that lead to such a variety of clinical presentations are very poorly understood. It is well established that much of the genetic diversity among different isolates of *C. jejuni* is found in putative virulence factors, in particular among surface structures (Gilbert et al., 2002; Dorrell et al., 2001; Karlyshev et al., 2005; Logan et al., 2002; Pearson et al., 2003; Taboada et al., 2004) that are thought to be under direct evolutionary pressure from the host's immune response (van der Woude and Baumler, 2004). However, much less is known about bacterial diversity in basic metabolic traits that may also be subject to host selection and may have a major influence in pathogenicity. Furthermore, it is not known how metabolic differences may affect the ability of pathogenic bacteria to colonize different tissues, since very little is known about specific requirements for growth and persistence in these environments.

*C. jejuni* cannot utilize sugars as a carbon source because it lacks the glycolytic enzyme phosphofructokinase (Parkhill et al., 2000; Velayudhan and Kelly, 2002). Consequently, the growth of *C. jejuni* is thought to depend mainly on the availability of free amino and keto acids scavenged from the host or from the intestinal microbial flora (Lee and Newell, 2006). Studies have shown that serine, aspartate, glutamate, and proline are preferentially used by *C. jejuni* as nutritional substrates in vitro (Leach et al., 1997; Elharrif and Megraud, 1986; Leon-Kempis Mdel et al., 2006; Velayudhan et al., 2004), and that serine catabolism as well as amino acid transporters are required for colonization of the intestinal tract (Velayudhan et al., 2004; Hendrixson and DiRita, 2004). Here we report that different strains of *C. jejuni* exhibit considerable diversity in their ability to utilize amino acids. We found that subtle genetic changes in a bacterial pathogen result in significant changes in its ability to utilize nutrients and colonize specific tissues in an animal model of infection.

## RESULTS

### *Campylobacter jejuni* 81-176 Exhibits Expanded Ability to Utilize Amino Acids

In an effort to understand pathogenic differences among *C. jejuni* strains, we compared the 81-176 strain, shown to be highly infectious in experimental infections in monkeys (Russell et al., 1989) and human volunteers (Black et al., 1988), with the reference NCTC 11168 strain, for their ability to utilize different amino acids. As recently reported for *C. jejuni* strain NCTC 11168 (Guccione et al., 2008), *C. jejuni* 81-176 was able to grow in the presence of aspartate, glutamate, proline, and serine (Figure 1). Surprisingly, however, *C. jejuni* 81-176 was also able to grow in the presence of glutamine and asparagine, which did not sustain the growth of the reference strain NCTC 11168 (Figure 1). These amino acids have not been previously reported to be substrates that can be utilized for growth by *C. jejuni*. These results indicate that *C. jejuni* 81-176 exhibits additional metabolic traits, which may enhance its virulence properties.

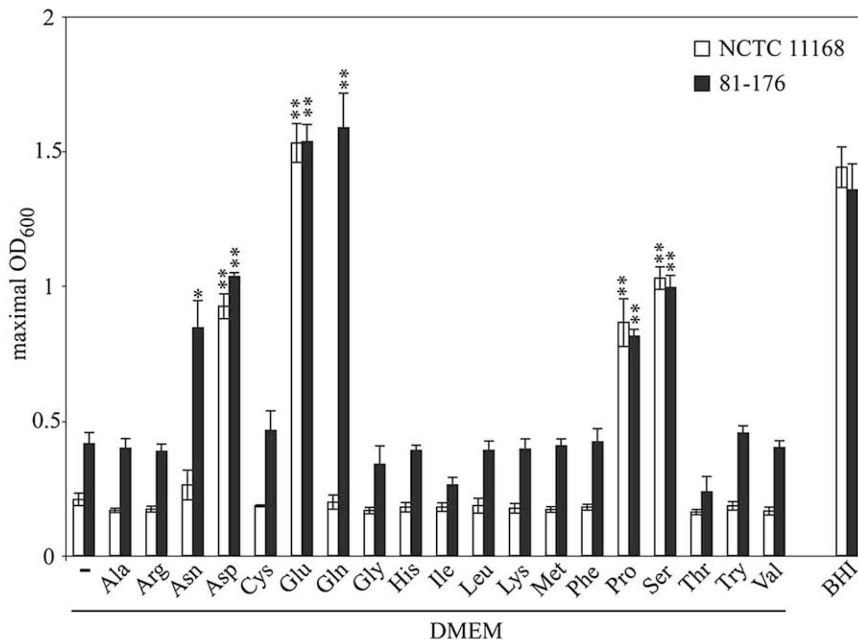


**Figure 1. Comparative Growth of *C. jejuni* 81-176 and NCTC 11168 in the Presence of Different Amino Acids**

Values are the mean ±SD of three determinations of the maximal optical density reached by *C. jejuni* 81-176 and NCTC 11168 after 24 hr of growth in DMEM, supplemented with different amino acids (20 mM) as indicated. Cultivation in BHI medium served as control. Stars denote statistically significant (**: $p < 0.0001$; *: $p < 0.05$) growth differences in comparison to growth in the basal medium without supplemental amino acids.

## A γ-glutamyltranspeptidase Confers on *C. jejuni* 81-176 the Ability to Utilize Glutamine and Glutathione

Since the ability to utilize glutamine was absent from the *C. jejuni* NCTC 11168 strain, we hypothesized that genetic determinants present solely in *C. jejuni* 81-176 must be responsible for this metabolic expansion. A *C. jejuni* 81-176 gene (cju06), which is absent from the NCTC 11168 strain, encodes a secreted γ-glutamyltranspeptidase (GGT) (Hofreuter et al., 2006). This enzyme is identical to the recently reported homolog of *C . jejuni* 81116 (Barnes et al., 2007), and exhibits high amino acid sequence similarity to the GGT of *Helicobacter pylori* (Chevalier et al., 1999) (Figure S1). Like its homolog in *H. pylori,* in *C. jejuni* GGT is produced as a 60 kDa proenzyme that is processed upon periplasmic secretion into an active enzyme consisting of 40 kDa and 20 kDa subunits (Figure 2A). GGT catalyzes the degradation of glutathione to glutamate and a cysteinylglycine dipeptide through a transpeptidation reaction that transfers the γ-glutamyl moiety to amino acids and peptides. In addition, this enzyme can hydrolyze glutathione and glutamine, releasing free glutamate (Tate and Meister, 1981). We therefore hypothesized that the hydrolytic activity of GGT may lead to the deamination of glutamine, and hence allow its utilization by *C. jejuni* 81-176 via glutamate, the end product of this reaction. Consistent with this hypothesis, the isogenic *ggt*::*kan* mutant of *C. jejuni* 81-176 was unable to utilize glutamine for growth in DMEM (Figure 2B).

Although the presence of glutathione in bacteria is well established (Fahey et al., 1978), analysis of the genome sequences of several *C. jejuni* strains (Parkhill et al., 2000; Fouts et al., 2005; Poly et al., 2007; Hofreuter et al., 2006; Pearson et al., 2007) has revealed that this pathogen does not encode homologs of the glutathione biosynthesis proteins. However, glutathione is readily available in mammalian tissues, since it is the most abundant source of intracellular reactive thiol groups, and plays a key role in detoxification of cellular free radicals, toxins, and carcinogens (Meister and Anderson, 1983). In the intestinal lumen, glutathione can originate from the diet, but it is also synthesized and secreted directly by the mucosal cells of the gut, or can reach the intestine via secreted bile (Aw, 2005). Glutathione has been shown to serve as a potential amino acid source for *E. coli* and *Neisseria meningitidis* in vitro (Takahashi et al., 2004; Suzuki et al., 1993), and since the hydrolytic activity of GGT leads to the degradation of the tripeptide glutathione and the release of glutamate (Tate and Meister, 1981), we hypothesized that this enzyme may allow *C. jejuni* to utilize glutathione as an amino acid source. Accordingly, we found that wild-type *C. jejuni* 81-176 showed significantly enhanced growth in DMEM supplemented with glutathione (Figure 2B) in a concentration-dependent manner (Figure S2). Addition of glutathione, however, did not enhance the growth of the *ggt*::*kan* mutant (Figure 2B), which indicates that GGT is required for glutathione utilization. Similar results were obtained when the experiments were carried out in rich BHI medium (Figure S3). These results are consistent with previous observations indicating that *H. pylori* can degrade glutathione in a *ggt*-dependent manner (Shibayama et al., 2007).

To further clarify the growth-enhancing properties of glutathione, we investigated the effect of adding its degradation products. Supplementation with the dipeptide cysteinylglycine did not enhance the growth of *C. jejuni* 81-176 (Figures 2B and S3). However, addition of glutamate or γ-glutamylcysteine enhanced the growth of *C. jejuni* 81-176 to levels equivalent to those resulting from the addition of glutathione (Figures 2B and S3). As expected, addition of γ-glutamylcysteine did not enhance the growth of the *C. jejuni* 81-176 *ggt*::*kan* mutant, although the growth of this strain was enhanced by the addition of glutamate, which does not require GGT for its utilization (Figures 2B and S3). To investigate the possibility that the growth-enhancing effect of glutathione and glutamine was due to the generation of glutamate after their GGT-mediated hydrolysis, we investigated the consequence of a mutation in *peb1A* (Cj0921c) in the utilization of these nutrients. Peb1A is an aspartate/glutamate-binding protein of an ABC transporter that is essential for the utilization of these amino acids by *C. jejuni* (Leon-Kempis Mdel et al., 2006; Pei and Blaser, 1993) and required for virulence (Pei et al., 1998). Disruption of *peb1A* effectively abrogated the ability of *C. jejuni* to utilize glutathione, glutamine, and the dipeptide γ-glutamylcysteine (Figures 2B and S3). Since disruption of *peb1A* did not affect the GGT activity of *C. jejuni* (Figure 2C), these results indicate that GGT allows the utilization

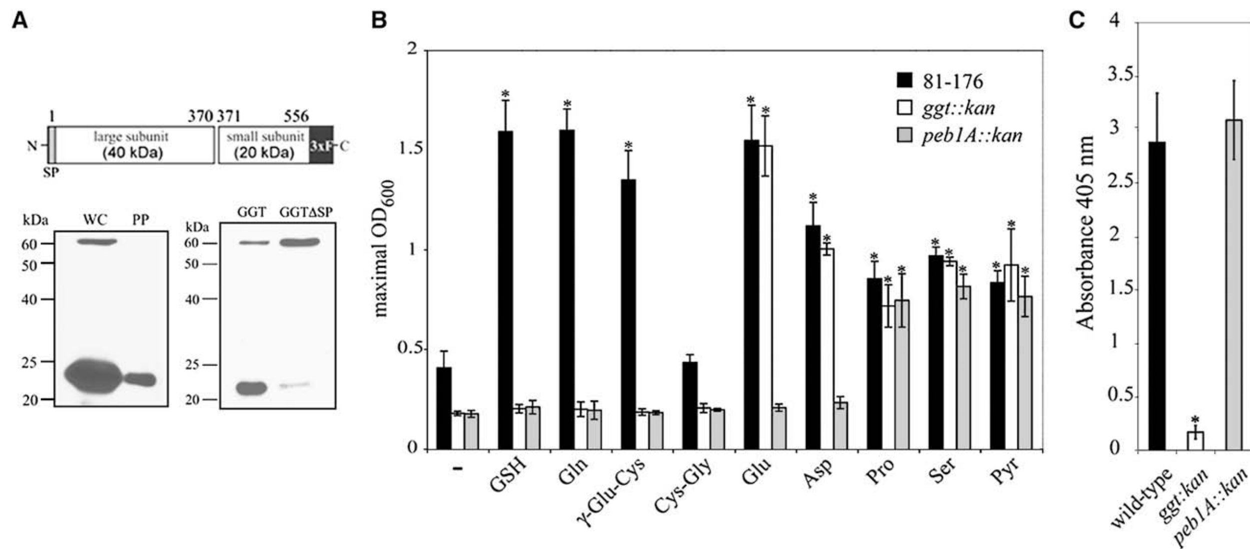


**Figure 2. *C. jejuni* γ-Glutamyltranspeptidase-Dependent Utilization of Glutamine and Glutathione**

(A) Expression, localization, and processing of *C. jejuni* 81-176 γ-glutamyltranspeptidase. A diagram of the organization of *C. jejuni* γ-glutamyltranspeptidase is shown. Whole cell (WC) and periplasmic (PP) fractions of *C. jejuni* expressing FLAG epitope-tagged γ-glutamyltranspeptidase examined by western blot analysis are shown in the left panel. FLAG epitope-tagged γ-glutamyltranspeptidase (GGT) or a mutant derivative lacking its putative *sec*-dependent secretion signal (GGTΔSP) were expressed in *C. jejuni* and examined by western blot analysis (right panel). Note that in the absence of the secretion signal, GGT is not efficiently processed into the 40 and 20 kDa subunits.

(B) Ability of *C. jejuni* 81-176 and mutant derivatives to utilize glutamine, glutathione, and compounds derived from the activity of γ-glutamyltranspeptidase. Values are the mean ±SD of three determinations of the maximal optical density reached by wild-type *C. jejuni* 81-176 and its isogenic *ggt*::*kan* and *peb1A*::*kan* mutants after 24 hr of growth in DMEM supplemented with the indicated compounds (20 mM). Stars denote statistically significant ($p < 0.0001$) growth differences in comparison to growth in the basal medium without supplements.

(C) γ-Glutamyltranspeptidase activity of wild-type *C. jejuni* 81-176 and its isogenic *ggt*::*kan* and *peb1A*::*kan* mutants. Activity is expressed as the absorbance $A_{405}$ of released p-nitroanilide. Values represent the mean ±SD of three independent determinations. Star denotes statistically significant ($p < 0.0001$) difference in comparison to the values of the wild-type strain.

of glutathione, glutamine, or the dipeptide γ-glutamylcysteine by generating glutamate, which is then taken up by the Peb1A-dependent transporter and used as a carbon source. These results show that *C. jejuni* is able to utilize glutamine and glutathione in a *ggt*-dependent manner, and extends previous biochemical observations in *H. pylori* indicating that this bacterium can take up glutamate released from glutathione or glutamine by its GGT activity (Shibayama et al., 2007). Our data also reveal significant variability in glutathione metabolism among different bacteria since, after GGT-dependent glutathione degradation, *E. coli* and *N. meningitidis* utilize the released cysteine residue rather than glutamate (Takahashi et al., 2004; Suzuki et al., 1993).

### A Secreted Asparaginase Confers on *C. jejuni* 81-176 the Ability to Utilize Asparagine

Based on studies of the metabolism of asparagine in other bacteria, it is predicted that the utilization of asparagine by *C. jejuni* 81-176 would require either a specific transporter for this amino acid (Strobel et al., 1989), or a periplasmic asparaginase capable of converting asparagine to aspartate (Singh and Rohm, 2008), which could then be taken up by *C. jejuni* in a Peb1A-dependent manner. No obvious homolog of an asparagine transporter was detected in any of the sequenced strains of *C. jejuni*, although all strains encode an L-asparaginase (annotated as Cj0029 in the genome of NCTC 11168 reference strain) with significant amino acid sequence similarity to *E. coli* AnsB. Interestingly, close inspection of the amino acid sequences of these asparaginases showed that, although highly related, their sequences exhibit intriguing differences. While the amino terminus of the *C. jejuni* 81-176 AnsB has a canonical *sec*-dependent secretion signal sequence, such a signal sequence is absent from the AnsB homolog of *C. jejuni* NCTC 11168 (Figure 3A). Indeed, comparison of the nucleotide sequence of the 5′ region of *ansB* in these strains revealed a striking sequence difference. In *C. jejuni* 81-176, this region shows the presence of an additional ~40 nucleotides adding a secretion signal to an otherwise cytoplasmic asparaginase (Figure 3A), resulting in a secreted form of this enzyme that allows the utilization of asparagine by *C. jejuni* 81-176. Introduction of a loss-of-function mutation in the *ansB* (Cj0029) homolog of *C. jejuni* 81-176, or exchanging the *ansB* allele with a homolog lacking the putative *sec*-dependent secretion signal, abolished the ability of this strain to utilize asparagine (Figure 3B). Conversely, the introduction of the *C. jejuni* 81-176 *ansB* (Cj0029) allele into the *C. jejuni* NCTC 11168 strain allowed this strain to utilize asparagine (Figure 3C). Furthermore, AnsB containing the putative secretion signal was detected in the periplasmic fraction of *C. jejuni* 81-176, while a mutant derivative expressing an allele lacking the putative secretion signal was detected only in the cytoplasmic fraction (Figure 3D). Moreover, utilization of asparagine by *C. jejuni* 81-176 required the Peb1A-dependent aspartate transporter (Figure 3B). Taken together, these results demonstrate that the addition of a secretion signal to an otherwise cytoplasmic asparaginase conferred to *C. jejuni* 81-176 the ability to deaminate asparagine to aspartate in the periplasm, thus allowing the utilization of asparagine as

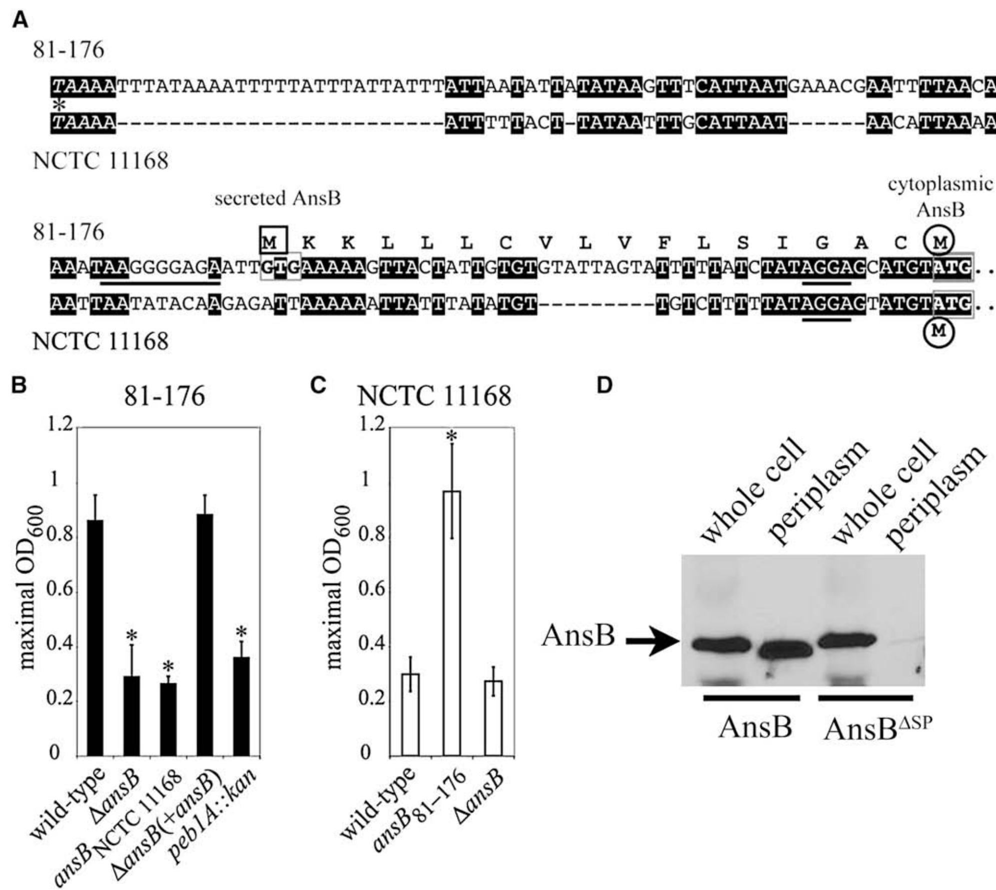


**Figure 3. Asparagine Utilization by *C. jejuni***

(A) Comparison of the 5′ nucleotide sequence of the *ansB* locus of *C. jejuni* 81-176 and NCTC 11168 strains. The putative initiation codons are indicated with a square (for the secreted form) and with a circle (for the cytoplasmic form) of AnsB.

(B) The ability of *C. jejuni* 81-176 to utilize asparagine is dependent upon secreted AnsB. Maximal optical density reached by the wild-type *C. jejuni* 81-176 and the isogenic mutants *ΔansB*, an isogenic strain expressing the *ansB* allele from *C. jejuni* NCTC11168, which lacks the secretion signal ($ansB_{NCTC11168}$), *ΔansB* complemented with a wild-type copy of the gene (*ΔansB* [+*ansB*]), and an isogenic mutant (*peb1A*::*kan*) defective in the dicarboxylic aspartate/glutamate transporter Peb1A, after 24 hr of growth in DMEM supplemented with 20 mM asparagine. Values represent the mean ±SD of three independent determinations. Stars denote statistically significant ($p < 0.0001$) growth differences in comparison to wild-type growth.

(C) Introduction of the *ansB* allele from *C. jejuni* 81-176 confers to *C. jejuni* NCTC 11168 the ability to utilize asparagine. Values are the mean ±SD of three determinations of the maximal optical density reached by wild-type *C. jejuni* NCTC 11168, the isogenic mutant *ΔansB*, and a derivative expressing the *ansB* allele from *C. jejuni* 81-176 ($ansB_{81\text{-}176}$), after 24 hr of growth in DMEM supplemented with 20 mM asparagine. Star denotes statistically significant ($p < 0.0001$) growth differences in comparison to the growth of wild-type NCTC 11168.

(D) Subcellular localization of AnsB. Cytoplasmic and periplasmic fractions of *C. jejuni* 81-176 strains expressing FLAG epitope-tagged AnsB (AnsB) or a mutant derivative expressing an *ansB* allele encoding a variant without the signal sequence ($AnsB^{\Delta sp}$) were analyzed by western immunoblot with an antibody directed to the epitope tag.

a nutritional source. The presence of a putative alternative start site downstream from the first initiation codon in the 81-176 *ansB* sequence (Figure 3A), however, could potentially generate a cytoplasmic form of this enzyme, which could presumably ensure the production of both secreted and cytoplasmic forms of this enzyme. The cytoplasmic form of AnsB could be involved in metabolizing asparagine that could be generated by the cytoplasmic degradation of small peptides imported by specific transporters.

### Clinical Isolates of *C. jejuni* Exhibit Significant Metabolic Diversity

To investigate whether the observations made in *C. jejuni* 81-176 extend to other isolates, we investigated the distribution of *ggt* in several clinical isolates of *C. jejuni*. We were able to detect the presence of *ggt* in 3 (ATCC 49943, 154.2067, and 240.0872) out of 15 clinical isolates tested. We determined the nucleotide sequence of *ggt* in these clinical isolates and found that they can encode full-length GGT (Figure S4). Furthermore, *ggt* is also present in *C. jejuni* 81116 (Barnes et al., 2007), whose genome sequence has recently been completed (Pearson et al., 2007), and in the unfinished genome sequences of the *C. jejuni* strains 260.94 and HB93-13, and in *C. jejuni* subspecies *doylei* 269.97. In all cases, the *ggt* locus is located in the vicinity of a highly conserved ribosomal RNA gene cluster (Figure S5), which is consistent with the hypothesis that this gene may have been acquired by horizontal gene transfer from another

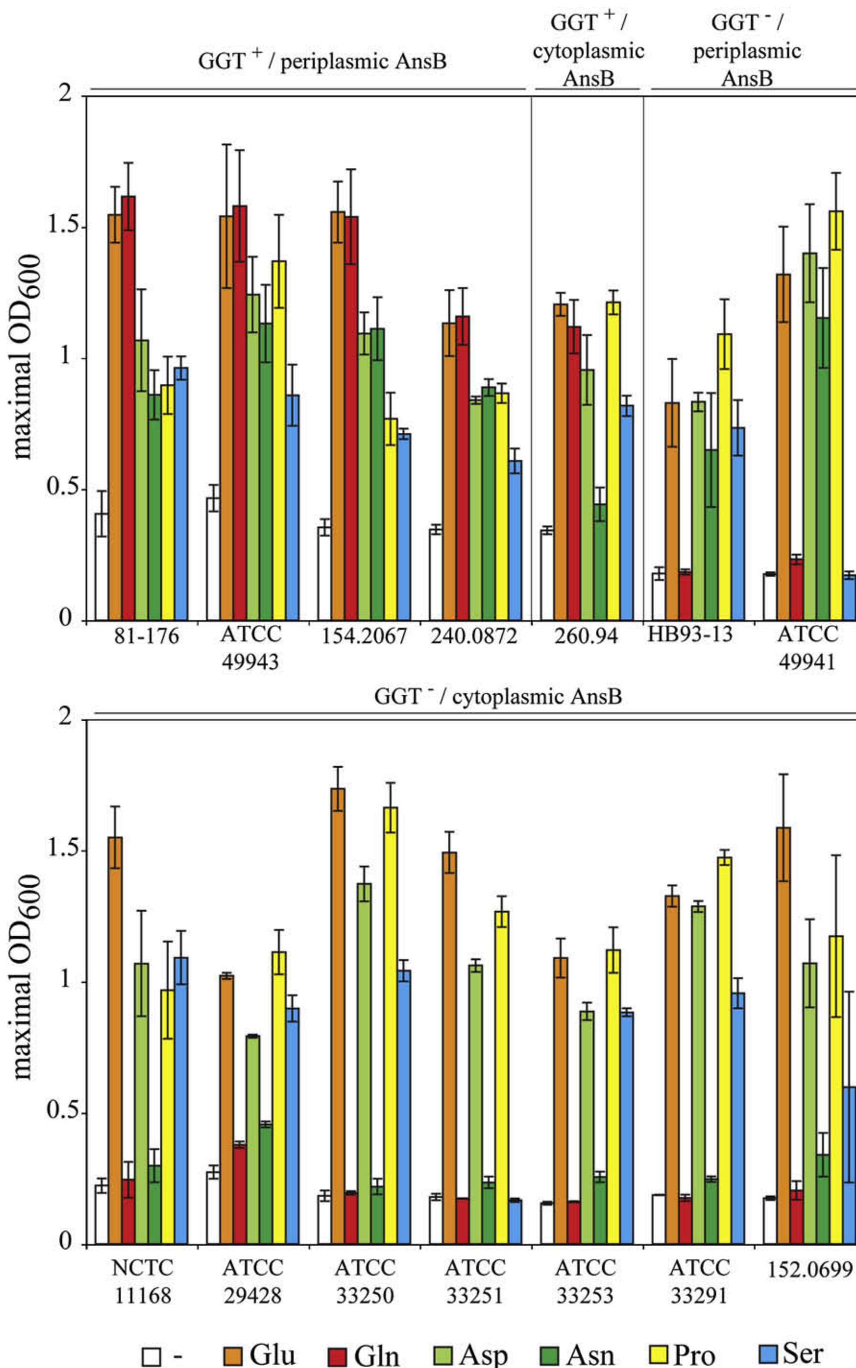


**Figure 4. Metabolic Diversity Among *C. jejuni* Isolates**

Values are the mean ±SD of three determinations of the maximal optical density reached by different wild-type *C. jejuni* strains after 24 hr of growth in DMEM supplemented with the indicated amino acids (20 mM). The strains are grouped according to their *ggt* and *ansB* genotypes as indicated.

source. We tested these clinical isolates for their ability to utilize glutamine, and found a strict correlation between the presence of *ggt* and the ability to utilize this nutrient (Figures 4 and S6A). In fact, introduction of an insertion mutation in the *ggt* locus of the *C. jejuni* strains ATCC 49943, 154.2067, and 240.0872 abolished their ability to utilize glutamine (Figure S6B). These results indicate that the *ggt*-mediated metabolic expansion is a common occurrence in clinical isolates of *C. jejuni*. We also examined the predicted amino acid sequence of AnsB encoded in the *C. jejuni* strains whose nucleotide genome sequences are available. The asparaginases of *C. jejuni* RM1221, CG8486, CF93-6, 84-25, and 260.94 showed no predicted N-terminal secretion signal and therefore are presumably cytoplasmic (Figure S7). In contrast, we found *sec*-dependent secretion signals in AnsB from *C. jejuni* 81116, HB93-13, and *C. jejuni subsp. doylei* 269.97 as well as *C. coli* RM2228, *C. lari* RM2100, and *C. upsaliensis* RM3195 (Figure S7). To expand the analysis of asparaginases to other *C. jejuni* strains, we determined the nucleotide sequence of *ansB* in several clinical isolates. In 5 out of 15 isolates, we found asparaginases containing a *sec*-dependent signal sequence (Figure S7). In all of these cases, the nucleotide sequence of the 5′ region of *ansB* was identical to that of *C. jejuni* 81-176, suggesting a common origin for the DNA segment encoding the secretion signal (Figure S8). We also tested these clinical isolates for their ability to utilize asparagine, and found a strict correlation between the presence of a *sec*-dependent secretion signal in the predicted sequences of AnsB and their ability to utilize this amino acid (Figure 4). In addition to the difference in the ability of *C. jejuni* strains to metabolize asparagine and glutamine, 1 (ATCC 33251) of the 13 *C. jejuni* isolates tested and *C. coli* ATCC 49941 were unable to grow with serine (Figure 4). This is of interest, as *C. jejuni* serine metabolism was shown to be important for the effective colonization of chicken (Velayudhan et al., 2004). Our comparison revealed that, of all the amino acids tested, proline was the only one that was able to support the growth of all tested strains. Taken together, these results revealed an unexpected diversity in basic metabolic traits of different clinical isolates of *C. jejuni*, which may affect their pathogenic potential.

## *C. jejuni* 81-176 Exhibits Differential Nutritional Requirements for the Efficient Colonization of Different Tissues

We have previously shown that a *ggt* mutant of *C. jejuni* 81-176 is defective in its ability to colonize the intestinal tract of $myd88^{-/-}$ mice after oral administration (Hofreuter et al., 2006), and it was recently demonstrated that a *ggt* mutant of *C. jejuni* 81116 was

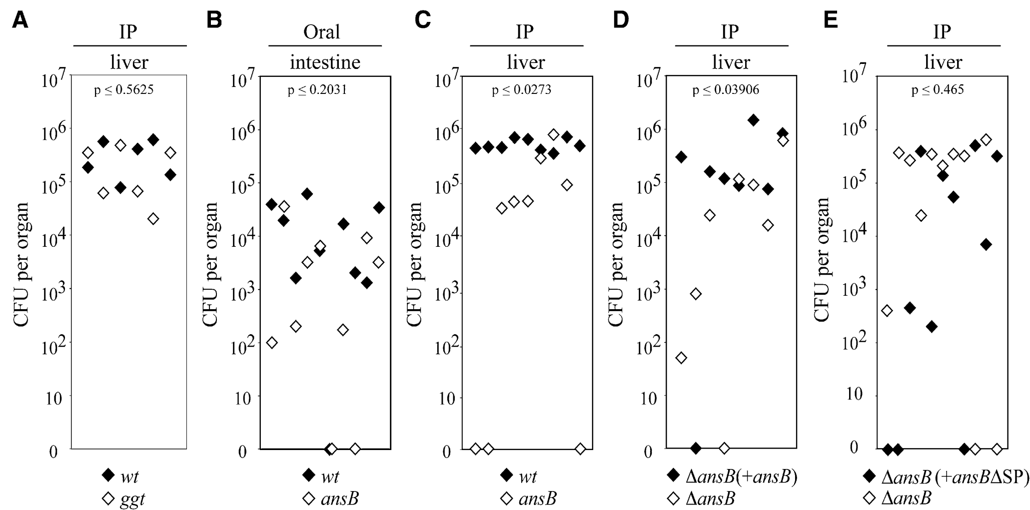


**Figure 5. Contribution of *ggt* and *ansB* to *C. jejuni* 81-176 Specific Tissue Colonization**

(A) *ggt* is not required for efficient liver colonization by *C. jejuni* 81-176. Liver colonization of wild-type *C. jejuni* and the *ggt*::*kan* mutant after IP administration. The colonization of the liver was evaluated 7 weeks after coinfection with equal numbers of wild-type and *ggt*::*kan C. jejuni* strains. Each pair of black and white diamonds represents the CFU of wild-type and the *ggt*::kan mutant, respectively, recovered from the liver of an individual animal. The *P* values for the statistical differences between the number of CFU of the two strains as analyzed by the Wilcoxon matched-pairs signed-rank test were not significant (p = 0. 563).

(B–E) Utilization of asparagine enhances the ability of *C. jejuni* 81-176 to colonize the liver but has no significant effect on its ability to colonize the intestine. Mice were coinfected orally (B) or intraperitoneally (C–E) with equal numbers of the indicated strains. Infected mice were sacrificed 6 weeks after infection and the number of CFU of the different strains in the indicated tissues was determined by plating in the appropriate media to distinguish the strains (note: orally infected mice had no recoverable CFU in the liver). Each pair of black and white diamonds represents the different strains (as indicated) recovered from an individual mouse. The *P* values for the statistical differences between the number of CFU of the different strains as analyzed by the Wilcoxon matched-pairs signed-ranks test are indicated. (Note: The *P* values for the statistical differences between the number of CFUs obtained in experiments shown in panels (C) and (D) combined is $p < 0.002$.)

defective for the intestinal colonization of chickens (Barnes et al., 2007). We investigated the potential role of GGT in the ability of *C. jejuni* to colonize the liver of *myd88*$^{-/-}$ mice in competition experiments, which are known to be able to resolve small differences in the colonization abilities of different bacterial strains. Surprisingly, we found that the *C. jejuni ggt* mutant strain colonized the liver of infected animals in a manner indistinguishable from wild-type (Figure 5A), suggesting that glutamine and/or glutathione may not be essential nutrients for *C. jejuni* growth in the liver. Combined with our previous findings (Hofreuter et al., 2006), and those of others (Barnes et al., 2007), these results revealed unique nutritional requirements for *C. jejuni* in different tissues, since glutathione and/or glutamine are important nutritional sources for *C. jejuni* in the intestinal tract but appear to be dispensable for its growth in the liver.

We tested whether asparagine, like glutamine and/or glutathione, may also be an important nutrient during infection by comparing the ability of wild-type *C. jejuni* 81-176 and the isogenic *ansB*::*kan* mutant to colonize mice in mixed infection experiments. Five weeks after oral administration of equal numbers of wild-type *C. jejuni* 81-176 and the isogenic *ansB*::*kan* mutant strain, both strains were recovered in similar numbers from intestinal tissues of colonized animals (Figure 5B). Notably, however, the *ansB::kan* mutant, as well as a mutant expressing an asparaginase without its secretion signal peptide (Figure S9), were significantly defective in their ability to colonize the liver of infected animals when administered systemically (Figure 5C). Such defect was fully restored by introduction of the wild-type allele (Figure 5D), but not by introduction of an allele lacking the secretion signal (Figure 5E). These results indicate that, in the liver but not in the intestine, asparagine, presumably through its metabolization into asparatate, is an important nutrient for *C. jejuni*. The reduced but detectable ability of the *ansB::kan* mutant of *C. jejuni* to colonize the liver suggests that nutrients other than asparagine may be also available at this site to support limited replication of this *C. jejuni* strain. Together with the observations made with the *ggt* mutant, these results show a remarkable specificity in *C. jejuni*'s nutritional requirements to efficiently colonize different tissues: glutathione or glutamine and the derived glutamate in the intestine and asparagine or aspartate in the liver.

## DISCUSSION

It is well established that different isolates of the same pathogen often display significant diversity within their repertoire of virulence factors (Joyce et al., 2002; Whittam and Bumbaugh, 2002), particularly in surface determinants, which are thought to be under short-term evolutionary pressure by the host's immune response (van der Woude and Baumler, 2004; Bentley et al., 2007). Less is known about diversity within basic metabolic traits, which are often considered more stable attributes of bacterial pathogens. However, pathogens that as part of their normal ecology inhabit different environments with different available nutrients are also likely to be under strong selective pressure to diversify and evolve their basic metabolic traits. We have shown here that *C. jejuni*, a bacterial pathogen that is capable of colonizing a diverse array of warm-blooded animals, and persists in various environmental habitats (Young et al.,

2007), exhibits significant diversity in its ability to utilize basic nutrients. This metabolic diversity translates in different capacity to colonize different tissues. We have also presented evidence indicating that such diversity is the result of the acquisition of new genetic traits or the modification of existing ones, presumably through the horizontal acquisition of genetic material. Thus, the ability of some clinical isolates to utilize glutamine and glutathione resulted from the acquisition of a gene encoding a $\gamma$-glutamiltranspeptidase. Furthermore, the acquisition of a DNA segment that added a *sec*-dependent secretion signal to a cytoplasmic asparaginase conferred on some clinical isolates the ability to utilize asparagine.

Our results indicate that the acquisition of specific metabolic pathways has enhanced the ability of a pathogen to colonize different tissues. It is unknown whether these results are significant to human infections or to infections of other vertebrate hosts, or whether they can be generalized to other strains of *C. jejuni*. In fact, our analysis has shown that many clinical isolates of *C. jejuni* do not encode GGT or a secreted form of AnsB, which suggests that the ability to utilize asparagine, glutathione, and/or glutamine is not essential for *C. jejuni* to cause disease in humans. Nevertheless, our findings do highlight the remarkable specificity of the nutritional requirements for a pathogen to colonize specific tissues in the animal model system utilized in these studies. *C. jejuni* 81-176 was isolated from an outbreak of diarrheal disease in schoolchildren (Korlath et al., 1985) and an infection dose of as little as 800 organisms was sufficient to cause illness in human volunteers (Black et al., 1988). It is possible that the expanded ability of this *C. jejuni* strain to utilize amino acids may contribute to its apparently increased infectivity. Furthermore, the ability of certain *C. jejuni* strains to utilize a broader range of carbon sources may confer an advantage during the commonly observed phenomenon of infection of a single host by multiple strains of *C. jejuni* (Godschalk et al., 2006; Koene et al., 2004; Shen et al., 2001), or may be beneficial for their survival in nutrient-poor environments.

Although bacterial metabolism has been the subject of intense study (Downs, 2006), knowledge of the nutritional requirements of bacterial pathogens within their hosts is one of the least-understood areas of bacterial pathogenesis (Muñoz-Elías and McKinney, 2006). This is an extremely important area of research, since understanding the in vivo nutritional requirements of bacterial pathogens can lead to the development of novel therapeutic strategies (Brown et al., 2008). The availability of the genome sequences of most bacterial pathogens has allowed the in silico reconstruction of their metabolic pathways (Marais et al., 1999; Doig et al., 1999; Bono et al., 1998; Feist and Palsson, 2008). However, this information, combined with traditional studies of bacterial physiology in artificial cultures, has significant limitations since the metabolism of pathogenic bacteria during infection can differ profoundly from their metabolism in vitro (Muñoz-Elías and McKinney, 2006). Only the understanding of the complex in vivo nutritional requirements of bacterial pathogens can lead to the effective design of new antimicrobials. Targeting metabolic pathways as an antimicrobial strategy could be challenging in the case of bacterial pathogens, such as *Salmonella enterica,* with complex and redundant metabolic pathways (Becker et al., 2006). However, in the case of nonsaccharolytic pathogens with more restricted metabolic capabilities, such as *Campylobacter jejuni*, this strategy offers more promising prospects. The findings reported here further emphasize the need for the study of metabolic requirements in the context of infection, and dramatically showcase a set of adaptations sculpted by evolutionary forces to maximize the fitness of a pathogen.

## EXPERIMENTAL PROCEDURES

### Bacterial Strains, Media, and Culture Conditions

The wild-type *C. jejuni* strains 81-176 and RM1221 were obtained from Patricia Guerry (Naval Medical Research Center) and Robert E. Mandrell (Agricultural Research Service, US Department of Agriculture; Albany, NY), respectively. Other clinical isolates from infected patients were obtained from Christine Ginocchio (North Shore-Long Island Jewish Health System) or from ATCC (Manassas, VA). Bacterial strains were grown routinely on brucella agar or tryptic soy agar plates supplemented with 5% defibrinated sheep blood in a 10% $CO_2$ atmosphere at 37°C. Selective plates were supplemented with chloramphenicol (7.5 mg/liter) or kanamycin (50 mg/liter). Brain heart infusion (BHI) medium was used to grow *C. jejuni* in liquid culture. Alternatively, *C. jejuni* strains were grown in the defined medium DMEM (GIBCO; catalogue number 11965) supplemented with $Fe^{2+}$ in the form of Iron(II)-ascorbate (Sigma; catalogue number AO207). Specific amino acids (Sigma) for supplementation of the DMEM were used in concentrations of 20 mM. For growth assays, *C. jejuni* cultures were adjusted to an $OD_{600}$ of 0.1–0.2 in 3 ml of the appropriate liquid medium and grown under an atmosphere of 10% $CO_2$ in a rotating wheel (50 rpm). For larger culture volumes, *C. jejuni* was grown in 125–250 ml Erlenmeyer flasks in 10% $CO_2$ atmosphere at 37°C with orbital shaking (200 rpm). Bacterial growth was monitored by measuring the optical density of the cultures at 600 nm ($OD_{600}$) with a spectrophotometer (Spectronic 20, Genesys). Statistical analysis of the results was carried out by Student's t test.

### Construction of *C. jejuni* Mutants

The *ggt::kan* mutant derivatives of the clinical isolates *C. jejuni* ATCC 49943, 154.2067, and 240.0872 were generated by transforming into these strains chromosomal DNA isolated from the previously described *ggt::kan* mutant derivative of *C. jejuni* 81-176 (Hofreuter et al., 2006), followed by selection on brucella agar plates supplemented with kanamycin. *C. jejuni* 81-176 *peb1A*::*kan* and *ansB*::*kan* (Cj0029) derivatives were constructed by allelic exchange following the standard procedures previously described (Hofreuter et al., 2006). Strains of *C. jejuni* expressing a FLAG epitope-tagged GGT or AnsB were generated by standard PCR, recombinant DNA, and allelic exchange procedures using a chloramphenicol resistance marker (lacking transcription terminator) placed immediately downstream from the tag for selection.

### Subcellular Fractionation of *C. jejuni*

Subcellular fractionation of *C. jejuni* was carried out as previously described (Leon-Kempis Mdel et al., 2006).

### GGT Activity

GGT activity was assayed as previously described (McGovern et al., 2001). Statistical analysis of the results was carried out by Student's t test.

### *C. jejuni* Mouse Infections

The mouse model for *C. jejuni* infection has been previously described (Watson et al., 2007). Age-matched (6–8 weeks) *myd88*$^{-/-}$ *nramp1*$^{-/-}$ male mice were infected orally or intraperitoneally with equal numbers ($10^9$ or $10^7$ CFU for oral or intraperitoneal [IP] infections, respectively) of the different *C. jejuni* strains. At the indicated times, animals were sacrificed, intestine and liver were removed and homogenized, and the bacterial loads in different tissues were determined by serial plating on brucella agar plates supplemented with *Campylobacter* selective medium (Oxoid), with or without the appropriate antibiotics (kanamycin, 50 $\mu$g ml$^{-1}$ or chloramphenicol, 7.5 $\mu$g ml$^{-1}$) to distinguish between wild-type and mutant strains. For IP coinfection of mice with the *C. jejuni* 81-176 wild-type strain and its *ggt* mutant, one experiment with six animals was performed (Figure 5A). Two independent experiments with five animals each were done for the oral coinfection of the wild-type strain and the $\Delta$*ansB* mutant (Figure 5B), and two independent experiments with three

and six animals each were done for the IP coinfection of mice with wild-type strain and the Δ*ansB* mutant (Figure 5C). For coinfection experiments of the Δ*ansB* mutant and its derivative complemented with a wild-type copy of *ansB*, two independent experiments were performed with four animals each (Figure 5D). Coinfection experiments with the Δ*ansB* mutant and its derivative-encoding AnsB lacking the secretion signal were conducted in two separate infections with five and six animals (Figure 5E). Statistical analysis of the results was carried out with the Wilcoxon matched-pairs signed-ranks test.

### Sequence Analysis and Strain Comparison

Predictions of open reading frames (ORFs) for the genome sequences available in the GenBank were performed with ARTEMIS (http://www.sanger.ac.uk/Software/Artemis), with a cutoff of 100 bp, and BLASTX (http://www.ncbi.nlm.nih.gov/BLAST). Homology searches were performed with BLASTP and BLASTN (http://www.ncbi.nlm.nih.gov/BLAST). CampyDB was used for specific comparison of the published genome sequences of *Campylobacter* (Chaudhuri and Pallen, 2006) (http://campy.bham.ac.uk). ClustalW (http://www.ebi.ac.uk/clustalw) was used for multiple DNA and protein sequence alignments.

## ACCESSION NUMBERS

Sequences of the *ggt* locus from *C. jejuni* isolates described in this study have been deposited in GenBank with the accession numbers FJ234213, FJ234214 and FJ234215 for the strains *C. jejuni* 154.2067, 240.0872, and ATCC 49943, respectively. Sequences of the different *C. jejuni ansB* loci described in these studies have been deposited in GenBank with the following accession numbers: FJ234222 (ATCC 33251), FJ234224 (152.0699), FJ234225 (153.1039), FJ234227 (234.1072), FJ234219 (ATCC 33253), FJ234220 (ATCC 29428), FJ234216 (ATCC 33250), FJ234217 (ATCC 33291), FJ234228 (M.D. Blood), FJ234223 (W6984), FJ234221 (ATCC 33560), FJ234218 (ATCC 49943), FJ234226 (154.2067), FJ234229 (240.0872). The sequence of the *ansB* locus from *Campylobacter coli* ATCC 49941 has been deposited in GenBank with the accession number FJ234230.

## SUPPLEMENTAL DATA

Supplemental Data include nine figures and can be found online at http://www.cell.com/cellhostandmicrobe/supplemental/S1931-3128(08)00309-0.

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