Quantitative Proteomics of Intracellular Campylobacter jejuni Reveals Metabolic Reprogramming

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Abstract

Campylobacter jejuni is the major cause of bacterial food-borne illness in the USA and Europe. An important virulence attribute of this bacterial pathogen is its ability to enter and survive within host cells. Here we show through a quantitative proteomic analysis that upon entry into host cells, C. jejuni undergoes a significant metabolic downshift. Furthermore, our results indicate that intracellular C. jejuni reprograms its respiration, favoring the respiration of fumarate. These results explain the poor ability of C. jejuni obtained from infected cells to grow under standard laboratory conditions and provide the bases for the development of novel anti microbial strategies that would target relevant metabolic pathways.

Introduction

Campylobacter jejuni is the leading cause of bacterial food-borne disease in the USA and Europe and a major cause of diarrheal disease in developing countries [1,2]. The typical route of C. jejuni transmission is through handling and ingesting contaminated food, milk, or water [3]. In many cases, poultry is a common source of food contamination, and the gastrointestinal tract of many species of birds often serves as the natural reservoir of C. jejuni [4]. Despite its important impact on public health, surprisingly little is known about its pathogenesis [5]. Biochemical analysis and comparative genomic studies of C. jejuni have revealed unique features in its metabolic pathways [6–8]. Unlike most enteric bacteria, C. jejuni lacks the key glycolytic enzyme 6-phosphofructokinase as well as alternative pathways for sugar catabolism. Consequently, C. jejuni utilizes amino acids and organic acids as the major carbon sources. In addition, C. jejuni possesses highly branched electron transport chains, which allow it to respire not only oxygen but also a variety of electron acceptors including fumarate, nitrate, nitrite, trimethylamine-N-oxide (TMAO), and dimethyl sulfide (DMSO) [9–12]. Such remarkable diversity in its respiratory pathways may contribute to its ability to survive or grow in substantially different environments [13].

We previously reported that C. jejuni survives within cultured intestinal mammalian cells by avoiding its delivery to lysosomes [14]. We also found that 24 hours after infection, C. jejuni recovered from cultured intestinal mammalian cells cannot be efficiently cultured under microaerophilic conditions unless it is pre-incubated under oxygen-limiting conditions. We hypothesized that within host cells C. jejuni may alter its physiologic and change its mode of respiration perhaps due to low level of oxygen within the C. jejuni-containing vacuole. Such metabolic change should most likely be reflected in alterations on the levels of different sets of metabolic enzymes.

In an effort to understand the physiological changes in intracellular C. jejuni we surveyed its proteome at different time after infection of cultured mammalian cells and compared it to the proteome of bacteria grown in vitro. Our data indicate that within cultured mammalian cells, C. jejuni undergoes a severe metabolic downshift and an apparent change of its respiration mode. These data provide major insight into the in vivo metabolism and pathogenesis of this important pathogen.

Results/Discussion

Overview of the proteomic analysis of intracellular C. jejuni

To obtain a snapshot of C. jejuni protein expression during infection, we developed a protocol to isolate bacteria away from mammalian host cell proteins by exploiting C. jejuni’s moderate resistance to detergent treatment. This protocol resulted in the presence of very low amounts (<15%) of host cell-derived proteins in the final preparation. COS-1 cells were infected with C. jejuni and 2 hs and 20 hs after infection, intracellular bacteria were isolated, and proteins from bacterial lysates were pre-fractionated by gel electrophoresis prior to LC-MS/MS analysis. We chose to compare these two time points because while at 2 hs after infection C. jejuni is readily culturable under microaerophilic conditions, at 20 hs after infection it is not [14]. We also reasoned that after 2 hs of infection, the C. jejuni proteome would closely resemble that of extracellular bacteria allowing the opportunity to examine the potential changes that may occur during the transition from an extracellular to an intracellular niche. More importantly, the comparison of proteomes of bacteria isolated by similar procedures minimized potential differences due to sample processing that could occur by comparing the proteomes of bacteria grown in culture with that of bacteria obtained from within mammalian cells. Samples generated with the outlined protocol (see Figure 1A) exhibited limited host-protein contamination when examined by both Gommmassie blue staining after SDS-PAGE (Figure 1B), and
mass spectrometry analysis (less than 15% of the identified proteins corresponded to the mammalian cell proteome). Through the assignments of 400,000 MS/MS spectra we were able to detect 1,428 C. jejuni proteins, which represent 86% of the entire C. jejuni proteome (see Dataset S1). We used spectral counting to assess the relative protein abundance in the different samples [15]. Most proteins in the dataset were assigned from multiple spectral counts, which combined with the numerous technical and biological replicates, provided strong confidence to the protein assignments. In addition we quantified a selected group of proteins by selective reaction monitoring (SRM) (see below) [16]. Our data showed that proteomic differences determined by spectral counting agreed well with those measured by SRM although in some cases (i.e., proteins present in large amounts) spectral counting slightly underestimated the extent of proteomic differences (reported as the fold changes) between samples (see below). Such observation is consistent with the fact the dynamic range of spectral counting is not as high as that of SRM [16]. To our knowledge, this represents the most comprehensive C. jejuni proteomic dataset available.

C. jejuni remodels its proteome within cultured mammalian cells

After applying the criteria described in Materials and Methods, we found 225 proteins whose levels at 20 h after infection differed significantly from those at 2 h after infection (Dataset S2). The vast majority of these proteins (211) showed significantly ($p<0.05$) reduced levels at 20 h after infection. Only a few proteins showed a very moderate increase in their levels at 20 h post-infection (Dataset S2). As predicted, the proteome of C. jejuni grown in vitro closely resembled the proteome of C. jejuni isolated from within cultured mammalian cells 2 h after infection (Dataset S3). We specifically examined the profile of proteins whose levels are known to increase as a consequence of general stress responses [17–19] and found that their levels were either unchanged or were even slightly decreased (Table 1). Furthermore, the levels of proteins that are thought or known to protect C. jejuni against oxidative stress [20] were also either decreased or unaltered (Table 1), although their levels were relatively high in all time points. These results suggest that C. jejuni may not be subject to environmental stress when located within cultured mammalian cells and have important implications for the understanding of C. jejuni intracellular stage during infection.

C. jejuni undergoes a metabolic downshift within cultured mammalian cells

Functional grouping of the proteins whose expression changed upon C. jejuni’s transition from the extracellular to the intracellular environment indicated an over representation of proteins involved in the fold changes) between samples (see below). Such observation is consistent with the fact the dynamic range of spectral counting is not as high as that of SRM [16]. To our knowledge, this represents the most comprehensive C. jejuni proteomic dataset available.

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in different metabolic pathways or in the transport of nutrients or compounds across the bacterial envelope (Figure 2). To better evaluate the potential physiological impact of the reduction of expression of proteins involved in metabolic pathways or transport mechanisms, we generated a graphical metabolic network database, using the Pathway Tools v14.0 [21]. For this purpose, we input the C. jejuni 81–176 genome sequence [22] to the Metacyc pathway database based on published literature, such as biochemical studies on metabolic enzymes, transporters or regulators specifically in Campylobacter species. C. jejuni has unique metabolic capabilities presumably to adapt to different environments, so we customized the C. jejuni pathway database by adding new pathways/reactions that are not present in Metacyc, or by linking related pathways to form super-pathways to better visualize the metabolite flow. Overall, this database could account for 155 metabolic pathways, 885 associated enzymatic reactions, 75 transporters and 49 transport reaction. In this graphic representation, C. jejuni proteins were mapped into individual pathways, and were differentially labeled based on whether they were detected in our analysis or whether their expression level was decreased or increased. Following this template, our proteomic analysis was able to monitor 151 metabolic pathways, 382 associated enzymes, 429 enzymatic reactions, and 41 transporters (Figure 3) (The entire database will be made available in the BioCyc Database collection web site [http://biocyc.org]). Evaluation of the proteins (and associated pathways) whose expression significantly decreased 20 hs after infection indicates that C. jejuni undergoes a significant metabolic downshift within host cells (Dataset S2). Several proteins associated with various anabolic pathways showed significantly reduced levels in samples obtained 20 hs after infection (Dataset S2 and Figure 3). This includes components of amino acid biosynthesis pathways such as those associated with histidine, lysine, valine, leucine, isoleucine, methionine, glycine, and alanine biosynthesis. Similarly, components of other important biosynthetic pathways including those associated with the synthesis of prosthetic groups and cofactors, fatty acids and lipids, pentose phosphate, as well as purine and pyrimidine nucleotides were also significantly down-regulated 20 hs after infection. In addition, the levels of several proteins associated with various catabolic pathways were significantly reduced in C. jejuni isolated from cells 20 hs after infection. These include pathways involved in the degradation and utilization of amino acids (e. g. proline, L-asparatate, lysine, serine and L-arginine), and C1 compounds. Pathways potentially involved in nutrient acquisition were also down-regulated, including several amino acid ABC-type transporters as well as transporters for phosphate, potassium, tungstate and molybdate. Overall, <50% of all C. jejuni proteins whose levels were decreased after 20 hs of infection were associated with metabolic pathways or transport reactions. Consistent with an overall metabolic downshift, the levels of several ribosomal protein subunits (e. g. 30S ribosomal proteins S6, S19, S15, and S20, and 50S ribosomal protein L9 and L24) were significantly decreased. This metabolic downshift is consistent with the observation that C. jejuni does not replicate within cultured mammalian cells but, rather, it seems to go into a dormant, non-culturable state [14]. In addition, the observed metabolic downshift resembles the one observed during C. jejuni’s

![Image](http://biocyc.org)

Table 1. Levels of C. jejuni proteins associated with oxidative stress.

| Gene ID     | Gene symbol | Protein name                | 2 h² | 20 h³ | Fold⁴ | p-value⁵ |
|------------|-------------|-----------------------------|------|-------|-------|---------|
| CBJ81176_1234 | groEL       | chaperonin GroEL            | 322  | 308   | −1.06 | 0.351   |
| CBJ81176_0775 | dnaK        | molecular chaperone DnaK    | 130  | 96    | −1.36 | <0.001  |
| CBJ81176_1242 | htrA        | protease DO                 | 101  | 81    | −1.24 | 0.004   |
| CBJ81176_1233 | groES       | co-chaperonin GroES         | 13   | 4     | −3.13 | 0.002   |
| CBJ81176_0774 | grpE        | co-chaperonin protein GrpE  | 4    | 4     | −1.04 | 0.660   |
| CBJ81176_0537 | clpB        | ATP-dependent chaperone protein ClpB | 32 | 19 | −1.66 | 0.001   |
| CBJ81176_1243 | dnaJ-1      | co-chaperonin protein DnaJ  | 4    | 2     | −1.64 | 0.125   |
| CBJ81176_1288 | spoT        | RelA/SpoT family protein   | <1   | 1     | n.a   | n.a     |
| CBJ81176_0356 | ahpC        | anti-oxidant AhpCTSA family protein | 126 | 110 | −1.15 | 0.017   |
| CBJ81176_0298 | ahpC        | anti-oxidant AhpCTSA family protein | 4  | 3     | −1.31 | 0.277   |
| CBJ81176_0205 | sodB        | superoxide dismutase, Fe    | 28   | 20    | −1.35 | 0.020   |
| CBJ81176_0800 | tpx         | thiol peroxidase            | 101  | 118   | 1.16  | 0.022   |
| CBJ81176_0183 | trx         | thioredoxin                 | 47   | 20    | −2.17 | 0.005   |
| CBJ81176_0291 | tpx         | thiol peroxidase            | 25   | 8     | −3.17 | <0.001  |
| CBJ81176_1387 | katA        | catalase                    | 11   | 15    | 1.59  | 0.023   |
| CBJ81176_0182 | trxB        | thioredoxin-disulfide reductase | 18  | 4     | −4.06 | 0.002   |
| CBJ81176_1519 | grpE        | co-chaperonin protein GrpE  | 56   | 38    | −1.53 | 0.004   |

1Protein abundance is indicated as averaged spectral counts from 7 biological replicates.
2C. jejuni samples isolated from host cells at 2 h of infection.
3C. jejuni samples isolated from host cells at 20 h of infection.
4Fold change in protein abundance; positive or negative values indicate higher or lower levels in the 20 h samples, respectively.
5p-values were calculated using the paired Student’s t-test.

*Data not available (due to low protein signal).*

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C. jejuni Reporogs Its Metabolism within Cells
Proteins were assigned to the different functional groups based on genome annotation and additional curation based on published literature.

C. jejuni. This hypothesis is also supported by the observation that a cytochrome c oxidase plays a more important role in aerobic respiration [28].

is consistent with the hypothesis that the cbb3-type cytochrome c oxidase could be hardly detected before and after infection, which 20 hs after infection (Table 2 and Table 3). The CioAB-type cytochrome c oxidase (CcoP and CcoO) decreased markedly oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6].

transition to late stationary phase [24]. However, unlike what was observed in C. jejuni stationary phase transcriptional reprogramming, we did not observe increased expression of heat shock proteins (e.g. GroEL, GroES, GrpE, ClpB) or proteins associated with oxidative stress resistance (e.g. AhpC, SodB, and Tpx). This suggests that the remodeling of the C. jejuni proteome that occurs within cultured mammalian cells is not simply the result of its transition to a different “growth phase” but most likely, the result of its specific adaptation to the intracellular environment.

C. jejuni reprograms respiration within cultured cells

The observation that intracellularly localized C. jejuni becomes culturable after incubation under very low oxygen conditions suggests at least two possibilities to explain its non-culturability under standard microaerophilic conditions. One possibility is that the intracellular remodeling of its proteome renders C. jejuni oxygen sensitive. This hypothesis would be supported by the observation that some proteins potentially involved in conferring protection against oxidative stress showed reduced levels at 20 hs after infection (Table 1). However, even though slightly reduced, the levels of these enzymes remained relatively high. Furthermore, no significant changes in the levels of proteins thought to be most important in oxidative stress protection (e.g. SodB and KatA) [25–27] were observed suggesting that other factors must account for C. jejuni’s inability to grow under microaerophilic conditions when directly obtained from cultured cells. Consistent with this hypothesis, examination of the proteome of intracellularly-localized C. jejuni suggests that it undergoes reprogramming of its respiration. Indeed, the levels of many proteins that are central to the main respiration pathways were significantly reduced in the 20 hs sample (Table 2), an observation confirmed by SRM experiments (Table 3). For example, key components of the aerobic respiration pathway [7] were greatly reduced. The C. jejuni genome only encodes two terminal oxidases, a cbb3-type cytochrome c oxidase and a CioAB-type (cyanide-insensitive oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6]. The expression levels of cbb3 type oxidase and a CioAB-type (cytochrome c oxidase) oxidase [6].

We have previously shown that intracellular C. jejuni at late time points of infection (e.g. >18 hs) can be rendered culturable under standard microaerophilic conditions if pre-incubated under oxygen-limiting conditions (O2<0.2%, CO2 ~6.5%) [14]. Therefore, to gain further insight into C. jejuni’s intracellular metabolic reprogramming, we carried out a proteomic analysis of C. jejuni
obtained immediately after its incubation under oxygen-limiting conditions. We reasoned that by examining changes induced by this incubation step, which render it culturable, we might be able to gain further insight into the changes undergone by C. jejuni when located within the cells, which render it non-culturable. Bacteria obtained from infected cells 20 h after infection and incubated for 48 h under low oxygen conditions were scraped from plates and their proteome was examined as described above (Dataset S3). We observed significantly increased levels of enzymes involved in known C. jejuni anaerobic respiratory pathways including FrdA, NapA, and NfrA [12] (Dataset S6). In addition, the levels of the anaerobic C4-dicarboxyate transporters DcuB and DcuA were also increased after incubation under low oxygen conditions. Strikingly, we also observed that the levels of several enzymes involved in aerobic respiration were also increased after incubation under oxygen-limiting conditions. All three subunits of ubiquinol-cytochrome c reductase (PetA, PetB and PetC) were markedly increased under these conditions. These results may provide an explanation for the observation that pre-incubation of intracellular C. jejuni under oxygen-limiting condition renders it ready for growth under microaerophilic environment, in which oxygen acts as the main electron acceptor [14]. Furthermore, it suggests the possibility that the low oxygen environment encountered by C. jejuni in the gut may also prepare the bacterium for its phase outside the host, in which oxygen levels are likely much higher. Interestingly, the ability to prepare for a change in a future environment has been previously observed in V. cholerae, in which genes required for the aquatic environment are induced within the intestinal track, where they are not needed [30].

To gain more insight into the potential respiratory reprogramming of C. jejuni within mammalian cells we specifically compared the relative abundance of key enzymes for different respiration pathways under the different conditions examined in this study. We specifically examined the levels of FdhA, PetC, CcoP and NfrA, which are central components in the respiration of fumarate, nitrate, nitrite and TMAO/DMSO, FrdA, NapA, NrfA, and TorA, which are central components in the anaerobic respiration pathways including FrdA, NapA, and NfrA [12] (Dataset S6). In addition, the levels of the anaerobic C4-dicarboxyate transporters DcuB and DcuA were also increased after incubation under low oxygen conditions.

Figure 3. Cellular overview of C. jejuni metabolic pathways. Metabolic pathways are grouped into different general functional categories as indicated. Proteins are located at the double peripheral lines representing the bacterial membrane structure. Within individual pathways, symbols represent metabolites and connecting lines denote respective enzymes that catalyze the inter-conversion of those metabolites. Most enzymes are color-coded in the following fashion: blue indicates mapped proteins in pathways present in the C. jejuni proteome, grey indicates those not found in the annotated genome, bold green represents those proteins whose expression levels were lower at 20 h of infection relative to 2 h of infection, and bold red corresponds to up-regulated proteins at 20 h of infection. Description of individually numbered pathways is as follows: 1. a, flavin biosynthesis; b, methylerythritol phosphate pathway; c, tetrapyrrole biosynthesis; d, ubiquinone-8 biosynthesis; e, di-trans, poly-cis-undecaprenyl phosphate biosynthesis; f, pantetheine biosynthesis I; g, chlorophyllide a biosynthesis I; h, TCA cycle; i, tetrahydrofolate biosynthesis; j, thiamine biosynthesis I; k, pyridoxal 5’-phosphate biosynthesis I; l, coenzyme M biosynthesis; m, 6-hydroxymethyl-dihydropterin diphostate biosynthesis I; n, NAD biosynthesis I; o, folate transformations II; p, phosphate acquisition I; q, NAD phosphorylation and dephosphorylation; r, α-ketol carrier protein metabolism; s, formyl THF biosynthesis I; t, thioredoxin pathway; u, NAD salvage pathway II; v, biotin biosynthesis I; w, menaquinone-8 biosynthesis; x, biotin biosynthesis; y, heme biosynthesis from uroporphyrinogen-III II; z, trans, trans-farnesyl diphostate biosynthesis; aa, glutathionylperoxidase biosynthesis; ab, NAD salvage pathway I; ac, geranyldiphosphate biosynthesis. 2. a, histidine biosynthesis I; b, lysine biosynthesis I; c, valine biosynthesis I; d, isoleucine biosynthesis I; e, threonine biosynthesis from homoserine I; f, homocysteine biosynthesis I; g, homoserine biosynthesis I; h, ornithine biosynthesis I; i, tryptophan biosynthesis I; j, proline biosynthesis III; k, alanine biosynthesis I; l, alanine biosynthesis III; m, serine biosynthesis I; n, tyrosine biosynthesis I; o, alanine biosynthesis I; p, serine biosynthesis I; q, protein citrullination I; r, cysteine biosynthesis I; s, L-glutamine biosynthesis I; u, glutamine biosynthesis I; t, pimelate biosynthesis I; u, CMP-KDO biosynthesis I; v, citrulline-carboxyl carrier protein assembly; w, fatty acid biosynthesis initiation III; x, CDP-diacylglycerol biosynthesis I; y, fatty acid biosynthesis initiation II; z, cyclopropane fatty acid (CFA) biosynthesis I; E, CDP-diacylglycerol biosynthesis II; f, cis-vaccenate biosynthesis I; j, phospholipid biosynthesis I; k, stearate biosynthesis I; l, fatty acid elongation-saturated. 4. a, (S)-carbapenem biosynthesis I; b, hyperforin biosynthesis I; c, myo-inositol biosynthesis I; d, arabinose (arabinobiose); e, aerobic respiration-electron donor II; f, homolactic fermentation I; g, (S)-acetoin biosynthesis I; h, (R)-acetoin biosynthesis I; i, mixed acid fermentation; j, pyruvate fermentation to acetate; k, pyruvate fermentation to lactate.
state. The levels of FrdA also remained unchanged in C. jejuni samples 2 and 20 hs after infection. However, in contrast to NrfA, the levels of FrdA were high, suggesting that fumarate may be an important electron acceptor during C. jejuni’s intracellular stage. Consistent with this hypothesis, the levels of AspA, which is an aspartase that can provide fumarate for respiration [33], showed a similar pattern to the levels of FrdA (Figure 4C), which were characterized by unchanged although high levels 20 hs after infection. Furthermore, the levels of AspA, FrdA and MfrA, another fumarate reductase [11], significantly increased after incubation under low oxygen conditions (Figure 4C).

To test the potential significance of fumarate respiration during C. jejuni intracellular survival, we constructed C. jejuni strains carrying mutations in aspA or the fumarate reductases frdA and/or mfrA and examined their viability 20 hs after infection. Since the absence of fumarate the C. jejuni aspA, frdA, or mfrA mutants have been shown to be defective for invasion, these mutant strains were grown and allowed to infect cells in the presence of fumarate, which results in wild type levels of bacterial internalization [34]. The aspA, frdA and frdA/mfrA mutants showed a significant decrease in viability 20 hs after infection (Figure 5, bottom graph) indicating that respiration of fumarate is central to the metabolism of intracellular C. jejuni. Complementation of the aspA mutant restored viability to wild-type levels. In contrast, the mfrA mutant showed intracellular survival close to wild type. This is consistent with the extremely low levels of MfrA (barely detectable by our LC-MS approach) observed

Table 2. Levels of C. jejuni proteins associated with respiration/electron transport chains.

| Protein complex | Gene ID | Gene symbol | Abundance<sup>1</sup> | Fold<sup>6</sup> | p-value<sup>7</sup> |
|-----------------|---------|-------------|------------------------|----------------|----------------|
| NADH dehydrogenase<sup>9</sup> | CJJ81176_1556 | nuoI | 7 5 4 7 | −1.27 | 0.049 |
|                 | CJJ81176_1558 | nuoG | 15 23 20 38 | −1.15 | 0.13 |
|                 | CJJ81176_1561 | nuoO | 7 5 4 5 | −1.18 | 0.536 |
| flavodoxin      | CJJ81176_1384 | fdA | 43 52 36 20 | −1.36 | 0.07 |
| cytochrome c oxidase, cbb3-type | CJJ81176_1479 | ccoP | 25 25 9 19 | −2.71 | <0.001 |
|                 | CJJ81176_1481 | ccoO | 26 25 12 19 | −2.05 | <0.001 |
| ubiquinol–cytochrome c reductase | CJJ81176_1199 | petC | 44 44 14 32 | −3.08 | <0.001 |
|                 | CJJ81176_1200 | petB | 0 <1 <1 5 | n.a<sup>3</sup> | n.a |
|                 | CJJ81176_1201 | petA | 17 15 4 10 | −3.62 | <0.001 |
| fumarate reductase | CJJ81176_0432 | frdC | 0 <1 <1 5 | n.a | n.a |
|                 | CJJ81176_0433 | frdA | 82 77 62 153 | −1.21 | 0.023 |
|                 | CJJ81176_0434 | frdB | 14 11 9 28 | −1.02 | 0.379 |
|                 | CJJ81176_0463 | mfrA | 2 1 <1 63 | n.a | n.a |
|                 | CJJ81176_0464 | mfrB | 0 <1 <1 37 | n.a | n.a |
| nitrate reductase | CJJ81176_0801 | napA | 60 51 9 85 | −5.47 | <0.001 |
|                 | CJJ81176_0804 | napB | 5 8 3 16 | −2.44 | <0.001 |
| nitrite reductase | CJJ81176_1359 | nfaA | 3 5 1 46 | −5.37 | 0.011 |
| sulfite reductase | CJJ81176_0403 | yedY | 4 4 2 3 | −1.59 | 0.05 |
| TMAO reductase | CJJ81176_0291 | torA | 28 25 8 37 | −3.17 | <0.001 |
|                 | CJJ81176_0292 | torC | 13 11 3 9 | −3.17 | <0.001 |
| cytochrome c551 peroxidase | CJJ81176_0382 | ccpA-2 | 13 12 1 8 | −7.32 | 0.001 |
|                 | CJJ81176_0047 | ccpA-1 | 7 7 1 2 | −7.45 | <0.001 |
| sulfite oxidoreductase | CJJ81176_0031 | sorA | 5 4 3 3 | −1.14 | 0.538 |
| gluconate dehydrogenase | CJJ81176_0438 | fdhC | 23 23 4 0 | −5.65 | <0.001 |
|                 | CJJ81176_0439 | fdhB | 86 74 29 5 | −2.49 | <0.001 |
| formate dehydrogenase | CJJ81176_1501 | fdhC | 0 0 0 4 | n.a | n.a |
|                 | CJJ81176_1502 | fdhB | 1 2 1 13 | −3.48 | <0.001 |
|                 | CJJ81176_1503 | fdhA | 28 28 5 46 | −5.15 | <0.001 |

1Protein abundance is indicated as averaged spectral counts.
2Extracellular C. jejuni samples obtained from in-vitro culture.
3C. jejuni samples isolated from host cells at 2 h of infection.
4C. jejuni samples isolated from host cells at 20 h of infection.
5Fold change in protein abundance; positive or negative values indicate higher or lower levels in the 20 h vs 2 h samples, respectively.
6p-values were calculated using the paired Student’s t-test.
7Other proteins encoded in the same operon (<nuoMLKJHCBA>) are not listed because they were not detected or detected at very low levels. This is also the case for other proteins relevant to respiration including CooQ, CioAB/CydAB, NapGHDLD, NrfH, YedZ, DmsABCD (dimethyl sulfoxide reductase), and SorB.

<sup>1</sup> Data not available (due to low protein signal).
in both extracellular and intracellular *C. jejuni* preparations. These data are also consistent with previous observations indicating that *C. jejuni* aspA or frdA mutant strains are impaired in chicken colonization while the mfrA mutant is not [30][28]. To further corroborate the hypothesis that fumarate respiration plays an important role in *C. jejuni* intracellular survival, we examined *C. jejuni* strains carrying mutations in napG, torA, cydA, and fdhB for their ability to survive within cultured mammalian cells. These genes are involved in aerobic and anaerobic respiration pathways [29] [32]. In contrast to mutants with impaired fumarate respiration, these mutant strains showed intracellular viability indistinguishable from that of the wild-type (Figure 5). Therefore, our proteomic and functional data indicate that fumarate respiration plays an important role in *C. jejuni* intracellular survival.

Conclusions

We have shown here through a detailed proteomic study that *C. jejuni* undergoes a significant metabolic reprogramming upon internalization within mammalian cells. A salient feature of this metabolic reprogramming is a significant metabolic downshift. This metabolic downshift is qualitatively different from the one associated with *C. jejuni*’s transition to stationary phase and therefore represents a unique metabolic state presumably associated with its adaptation to the intracellular environment. It is also consistent with the observation that although *C. jejuni* survives within mammalian cells, it does not replicate [14]. It is therefore possible that the intracellular environment, presumably shielded from many innate immune defense mechanisms, may serve as a reservoir for this pathogen but not necessarily as a replication site. The proteomic profile indicates that although non-replicating, *C. jejuni* is certainly not subject to environmental stress in this intracellular environment since none of the proteins usually associated with responses to different environmental stress showed increased levels. Another salient feature of the intracellular metabolic reprogramming is a change in respiration mode, which may be responsible for its decreased culturability under

Table 3. SRM measurements of a subset of *C. jejuni* metabolic enzymes.

| Protein | Peptide | Transitions<sup>1</sup> | 2 h | 20 h | Fold<sup>3</sup> | 2 h | 20 h | Fold<sup>3</sup> |
|---------|---------|------------------------|-----|------|----------|-----|------|----------|
| CcoP    | TANENLVAK | 480.4 787.5 1.25E7 5.15E6 | -2.4 | 22 | 10.7 | -2.1 |
| NrfA    | KISEELK | 424.2 605.6 2.30E5 4.30E4 | -5.3 | 1.7 | 0.7 | -2.4 |
| AspA    | VADIALER | 444.0 716.5 9.10E6 3.40E6 | -2.7 | 28 | 18 | -1.6 |
| CcpA-2  | NSGLVALPK | 449.9 441.0 5.70E5 | N.D. | 6 | N.D. | 7 8.3 | 2 | -4.2 |
| PetC    | VGLTEAAQAK | 494.5 485.5 5.00E6 | 3.00E5 | -16.7 | 32.3 | 13.3 | -2.4 |
| FdhA    | FGGGVNILR | 467.1 515.3 2.20E5 | 4.90E4 | -4.5 | 13.3 | 4.3 | -3.1 |
| FrdA    | SLVDKEGK | 438.8 675.4 3.50E5 | 4.60E5 | 1.3 | 41 | 42.3 | 1.0 |
| NapA    | LPADMVVANPK | 586.1 529.3 1.90E5 | 5.00E4 | -3.8 | 38.3 | 7.3 | -5.2 |
| TorA    | IAWTSNEQR | 552.9 460.9 4.60E5 | 7.10E4 | -6.5 | 19.3 | 11.3 | -1.7 |

1 For each peptide, three SRM transitions were measured.
2 Intensity values were represented by integrated peak area obtained from extracted ion chromatograms.
3 Fold change of fragment ion intensities between the 2 h and 20 h samples; negative values denote decreased levels in the 20 h samples.
4 Spectral counts obtained for the same biological replicate.
5 Fold change of peptide spectral counts between the 2 h and 20 h samples; negative values denote decreased levels in the 20 h samples.
6 Peak not detected due to low peptide ion signals.
7 Fold change not determined. In this case, a large negative fold value would be expected.

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microaerophilic conditions in which oxygen is the main electron acceptor. Indeed, our data suggest that *C. jejuni* may favor fumarate respiration inside mammalian cells. Consistent with our data, *C. jejuni aspA* and *frdA* mutants, which are defective for fumarate respiration, were shown to be significantly impaired for chicken colonization [30][28], while mutations in genes essential for other respiration pathways were not [Olson, Appl Environ Microbiol. 2008 Mar;74(5):1367–75]. Therefore, this study highlights the importance of metabolic reprogramming in the biology of *C. jejuni* and may help the development of novel antimicrobial strategies that would target relevant metabolic pathways.

**Materials and Methods**

**Bacterial strains, cell lines, and culture conditions**

The *C. jejuni* 81–176 wild-type strain, its *aspA* mutant, and the complemented *aspA* mutant derivative have been previously described [34,35]. All other mutant strains were constructed as previously described [22]. Bacteria were routinely grown on blood agar plates (tryptic soy broth agar supplemented with 5% sheep blood) or in brain heart infusion (BHI) broth at 37°C under 10% CO₂ (microaerophilic) or low oxygen conditions (GasPak Plus, BD-Diagnostic Systems, New Jersey). All *C. jejuni* strains were stored at −80°C in BHI broth containing 30% glycerol. COS-1 (African green monkey kidney fibroblast-like cell line) cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% bovine calf serum (BCS). All cell lines were grown under an atmosphere of 5% CO₂.

**C. jejuni infection of mammalian cells and isolation of intracellular bacteria**

In order to obtain sufficient amounts of bacterial proteins for mass spectrometric analysis (see strategy described below), we determined that 5 × 10⁷ to 10⁸ bacteria (equivalent to 50–100 μg of protein yield) would be needed for proteomic analysis. To obtain this number of bacteria *C. jejuni* infections were carried out in 15-cm plates as previously described [34] with a multiplicity of infection of 1000. Infection was allowed to proceed for 2 hs in HBSS medium. Subsequently, cell monolayers were washed extensively with pre-warmed HBSS and incubated in gentamicin-containing (100 μg/mL) DMEM media for another 2 hs to kill extracellular bacteria. After gentamicin treatment, cells were washed and lysed to release intracellular bacteria. Alternatively, the culture media was replaced with fresh DMEM containing lower concentration of gentamicin (10 μg/mL) and cells were further incubated for 16 hs before releasing intracellular bacteria.

A two-step differential centrifugation strategy was developed to separate intracellular bacteria from host cell debris. COS-1 cells were lysed in a buffer containing 0.5% Triton X-100, 20 mM Tris-HCl (pH 7.6) and 150 mM NaCl. The sample was first pelleted at 3000 g for 5 min to remove host cell nuclei, and then the post-nuclear supernatant was centrifuged again at 30000 g for 20 min. These conditions resulted in the recovery of most of the bacteria in the second pellet as shown by CFU assays. The resulting bacterial pellets were washed extensively with RIPA buffer to minimize host-protein contamination.

**A general proteomic strategy for profiling *C. jejuni* proteins**

We carried out proteomic analysis of proteins extracted from different *C. jejuni* samples by LC-MS/MS. The experimental design is depicted in Figure 1. Briefly, intracellular *C. jejuni* were harvested from mammalian cells at 2 hs and 20 hs of infection by differential centrifugation as described above. Bacterial cell lysates were separated by 10% SDS-PAGE to pre-fractionate the bacterial proteins. Electrophoresis was stopped when the dye

**Figure 4. Protein abundance of respiration/electron transport chain components of *C. jejuni* in four distinct environments.**

Protein abundance (expressed as spectral counts) in *C. jejuni* samples obtained after growth in BHI, after 2 and 20 hs after infection, and after exposure to oxygen limiting conditions subsequently to their recovery 20 hs after infection (indicated as O₂-limiting). A Protein abundance of representative enzymes involved in aerobic respiration. B Protein abundance of representative enzymes involved in anaerobic respiration. C Protein abundance of enzymes relevant to fumarate respiration. Values represent the mean ± standard error of the mean of three determinations. doi:10.1371/journal.ppat.1002562.g004

front reached 1.5 cm below the stacking gel. Subsequently, the gel was divided equally into three slices, each slice was subjected to in-gel protein digestion (see below), and extracted peptide samples were analyzed by LC-MS/MS for protein identification. Spectral counts associated with protein assignments were utilized to quantitatively assess their relative abundance in each sample. Triplicate LC-MS/MS runs were conducted for each sample, thereby allowing the assessment of the technical reproducibility of the measurements. Statistical analysis was performed on data obtained from 7 biological replicates. Each replicate was comprised of two samples corresponding to two bacterial populations (from 2 hs and 20 hs of infection). Since each bacterial sample was divided into three gel fractions and each fraction was run in triplicate, in total 126 LC-MS/MS experiments were carried out to obtain the entire data set. Protein and peptide assignments from different gel fractions were pooled within the same bacterial sample. All mass spectrometric data have been deposited at the public data base PRIDE hosted by EMBL-EBI (http://www.ebi.ac.uk/pride/)

**In-gel digestion of protein samples**

Gel slices were excised into small (~1×1 mm) cubes and transferred into sample tubes. Protein disulfide bonds were reduced with 10 mM DTT in 100 mM NH₄HCO₃ at 56°C for 30 min and subsequently alkylated with 55 mM iodoacetamide (IAM) in 100 mM NH₄HCO₃ at room temperature for 20 min (in complete darkness). Upon alkylation, the gel samples were destained with 50% acetonitrile (ACN) in 50 mM NH₄HCO₃ and dehydrated by addition of neat ACN. After removal of the destaining buffer and ACN, the samples were subjected to in-gel digestion. The digestion buffer contained 10 ng/μL trypsin in 50 mM NH₄HCO₃ and the enzymatic reaction was allowed to proceed at 37°C for 20 h.

**Figure 5. Intracellular survival of C. jejuni mutant strains defective in specific respiration pathways.** Cultured mammalian cells were infected with the wild type C. jejuni 81–176 strain (WT) and the indicated mutant derivatives at a MOI of 100 for 2 hs, followed by 2 and 18 hs incubation in the presence of gentamicin. For each strain, levels of intracellular bacteria at 20 hs after infection are shown relative to the levels obtained at 2 hs after infection. The value of the ratio in wild type was set at 100%. The error bars represent the standard deviation of three independent determinations. The survival values at 20 hs of the aspA, frdA, or frdA mfrA double mutant strains were statistically significantly different (P<0.05, Student t test) from those of wild type.

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proceed overnight at 37°C. The resulting tryptic peptides were extracted from the gel matrix by equilibrating the samples with 50% ACN and 5% formic acid (FA). Finally the extracted peptides were vacuum dried prior to LC-MS/MS analysis.

**Nanoflow LC-MS/MS analysis**

Nanoflow reverse-phase LC separation was carried out on a Proxeon EASY-nLC System (Thermo Scientific). The capillary column (75 μm x 150 mm, PICOFRIT, New Objective, Woburn, MA) was packed in-house. Briefly, a methanol slurry containing 5 μm, 100 Å Magic C18AQ silica-based particles (Microm BioResources Inc., Auburn, CA) was forced to pass through an empty capillary (with a frit in the end) using a pressurized device. Tryptic peptides were dissolved in HPLC-grade water and ~200 ng of samples were loaded onto the analytical column in a single LC-MS/MS experiment. The mobile phase was comprised of solvent A (97% H2O, 3% ACN, and 0.1% FA) and solvent B (100% ACN and 0.1% FA). The LC separation was carried out with the following gradient: solvent B was started at 7% for 3 min, and then raised to 35% in 40 min; subsequently, solvent B was rapidly increased to 90% in 2 min and maintained for 10 min before 100% solvent A was used for column equilibration. Peptides eluted from the capillary column were electrosprayed directly onto a linear ion trap mass spectrometer (LTQ Velos, ThermoElectron, San Jose, CA) for MS/MS analysis. A data-dependent mode was enabled for peptide fragmentation. One full MS scan was followed by fragmentation of the top 10 most intense ions by collision-induced dissociation (CID). Dynamic exclusion (with a duration of 6 seconds) was enabled to preclude repeated analyses of the same precursor ion. Both pre-column and analytical column were washed intensively and incubated overnight with DMEM containing 150 mM NaCl, and the number of intracellular bacteria was assessed by CFU assays. Alternatively, infected cells were washed extensively and incubated overnight with DMEM containing 10 μg/ml of gentamicin. Cells were then lysed as described above and the released intracellular bacteria were plated and incubated under low-oxygen conditions for 2 days before further incubation under microaerophilic conditions as previously described [14]. The ability of *C. jejuni* to survive intracellularly was assessed as a percentage of bacteria recovered after 20 h incubation relative to those recovered 2 hours after infection.

**Supporting Information**

**Dataset S1** Spectral counts of *C. jejuni* proteins detected by LC-MS/MS. (XLS)

**Dataset S2** *C. jejuni* proteins whose levels exhibit significant changes between the 2 h and 20 h samples. (XLS)

**Dataset S3** *C. jejuni* proteins detected in in-vitro grown or obtained from infected cells 2 hs after infection. (XLS)

**Dataset S4** Spectral counts of *C. jejuni* proteins detected by LC-MS/MS after exposure to infection medium. (XLS)

**Dataset S5** *C. jejuni* proteins detected 20 hs after infection or after incubation under oxygen-limiting conditions. (XLS)

**Dataset S6** *C. jejuni* proteins whose levels exhibit significant changes between 20 h after infection and after incubation under low oxygen conditions. (XLS)

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**Author Contributions**

Conceived and designed the experiments: XL BG JEG. Performed the experiments: XL BG. Analyzed the data: XL BG JEG. Contributed reagents/materials/analysis tools: VN. Wrote the paper: XL BG JEG.
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