Metabolite and transcriptome analysis of *Campylobacter jejuni* in vitro growth reveals a stationary-phase physiological switch

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*Campylobacter jejuni* is a prevalent cause of food-borne diarrhoeal illness in humans. Understanding of the physiological and metabolic capabilities of the organism is limited. We report a detailed analysis of the *C. jejuni* growth cycle in batch culture. Combined transcriptomic, phenotypic and metabolic analysis demonstrates a highly dynamic ‘stationary phase’, characterized by a peak in motility, numerous gene expression changes and substrate switching, despite transcript changes that indicate a metabolic downshift upon the onset of stationary phase. Video tracking of bacterial motility identifies peak activity during stationary phase. Amino acid analysis of culture supernatants shows a preferential order of amino acid utilization. Proton NMR (1H-NMR) highlights an acetate switch mechanism whereby bacteria change from acetate excretion to acetate uptake, most probably in response to depletion of other substrates. Acetate production requires *pta* (*Cj0688*) and *ackA* (*Cj0689*), although the *acs* homologue (*Cj1537c*) is not required. Insertion mutants in *Cj0688* and *Cj0689* maintain viability less well during the stationary and decline phases of the growth cycle than wild-type *C. jejuni*, suggesting that these genes, and the acetate pathway, are important for survival.

INTRODUCTION

*Campylobacter jejuni* is the major cause of food-borne bacterial gastroenteritis worldwide, with an estimated 1 in 100 individuals in both the USA and the UK developing *Campylobacter*-related illness each year (Gaynor et al., 2004; Gillespie et al., 2002). Although most cases are self-limiting, *C. jejuni* can cause severe post-infection complications including the peripheral neuropathies Guillain–Barré and Miller–Fisher syndromes (Nachamkin et al., 2000). Despite the significance of *C. jejuni* as a food-borne pathogen, our understanding of its basic biochemistry and physiology, gene regulation, colonization, virulence and environmental survival mechanisms lags behind that of many other pathogenic and non-pathogenic bacteria, and many questions regarding *C. jejuni* physiology and pathogenesis remain (Young et al., 2007). Research has been hampered by a lack of genetic tools, the difficulty of growing the bacteria in the laboratory and the absence of a suitable animal model that mimics human disease.

*C. jejuni* is a fastidious bacterium, with a limited capacity for biosynthesis, and requires complex growth media (Kelly, 2001). Establishing the metabolic capabilities of *C. jejuni* is central to comprehension of environmental persistence and host colonization. The microaerophilic nature, complex nutritional requirements and relative difficulty of culturing *C. jejuni* have all contributed to the limited understanding of metabolism (Kelly, 2001, 2005; Sellars et al., 2002). *C. jejuni* is unable to catabolize glucose and other hexose sugars due to the absence of the key glycolytic enzyme 6-phosphofructokinase (Parkhill et al., 2000; Velayudhan & Kelly, 2002). Amino acids serve as...
major substrates for *C. jejuni*, and molecular detail concerning the utilization of L-serine, L-aspartate, L-proline and L-glutamate has begun to emerge (Guccione et al., 2008; Joshua et al., 2006; Kelly, 2005; Leach et al., 1997; Del Recio Leon-Kempis et al., 2006; Velayudhan et al., 2004). The importance of each amino acid in *C. jejuni* metabolism is yet to be fully established, however, and information on the carbon and nitrogen flow in this organism remains limited.

Detailed understanding of the growth dynamics of *C. jejuni* during *in vitro* culture is lacking, and methods for growing this species are diverse and occasionally ill-considered. Batch culture remains an extremely common method of growing *C. jejuni in vitro* and is itself a common experimental system (Corcoran & Moran, 2007; Mohammed et al., 2005). In light of this, in addition to informing the design and interpretation of studies that employ *C. jejuni* grown *in vitro*, greater understanding of the growth cycle presents a sensible aim in itself.

This report describes a detailed analysis of the *C. jejuni* growth cycle from transcriptomic, phenotypic and metabolic perspectives. We show that *C. jejuni* exhibits a dynamic stationary phase, characterized by switches in motility, substrate utilization and production accompanied by concurrent changes in gene expression. A stationary-phase acetate switch, whereby *C. jejuni* changes from a mode of acetate production to utilization, is identified, and roles for *Cj0688* and *Cj0689* in this process are demonstrated. Furthermore, a potential role for the acetate pathway in the stationary phase survival of *C. jejuni* is identified.

**METHODS**

**Bacterial strains and culture conditions.** All bacterial strains used in this study are listed in Table 1. *C. jejuni* was routinely cultured on Mueller–Hinton (MH) agar (Oxoid) supplemented with 5% defibrinated horse blood (hereafter referred to as MH blood agar plates) at 42 °C under microaerobic conditions (5% O2, 5% CO2, 5% H2, 85% N2) in a MACS VA500 variable atmosphere workstation (Don Whitley). Liquid cultures of *C. jejuni* were grown in brain heart infusion (BHI) broth (Oxoid) at 42 °C under microaerobic conditions with agitation at 150 r.p.m. for 66 h. Growth was monitored by recording OD600, and by determining viable c.f.u. ml–1 by serial 10-fold dilution of cultures in BHI broth and spot-plating 30 μl onto MH agar supplemented with 5 μg trimethoprim ml–1. Plates were incubated microaerobically at 42 °C for 48 h before colonies were counted.

**Growth dynamics of *C. jejuni*.** *C. jejuni* strains were cultured for 48 h on MH blood agar plates with antibiotics added as appropriate (10 μg chloramphenicol ml–1, 35 μg kanamycin ml–1 or 5 μg trimethoprim ml–1). Bacterial lawns were harvested in 1 ml BHI broth, 50 μl of which was inoculated into 10 ml BHI broth in a 50 ml Falcon centrifuge tube with a loosened cap, and grown for 16 h at 42 °C under microaerobic conditions with agitation at 150 r.p.m. to generate a starter culture. The OD600 of starter cultures was taken using a 6305 UV/visible spectrophotometer (Jenway). Starter cultures were diluted appropriately in BHI broth and used to inoculate 250 ml BHI broth containing 5 μg trimethoprim ml–1 in a 500 ml flask (previously equilibrated at 42 °C in microaerobic conditions for 16 h) to a calculated OD600 of 0.00002, corresponding to ~1 × 107 c.f.u. ml–1. Once cultures were placed in the MACS VA500 for equilibration, they were never subsequently removed from the cabinet until the end of the experiment. Cultures were grown at 42 °C under microaerobic conditions with agitation at 150 r.p.m. for 66 h. Growth was monitored by recording OD600, and by determining viable c.f.u. ml–1 by serial 10-fold dilution of cultures in BHI broth and spot-plating 30 μl onto MH agar supplemented with 5 μg trimethoprim ml–1. Plates were incubated microaerobically at 42 °C for 48 h before colonies were counted.

**Statistical testing of stationary-phase survival.** Statistical testing for a difference between treatment effects across NCTC11168, 11168H, 11168H derivative, insertion in *Cj0688* and 11168H, defined as the difference between viable counts at 20 and 66 h, was performed using two-sample paired *t*-tests with Holm–Bonferroni multiple testing correction (Altman, 1991).

**Extraction of total RNA from *C. jejuni*.** Aliquots of bacterial culture (5–20 ml) were removed from *C. jejuni* cultures at specific times during batch culture in BHI, and immediately added to two volumes of RNAprotect bacterial reagent (Qiagen) under microaerobic conditions at 42 °C. The mixture was vortexed, incubated for 5 min at room temperature then centrifuged for 10 min at 3220 g in a 5810R centrifuge (Eppendorf). The supernatant was removed, and pellets were stored at −80 °C until RNA extraction was performed. Total RNA was extracted from stabilized *C. jejuni* pellets using an RNeasy Mini kit (Qiagen) with additional homogenization with QIAshredder columns (Qiagen). RNA was eluted in 100 μl nuclease-free water (Ambion). Total RNA was treated with recombinant DNase I (RNase-free) (Ambion) to remove any remaining DNA. Digests were carried out according to the manufacturer’s instructions. Following digestion, reactions were cleaned using an RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions and the RNA was eluted in 100 μl nuclease-free water (Ambion). When necessary, RNA was concentrated by rotary evaporation to a volume of 10 μl using a 5301 concentrator (Eppendorf). The integrity and quantity of RNA preparations were assessed using an RNA Nano 6000 LabChip (Agilent Technologies) on a 2100 bioanalyzer (Agilent Technologies) according to manufacturer’s directions, and by spectrophotometry using an ND-1000 spectrophotometer (NanoDrop Technologies).

**Microarray hybridizations.** DNA microarrays covering the whole genome of *C. jejuni* NCTC11168 were designed and produced by

<table>
<thead>
<tr>
<th>Table 1. <em>C. jejuni</em> strains used in this study</th>
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<tr>
<td><strong>C. jejuni strain</strong></td>
</tr>
<tr>
<td>NCTC11168</td>
</tr>
<tr>
<td>11168H</td>
</tr>
<tr>
<td>AG63</td>
</tr>
<tr>
<td>11168H <em>Cj0688</em></td>
</tr>
<tr>
<td>11168H <em>Cj0689</em></td>
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</tbody>
</table>

*The Tn insertion is located at position 1470298 in the NCTC11168 genome (http://www.sanger.ac.uk/Projects/C_jejuni/).
Oxford Gene Technology. Microarrays were composed of optimized 60mer oligonucleotide probes, printed in triplicate, matching each gene (as determined from the original annotation of C. jejuni NCTC11168) (Parkhill et al., 2000). One gene (Cj1360c) was omitted due to a sequence length of only 45 bp.

Fluorescently labelled probes for microarray hybridization were generated from 3 μg total RNA using a CyScribe post-labelling kit (GE Healthcare) according to the manufacturer’s instructions. Table 2 details the sample combinations that were hybridized together to the DNA microarrays, for each biological repeat. Three independent biological repeats were performed of five time-point comparisons with dye reversals of each, giving a total of 30 microarray hybridizations. Cy3- and Cy5-labelled probes were mixed together in the appropriate combinations, 10 μl 236 pM control oligonucleotide, designated MSHR, was added and the final volume adjusted to 94 μl. This was added to 156 μl microarray hybridization buffer (1 M NaCl, 50 mM MES, 20 % formamide, 1 % Triton X-100, 20 mM EDTA, pH 7). This hybridization mixture was applied to the array in a BC type DNA microarray hybridization chamber (Agilent Technologies). Microarrays were incubated at 55 °C in a hybridization oven (Agilent Technologies) with rotation at 4 r.p.m. for 60 h.

Following hybridization, microarrays were washed for 5 min at room temperature in 6 x saline-sodium phosphate-EDTA (SSPE), 0.005 % N-lauryl sarcosine, followed by a second 5 min wash in 0.06 x SSPE, 0.18 % PEG 200, at room temperature. Microarrays were immersed briefly in ether, and dried under a stream of air. Washed microarrays were scanned using a DNA microarray scanner (Agilent Technologies) and spots were identified using Feature Extraction Software A7.5.1 (Agilent Technologies) with local background subtraction. Control probes were excluded from any further data analysis at this stage.

**Microarray data analysis.** Initial data analysis was performed using GeneSpring GX 7.3 software (Agilent Technologies). Replicates were averaged and the signal channel and control channel measurements for each time point were filtered on confidence by applying a t test with a P-value cut-off of 0.05, with Benjamini and Hochberg multiple testing correction (Benjamini & Hochberg, 1995). Genes passing this restriction were then filtered to identify those genes whose normalized expression levels changed 1.5-fold or more between time points. Clustering of microarray data was carried out using the Short Time-series Expression Miner (STEM) 1.2.2.b software package (Ernst et al., 2005; Ernst & Bar-Joseph, 2006), freely available to academic users at http://www.cs.cmu.edu/~jernst/stem/. Normalized expression ratios for each gene from each individual microarray for the time points 16 and 20 h, 20 and 40 h, 40 and 46 h and 46 and 54 h were exported as a text file using GeneSpring 7.3. STEM is unable to handle sequential time point design experiments; therefore, data were converted to cumulative expression changes from the 16 h time point by transitive inference using Microsoft Excel. Expression ratios were log-transformed and filtered to include only genes that exhibited a 0.585 log, expression change (equivalent to 1.5-fold) for at least one time point. The maximum unit change between successive time points, c, for model profiles was set to 2, and the maximum number of model profiles at 50. The significance level for expression profiles was set at 0.05 with Bonferroni correction, and profiles were grouped into clusters if the correlation between them exceeded 0.7.

### Quantitative RT-PCR (QRT-PCR).

The oligonucleotide primers used for QRT-PCR are listed in Table 3. All primers were supplied by Sigma-Genosys. Sequences for primers QJAW080 to QJAW083 were taken from Woodall et al. (2005). The remaining primers were designed using Primer3, freely available at http://frodo.wi.mit.edu/ (Rozen & Skaletsky, 2000). Primers were assessed for secondary structure using NetPrimer (Premier Biosoft).

The QuantiTect SYBR Green RT-PCR kit (Qiagen) was used to perform one-step QRT-PCR. Reactions were carried out in 25 μl volumes in 0.1 ml tubes (Corbett Research) in a Rotorgene 3000 real-time thermal cycler (Corbett Research). Reactions contained 1 × QuantiTect SYBR Green (Qiagen), 0.5 μM each primer (Sigma-Genosys), 1 μl template RNA in nuclease-free water, 0.25 μl QuantiTect RT mix (Qiagen) and nuclease-free water (Ambion) to 25 μl. Reaction conditions were: 50 °C, 30 min (reverse transcription), 95 °C, 15 min (polymerase activation), 40 cycles of 94 °C for 15 s (denature), ‘X’ °C for 30 s (anneal, see below) and 72 °C for 30 s (extend). The melting curve was obtained between 65 and 95 °C, rising 1 °C every 5 s.

Optimal annealing temperatures (‘X’ °C) were determined for each primer pair using temperature-gradient PCR on template DNA with HotStarTaq DNA polymerase (Qiagen) according to the manufacturer’s instructions. As a result of this optimization, annealing temperatures of 54, 58 or 60 °C were used. Negative controls, with nuclease-free water replacing template RNA, and reverse-transcriptase-negative controls, with HotStarTaq DNA polymerase replacing QuantiTect RT Mix, were included in each run. To determine reaction efficiency, 10-fold dilution series, spanning a 10^{-6}-fold range, of an appropriate sample of RNA template were included in each run to enable construction of a standard curve. Dilution series and sample QRT-PCRs were carried out in triplicate.

### Validation of microarray data by QRT-PCR relative quantification of selected target genes.

Rotor-Gene 6 software (Corbett Research) was used to visualize QRT-PCR and to calculate the fluorescence threshold which gave the greatest efficiency from the standard dilution series. Raw expression ratios were determined using a mathematical model which included efficiency correction for the QRT-PCR efficiency of each target transcript (Pfaffl, 2001). The raw expression ratio was calculated as follows: raw ratio\(=\frac{E_{\text{target}}}{E_{\text{M technique target (control sample)}}}\), where $E_{\text{QRT-PCR efficiency}} \approx 10^{-\frac{\Delta C_t}{C_t}}$ (slope of standard curve) and $\Delta C_t$= difference in cycle number at calculated threshold. Raw ratios were calculated for each transcript at each time point against the adjacent time point, with the earlier time point as the control. The transcript expression stability of the selected reference transcripts was evaluated using the gNORM application running in Microsoft Excel.

<table>
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<th>Hybridization pair</th>
<th>Cy3-labelled RNA sample</th>
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<tr>
<td>1</td>
<td>16 h</td>
<td>20 h</td>
</tr>
<tr>
<td>2</td>
<td>20 h</td>
<td>40 h</td>
</tr>
<tr>
<td>3</td>
<td>40 h</td>
<td>46 h</td>
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<tr>
<td>4</td>
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</tr>
<tr>
<td>5</td>
<td>16 h</td>
<td>54 h</td>
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<tr>
<td>6</td>
<td>20 h</td>
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<td>7</td>
<td>40 h</td>
<td>20 h</td>
</tr>
<tr>
<td>8</td>
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<td>9</td>
<td>54 h</td>
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<tr>
<td>10</td>
<td>54 h</td>
<td>16 h</td>
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Table 3. Oligonucleotide primers used in this study for QRT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Target gene</th>
<th>Product size (bp)</th>
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<td>dcuA (cj0888)</td>
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<td>QJAW083</td>
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<td>CGGCGATAGCGCGATGAAA</td>
<td>ackA (cj0689)</td>
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<td>QJAW153</td>
<td>GTGCCGATAGGCGTCTTTT</td>
<td>acs (cj1537c)</td>
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<td>QJAW154</td>
<td>GCTTTCAATTTCAAGCGGCTTC</td>
<td>acs (cj1537c)</td>
<td></td>
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</tbody>
</table>

(Vandesompele et al., 2002). The geometric mean of the raw expression ratios of the four most stably expressed reference transcripts, identified as recA, hemN, secD and guaB, was then used to determine normalized expression ratios for the test genes, as follows: normalized ratio=[(Etarget)ACt target / ref − sample])/[(ErefACt ref − sample)/], where ref=geometric mean of selected reference transcripts (Pfaffl, 2001). Normalized ratios were calculated for each transcript at each time point against the adjacent time point, with the earlier time point as the control.

**Live video-phase-contrast microscopy and bacterial motility tracking.** Aliquots of C. jejuni batch culture in BHI were removed from the culture at specific time points; where necessary the culture was diluted 1:10 in culture supernatant. A 10 μl volume was spotted onto a microscope slide, pre-warmed to 42 °C. A glass 22 × 32 mm coverslip was placed over the culture and sealed with nail varnish. The slide was observed using a DM6000B digital microscope (Leica Microsystems) under phase-contrast illumination at ×1000 magnification. A V-1070 high-resolution monochrome charge-coupled device (CCD) camera (Marshall Electronics) was used to capture video to computer using Scion Image software. Videos of between nine and 13 frames per second of either 90 or 120 frames in length were captured, for between five and 10 fields, depending on bacterial density. Videos were saved frame by frame as TIFF using Scion Image and then reassembled into AVI files with INDEO Video 5.10 compression at the correct frame rate using VirtualDub version 1.6.16 software (http://www.virtualdub.org/). It was not possible to track sufficient numbers of motile bacteria at 8 and 66 h due to the low density of the culture, and the low levels of motility, respectively. Motile C. jejuni cells were tracked using Bacterial Tracking Software running in MATLAB version 14.1 (The Mathworks) (Darnton et al., 2004; Jaffe et al., 2004). The software was set to report speed of movement for bacteria that appeared in more than three contiguous frames, whose velocity exceeded a threshold calculated from the maximum recorded movement of heat-killed C. jejuni. The software was only able to track bacteria while they were in a plane of focus that provided sufficient contrast for the software to identify them as an object. The percentage of motile bacteria was counted by eye for this reason, by calculating the number of static and motile bacteria (defined as those with obvious directional motility) present in the first frame of each video.

**Metabolite analysis of culture supernatants.** Aliquots (2 ml) removed from batch cultures of C. jejuni were centrifuged at 16 100 g for 5 min. The supernatant was then passed through a Millex GS 25 mm 0.22 μm pore-size syringe filter unit (Millipore) into a sterile 2 ml microcentrifuge tube, snap-frozen on dry ice and stored at −20 °C. Free amino acid analysis of spent BHI media was carried out by the Protein and Nucleic Acid Chemistry Facility at the Department of Biochemistry, University of Cambridge. This entailed derivatization with 6-aminouinolyl-N-hydroxysuccinimidyl carbamate (AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate).
or AccQ-Tag) fluorescent labelling reagent followed by reverse-phase HPLC separation. $^1$H-NMR was carried out as described previously (Del Recio Leon-Kempis et al., 2006). Acetate determination was performed by Analytical Data Services. Briefly, acetate was separated from interfering species by ion-exclusion chromatography on a Dionex IonPac ICE-AS1 separator column. Samples were run in 1 mM octane sulphonic acid eluant. Acetate was detected by suppressed conductivity using a Dionex AMMS-ICE II suppressor column and a Dionex CD20 conductivity detector. Measurements were calibrated with reference to a dilution series of glacial acetic acid.

RESULTS

C. jejuni growth dynamics

In light of the wide range of growth conditions and media used to cultivate C. jejuni, we aimed to determine a set of standard growth conditions which fulfilled the following criteria: (a) satisfactory reproducible growth of the organism; (b) an extended, well-defined growth cycle; and (c) conditions used widely within the research community. In this study, C. jejuni was grown in BHI broth at 42 °C under microaerobic conditions for 66 h (Fig. 1, see Methods). Exponential (0–16 h), retardation (16–20 h), stationary (20–40 h) and decline (40–66 h) phases are visible from the viable count data. OD provides a less clear measure of bacterial growth (Fig. 1), increasing rapidly throughout exponential phase, and then continuing to increase, albeit at a slower rate, during stationary phase. Only a moderate fall in OD was observed during the period corresponding to the decline phase of viable counts. This indicates that OD is not a useful measure of viable bacterial numbers in C. jejuni cultures after exponential phase. A variety of morphological forms of C. jejuni were observed under fluorescence microscopy in stationary phase, including spiral, coccoid and extended spiral cells. The variation in C. jejuni morphology has been described extensively elsewhere, and may provide an explanation for the lack of correlation between OD and viable counts, along with the contribution of any bacterial debris (Hazeleger et al., 1995; Thomas et al., 1999). Extended growth curves, over a period of 127 h, were also performed and indicated a two-peak pattern (data not shown) characterized by a second period of growth following the initial decline phase, in agreement with previous reports (Martinez-Rodriguez et al., 2004).

Microarray analysis

We measured gene expression changes in the C. jejuni transcriptome between different phases of growth using a loop design (Townsend, 2003) (Fig. 2a). Sufficient RNA could only be recovered from cultures from late exponential phase onwards; therefore, time points were selected to represent late exponential (16 h), early stationary (20 h), late stationary (40 h), early decline (46 h) and mid decline (54 h) phases of the growth cycle. Hybridizations were performed using total RNA extracted from three independent cultures, with two dye-reversal technical replicates for each biological replicate, giving a total of 30 microarray hybridizations. Microarray replicates were averaged and the dataset was filtered to identify genes that were significantly differentially expressed by 1.5-fold or greater. The dataset is available through the ArrayExpress Database.
The vast majority of significant gene expression changes identified by filtering occurred between 16 and 40 h, spanning late exponential to late stationary phase (the full list of gene expression changes is detailed in Supplementary Table S1). The large quantity of expression changes during stationary phase (133 genes increase in expression, 151 genes decrease) identifies this period as one of substantial bacterial activity despite there being no net bacterial growth. The STEM clustering algorithm (Ernst et al., 2005; Ernst & Bar-Joseph, 2006) identified 11 distinct gene expression profiles which contained significantly more genes than expected due to chance, representing major expression trends within the data. These profiles were grouped further into six clusters, each containing similar profiles (Fig. 2b, Supplementary Table S2).

There were a number of striking expression trends within the data. These included significant downregulation of 35 genes predicted to encode ribosomal subunits. These genes showed reduced expression of between 1.5- and 3.58-fold between 16 and 20 h, a period representing entry into stationary phase. This apparent downregulation of ribosomal function fits with the switch from rapid cell division to a more static population that characterizes this transition between phases. Consistent with metabolic downshift in stationary phase, downregulation of large numbers of genes involved in electron transport was also observed. Eight genes in the *nuo* operon, which encode components of a respiratory complex I that utilizes flavodoxin rather than NADH, are downregulated between 2.21- and 3.52-fold during stationary phase, between 20 and 40 h (Weerakoon & Olson, 2008). In addition, the *sdhABC* operon, predicted to encode succinate dehydrogenase, shows reduced expression of between 4.35- and 6.25-fold, and four nitrate reductase enzymes, *napA, napB, napG* and *napH*, are downregulated from 2.33- to 3.77-fold between 20 and 40 h (Parkhill et al., 2000; Pittman & Kelly, 2005). All of these electron transport chain components fall into cluster 1 (Fig. 2b), which is characterized by continually declining expression from the onset of stationary phase.

A number of heat shock-response genes were upregulated, falling into cluster 2, which is characterized by increased expression from the onset of stationary phase onwards, including *groEL, groES, grpE, cdpB, hspR* and *hrcA*. The *groEL, groES* and *grpE* genes are all significantly upregulated by between 2.86- and 3.34-fold. GroEL and GroES have been shown to be induced in response to heat shock and alkaline shock in *C. jejuni* (Wu et al., 1994). Indeed, in extended 127 h growth curves, pH increased from a mean of 6.99 (±0.06) to 8.13 (±0.03), in agreement with earlier studies (Rollins et al., 1983), and is most likely a result of deamination of amino acids and subsequent release of ammonia (Leach et al., 1997; Velayudhan et al., 2004). In addition, a number of genes with putative functions in oxidative stress resistance, including *ahpC*, *sodB*, *tpx* and *perR*, show significantly increased expression from 20 to 40 h, by between 1.64- and 2.65-fold.

**Validation of microarray expression data**

The inclusion of a ‘loop’ hybridization in the microarray experiment (Fig. 2a) enabled the measurement of cumulative expression changes across the time-course both directly and indirectly. The microarray hybridizations comparing the 16 and 54 h time points provide a direct measure of the cumulative change, but this can also be calculated by transitive inference from the sequential hybridizations (Townsend, 2003). In order to assess the correlation between the two sets of measurements, the log2-transformed ratios of the direct and inferred values for all the genes on the microarray were taken and plotted against each other (Fig. 3a). There is a strong linear correlation between direct and indirect measures of the cumulative gene expression changes throughout the time-course. Given the additional variation introduced into the inferred data by calculating the cumulative ratios using several hybridizations, the strength of this correlation indicates internal consistency in the dataset.

The microarray data were also independently validated using QRT-PCR to measure the transcript levels of nine genes across the five time points. These nine genes were shown by microarray analysis to be significantly differentially regulated by varying levels across the time points, and were chosen to have diverse functions to avoid bias towards any particular regulon. QRT-PCR expression ratios were calculated for each of the time point comparisons and normalized to the geometric mean of four stably expressed genes, identified from the microarray data and independently verified using the geNORM method (Vandesompele et al., 2002). The log2-transformed microarray expression and QRT-PCR expression ratios were plotted against each other to assess the strength of the correlation between the measurements obtained by the two methods. The 16–54 h ‘loop’ comparison (Fig. 3b) shows a strong correlation between the measurements, with an *R*² value of 0.9635, and the slope of the correlation is close to 1, indicating that the magnitude of expression changes detected by each platform is highly similar.

**Bacterial motility peaks during stationary phase**

*C. jejuni* is a motile species driven by polar flagella. The *C. jejuni* flagellar system involves over 40 structural and regulatory genes, and includes a glycosylation system that post-translationally modifies the flagellin subunits (Guerry et al., 2006; Jangannathan & Penn, 2005; Parkhill et al., 2000). The flagella are required for invasion of human epithelial cells *in vitro* and for adhesion, invasion and colonization in a range of animal models, with strong evidence for an important role for this organelle in colonization and virulence (Black et al., 1988; Caldwell et al., 1989).
growth curve using phase-contrast video microscopy and particle tracking software (Darnton et al., 2004). This enabled the calculation of the mean velocity of motile bacteria and the percentage motility between 16 and 54 h of growth (Fig. 4). Motility, in terms of both the percentage of motile cells and the average velocity of these cells, peaks during stationary phase, at 28 h (Fig. 4a), when the maximal percentage of motile bacteria is ~55% and the highest mean velocity reached is 35 μm s⁻¹, in good agreement with other reports (Karim et al., 1998). Despite being in stationary phase, the bacteria remain highly active, exhibiting high levels of motility during this phase.

C. jejuni NCTC11168 genome has only three predicted sigma factors: σ^{70} (encoded by rpoD), σ^{54} (rpoN) and σ^{28} (fliA) (Parkhill et al., 2000). All three sigma factors are involved in the regulation of flagellar genes, with σ^{70} believed to regulate early ‘class I’ genes that are involved in assembly of the basal body and export apparatus, σ^{54} regulating ‘class II’ genes that are involved in basal body, hook and filament biosynthesis, and σ^{28} regulating ‘class III’ genes that are involved in filament biosynthesis and capping and flagellin post-translational modification (Carrillo et al., 2004; Kamal et al., 2007). In order to examine whether bacterial motility correlates with the transcription of flagellar genes, we compared expression data with the motility of C. jejuni throughout the growth curve and identified two main relationships. Genes grouped into cluster 1 (Fig. 4b) are predicted to be regulated by σ^{70}, and are characterized by a pattern of downregulation throughout stationary phase and onwards (Carrillo et al., 2004; Hendrixson & DiRita, 2003; Petersen et al., 2003). This expression pattern coincides with motility, which increases from 20 to 28 h but falls overall during stationary phase from 20 to 40 h (Fig. 4a) in both velocity and percentage of motile cells, and continues to fall throughout the decline phase. Functionally, these genes are predicted to encode components of the export machinery, the C ring motor/switch and the MS ring stator (Carrillo et al., 2004; Jangannathan & Penn, 2005; Parkhill et al., 2000). A distinct subset of genes follows a different expression profile of sustained or upregulated gene expression throughout stationary phase and this subset is grouped into cluster 3 (Fig. 4b). These genes are predicted to have σ^{54}- and σ^{28}-regulated promoters (Carrillo et al., 2004; Hendrixson & DiRita, 2003; Petersen et al., 2003). They putatively encode proteins that form the hook, rod and P ring (Carrillo et al., 2004; Parkhill et al., 2000), suggesting that these structures may be maintained throughout the later part of stationary phase, despite declining motility.

C. jejuni exhibits a preferential order of amino acid utilization

The ability of amino acids to support growth of C. jejuni has been recognized for several decades (Kiggins & Plastridge, 1958; Smibert, 1978). Examination of the C. jejuni NCTC11168 genome suggests the presence of
enzymes to enable the catabolism of L-aspartate, L-asparagine, L-glutamate, L-glutamine, L-serine and L-proline, which correlates well with the available data on amino acid utilization and also reflects the most abundant amino acids in chicken excreta (Guccione et al., 2008; Kelly, 2005; Kiggins & Plastridge, 1958; Leach et al., 1997; Parkhill et al., 2000; Smibert, 1978). The amino acids that are catabolized are likely to be deaminated by C. jejuni into intermediates that can then enter straight into the tricarboxylic acid (TCA) cycle. For example, pyruvate can be derived from L-serine and L-alanine, oxaloacetate from L-aspartate, and L-glutamate can be used to produce 2-oxoglutarate (Velayudhan et al., 2004). Important catabolic roles for an sdaA-encoded serine dehydratase and the aspA-encoded aspartase, in vitro and in chicken colonization, have been identified (Guccione et al., 2008; Velayudhan et al., 2004). Despite the significance of amino acids in C. jejuni physiology, the relative importance of individual amino acids is yet to be fully established, and their utilization during different phases of batch growth in widely used bacteriological growth media has not been examined. Therefore, to determine the preferred amino acid utilization and the timing of substrate switching, and to link metabolite and transcriptome data, amino acid profiling of culture supernatants by reverse-phase HPLC was performed at nine time points during batch growth of C. jejuni. Utilization curves for the six most significantly depleted amino acids are shown in Fig. 5; data for 13 further amino acids are shown in Supplementary Table S3. In agreement with previously published data in different culture media (Leach et al., 1997), L-aspartate, L-serine, L-asparagine and L-glutamate are utilized most rapidly, with L-aspartate, L-serine and L-asparagine disappearing completely from the BHI medium by 28 h, the mid-point of stationary phase. L-Glutamate is depleted less rapidly, remaining in the culture supernatant until the decline phase, at 54 h. L-Proline appears to represent a less preferred substrate for C. jejuni growth, and is not exhausted completely over the growth cycle. Moreover, utilization apparently only begins to accelerate during stationary phase. The decrease in L-threonine, as observed elsewhere, may result from the activity of the biosynthetic threonine dehydratase, IlvA, and is unlikely to be catabolic (Guccione et al., 2008).

The putA and putP genes, which are predicted to encode the enzymes responsible for the oxidation of L-proline to L-glutamate and the major L-proline permease, respectively (Cairney et al., 1984; Parkhill et al., 2000), are significantly upregulated 4.90- and 3.30-fold, respectively, during the stationary phase, from 20 to 40 h of growth. Levels of L-proline remain relatively constant in the culture supernatant at around 0.7 mM up until 20 h (Fig. 5). Throughout stationary phase, L-proline levels then decline, falling to

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**Fig. 4.** (a) Velocity (■) and percentage motility (□) of C. jejuni during growth in BHI broth. The graph shows a representative example of a single culture; the velocity data points represent the mean value of tracked bacteria and error bars show SEM. (b) Representation of microarray expression data of flagellar genes, with the fold change in expression between each time point indicated by a coloured box, with the colour scale shown. The clusters (Fig. 2b, Supplementary Table S2) into which these genes were grouped by STEM analysis are indicated. Significant expression changes greater than the 1.5-fold change threshold are indicated (*). The predicted sigma factors for the genes are indicated: σ^70 (●), σ^54 (○), σ^28 (■) (Carrillo et al., 2004; Hendrixson & DiRita, 2003; Kamal et al., 2007; Petersen et al., 2003).
0.42 mM by 28 h and reaching 0.21 mM by the end of the growth curve, corresponding with the putAP expression trend.

pebC and peb1A are induced 1.97- and 1.6-fold respectively throughout stationary phase, and their profiles show a pattern of upregulation throughout the entire growth cycle. peb1A encodes a periplasmic binding protein for L-aspartate and L-glutamate that is required for most of the transport of, and microaerobic growth on, these amino acids (Del Recio Leon-Kempis et al., 2006). The pebC gene encodes an ATP binding protein that forms another component of this transporter (Del Recio Leon-Kempis et al., 2006). Both L-aspartate and L-glutamate are utilized by C. jejuni during growth in BHI broth, and both amino acids are completely depleted from the medium during the growth cycle (Fig. 5). Thus, the upregulation of the peb1a and pebC transcripts coincides with increased utilization of L-glutamate, suggesting that assimilation of this amino acid during growth in BHI proceeds via this transport system. In a similar manner to L-proline, L-glutamate is most rapidly metabolized during stationary phase, following the exhaustion of a number of other amino acids, and may represent a substrate used by C. jejuni to extend stationary phase survival.

1H-NMR resonance analysis reveals evidence for an ‘acetate switch’

NMR has been successfully applied to culture supernatants and bacterial suspensions and lysates of C. jejuni to monitor utilization and production of a variety of
metabolites (Del Recio Leon-Kemps et al., 2006; Mendz et al., 1997; Smith et al., 1999; Velayudhan et al., 2004). In this study, $^1$H-NMR was used to analyse supernatants throughout the 66 h time-course with the original aim of determining amino acid utilization. However, clear changes in only a small number of resonance peaks were observed throughout the time-course (Fig. 6). Peaks indicated by grey arrows at 2.06 and 2.34 p.p.m. correspond to two major l-proline resonances, confirming the depletion of l-proline throughout the time-course, in agreement with the metabolite profile (Fig. 5).

The most clearly visible change in the $^1$H-NMR spectra across the time-course is the resonance peak indicated by a black arrow (Fig. 6) at 1.77 p.p.m., which corresponds to acetate. The pattern of acetate use by C. jejuni during batch growth is one of continual production and excretion until the mid-point of stationary phase, which occurs at 28 h in this system, followed by utilization after this point, throughout the later part of stationary phase and the decline phase. This pattern of acetate production followed by utilization is characteristic of a bacterial mechanism known as the ‘acetate switch’ which occurs when bacteria deplete their environment of acetogenic carbon sources, such as l-serine or pyruvate, and begin to scavenge for acetate (Wolfe, 2005). The production of acetate by C. jejuni as a result of l-serine and pyruvate catabolism has been reported elsewhere (Mendz et al., 1997). However, to our knowledge, this is the first time that subsequent reassimilation of acetate during the later stages of the C. jejuni growth cycle has been described.

Identification of a role for Cj0688 and Cj0689 in the acetate switch

The production of acetate is indicative of the presence of a mixed acid fermentation pathway in C. jejuni (Mendz et al., 1997). Acetate excretion is thought to result from the need to recycle CoA, required for the conversion of pyruvate into acetyl-CoA. Acetate is produced from acetyl-CoA via the Pta–AckA pathway in Escherichia coli, which involves the pta-encoded phosphotransacetylase and the ackA-encoded acetate kinase (Wolfe, 2005). This pathway is expressed constitutively and is reversible. Pta converts acetyl-CoA and inorganic phosphate to acetyl-phosphate and coenzyme A. AckA catalyses the conversion of acetyl-phosphate and ADP to acetate and ATP. Acetate is converted back to acetyl-CoA via acetyl-CoA synthetase, encoded by the ackA gene, or back through Pta–AckA (Chang et al., 1999; Wolfe, 2005). The C. jejuni NCTC11168 genome contains homologues of pta, ackA and ackB encoded by Cj0688, Cj0689 and Cj1537c, respectively (Parkhill et al., 2000). In order to characterize the role of Cj0688, Cj0689 and Cj1537c in the growth of C. jejuni, mutants were obtained with insertions in each of these genes.

AG63 (Cj1537c::Tn) grew almost identically to wild-type NCTC11168 (data not shown). Strains 11168H Cj0688 and 11168H Cj0689, containing kanamycin-resistance cassette insertions in Cj0688 and Cj0689, respectively (Joshua et al., 2006), exhibited growth dynamics that differed from those of the 11168H (Jones et al., 2004; Karlyshev et al., 2002) parent strain when assessed by viable counts, exhibiting poorer stationary phase survival (Fig. 7a). The difference in survival of these three strains from the onset of stationary phase, at 20 h, until late decline phase, at 66 h, was assessed using two-sample paired $t$ tests. Significant differences between the survival of 11168H and 11168H Cj0689 ($P=0.019$) and 11168H Cj0688 and 11168H Cj0689 ($P=0.049$) were identified, although the difference between 11168H and Cj0688 was not significant ($P=0.101$) (Fig. 7a). Cj0689, encoding the ackA homologue, is required to maintain wild-type levels of survival from stationary phase onwards. Although falling short of statistical significance, the data are suggestive of a survival defect in 11168H Cj0688, indicating that the pta homologue may also be required.

Given the reduced survival of two of these mutant strains, it was important to determine whether acetate production
and utilization were affected and could be responsible for the observed phenotype. Therefore, we measured acetate concentrations in culture supernatants of NCTC11168, AG63, 11168H, 11168H Cj0688 and 11168H Cj0689 using anion-exclusion chromatography. Fig. 7(b) shows the acetate measurements taken from culture supernatants of 11168H, 11168H Cj0688 and 11168H Cj0689. NCTC11168 and AG63 show near identical acetate production and utilization trends (data not shown), with peak acetate concentration after 28 h of growth, at the mid-point of stationary phase, confirming the acetate switch pattern shown by 1H-NMR (Fig. 6). The data thus indicate that Cj1537c is not involved in acetate production or assimilation under these growth conditions. 11168H shows the same characteristic pattern of acetate production and utilization that defines the acetate switch (Fig. 7b). However, the timing of the switch differs from that observed with NCTC11168, occurring at around 40–46 h rather than 28 h (Figs 6 and 7b). This may reflect a degree of variation in timing of the switch, or may be accounted for by strain differences linked to the undefined hypermobility phenotype of 11168H (Karlyshev et al., 2002). Strains 11168H Cj0688 and 11168H Cj0689 are defective in acetate production (Fig. 7b). Although some accumulation of acetate is evident, it is substantially lower in these strains than in the wild-type, peaking at 4.15 and 7.19 mM for 11168H Cj0688 and 11168H Cj0689, respectively, compared with 16.59 mM for 11168H. In addition, the dramatic switch from acetate dissimilation to assimilation is not observed in these two mutant strains. A reduced quantity of acetate is excreted up until the end of stationary phase, at 40 h, followed by a stabilization in acetate concentration thereafter. The Cj0688 and Cj0689 mutants were not complemented, and thus a polar effect of the Cj0689 insertion on Cj0689 cannot be definitively ruled out. However, the distinct phenotypes in biofilm formation and hydrophobicity of these two mutants reported elsewhere indicate that the mutations are non-polar and have discrete effects (Joshua et al., 2006).

**DISCUSSION**

Examination of the C. jejuni growth cycle from transcriptomic and metabolic perspectives has revealed a stationary phase that is highly dynamic. Microarray analysis identifies a large number of transcriptional changes between late exponential and late stationary phase, between 16 and 40 h. The absence of significant changes after 40 h does not provide evidence for any coordinated transcriptional response in the transition to a ‘non-culturable’ state, viable or otherwise, during the decline phase. This is supported by the observation that inhibition of protein synthesis using chloramphenicol does not affect the transition to ‘non-culturable’ coccoid forms of C. jejuni during long-term incubation at a range of temperatures in BHI broth (Hazeleger et al., 1995).

Decreased expression of a range of genes involved in key metabolic functions, such as protein synthesis and electron transport, during the transition from exponential to stationary phases is consistent with the idea of a metabolic downshift that accompanies reduced cell division and no net bacterial growth. Indeed, a similar microarray study in the related pathogen Helicobacter pylori found that 15 ribosomal subunit genes were repressed between mid-exponential and stationary phase (Thompson et al., 2003). Genes encoding ribosomal proteins are upregulated in C. jejuni in the rabbit ileal loop model of infection, suggesting that in this *in vivo* situation, C. jejuni is encountering an environment more similar to exponential phase than stationary phase, at least with respect to ribosome expression (Stintzi et al., 2005). Components of the *nuo*

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**Fig. 7.** (a) Growth curves of *C. jejuni* strains 11168H (■), 11168H cj0688 (●) and 11168H cj0689 (△) in BHI broth, monitored by viable counts. The plotted values are mean measurements from three independent cultures; error bars show SEM. In some cases, error bars are too small to be visible. *Statistically significant difference between the survival of the indicated strains between 16 and 66 h (P<0.05).* (b) Graph indicating the acetate concentration of culture supernatants taken during the growth of *C. jejuni* 11168H (■), 11168H Cj0688 (●) and 11168H Cj0689 (△) in BHI broth. Plotted points are mean values from two measurements from supernatants taken from a single growth curve. The error bars, corresponding to the SEM, are too small to be visible.

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and nap operons show reduced expression in ΔspoT mutants, suggesting that the stringent response may be involved in regulating these genes (Gaynor et al., 2005). A number of electron transport genes, including sdhABC and nuoGHL, are also downregulated in the rabbit ileal loop model, but sdhABC and napA, napB and napG are upregulated during chick colonization (Stintzi et al., 2005; Woodall et al., 2005). This may indicate alteration of electron transport in response to different in vivo environments, or alternatively may reflect differences in the in vitro cultured reference samples used in these studies, emphasizing the importance of consideration of growth phase in experimental design.

Increased expression of a number of heat shock-response genes, including groES, groEL and grpE, is suggestive of the induction of a stress response during stationary phase. Induction of ahpC, sodB and tpx, which have functions in oxidative and nitrosative stress resistance, provides further examples of the upregulation of stress response genes during stationary phase (Atack et al., 2008; Baillon et al., 1999; Purdy et al., 1999). Protein damage in the form of carboxylation and disulphide bond formation is known to accumulate in E. coli stationary-phase cultures (Dukan & Nystrom, 1998). Heat-shock proteins are induced during stationary phase in this organism via the rpoH-encoded alternative sigma factor (Jenkins et al., 1991), and the action of DnaK/DnaJ and GroEL/GroES has been shown to protect against such damage through stabilization of polypeptides and assisting in their folding (Fredriksson et al., 2005; Siegenthaler & Christen, 2005; Tang et al., 2006).

Although C. jejuni lacks any homologue of rpoH, the stationary-phase induction of heat shock genes may represent a similar response to protein damage, albeit regulated by a different mechanism. Similarly, oxidative stress-resistance genes, such as sodB and tpx, are important in resisting the types of protein damage that are known to accumulate in stationary phase cultures (Atack et al., 2008; Dukan & Nystrom, 1999). Interestingly, AhpC and Tpx have been shown by proteomic methods to be upregulated in C. jejuni biofilms (Kalmokoff et al., 2006). Increased expression of perR, which is known to negatively regulate ahpC, is somewhat paradoxical, and may indicate the existence of alternative regulators of ahpC (van Vliet et al., 1999). Recent microarray data suggest that the luxS gene, putatively encoding an autoinducer-2 biosynthetic enzyme, increases the expression of ahpC and tpx, suggesting that these genes could be under the control of quorum-sensing signals (He et al., 2008). Phenotypic studies of C. jejuni stress responses in different growth phases have, however, proved inconclusive and further work is needed to establish the details of C. jejuni stress responses during stationary phase and their regulation (Kelly et al., 2001; Klančnik et al., 2006; Murphy et al., 2003, 2005).

In contrast to the pattern of reduced activity suggested by downregulation of ribosomal and electron transport genes, motility increases during the early part of stationary phase, reaching a peak at 28 h. Expression of flagellar genes follows two patterns. One coincides with motility, declining overall across stationary phase; the other exhibits sustained or upregulated expression throughout stationary phase, and includes genes that putatively encode proteins that form the hook, rod and P ring (Carrillo et al., 2004; Parkhill et al., 2000). This indicates that these structures may be maintained during the later part of stationary phase, despite declining motility. Earlier studies have shown that mutants in various components of the flagellar apparatus fail to secrete a set of proteins, including Campylobacter invasion antigens (Cia), that are important for in vitro invasion during co-culture with epithelial cells (Guerry, 2007; Konkel et al., 2004). In addition to at least one of the flagellin components, the rod genes flgC and flgD and the flgE2 hook component are required for secretion (Konkel et al., 2004). It is tempting to speculate that these structures are maintained throughout stationary phase, despite declining motility, to fulfill a secretory function. Similar motility analysis of H. pylori has shown that the percentage of motile cells peaks earlier than for C. jejuni, at the transition between exponential and stationary phase, and that velocity peaks at a similar time, in early to mid stationary phase (Thompson et al., 2003). Again, clustering techniques separate the flagellar genes of H. pylori into distinct sets that correspond to class I, II and III, and indicate that flagellar gene regulation is likely to be similar in these two related pathogens (Thompson et al., 2003).

Analysis of amino acid utilization during growth fits well with previous data in different growth media (Guccione et al., 2008; Leach et al., 1997), showing that L-aspartate, L-serine, L-asparagine and L-glutamate are all completely utilized during the growth cycle. The timing of amino acid utilization has not been previously examined in C. jejuni. L-Proline appears to represent a less preferred substrate for C. jejuni growth, and is not exhausted completely over the growth cycle, and utilization apparently only begins to accelerate during stationary phase. It is possible that this amino acid represents a ‘secondary’ substrate for C. jejuni, which is catabolized when more favourable amino acids are exhausted. These patterns suggest a preferential order of amino acid utilization, and that some may support growth during exponential phase, whereas others play a role in survival throughout stationary phase. The apparent switching of amino acid substrates during stationary phase indicates that C. jejuni is physiologically active in this period, responding to altering conditions to prolong survival.

Consistent with the switching of substrates, 1H-NMR and ion-exclusion chromatography of culture supernatants have identified an acetate switch mechanism in C. jejuni, with bacteria changing from acetate excretion to assimilation in mid to late stationary phase. Our data indicate that acetate production proceeds through the Pta–AckA pathway. Assimilation may also proceed through this pathway, given that acetyl-CoA synthetase is not involved, although this has not been distinctly expressed. Some
acetate does not accumulate in the pta and ackA mutants, and is highest in the 11168H Cj0689 (ackA) mutant, reaching 7.19 mM, although no switch is observed. Indeed, ackA mutants of E. coli are known to accumulate small amounts of acetate, in contrast to pta mutants, in which acetate production is ablated. The acetyl-phosphate produced by pta is labile around physiological pH; thus, some may be converted to acetate extracellularly (Wolfe, 2005). The source of the small amount of acetate produced by the pta mutant (reaching 4.15 mM) is unknown. It is tempting to speculate that failure to produce or utilize acetate explains the poorer survival of these strains from late stationary phase onwards.

This investigation, which characterized C. jejuni growth in batch culture, has revealed new aspects of the physiology of this important pathogen. C. jejuni is highly active during stationary phase, responding to changing conditions by altering gene expression to increase motility, switching to alternative amino acid substrates and scavenging to use molecules, such as acetate, previously excreted as waste. Investigation of the metabolic and physiological capabilities of C. jejuni in this simple system represents a potent way of exploring novel aspects of metabolism. More extensive phenotypic and metabolic analysis of this system, considered alongside the substantial gene expression data presented here, will be a powerful approach for understanding this organism.

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