



Gene expression and phenotypic profiling of *Campylobacter jejuni* during *in vitro* growth.



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ABSTRACT

Infections with bacteria of the genus *Campylobacter* represent the most common food borne cause of gastroenteritis in industrialised nations, and 93% of cases are attributed to *Campylobacter jejuni*. In the U.K., reported cases of *Campylobacter* infection exceeded 60,000 in 1999, although this is believed to reflect only a small portion of the true number of cases, which is thought to be closer to 500,000 annually.¹ In the United States the number of estimated annual cases of infection caused by *Campylobacter* is closer to 2,500,000.² Despite the public health importance of this pathogen, some of the most fundamental aspects of its physiology remain poorly understood, including the growth of *C. jejuni* in liquid culture, which is central to a large portion of research on this bacterium. The significance of different morphological forms, and their association with a 'Viable Non-Culturable' state remains unclear. We report a phenotypic and transcriptional investigation of the growth of *C. jejuni* NCTC11168 in BHI medium. High density ink-jet *in situ* synthesised oligonucleotide microarrays were used to monitor the gene expression status of *C. jejuni* at five time points, in different phases of growth. Live phase contrast microscopy was carried out, to measure motility and fluorescent staining techniques were used to assess the metabolic activity and morphology of the bacteria. Substantial changes in the gene expression profile of *C. jejuni* were observed, alongside a reduction in bacterial motility and metabolic activity from mid-stationary phase, and a concurrent increase in the proportion of coccoid and extended spiral cells.

INTRODUCTION

The publication of the genome sequence of *C. jejuni* strain NCTC11168 in 2000, and more recently of numerous other strains of *Campylobacter* has enabled the application of a variety of post-genomic techniques to study these bacteria.^{3,4} These have included *C. jejuni* microarray experiments encompassing investigations into the effects of temperature and oxygen tension, iron limitation, and gene expression in rabbit and chick *in vivo* models.^{5,6,7,8,9} Furthermore, microarray analysis has also been utilised to identify genes controlled by regulatory systems, such as the DccRS two component regulator, the heat shock response regulator HspR, and the stringent response regulator SpoT.^{10,11,12} These studies have all used *in vitro* cultured bacteria, yet a detailed investigation into *in vitro* growth of *C. jejuni* has yet to be undertaken. Such a study would better inform microarray studies, and other work involving *in vitro* grown *C. jejuni*, for example, investigations into stress responses, physiology and motility. To this end, we have undertaken a study, in collaboration with Oxford Gene Technology Services (OGT), to profile the phenotypic and transcriptional status of *C. jejuni* NCTC11168 during 'standard' *in vitro* laboratory growth in Brain Heart Infusion broth at 42°C.

C. jejuni undergoes a series of interesting changes during the *in vitro* growth cycle. As cultures progress through the growth curve, levels of metabolic activity and motility alter, declining throughout stationary phase as available nutrients are depleted. Typical spiral forms of the bacteria are replaced by small coccoid forms as culturability decreases. These coccoid forms have been associated with a 'Viable Non-Culturable' form of *C. jejuni*, which may represent a dormant but potentially infectious form. We are using various forms of microscopy to follow and quantify such changes through the growth curve, from late exponential phase through to the decline in culturability, combined with microarray analysis to monitor concurrent gene expression changes.

METHODS

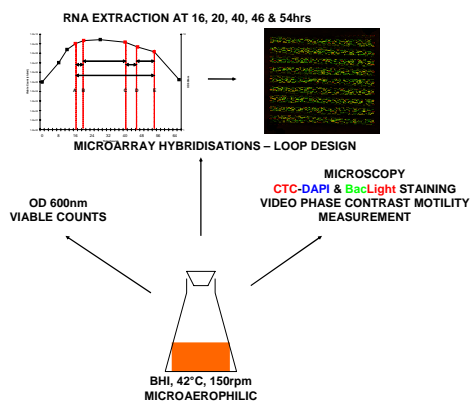


Figure 1. Overview of experimental design. *C. jejuni* NCTC11168 was grown *in vitro* as shown, and the indicated measurements taken throughout the growth curve. RNA was extracted at the five time points, and adjacent time points hybridised to each other. The first and final time points were also hybridised against each other, in a 'loop' design. Three growth curves were carried out to provide biological replicates and dye reversals were performed. Data was analysed using GeneSpring 7.2 (Agilent). Replicates were averaged and the signal channel and control channel measurements for dye reversal samples inverted. A Lowess curve was fit to the log-intensity versus log-ratio plot using 50% of the data to calculate the Lowess fit at each point. The data from each pairwise comparison of time points was filtered by confidence by applying a t-test with a p-value cut off of 0.05, with Benjamini and Hochberg multiple testing correction. Genes passing this restriction were then filtered to identify those genes whose normalised expression levels changed 1.5 fold or more between time points.

RESULTS

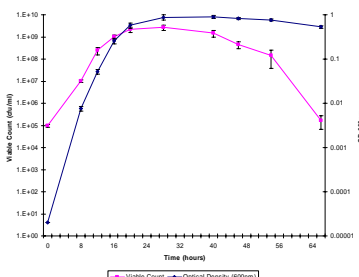


Figure 2. Growth of *C. jejuni* in BHI was monitored in BHI through viable counts and OD at 600nm. Graph shows the average of 5 replicates, error bars show standard error.

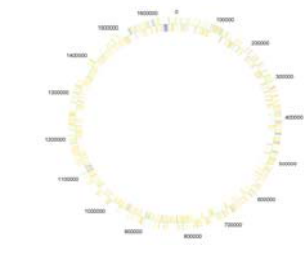


Figure 3. Physical view from GeneSpring of gene expression changes between 16 and 20 hours mapped onto the *C. jejuni* NCTC11168 chromosome. Red colouring indicates an increase in expression, blue indicates decrease, and yellow indicates no change.

RESULTS

The majority of gene expression changes occur between 16 and 40 hours, representing late log to late stationary phases.

Comparison of time points	Genes Up	% Genes Up	Genes Down	% Genes Down
16hrs - 20hrs	62	3.75	134	8.10
20hrs - 40hrs	135	8.16	151	9.13
40hrs - 46hrs	1	0.06	1	0.06
46hrs - 54hrs	1	0.06	3	0.18
16hrs - 54hrs	146	8.83	214	12.94

Table 1. Number of significant genes 1.5 fold up and down regulated between compared time points.

Large numbers of genes involved in protein synthesis decrease expression upon entry into stationary phase, with 35 genes predicted to encode ribosomal proteins significantly downregulated from 1.5 to 3.58 fold between 16 and 20 hours.

Changes in genes whose protein products are predicted to be involved in electron transport and energy metabolism genes are also observed, throughout late log and stationary phase. These include the *petABC* (ubiquinol-cytochrome-c reductase) operon, which decreases expression from 16-20 hours, and *nuoDGHJLN* (NADH dehydrogenase), *napABH* (nitrate reduction) and *sdhABC* (succinate dehydrogenase), which are downregulated during stationary phase, from 20-40 hours. The downregulation of these genes is reflected in the reduction of the proportion of bacteria indicated to be metabolically active by CTC staining throughout stationary phase and onwards.

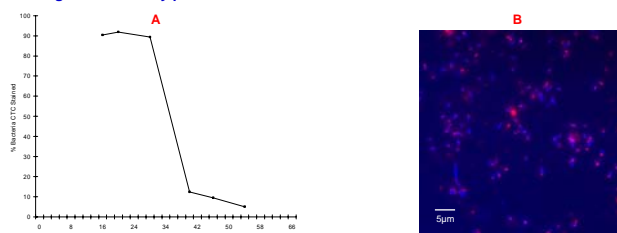


Figure 4A. Percentage of bacteria stained with DAPI that are also stained with CTC, a fluorescent redox dye that acts as an electron acceptor and accumulates in metabolically active bacteria. B. Fluorescent microscopy image of CTC-DAPI stained *C. jejuni* culture, at 1000x magnification.

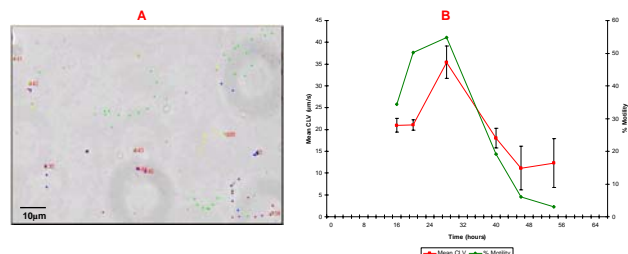


Figure 5A. Screenshot of BacTrack bacterial motility tracking software running in MATLAB 14.1, image was taken by phase contrast microscopy at 1000x magnification. B. Motility of *C. jejuni* during growth in BHI. BacTrack software was used to determine the mean curvilinear velocity (CLV) of motile bacteria from phase contrast microscopy movies¹³. Motility movies were also inspected by eye, and the percentage of motile bacteria displaying clear directional motility was calculated for each time point.

Motility gene expression changes are associated with alterations in bacterial motility. These include changes in flagellar switch genes *fljG*, *fljM*, *fljN* and *fljY*, basal body and associated genes *flgE2*, *flgG2*, *flgB*, *flgI* and *fljL* and putative flagellar biosynthesis genes *fljP* and *fljQ*.

Comparison of time points	Genes Up	Expression Change	Genes Down	Expression Change
16-20hrs	<i>fljE</i>	1.603		
	<i>fljN</i>	1.539		
			<i>fljG</i>	1.838
20-40hrs	<i>flgG2</i>	4.014	<i>fljY</i>	1.887
	<i>flgB</i>	3.308	<i>fljM</i>	1.992
	<i>flgI</i>	2.001	<i>fljP</i>	2.039
16-54hrs			<i>fljQ</i>	1.885
			<i>fljI</i>	1.818
			<i>fljY</i>	1.692
			<i>flgE2</i>	1.584
			<i>fljL</i>	1.521

Figure 6. GeneSpring graph of motility genes whose expression changes by 1.5 fold or more throughout the growth curve. At each point the difference in expression between adjacent time points is plotted.

CONCLUSIONS

We have carried out microarray and phenotypic analysis of *C. jejuni* NCTC11168 during growth in BHI broth. Large numbers of genes alter their expression during different phases of growth, with the majority of changes occurring between late log and late stationary phase.

Gene expression changes are reflected by phenotypic alterations. Extensive downregulation of ribosomal genes is observed upon entry into stationary phase. Fluctuations in motility are matched by changes in putative flagellar switch and biosynthesis genes, and the decrease in metabolic activity as cultures age is accompanied by downregulated expression of genes associated with these processes.

This study provides a platform for more detailed investigation of numerous facets of the physiology of *C. jejuni*, and should help inform the design of microarray experiments, and of other studies that use *in vitro* cultured *C. jejuni*.

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