Transcriptional and translational expression patterns associated with immobilized growth of *Campylobacter jejuni*

Balamurugan Sampathkumar,^{3†} Scott Napper,^{1†} Catherine D. Carrillo,² Philip Willson,¹ Eduardo Taboada,² John H. E. Nash,² Andrew A. Potter,¹ Lorne A. Babiuk¹ and Brenda J. Allan¹

Correspondence

Brenda J. Allan

brenda.allan@usask.ca

¹Vaccine and Infectious Disease Organization, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 5E3

²Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Road, Ottawa, Ontario, Canada K1A 0R6

³Agriculture and Agri-Food Canada, Lacombe Research Centre, 6000 C&E Trail, Lacombe, Alberta, Canada T4L 1W1

Although *Campylobacter jejuni* is a leading cause of food-borne illness, little is known about the mechanisms by which this pathogen mediates prolonged environmental survival or host cell virulence. Although these behaviours represent distinct phenotypes, they share a common requirement of an immobilized state. In order to understand the cellular mechanisms that facilitate a surface-associated lifestyle, transcriptional and translational expression profiles were determined for sessile and planktonic *C. jejuni*. These investigations indicate that the immobilized bacteria undergo a shift in cellular priorities away from metabolic, motility and protein synthesis capabilities towards emphasis on iron uptake, oxidative stress defence and membrane transport. This pattern of expression partially overlaps those reported for *Campylobacter* during host colonization, as well as for other species of bacteria involved in biofilms, highlighting common adaptive responses to the conserved challenges within each of these phenotypes. The adaptation of *Campylobacter* to immobilized growth may represent a quasi-differentiated state that functions as a foundation for further specialization towards phenotypes such as biofilm formation or host cell virulence.

Received 3 August 2005

Revised 21 October 2005

Accepted 10 November 2005

INTRODUCTION

Campylobacter jejuni is the leading cause of bacterial food-borne illness in humans (Ketley, 1997), with contaminated poultry products representing the leading cause of human infection (Berndtson *et al.*, 1996). Acute *C. jejuni* infection results in watery to bloody diarrhoea, fever, nausea and vomiting. Although the majority of infections are self-limiting, more serious medical conditions such as reactive arthritis, inflammatory bowel syndrome, Miller–Fisher syndrome (MFS) and Guillain–Barré syndrome (GBS) (Nachamkin *et al.*, 1998) can result.

Unlike many bacterial pathogens, *C. jejuni* does not grow effectively outside the host. The ability of this bacterium to remain as the most frequent cause of food-borne illness, despite these fastidious growth requirements, is referred to

as the *Campylobacter* conundrum (Jones, 2001). Of particular relevance to food-borne infections is that the growth requirements of *C. jejuni* do not permit proliferation on contaminated foods under normal storage conditions (Park, 2002). While limited in its ability to proliferate, *Campylobacter* can survive for extended periods in the environment (Park, 2002) and has been isolated from a number of ecosystems (Altekruse *et al.*, 1999; Bolton *et al.*, 1987; Jones & Bradshaw, 1996; Pearson *et al.*, 1993; Stanley *et al.*, 1998).

For *Campylobacter*, and other bacteria, the vast majority of growth within natural ecosystems occurs in association with a surface (Kirchman & Mitchell, 1982; Kuhn *et al.*, 1987). In the ecosystem, surface-associated growth can lead to the formation of highly structured, sessile microbial communities known as biofilms. Biofilm development is believed to occur through a series of coordinated stages including (i) transport of micro-organisms to a surface, (ii) initial attachment, (iii) formation of microcolonies, and (iv) formation of mature biofilms (van Loosdrecht *et al.*,

†These authors contributed equally to this work.

The Gene Expression Omnibus Repository accession number for the microarray experiments reported in this paper is GSE3028.

1990). Growth on an agar surface, with increased population densities and the associated challenges, bears functional resemblance to the second and third stages of biofilm formation, prior to the encapsulation that defines the biofilm phenotype.

There have been numerous genomic and proteomic investigations of biofilms involving various species of bacteria (Lazazzera, 2005). While these investigations do not identify a consensus pattern of gene expression, they do present a relatively cohesive picture of the adaptations underlying the phenotype. As considerable variation exists in the specifics of each biofilm, inconsistencies in expressional response are anticipated. Investigations of phenotypic features that are conserved throughout all biofilms, such as immobilization, are more likely to identify conserved cellular response elements.

Establishment of infection is also dependent upon immobilization during adhesion to intestinal cells. As the ability to grow on surfaces and adhere to intestinal epithelial cells is necessary for production of disease (de Melo & Pechere, 1990; Russell & Blake, 1994), growth on the surface of agar has been applied as a model for gene expression during initial stages of host infection (Wang *et al.*, 2004). In these investigations *Salmonella typhimurium* grown on conventional agar showed surface-specific upregulation of genes associated with virulence, including those for lipopolysaccharide synthesis, iron metabolism and type III secretion (Wang *et al.*, 2004).

Complex phenotypes, including virulence and biofilm formation, are often the cumulative result of parallel responses to different facets of a particular challenge. These phenotypes can be dissected, and associated responses partitioned, through models that incorporate a limited number of the associated stimuli. For example, through examination of responses to surface-associated growth, adaptations associated with immobilization can be distinguished from responses unique to specialized phenotypes such as biofilms.

In this investigation genomic and proteomic approaches were utilized to characterize differential patterns of expression of *C. jejuni* in response to immobilized growth conditions. Growth on agar resulted in the induction of a discrete number of functional systems directed towards the biological objectives of (i) iron acquisition, (ii) management of oxidative stress, (iii) membrane modifications and (iv) primary active-transport systems. Surface-associated growth also demonstrated a corresponding repression of systems involved in (i) energy production, (ii) motility, (iii) protein synthesis and (iv) non-essential iron-requiring proteins.

METHODS

Bacteria and culture conditions. *Campylobacter jejuni* NCTC 11168, obtained from the American Type Culture Collection (ATCC 700819), was used. A loopful of frozen (-80°C) culture was

streaked on Mueller–Hinton (MH) agar plates and incubated at 37°C for 24 h under microaerophilic conditions (10% CO_2 , 5% O_2 , 85% N_2) created using a CampyPack plus (Becton Dickinson Microbiology Systems). Following incubation, cells were collected using a sterile loop, resuspended in 1.0 ml MH broth and transferred to 50 ml sterile MH broth in 25 cm^2 tissue culture flasks with $0.4\text{ }\mu\text{m}$ filter caps, followed by incubation for 24 h in the above-mentioned conditions. The resulting culture was used as a stock culture for growth on agar and in broth. Growth in broth was achieved by transferring 1.0 ml of the stock culture to 50 ml MH broth in tissue culture flasks (described above) and incubating microaerobically. At various times, samples were removed to determine the viable cell counts by plating serial dilutions of the culture on MH agar. Growth on agar was achieved by transferring 1.0 ml stock culture onto a fresh MH agar plate, spreading the culture to produce a lawn, and incubating microaerobically. Samples were harvested by washing cells from the plates with 2.0 ml 0.1 M phosphate-buffered saline (pH 7.4). Viable cell counts were determined as described above.

Genomic analysis. *C. jejuni* cells were harvested after 16 h growth on agar or in broth and homogenized in TRIzol (Invitrogen) by passing the mixture through a syringe. Nucleic acids (RNA and DNA) were isolated as recommended by the manufacturer. RNA was quantified spectrophotometrically at 260 nm using an ND-1000 spectrophotometer (Nanodrop). Preparation of complementary DNA (cDNA) and indirect labelling with Cy3 and Cy5 were performed as previously described (Carrillo *et al.*, 2004). Samples were probed with the whole-genome *C. jejuni* NCTC 11168 microarray (version 3.0). Details of the construction and content of the microarray are available at (http://ibs-isb.nrc-cnrc.gc.ca/ibs/immunochemistry/campychips_e.html). Slides were scanned using a Chipreader (Bio-Rad), and image analysis, signal normalization and data visualization were performed using Array Pro Analyser 4.5 (Media Cybernetics). Net signal intensities were obtained by performing local-ring background subtraction and spots with a signal less than 3SD of background in both channels were excluded from the analysis. The mean signal intensities for duplicate spots were averaged and data from each channel were adjusted by subarray normalization using cross-channel Lowess regression.

ArrayStat (Imaging Research) was used for statistical analysis of the replicated data. A proportional model with offsets, for dependent data, was selected and statistical significance was determined using the pooled common error method with the false discovery rate multiple test correction (nominal $\alpha = 0.05$). Four biological replicates were tested, including one comparison in which the Cy3 and Cy5 dyes were swapped to compensate for biases caused by differing chemical properties of the fluorescent dye molecules. Complete results for these experiments are available at the Gene Expression Omnibus Repository (NCBI, <http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE3028.

Proteomic analysis

SDS-PAGE of total cellular and detergent-insoluble outer-membrane proteins. Total cellular proteins were obtained by sonicating cells in 50 mM HEPES buffer (pH 7.4). Detergent-insoluble outer-membrane proteins from the cell pellets were obtained as described previously (Sampathkumar *et al.*, 2004). The extracted proteins were quantified using the dye-binding assay of Bradford (1976) and $8.0\text{ }\mu\text{g}$ of the protein preparation was separated by SDS-PAGE (4.0% stacking gel and 12.0% resolving gel) using a Bio-Rad Protean II electrophoresis system. The gels were silver stained according to the manufacturer's instructions (Amersham Biosciences).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of total cellular proteins. *Sample preparation.* Total cellular protein was extracted by sonicating cell pellets in 1.0 ml lysis buffer [8 M urea, 4.0 % (w/v) CHAPS, 40 mM DTT and 2.0 % (v/v) IPG pH 3–10 buffer] once for 15 s at 40 Ω amplitude on ice. The extracts were centrifuged at 16 000 g for 10 min to remove cell debris. The resulting supernatant was treated with 0.1 vol. buffer containing 50 mM $MgCl_2$, 1 mg DNase I ml^{-1} and 0.25 mg RNase A ml^{-1} . The reaction was stopped after 15 min at 4 °C with 3 vols ice-cold acetone. Proteins were then precipitated for 2 h at –20 °C. The precipitate was collected by centrifugation at 3700 g for 15 min and resuspended in IPG gel rehydration solution [8.0 M urea, 2 % (w/v) CHAPS, 40 mM DTT, 0.5 % (v/v) pH 3.0–10.0 IPG buffer and 0.01 % (w/v) bromophenol blue]. The dye-binding assay of Bradford (1976) was then performed to quantify the protein concentration.

Isoelectric focusing. Protein extracts were subjected to high-resolution 2D-PAGE according to the method described by O'Farrell (1975) and modified by Görg *et al.* (2000). Equal amounts of proteins (450 μ g) were loaded onto a first-dimension gel strip. Isoelectric focusing was performed using an IPGphor electrophoresis unit with the Immobiline DryStrip Kit (Amersham Biosciences). Thirteen-centimetre Immobiline DryStrip gels (pH 4.0–7.0) were rehydrated in 250 μ l IPG gel rehydration solution (composition as above except that IPG buffer pH 4.0–7.0 was used) containing 450 μ g protein for 16 h at room temperature. Isoelectric focusing was achieved when the total running time yielded 60 kVh at 20 °C. Following isoelectric focusing, the gels were equilibrated twice for 15 min each in 10.0 ml isoelectric focusing gel equilibration buffer [50 mM Tris/HCl (pH 8.8), 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS and 0.002 % (w/v) bromophenol blue] containing 100 mg DTT for the first equilibration and 250 mg iodoacetamide for the second equilibration.

SDS-PAGE. Equilibrated isoelectric-focused strips were placed on top of a uniform 14 % SDS-polyacrylamide gel for second-dimension electrophoresis with the Bio-Rad Protean II xi electrophoresis system. Second-dimension separation was carried out at 25 mA per gel constant current at 4 °C. The gels were stained with Bio-Safe colloidal Coomassie blue G-250 stain according to the manufacturer's (Bio-Rad) instructions.

Analysis of protein spots on 2D-PAGE gels. Individual gels were scanned using an Epson Expression 1680 scanner with a transparency adapter as 8-bit greyscale 300 d.p.i. images. Differentially expressed proteins were then detected and quantified from the images with PDQuest 2-D Analysis Software (Bio-Rad). A twofold difference in expression was set as a threshold during image analysis to detect proteins that were differentially expressed.

Mass spectrometry of proteins. Protein spots of interest were excised from the gel, destained and subjected to in-gel trypsin digestion according to the established protocols for the MassPrep robotic workstation (Water/Micromass). The samples were then dried in a speed-vac and reconstituted in 75 % acetonitrile containing 5 mg α -cyano-4-hydroxycinnamic acid ml^{-1} and eluted directly onto the MALDI plate.

The MALDI-TOF-MS (Voyager DE-STR; Applied Biosystems) was operated in the positive-ion reflectron mode. Four hundred laser shots were averaged and processed with Data Explorer software (Applied Biosystems). The samples were internally calibrated using trypsin autolytic fragments, and database searches carried out with Protein Prospector (UCSF Mass Spectrometry Facility, San Francisco, CA, USA). Sample preparation and MS analysis were performed by the Mass Spectrometry facility at the National Research Council, Plant Biotechnology Institute (Saskatoon, Canada).

RESULTS AND DISCUSSION

Growth characteristics

An essential component of genomic or proteomic comparative analysis is the establishment of differentially expressed genes in reference to an appropriate and reproducible control point or growth condition. Bacterial patterns of expression are highly dependent upon growth phase, with dramatic changes accompanying the transition from exponential to stationary-phase growth. As noted by others (Lazazzera, 2005), many of the seemingly contradictory results that have emerged from biofilm expression investigations are due to differences in the growth phase. Careful consideration was therefore given to ensuring comparison of bacteria that were at a common stage of growth stage progression.

The growth curve of *C. jejuni* grown on agar or in broth was determined by plating aliquots from predetermined time points on MH agar and expressed as c.f.u. per 25 ml medium and c.f.u. ml^{-1} respectively. As shown in Fig. 1, the growth profiles were very similar for both growth conditions. Cell densities in both growth conditions increased steadily up to 12 h and maximum cell densities were achieved at 18 h, following which cell death started to occur (Fig. 1). Thus *C. jejuni* cells at 16 h of incubation were used in all studies.

Gene and two-dimensional protein expression analysis

Growth on agar plates. Surface-associated growth of *C. jejuni* results in a distinct pattern of expression at the levels of transcription and translation relative to growth in broth, with over 90 genes or proteins having altered levels of expression. The results from genomic and proteomic components of this investigation are complementary in that they identify the same functional groupings of biomolecules (Table 1). The absence of a more definitive

Fig. 1. Growth curves of *C. jejuni* grown in broth (■) or on agar (▲). At the indicated times, samples were removed to determine the viable cell counts present by plating serial dilutions on MH agar.

Table 1. Number of quantitative changes, and associated cellular function, identified through proteomic and genomic analysis

	Proteomic analysis	Genomic analysis
Upregulated on agar		
Iron metabolism	5	3
Oxidative defence	4	3
Energy metabolism	2	7
Protein synthesis	1	
Membrane modification	1	13
Upregulated in broth		
Energy metabolism	7	13
Motility		3
Protein synthesis		29

correlation between the genes and proteins identified is likely to be a consequence of post-transcriptional regulatory events.

Many of the proteins found to be induced as a consequence of immobilized growth are membrane-associated and therefore problematic for separation via two-dimensional gel electrophoresis. A proteomic comparison of both total cell protein and detergent-insoluble outer-membrane proteins was performed through one-dimensional SDS-PAGE analysis (Fig. 2). It is of significance that the proteins identified as being differentially expressed as a consequence of growth on agar through one-dimensional SDS-PAGE are in agreement with the results obtained from the two-dimensional proteomic analysis (Fig. 3). The nine proteins identified as undergoing differential patterns of expression were also identified within the large-scale proteomic analysis (Tables 2 and 4). An additional detergent-insoluble protein of approximately 27 kDa was uniquely expressed during growth on agar; however this protein could not be positively identified.

Induced systems

Iron uptake. Bioavailability of iron represents a significant challenge to the survival of bacteria in the environment and during infection of a host. Like most organisms, Campylobacters have an absolute requirement for iron and have adapted iron-acquisition strategies to cope with dynamic patterns of iron availability. Campylobacters have two distinct but overlapping iron-responsive regulatory systems: Fur, which regulates iron-uptake genes, and the Fur homologue, PerR, which regulates oxidative-stress defence (Baillon *et al.*, 1999; Holmes *et al.*, 2005; Palyada *et al.*, 2004; Ratledge & Dover, 2000; van Vliet *et al.*, 1998). In many species of bacteria Fur is solely responsible for the regulation of iron homeostasis as well as oxidative-stress defence. That *C. jejuni*, which contains relatively few regulatory proteins, has evolved two separate systems for iron acquisition and prevention of oxidative damage

Fig. 2. Silver-stained SDS-PAGE profile of detergent-insoluble and total cellular proteins from *C. jejuni* NCTC 11168. Proteins identified as being differentially expressed are numbered. Lanes: A, molecular mass markers; B, C, detergent-insoluble proteins from growth on agar (B) or broth (C); D, E, total cellular proteins from growth on agar (D) or broth (E).

indicates the central role that iron plays in its gene regulation and virulence.

Both proteomic (Table 2) and microarray data (Table 3) demonstrate significant upregulation of genes and proteins with involvement in iron acquisition. These include ChuA, a haemin/haemoglobin uptake system (Cj1614), a possible outer membrane siderophore receptor (Cj0178), components of putative iron-uptake ABC transport systems (Cj0175c, Cj0174c and Cj1663), cfrA, a putative iron-uptake protein (Cj0755) and the periplasmic protein p19 (Cj1659). The induction of iron-uptake molecules, at the levels of both transcription and translation, as a consequence of growth on agar represents the most dramatic alteration in expression; at the level of transcription the three most strongly induced genes code for iron-uptake proteins (Table 3) and a parallel pattern is seen at the level of translation with the unique or highly induced expression of several iron-uptake proteins (Figs 3 and 4).

From the perspective of immobilized growth, rather than growth in broth, representing the most physiologically

Fig. 3. Protein expression profiles of *C. jejuni* NCTC 11168 grown on MH agar (a) or in MH broth (b). Labelled spots indicate those identified as being either uniquely or strongly induced under the growth condition. Spots whose number is prefixed with 'A' are induced during growth on agar; those prefixed with 'B' are induced during growth in broth.

appropriate baseline, the induction of iron-uptake systems during surface-associated growth may be more appropriately viewed as a repression of these systems during growth in liquid media.

The increased requirement for iron during immobilized growth, whether within a biofilm or surface-associated growth, is probably a consequence of the increased cell densities depleting locally available iron stores. These

iron-uptake systems show a similar pattern of induction for *C. jejuni* under iron-limiting conditions (Holmes *et al.*, 2005), as well as during growth within a host (Stintzi *et al.*, 2005).

Induction of iron-uptake systems has not emerged as a conserved response within investigations of biofilms, although it has been reported for select biofilms (Pysz *et al.*, 2004). The availability of iron does however represent a central

Table 2. Proteins induced during growth of *C. jejuni* NCTC 11168 on MH agar

Function	Spot ID	Cj no.	Gene	Description	Expression
Iron metabolism	1	Cj1614	<i>chuA</i>	Haemin uptake system outer-membrane receptor	Unique
	A01	Cj0755	<i>cfrA</i>	Putative iron-uptake protein	Unique
	4	Cj0175c	–	Putative iron-uptake ABC transport system periplasmic iron-binding protein	Upregulated
	A07	Cj1663	–	Putative ABC transport system ATP-binding protein	Unique
	A09	Cj1659	P19	Periplasmic protein p19	Upregulated
Oxidative defence	6/A08	Cj0334	<i>ahpC</i>	Alkyl hydroperoxide reductase	Upregulated
	2/A02	Cj0193c	<i>tig</i>	Trigger factor (peptidyl-prolyl <i>cis/trans</i> isomerase, chaperone)	Upregulated
	A05	Cj0559	–	Oxidoreductase	Upregulated
	A06	Cj0146c	<i>trxB</i>	Thioredoxin reductase	Upregulated
Metabolism	A03	Cj1096c	<i>metK</i>	S-Adenosylmethionine synthetase	Upregulated
	A04	Cj0533	<i>sucC</i>	Succinyl-CoA synthetase β chain	Upregulated
	5	Cj0982c	–	Putative amino-acid transporter periplasmic solute-binding protein	Upregulated
Protein synthesis	A10	Cj0477	<i>rpL</i>	50S ribosomal protein L7/L12	Upregulated
Signal transduction	A04	Cj0448c	–	Putative MCP-type signal transduction protein	Upregulated

Table 3. Genes induced during growth of *C. jejuni* NCTC 11168 on MH agar

Function	Cj no.	Gene	Description	log (Mean ratio)	Z-score	P (2-tailed)
Iron uptake	Cj0174c	–	Putative iron-uptake ABC transport system permease protein	0.258	8.13	4.44E–16
	Cj1614	<i>chuA</i>	Haemin uptake system outer-membrane receptor	1.012	24.13	0
	Cj0178	–	Putative outer-membrane siderophore receptor	0.876	20.90	0
Oxidative defence	Cj1385	<i>katA</i>	Catalase	1.096	26.14	0
	Cj0393c	–	Putative oxidoreductase	0.176	5.55	2.82E–08
	Cj1287c	–	Malate oxidoreductase	0.227	7.16	8.28E–13
Metabolism	Cj0347	<i>trpF</i>	N-(5'-Phosphoribosyl)anthranilate isomerase	0.191	6.03	1.61E–09
	Cj0348	<i>trpB</i>	Tryptophan synthase β chain	0.329	10.39	0
	Cj0689	<i>ackA</i>	Acetate kinase	0.184	5.81	6.16E–09
	Cj0483	<i>uxaA'</i>	Putative altronate hydrolase C-terminus	0.244	7.69	1.53E–14
Membrane	Cj0346	<i>trpD</i>	Anthranilate synthase component II	0.178	5.63	1.79E–08
	Cj0811	<i>lpxK</i>	Putative tetraacyldisaccharide 4'-kinase	0.180	5.25	1.49E–07
	Cj1151c	<i>waaD</i>	ADP-L-glycero-D-manno-heptose-6-epimerase	0.271	7.93	2.22E–15
	Cj1133	<i>waaC</i>	Putative lipopolysaccharide heptosyltransferase	0.410	12.95	0
Transport systems	Cj0922c	<i>pebC</i>	ABC-type amino-acid transporter ATP-binding protein	0.185	5.42	6.03E–08
	Cj0613	<i>pstS</i>	Possible periplasmic phosphate-binding protein	0.184	5.38	7.52E–08
	Cj0920c	–	Putative ABC-type amino-acid transporter permease protein	0.283	6.16	7.26E–10
	Cj0300c	<i>modC</i>	Putative molybdenum-transport ATP-binding protein	0.255	8.03	8.88E–16
	Cj0919c	–	Putative ABC-type amino-acid transporter permease protein	0.283	7.54	4.64E–14
	Cj0303c	<i>modA</i>	Putative molybdate-binding lipoprotein	0.298	8.70	0
	Cj0730	–	Putative ABC transport system permease	0.409	9.76	0
	Cj0982c	–	Putative amino-acid transporter periplasmic solute-binding protein	0.714	14.74	0
	Cj1163c	–	Putative cation-transport protein	0.216	6.80	1.03E–11
	Cj1502c	<i>putP</i>	Sodium/proline symporter	0.508	16.03	0

Fig. 4. Proteins induced as a consequence of immobilized growth of *C. jejuni* NCTC 11168 on MH agar: (a) ferric receptor (A01), (b) 19 kDa periplasmic protein (A09) and (c) putative ABC transport system ATP-binding protein (A07).

consideration in the decision to commit to biofilm formation; iron chelation through lactoferrin has been shown to discourage *Pseudomonas aeruginosa* biofilm formation. The repression of biofilm formation is hypothesized to occur due to the insufficient iron responses to match the localized increases in demand for iron by biofilm bacteria. This appears to be a general phenomenon, with the adhesion of various species of bacteria to biomaterials being suppressed by iron chelators at concentrations which are unable to influence growth in the vegetative state (Ardehali *et al.*, 2002).

Oxidative defence. In addition to representing a limiting nutrient to bacterial survival, iron imposes an additional challenge to living systems through its involvement in the generation of toxic molecules. Iron can react with oxygen to produce reactive oxygen species like superoxide anions (O_2^-), peroxides (RO_2) and hydroxyl radicals ($OH^\cdot$) through the Haber–Weiss ($Fe_2^{+} + O_2 \rightarrow Fe_3^{+} + O_2^-$) and Fenton ($Fe_2^{+} + H_2O_2 \rightarrow Fe_3^{+} + OH^- + OH^\cdot$) reactions. The absolute requirement for iron for electron transport of microaerophilic organisms like *C. jejuni* therefore requires the bacteria to maintain a proper iron homeostasis and to coordinate expression of iron-uptake and oxidative stress systems. In contrast to other Gram-negative bacteria, oxidative stress and iron acquisition are regulated separately in *C. jejuni* (van Vliet *et al.*, 1998). This is of

Table 4. Proteins induced during growth of *C. jejuni* NCTC 11168 in MH broth

These proteins all function in energy metabolism.

Spot ID	Cj no.	Gene	Description	Expression
3	Cj0264c	–	Molybdopterin-containing oxidoreductase	Unique
8	Cj0780	<i>napA</i>	Periplasmic nitrate reductase	Upregulated
B01	Cj0437	<i>sdhA</i>	Succinate dehydrogenase flavoprotein subunit	Unique
B02	Cj1267c	<i>hydA</i>	Ni/Fe-hydrogenase small chain	Upregulated
B03	Cj1510c	<i>fdhB</i>	Putative formate dehydrogenase iron–sulfur subunit	Upregulated
B04	Cj0012c	–	Non-haem iron protein	Upregulated
9	Cj0358	–	Putative cytochrome <i>c</i> ₅₅₁ peroxidase	Upregulated

particular significance given the limited number of regulatory systems present within the bacteria.

Oxidative stress proteins often show distinct patterns of induction in response to different kinds of oxidative stress: superoxide stress and peroxide stress. The superoxide stress proteins include manganese-containing superoxide dismutase and endonuclease IV; the peroxide regulon contains catalase (*KatA*) and alkyl hydroperoxide reductase (*AhpC*). The regulation of *AhpC* and *KatA* expression in *C. jejuni* has a Fur-independent component, as the expression of these proteins is only partially affected by the mutation of Fur. A second Fur homologue, the peroxide stress regulator *PerR*, functions to co-regulate *ahpC* and *katA* expression (van Vliet *et al.*, 1998).

Parallel to the observation that iron functions as both a signal and an ongoing challenge for formation and survival within a biofilm, oxidative stress also appears to serve as both a trigger and a required adaptive response for biofilm formation and survival. Biofilm formation has been observed in response to oxidative stress in the hyperthermophilic archaeon *Archaeoglobus fulgidus* (LaPaglia & Hartzell, 1997) and certain aerobic mesophilic biofilms showed increased expression of oxidative stress genes (Golovlev, 2002; Schembri *et al.*, 2003).

As a consequence of growth on agar there was increased expression of the primary oxidative stress defence components, alkyl hydroperoxide reductase (*ahpC*) and catalase (*katA*). Increased expression of additional proteins with known involvement in the minimization of oxidative damage, including oxidoreductase (Cj0559), a putative oxidoreductase (Cj0393c) and malate oxidoreductase (Cj1287c), was also observed. The ribosomally associated stress response chaperone, trigger factor (Cj0193c), which has also been implicated in oxidative stress response, exhibited increased expression (Tables 2 and 3).

Membrane modification. Our characterization of sessile *C. jejuni* indicates the upregulation of enzymes involved in modifications of the lipid, carbohydrate and protein components of the membrane. These include the upregulation of ADP-L-glycero-D-manno-heptose-6-epimerase (*waaD*), as well as two putative membrane-modification enzymes,

tetraacyldisaccharide 4'-kinase (*lpxK*) and lipopolysaccharide heptosyltransferase (*waaC*) (Table 3). Notably, our analysis does not reveal the induction of a large number of the enzymes with established roles in the formation of the biofilm-associated extracellular matrix, supportive of growth on agar as a model of mid-to-late-phase biofilm formation.

ABC transport systems. Many of the membrane proteins that undergo differential expression as a consequence of growth on agar are associated with the primary active transport of amino acids. To our knowledge there have been no previous connections proposed between either low-iron adaptation or biofilm formation and amino acid transport and metabolism, although amino acid starvation was identified as a trigger for biofilm formation for *Candida albicans* (Garcia-Sanchez *et al.*, 2004). The induction of ABC transport systems which are not specific to amino acid transport has been reported as a consequence of biofilm formation (Pysz *et al.*, 2004) although the functional relevance remains unclear.

As many of the induced transporters identified in our investigation are ABC-type primary active-transport systems one possibility is that they may have roles in mediating the efflux of antibiotics and antimicrobial agents such that the induction of these systems may be related to the increased resistance to antibiotics which is a hallmark of biofilm bacteria. While the sheltering of bacteria within the biofilm extracellular matrix may provide the initial line of defence against antibiotics this may be supplemented through the ability to actively transport antibiotics out of the bacteria.

Other transport systems. In addition to the ABC transport systems, growth on agar also resulted in the induction of both phosphate and molybdate-transport systems (Table 3).

The induction of molybdate uptake systems, and corresponding repression of molybdate-requiring enzymes such as periplasmic nitrate reductase, has been reported from investigations of *C. jejuni* in iron-limiting conditions (Holmes *et al.*, 2005; Palyada *et al.*, 2004). This parallels our observation of induction of a putative molybdenum-transport

ATP-binding protein, a molybdate-binding lipoprotein and a periplasmic phosphate-binding protein, as well as the repression of periplasmic nitrate reductase and molybdopterin oxidoreductase under sessile growth conditions. Molybdate metabolism appears to be closely coordinated with that of iron, so the patterns of expression we observe may be a secondary consequence of iron limitation during immobilized growth.

Systems repressed during growth on agar

Growth on agar results in the repression of a variety of cellular systems as determined by their induction during growth in broth. The overall objectives represented by these systems indicate a trend towards a reduction in metabolism, motility and protein synthesis. There is also a general repression of non-essential, iron-containing enzymes, presumably as a measure to conserve iron for essential processes.

Non-essential iron-containing proteins. Levels of several non-essential iron-containing proteins were decreased in iron-limiting conditions at the levels of transcription (Table 5) and translation (Table 4). For example, non-haem protein (Cj0012c), Ni/Fe hydrogenase (Cj1267c), ferredoxin, cytochrome *c* peroxidase and a member of the succinate dehydrogenase complex (Cj0437) all show decreased levels of expression. The alterations in levels of expression of these proteins were some of the most dramatically observed at the level of translation (Fig. 5). The pattern of genes induced in broth bears close resemblance to those reported to be induced upon the addition

of iron to *C. jejuni* which have been cultured in iron-limited media (Holmes *et al.*, 2005; Palyada *et al.*, 2004).

Energy metabolism. Growth on agar results in the repression of genes involved in metabolic processes, in particular respiratory and iron-containing enzymes (Tables 4 and 5). This probably serves a dual function in conservation of bioavailable iron, as well as in the minimization of reactive by-products of oxidative metabolism. This is consistent with patterns of expression reported by others under low-iron conditions.

Motility. The repression of genes involved in motility is a logical consequence of an immobilized growth state and has been observed during biofilm formation for both *P. aeruginosa* (Whitely *et al.*, 2001) and *Bacillus subtilis* (Stanley *et al.*, 2003). In our investigation we report the repression of flagellin (FlaB), the flagellar biosynthesis protein (FlhA) and a putative RNA polymerase sigma factor for the flagellar operon (FliA) in the sessile state (Tables 4 and 5). The repression of biomolecules involved in motility would function to conserve the significant energy expenditure associated with flagella production, consistent with the general downregulation of energy-production systems observed during immobilized growth.

While the repression of motility genes during immobilized growth is consistent with the majority of biofilm expression studies, this has not been a unanimous conclusion. Indeed some groups have reported the induction of motility biomolecules during biofilm formation. There are suggestions however that the lack of repression of motility systems

Table 5. Genes induced during growth of *C. jejuni* NCTC 11168 in MH broth

Function	Cj no.	Gene	Description	log(Mean ratio)	Z-score	P (2-tailed)
Oxidative phosphorylation	Cj1489c	<i>ccoO</i>	<i>cb</i> -type cytochrome <i>c</i> oxidase subunit II	−0.183	−5.79	6.97E−09
	Cj1488c	<i>ccoQ</i>	<i>cb</i> -type cytochrome <i>c</i> oxidase subunit IV	−0.231	−7.28	3.24E−13
	Cj1185c	<i>petB</i>	Putative ubiquinol-cytochrome <i>c</i> reductase cytochrome <i>b</i> subunit	−0.488	−11.65	0
	Cj1184c	<i>petC</i>	Putative ubiquinol-cytochrome <i>c</i> reductase cytochrome <i>c</i> subunit	−0.279	−8.15	4.44E−16
Nitrogen metabolism	Cj0102	<i>atpF'</i>	ATP synthase F ₀ sector B' subunit	−0.241	−7.05	1.77E−12
	Cj0780	<i>napA</i>	Periplasmic nitrate reductase	−0.291	−8.49	0
	Cj0783	<i>napB</i>	Periplasmic nitrate reductase small subunit (cytochrome <i>c</i> -type protein)	−0.298	−8.70	0
	Cj0781	<i>napG</i>	Putative ferredoxin	−0.363	−11.46	0
	Cj0782	<i>napH</i>	Putative ferredoxin	−0.195	−4.03	5.65E−05
Energy metabolism	Cj1511c	<i>fdhA</i>	Putative formate dehydrogenase large subunit (selenocysteine containing)	−0.198	−5.78	7.60E−09
Motility	Cj1338c	<i>flaB</i>	Flagellin	−0.205	−6.48	9.05E−11
	Cj0882c	<i>flhA</i>	Flagellar biosynthesis protein	−0.183	−5.36	8.53E−08
	Cj0061c	<i>fliA</i>	Putative RNA polymerase sigma factor for flagellar operon (sigma-28 factor)	−0.527	−12.57	0

Fig. 5. Proteins induced as a consequence of growth of *C. jejuni* NCTC 11168 in MH broth: (a) succinate dehydrogenase flavoprotein subunit (B01), (b) Ni/Fe hydrogenase small chain (B02) and (c) putative formate dehydrogenase iron-sulfur subunit (B03).

in these investigations reflects differences in experimental design rather than discrepancies in biological response. For studies of *B. subtilis* biofilms, in which one of the two investigations observed repression of flagellar genes, the biofilms were grown in similar conditions; however, the planktonic cells were not (Ren *et al.*, 2004a; Stanley *et al.*, 2003). Similarly, investigations of *Escherichia coli* biofilms that did not observe repression of motility genes through microarray analysis (Beloin & Ghigo, 2005; Ren *et al.*, 2004b; Schembri *et al.*, 2003) did show repression when assayed with a *lacZ* transcriptional fusion (Prigent-Combaret *et al.*, 1999). Thus, repression of genes involved in motility probably represents a conserved theme amongst biofilms.

Ribosomal synthesis. Repression of ribosomal proteins during immobilized growth emerges as one of the most definitive conclusions from our investigations, with 30 of the 53 ribosomal proteins identified as being repressed (Table 6). This is consistent with the overall trend of adaptation in the sessile state towards a dormant lifestyle with reduced energy production and expenditure.

Amongst biofilm investigations the levels of expression of ribosomal proteins are an interesting point of contention, with equal but contradicting opinions of whether these proteins are induced or repressed. These contradictory findings are probably a consequence of the growth phase of the planktonic cells to which biofilm cells are compared.

This is best exemplified by a study of *E. coli* biofilms which identified ribosomal proteins as being more highly expressed in biofilm cells when compared with stationary-phase cells, but not when compared with exponential-phase planktonic cells (Schembri *et al.*, 2003). The suggestion that the expression of ribosomal proteins is activated in biofilms is surprising as this presumably reflects increased growth rate.

Conclusions

While growth within the permissive conditions of liquid media is often taken as the reference point of bacterial behaviour these conditions do not reflect the challenges encountered by bacteria in the natural environment. There is considerable evidence that bacteria spend a significant portion of their natural existence in an immobilized state through the formation of microcolonies and biofilms. The cellular adaptations that accompany survival in a sedentary state are therefore of significant biological interest. For pathogenic bacteria, such as *C. jejuni*, immobilization takes on further significance as it represents a prerequisite step to the formation of biofilms, as well as in the establishment of the early stages of virulence. Growth on agar is proposed to function as a limited model of a developmental stage towards each of these phenotypes.

Through genomic and proteomic analysis we have established expression profiles for *C. jejuni* during immobilized and planktonic growth. Relative to growth in broth, growth on agar results in the induction of systems directed towards the biological objectives of iron acquisition, management of oxidative stress and modifications to both the lipid and protein composition of the membranes. During growth on agar there is a corresponding repression of systems involved in energy production, motility and protein synthesis, in particular of non-essential iron-requiring proteins.

The ability of a single growth condition to function as a model for two distinct phenotypes is a consequence of the common denominator within each of these processes in the requirement for an immobilized growth state. This is consistent with the emerging hypothesis that specific bacterial phenotypes do not result from the expression of phenotype-specific genes but rather from the expression of particular subsets of genes that can be induced under a variety of conditions (Lazazzera, 2005).

Food-borne pathogenic bacteria such as *C. jejuni* can experience rapid transitions from a relatively dormant phase of environmental survival to life within a host. This transition is associated with dramatic shifts in nutrient availability as well as host immune defences. The systems induced during surface-associated growth bestow a phenotype that facilitates survival both within the environment, as well as upon encountering the host, through the prioritizing of systems to withstand two of the most significant challenges associated with the host: iron limitation and oxidative stress.

Table 6. Ribosomal genes induced during growth of *C. jejuni* NCTC 11168 in MH broth

Cj no.	Gene	Description*	log(Mean ratio)	Z-score	P (2-tailed)
Cj1704c	<i>rplB</i>	50S RP L2	-0.262	-8.26	2.22E-16
Cj1707c	<i>rplC</i>	50S RP L3	-0.214	-6.75	1.50E-11
Cj1706c	<i>rplD</i>	50S RP L4	-0.201	-5.87	4.41E-09
Cj0476	<i>rplJ</i>	50S RP L10	-0.250	-7.90	2.66E-15
Cj1480c	<i>rplM</i>	50S RP L13	-0.187	-5.91	3.48E-09
Cj1689c	<i>rplO</i>	50S RP L15	-0.198	-5.79	7.16E-09
Cj1700c	<i>rplP</i>	50S RP L16	-0.208	-6.56	5.44E-11
Cj1691c	<i>rplR</i>	50S RP L18	-0.214	-6.75	1.49E-11
Cj0245	<i>rplT</i>	50S RP L20	-0.204	-6.45	1.13E-10
Cj1702c	<i>rplV</i>	50S RP L22	-0.207	-6.54	6.09E-11
Cj1705c	<i>rplW</i>	50S RP L23	-0.203	-6.42	1.36E-10
Cj1696c	<i>rplX</i>	50S RP L24	-0.205	-6.46	1.02E-10
Cj1699c	<i>rpmC</i>	50S RP L29	-0.230	-7.24	4.41E-13
Cj0330c	<i>rpmF</i>	50S RP L32	-0.329	-9.62	0
Cj0244	<i>rpmI</i>	50s RP L35	-0.272	-8.59	0
Cj1591	<i>rpmJ</i>	50S RP L36	-0.241	-7.03	2.06E-12
Cj1182c	<i>rpsB</i>	30S RP S2	-0.243	-7.66	1.82E-14
Cj1701c	<i>rpsC</i>	30S RP S3	-0.256	-8.09	6.66E-16
Cj1594	<i>rpsD</i>	30S RP S4	-0.262	-8.27	2.22E-16
Cj1070	<i>rpsF</i>	30S RP S6	-0.197	-6.20	5.63E-10
Cj1693c	<i>rpsH</i>	30S RP S8	-0.266	-7.77	7.77E-15
Cj1708c	<i>rpsJ</i>	30S RP S10	-0.238	-6.94	3.97E-12
Cj1593	<i>rpsK</i>	30S RP S11	-0.304	-9.58	0
Cj1592	<i>rpsM</i>	30S RP S13	-0.333	-10.51	0
Cj1694c	<i>rpsN</i>	30S RP S14	-0.211	-6.65	2.86E-11
Cj1698c	<i>rpsQ</i>	30S RP S17	-0.228	-7.18	6.87E-13
Cj1072	<i>rpsR</i>	30S RP S18	-0.181	-5.72	1.05E-08
Cj1703c	<i>rpsS</i>	30S RP S19	-0.237	-7.47	8.17E-14
Cj1611	<i>rpsT</i>	30S RP S20	-0.204	-6.45	1.12E-10

*RP, ribosomal protein.

ACKNOWLEDGEMENTS

This work was supported by Genome Prairie, Genome BC and Inimex Pharmaceuticals through the 'Functional Pathogenomics of Mucosal Immunity' project as well as through funding from the Canadian Bacterial Diseases Network (CBDN) and the National Research Council's Genome and Health Initiative. L.A.B. is a holder of the Canada Research Chair in Vaccinology and A.A.P. is a holder of the NSERC/Bioniche Industrial Research Chair in Food Safety. DNA microarray hybridizations were performed by Oksana Mykytczuk.

Published with permission of the Director of VIDO as manuscript no. 411.

REFERENCES

- Altekruse, S. F., Stern, N. J., Fields, P. I. & Sverdlow, D. L. (1999). *Campylobacter jejuni* – an emerging foodborne pathogen. *Emerg Infect Dis* **5**, 28–35.
- Ardehali, R., Shi, L., Janatova, J., Mohammad, S. F. & Burns, G. L. (2002). The effect of apo-transferrin on bacterial adhesion to biomaterials. *Artif Organs* **26**, 512–520.
- Baillon, M. L., van Vliet, A. H., Ketley, J. M., Constantinidou, C. & Penn, C. W. (1999). An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. *J Bacteriol* **181**, 4798–4804.
- Beloin, C. & Ghigo, J. M. (2005). Finding gene-expression patterns in bacterial biofilms. *Trends Microbiol* **13**, 16–19.
- Berndtson, E., Danielsson-Tham, M. L. & Engvall, A. (1996). *Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process. *Int J Food Microbiol* **32**, 35–47.
- Bolton, F. J., Coates, D., Hutchinson, D. N. & Godfree, A. F. (1987). A study of thermophilic campylobacters in a river system. *J Appl Bacteriol* **62**, 167–176.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Carrillo, C. D., Taboada, E., Nash, J. H. E. & 15 other authors (2004). Genome-wide expression analyses of *Campylobacter jejuni* NCTC 11168 reveals coordinate regulation of motility and virulence by *flhA*. *J Biol Chem* **279**, 20327–20338.

- de Melo, M. A. & Pechere, J. C. (1990). Identification of *Campylobacter jejuni* surface proteins that bind to eucaryotic cells *in vitro*. *Infect Immun* **58**, 1749–1756.
- Garcia-Sanchez, S., Aubert, S., Iraqui, I., Janbon, G., Ghigo, J. M. & d'Enfert, C. (2004). *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot Cell* **3**, 536–545.
- Golovlev, E. L. (2002). The mechanism of formation of *Pseudomonas aeruginosa* biofilm, a type of structured population. *Microbiology* **71**, 249–254.
- Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R. & Weiss, W. (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **6**, 1037–1053.
- Holmes, K., Mulholland, F., Pearson, B. M., Pin, C., McNichol-Kennedy, J., Ketely, J. M. & Wells, J. M. (2005). *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. *Microbiology* **151**, 243–257.
- Jones, K. (2001). The *Campylobacter* conundrum. *Trends Microbiol* **9**, 365–366.
- Jones, K. & Bradshaw, S. B. (1996). Biofilm formation by Enterobacteriaceae: a comparison between *Salmonella enteritidis*, *E. coli* and a nitrogen fixing strain of *Klebsiella pneumoniae*. *J Appl Bacteriol* **80**, 458–464.
- Ketley, J. M. (1997). Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* **143**, 5–21.
- Kirchman, D. & Mitchell, R. (1982). Contribution of particle-bound bacteria to total microheterotrophic activity in five ponds and two marshes. *Water Res* **21**, 1237–1248.
- Kuhn, E., van Loosdrecht, M. C. M., Giger, W. & Schwarzenbach, R. P. (1987). Microbial degradation of nitrilotriacetate (NTA) during riverwater/groundwater infiltration: laboratory column studies. *Water Res* **21**, 1237–1248.
- LaPaglia, C. & Hartzell, P. L. (1997). Stress-induced production of biofilm in the hyperthermophile *Archaeoglobus fulgidus*. *Appl Environ Microbiol* **63**, 3158–3163.
- Lazazzera, B. A. (2005). Lessons from DNA microarray analysis: the gene expression profile of biofilms. *Curr Opin Microbiol* **8**, 222–227.
- Nachamkin, I., Allos, B. M. & Ho, T. (1998). *Campylobacter* species and Guillain-Barré syndrome. *Clin Microbiol Rev* **11**, 555–567.
- O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**, 4007–4021.
- Palyada, K., Threadgill, D. & Stintzi, A. (2004). Iron acquisition and regulation in *Campylobacter jejuni*. *J Bacteriol* **186**, 4714–4729.
- Park, S. F. (2002). The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int J Food Microbiol* **74**, 177–188.
- Pearson, A. D., Greenwood, M., Healing, T. D., Rollins, D., Shamat, M., Donaldson, J. & Colwell, R. R. (1993). Colonization of broiler chickens by waterborne *Campylobacter jejuni*. *Appl Environ Microbiol* **59**, 987–996.
- Prigent-Combaret, C., Vidal, O., Dorel, C. & Lejeune, P. (1999). Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *J Bacteriol* **181**, 5993–6002.
- Pysz, M. A., Conners, S. B., Montero, C. I., Shockley, K. R., Johnson, M. R., Ward, D. E. & Kelly, R. M. (2004). Transcriptional analysis of biofilm formation processes in the anaerobic, hyperthermophilic bacterium *Thermotoga maritima*. *Appl Environ Microbiol* **70**, 6098–6112.
- Ratledge, C. & Dover, L. G. (2000). Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol* **54**, 881–941.
- Ren, D., Bedzyk, L. A., Setlow, P., Thomas, S. M., Ye, R. W. & Wood, T. K. (2004a). Gene expression in *Bacillus subtilis* surface biofilms with and without sporulation and the importance of *yveR* for biofilm maintenance. *Biotechnol Bioeng* **86**, 344–364.
- Ren, D., Bedzyk, L. A., Thomas, S. M., Ye, R. W. & Wood, T. K. (2004b). Gene expression in *Escherichia coli* biofilms. *Appl Environ Microbiol* **64**, 515–524.
- Russell, R. G. & Blake, D. C., Jr (1994). Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. *Infect Immun* **62**, 3773–3779.
- Sampathkumar, B., Khachatourians, G. G. & Korber, D. R. (2004). Treatment of *Salmonella enterica* serovar Enteritidis with a sublethal concentration of trisodium phosphate or alkaline pH induces thermotolerance. *Appl Environ Microbiol* **70**, 4613–4620.
- Schembri, M., Kjaergaard, K. & Klemm, P. (2003). Global gene expression in *Escherichia coli* biofilms. *Mol Microbiol* **48**, 253–267.
- Stanley, K., Cunningham, R. & Jones, K. (1998). Isolation of *Campylobacter jejuni* from ground water. *J Appl Microbiol* **85**, 187–191.
- Stanley, N. R., Britton, R. A., Grossman, A. D. & Lazazzera, B. A. (2003). Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. *J Bacteriol* **185**, 1951–1957.
- Stintzi, A., Marlow, D., Palyada, K., Naikare, H., Panciera, R., Whitworth, L. & Clarke, C. (2005). Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. *Infect Immun* **73**, 1797–1810.
- van Loosdrecht, M. C., Lyklema, J., Norde, W. & Zehnder, A. J. (1990). Influence of interfaces on microbial activity. *Microbiol Rev* **54**, 73–87.
- van Vliet, A. H., Wooldridge, K. G. & Ketley, J. M. (1998). Iron-responsive gene regulation in a *Campylobacter jejuni* *fur* mutant. *J Bacteriol* **180**, 5291–5298.
- Wang, Q., Frye, J. G., McClelland, M. & Harshey, R. M. (2004). Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. *Mol Microbiol* **52**, 169–187.
- Whitely, M., Banger, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S. & Greenberg, E. P. (2001). Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**, 860–864.