

RESPONSE OF *CAMPYLOBACTER JEJUNI*  
TO pH STRESS

By

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RESPONSE OF *CAMPYLOBACTER JEJUNI*  
TO pH STRESS

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## Chapter I

### Introduction

A most remarkable property of bacteria is their capacity to weather extreme environmental stresses. Not only do they tolerate environmental stress but bacteria also thrive in environments that are extremes of pressure, temperature and salinity. These exceptional characteristics of bacteria beg questions to the curious. Why do bacteria grow in such environments? How do they do so? What general principles of biology do these bizarre lifestyles teach us? How can we exploit our knowledge of these responses to stresses, such as pH, to improve or indeed delay the growth of bacteria in such extremes? (9).

*Campylobacter jejuni* is from the delta-epsilon group of proteobacteria, and has microaerophilic, gram-negative, flagellate, and spiral bacterium- properties that it shares with the related pathogen *Helicobacter pylori* (8). Human infection is usually acquired by the consumption of contaminated food (especially poultry) or water (8). *Campylobacter jejuni* causes an acute diarrheal disease with a variety of clinical symptoms, such as fever, diarrhea, headache, abdominal pain, myalgia, vomiting, and blood in feces (5). In addition, this bacterium is associated with the development of Guillain-Barré syndrome, an autoimmune-mediated disorder of the peripheral nervous system (5). In England and the United States, *campylobacter* species are isolated from about 5% of patients with



diarrhea and the annual incidence of infection is estimated to be 50/100,000, an isolation rate which exceeds even that reported for *Salmonella* (10)

Most food borne pathogens appear to be relatively robust and have the ability to survive environmental stress (4). However, *Campylobacter* spp. are very fragile organisms that lack the genes encoding the RpoS global stress response mechanism, the oxidative stress response factor SoxRS (positive regulators for the response to superoxide stress) as well as other stress response factors such as Lrp (global regulator of metabolism), ProU (high affinity osmoregulatory uptake of compatible solutes), CspA (major cold shock protein) and RpoH (alternative sigma factor regulating the heat shock response) (7).

Enteric bacteria encounter a wide range of external pHs in their natural habitats, as well as the human digestive tract (1). To colonize the gastrointestinal tract, *Campylobacter* must be able to compete successfully in a complex and dynamic environment for nutrients and to tolerate a series of environmental insults, like acid stress in the stomach, to overcome the conditions encountered during invasion of epithelial cells.(6). Gastric acidity is a barrier through which all microbial food-borne pathogens must pass. With pH values as low as 1.5 – 2.5, the stomach is one of the most inhospitable environments of the mammalian anatomy(2, 3)

Despite the significance of *Campylobacter* species as food-borne pathogens, little is known of the response to these organisms to stressful environmental conditions, especially acid stress. This study of campylobacter stress response will utilize the enormous gene and protein database generated by the sequencing project of

*Campylobacter jejuni* NCTC 11168 genome (8). Therefore, the aim of the present study is to investigate acid stress and survival in *Campylobacter jejuni*.

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## Chapter II

### Literature Review

#### 1. Definition of acid stress

Acid stress is the cumulative biological effect of low pH and weak (organic) acids present in the environment. Examples of weak acids include volatile fatty acids (VFAs) like butyrate, propionate and acetate produced due to fermentation (6). Weak acids are toxic as a result of their uncharged, protonated forms, which can diffuse across the plasma membrane, dissociate and release a proton and lower internal pH ( $\text{pH}_i$ ) in the process (6, 44).

#### 2. Protective mechanism employed by *Escherichia coli*

Over recent years, considerable knowledge has accumulated about the systems that *E.coli* uses to survive acid stress and the regulatory network that controls their expression (16).

*E.coli* has four known inducible acid resistance mechanisms in place:

1. Acid resistance system 1 (AR 1)/Oxidative or Glucose repressed
2. Acid resistance system 2 (AR 2)/Glutamate dependent
3. Acid resistance system 3 (AR 3)/Arginine dependent
4. Lysine dependent

## **2.1 Acid resistance system 1 (AR 1) /Oxidative or Glucose repressed**

AR 1, also known as the oxidative or glucose repressed system, is expressed when cells are grown to stationary phase in Luria-Bertani broth (15). Upon the induction of this system, *E.coli* cells can survive exposure to pH 2.5 conditions. The expression of AR 1 is heavily dependent on the alternative sigma factor  $\sigma^s$  (also known as RpoS). However, the manner in which acid resistance is conferred is unknown (1, 13). Interestingly, Castine-Cornet *et al.*, (13) suggested that when *E.coli* cultures are grown at pH 8, they fail to exhibit AR 1 because these cultures appear to synthesize and secrete an inhibitor of the oxidative AR 1 system. This inhibitor is not synthesized at pH 5. Furthermore, the AR 1 system can be reactivated by washing the pH 8 grown culture or by adding glutamate or glutamine (1, 12, 13). It appears that the phenotypic recovery requires the presence of the GadC glutamate/GABA antiporter, suggesting that there may be an overlap between the oxidative and the glutamate-dependent system (1). However, the oxidative or glucose-repressed AR 1 system remains largely undefined.

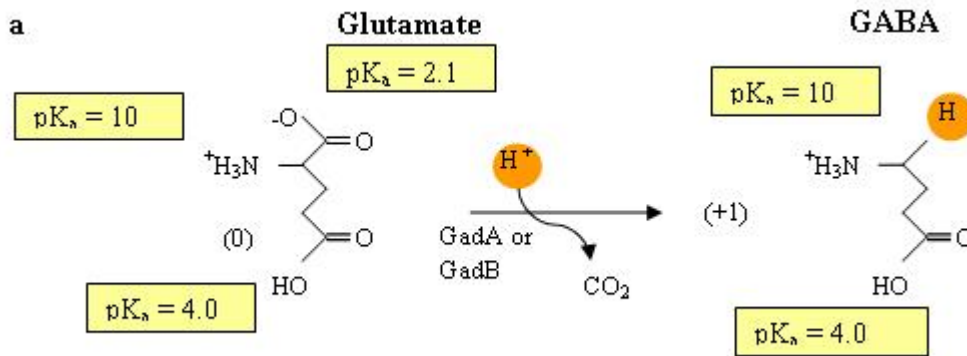
## **2.2 Acid resistance system 2 (AR 2)/ Glutamate dependent**

Of the four systems listed above, AR 2 has been the most intensely studied. AR 2 provides the highest level of acid resistance, allowing cells to survive extremely low pH challenge (pH 2) for several hours if glutamate is present in the challenge medium (13). The glutamate decarboxylase isozymes, GadA and GadB, are pyridoxal phosphate-containing enzymes that replace the  $\alpha$ -carboxyl groups of their amino acid substrates with

a proton that is commissioned from the cytoplasm (Figure 1), thus glutamate decarboxylase converts glutamic acid to  $\gamma$ -aminobutyric acid (GABA).

**Figure 1** Consumption of protons during decarboxylation of glutamate (a)

Numbers in yellow indicate  $pK_a$  values of ionizable groups. Numbers in parentheses represent the charge of the compounds during the process. Orange circles mark locations of proton addition. GABA,  $\gamma$ -amino butyric acid. Modified and adapted from (16).



The end product GABA is then exchanged via the GadC antiporter for another molecule of glutamate. The net result of the AR 2 system provides the organism with a mechanism to raise internal pH ( $pH_i$ ) as a result of the acid stress as well as contributing to an increase in the alkalization of the media by consuming protons during the conversion of glutamate to GABA. However, regulation of this system is complex and varies according to whether cells are cultured in minimal or complex media (1). At present, at least 11 regulatory proteins have been identified in the induction of AR 2 system (17).

The main activator is GadE (formerly known as YhiE). GadE binds to a 20-bp sequence called the *gad* box, which is situated 63-bp upstream of the transcription start site of *gadA* and *gadBC*(16). The *gad* box and the GadE activator are necessary for the expression of both *gadA* and *gadBC* to induce acid resistance (16). The other 10 regulators (RpoS, EvgAS, YdeO, GadE, RpoD, GadW, Crp, TrmE, HNS and TorR) change with growth phase and media composition (16).

**Table 1:** Genes involved in regulating glutamate-dependent acid resistance (16).

| <b>Protein</b> | <b>Descriptor</b>                    | <b>Function in acid resistance</b>   |
|----------------|--------------------------------------|--|
| RpoS           | $\sigma^{38}$                        | Transcription of <i>gadX</i>   |
| RpoD           | $\sigma^{70}$                        | Transcription of <i>gadA/BC</i>  |
| EvgAS          | Two-component signal transduction    | Activates <i>ydeO</i> and <i>gadE</i> transcription  |
| YdeO           | AraC-like regulator                  | Activates <i>gadE</i> transcription  |
| GadE           | LuxR-related activator               | Required for acid resistance, binds to <i>gad</i> box, activates transcription of <i>gadA/BC</i> , autoactivates <i>gadE</i> , represses <i>ydeO</i> |
| GadX           | AraC-like regulator                  | Activator of <i>gadE</i> , co-activator of <i>gadA/BC</i> , represses <i>gadW</i>  |
| GadW           | AraC-like regulator                  | Inhibits RpoS production, activator of <i>gadE</i> , can co-activate <i>gadA/BC</i> at pH 8  |
| Crp            | cAMP-receptor protein                | Inhibits RpoS production   |
| TrmE           | Era-like GTPase                      | Activates <i>gadE</i> mRNA production, stimulates translation of <i>gadA</i> and <i>gadB</i> mRNA  |
| HNS            | Histone-like protein                 | Activates <i>gadE</i> mRNA production, stimulates translation of <i>gadA</i> and <i>gadB</i> mRNA  |
| TorR           | Response regulator of TMAO reductase | Negative regulator of <i>gadE</i>  |

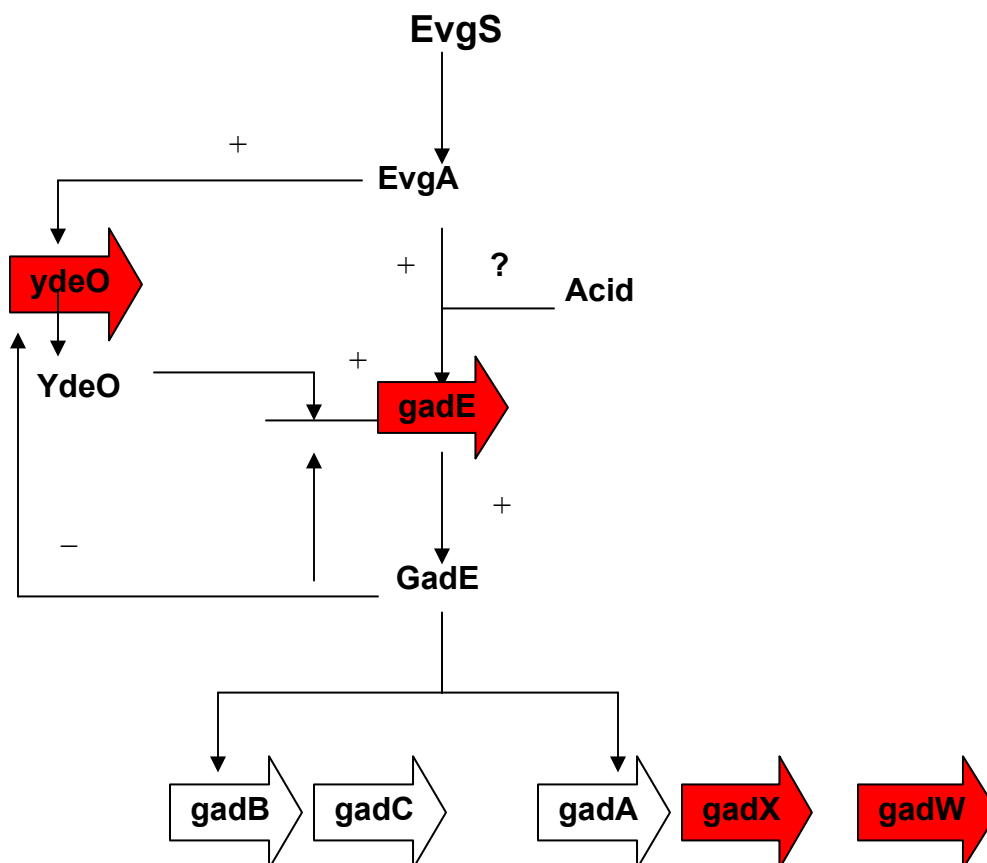


The 10 regulators form reiterative control circuits that primarily regulate expression of the crucial *GadE* activator (Table 1). The relative importance of the different circuits changes with the physiological status of the cell and the chances of experiencing extreme acid stress (pH 2.5 or less). For example, the *gadA/BC* loci can be induced during exponential growth in acidic minimal media (pH 5.5) or in stationary phase irrespective of the pH (16). Nevertheless, in complex media such as LB (Luria-Bertani broth), neither locus is induced until the culture enters stationary phase (16). In order to meet the demands of the different physiological needs, one *gadE* activation circuit functions in minimal glucose media, whereas two, three, or perhaps, other circuits regulate production of *GadE* in LB. The activation of the *gadA* and *gadBC* genes is not dependent on when transcription is initiated, rather that once it is induced, the system will provide acid resistance to pH values as low as 2.5. However, stationary phase cells display the most robust survival (16).

Work by Masuda and Church (32, 33) implicated *GadE* activation circuits in AR using microarray studies ( Figure 2) (29). The circuit pathway includes the membrane-bound sensor kinase *EvgS*, the response regulator *EvgA* and an *AraC*-like regulator known as *YdeO* (16). *EvgAS* is a two-component regulatory system with unknown function but has been the subject of several studies designed to determine its physiological role (22, 29). In particular, cells over-expressing *EvgA/YdeO* were shown to become acid resistant, even after growth at neutral pH (16). Follow-up microarray transcription profiles showed that the over expressed *EvgA* response regulator indirectly activated acid resistance by first activating transcription of *YdeO*. More recently, Foster et al, (29) have shown that

*YdeO* activates *gadE* transcription via a cascading pathway – *EvgA*-*YdeO*-*GadE* → acid resistance (29). Evidently, *EvgA* can also activate *YdeO* (29). Furthermore, data indicates that *GadE* activates itself and represses *YdeO* in a partial feedback loop. Hence, as *GadE* is produced, it begins to shut down the *YdeO* activation pathway but stimulates its own synthesis (29). Even though *EvgAS* and *YdeO* are important for inducing acid resistance during exponential growth in minimal media, these regulators contribute nothing during stationary phase. In this case, the other circuits become more important (29).

**Figure 2:** *EvgA*-*ydeO*-*gadE* branched pathway regulating glutamate-dependent AR  
Modified and adapted from (29).



The second circuit which activates the *GadE* circuit is comprised of CRP, *RpoS* and two *AraC*-like regulators, *GadX* and *GadW* (Figure 3) (16). This circuit has a strategic role in cells that have been grown in complex media but can also influence expression of *GadE* in minimal media (16). The two *AraC* like regulators, *GadX* and *GadW*, are located downstream of *gadA* but are transcribed, for the most part, by independent promoters (29). Nevertheless, *GadX* and *GadW* also bind to the *gadA* and *gadBC* *gad* box sequence and appear to repress the *gadA* and *gadBC* promoters (29). This double role of *GadX/W* aids in explaining the paradoxical reports that indicated that the *GadX/W* proteins were activators of glutamate decarboxylase under the some conditions but repressors under others (43). *GadW* in this regulatory circuit inhibits synthesis of  $\sigma^s$ , the alternative sigma factor sequence for expression of the *GadX* activator (31, 46). Therefore, a mutant lacking *GadW* over expressed *GadX*, which, in turn, stimulates transcription of *gadE* and the decarboxylase operons (31). Thus, the *GadX/W* circuit is completed by the action of *GadX*, which represses the transcription of *GadW* (16). This is most apparent during exponential growth in complex media, where *GadX* and *GadW* seem to tightly control each other (16). However, a mutant lacking *GadX* activator still shows an increase in *gadA/BC* expression at pH 8 in exponential phase due to overproduction of *GadW*, which in turn activates the transcription of *gadE* (31, 46).

The balance in this circuit is contributed by cAMP and CRP (cAMP receptor protein), which together inhibits the synthesis of *RpoS* (27). Growth in acidic conditions reduces the concentration of cAMP in the cell (30). Decreasing cAMP concentration initiates a cascade that increases expression of *RpoS*, which subsequently increases the synthesis of *GadX*. This increase then stimulates transcription of *gadE* and the decarboxylase genes in

addition to down regulating the GadX part of the circuit (30). All this evidence helps to shed some light on acid induction and regulation of the AR 2 system.

The accumulation of evidence so far suggests that acidic conditions activate *gadA/BC* transcription in at least 3 ways – by expression of the activators *GadE* and *GadX*, and by altering the activity of *GadW* (16). Foster and colleagues have proposed that *GadX*, *GadW* and CRP sense different chemical signals in the cell and that the intracellular ratio of these signals alter the balance in the regulatory loop.

The third factor that affects the expression of the glutamate decarboxylase system was recently discovered during a brute-force screen for acid sensitive mutants *in E.coli* (10). Mutation of the GTPase protein *TrmE* (also known as *MnmE*) prevented synthesis of the *gadA* and *gadBC* gene products during growth in LB containing glucose (10). No change was observed when cultures were grown in buffered LB lacking glucose and a moderate effect was observed after growth in minimal glucose media. The function of TrmE in the cell is not fully defined but it has a clear effect on tRNA modification (11, 21, 40, 50).

So far, 5-7 regulators are thought to adhere on the *gadE* regulatory region, including *EvgA*, *YdeO*, *GadE*, *GadX* and *GadW*. This is apparent since almost 800bp of non-coding DNA is located upstream of *gadE*, most of which is used to control transcription (29).

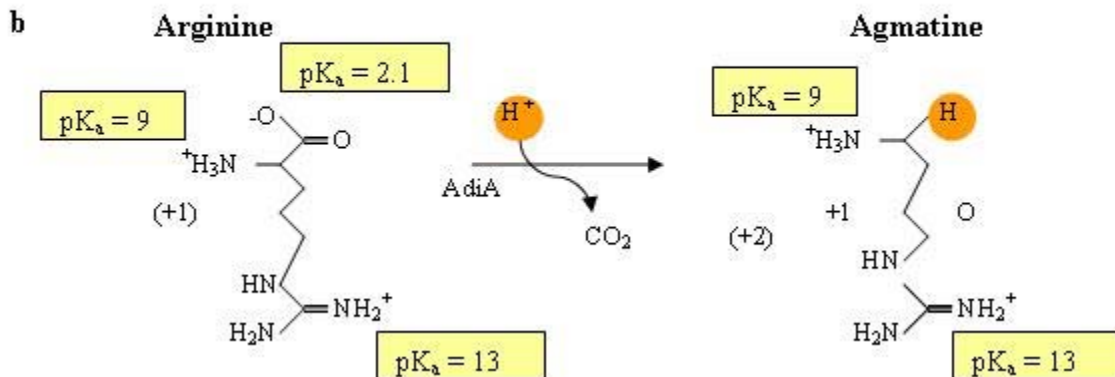
### 2.3 Acid resistance system 3 (AR 3)/Arginine

The AR3 system is not as well studied as the AR 2/Gad system. However, regulation of the arginine dependent AR 3 system seems no less complex. Below is a diagram that illustrates how protons are consumed for the decarboxylation arginine (Figure 3).

**Figure 3:** Consumption of protons during decarboxylation of arginine (b)

Numbers in yellow indicate  $pK_a$  values of ionizable groups. Numbers in parentheses represent the charge of the compounds during the process. Orange circles mark locations of proton addition.

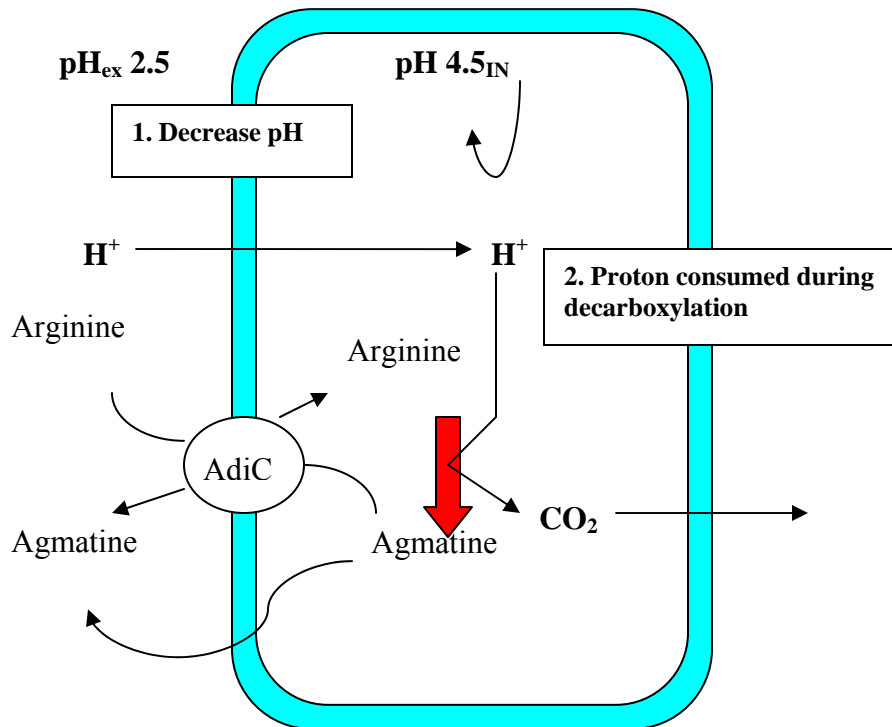
Modified and adapted from (16).



The adenine decarboxylase (*adiA*) gene is located upstream of *adiC* (agmatine antiporter), yet the 2 genes are separated by a third gene, *adiY*, which encodes another *AraC*-like regulator. *AdiY* has been implicated in activating the expression of *adiA* and *adiC*, but only when it is over expressed (45). It is likely that the environmental conditions for which *AdiY* is required have not yet been identified (16). The *adiA/C* genes are only transcribed under anaerobic conditions at low pH in complex media (16). CysB (part of the cystine biosynthesis pathway) might be an important sensor of these factors (42). The alternative sigma factor  $\sigma^s$  (*RpoS*) also has a role but like the *Gad* system, it is not required for transcription of these genes (16).

**Figure 4:** Below is a model for arginine -dependent acid resistance. Acid stress at pH 2.5 results in illicit entry of  $H^+$ , which decreases pH. As  $pH_i$  drops to around 5, arginine carboxylase will start to consume protons and convert arginine to agmatine. In this model, production of  $CO_2$  does not contribute toward internal pH or charge.

Modified and adapted from (35).



#### 2.4 Acid resistance system 4 (AR 4)/Lysine dependent

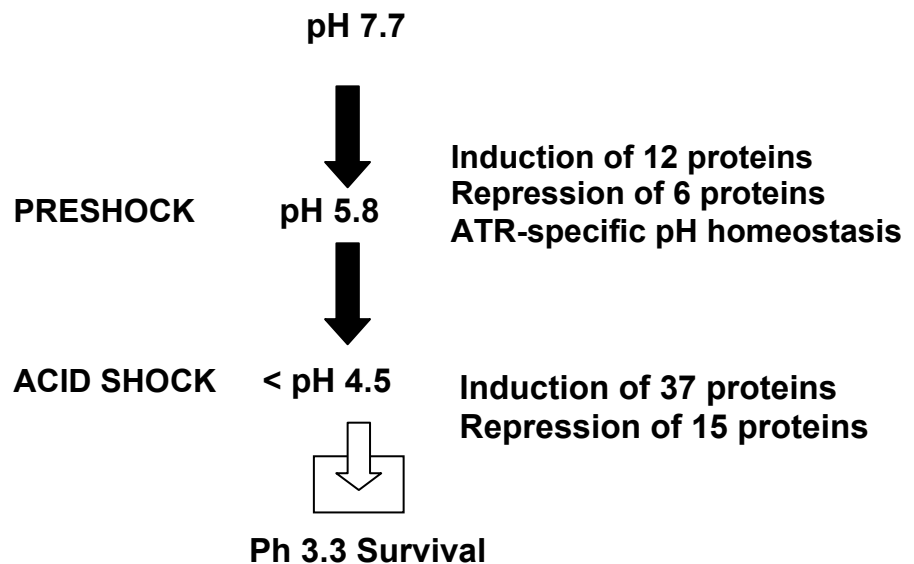
The fourth system for protecting *E. coli* against acid stress is a much less efficient mechanism that relies on lysine and its associated decarboxylase/antiporter proteins (25). *E. coli* uses lysine decarboxylase and its accompanying lysine-cadaverine exchanger, the CadBA system, to protect against mild shock. An identical set of genes work analogously in *Salmonella enterica* serovar *Typhimurium*, providing protection at pH 3.

***Salmonellae Enterica Serovar Typhimurium***

*S. typhimurium* is a neutralophilic, facultative intracellular parasite that has evolved intricate schemes to survive acid conditions (1). Serovar *typhimurium* possesses both log-phase and stationary phase acid tolerance response that protect the cells at pH 3 for several hours. This inducible acid tolerance response (ATR) is a two-stage process (Figure 6) involving overlapping acid protection systems triggered at different levels of acidity. Encounters with pH<sub>o</sub> 6 sets off the first stage (pre acid shock) involving the synthesis of emergency pH homeostasis systems that alkalinize the cytoplasm during periods of extreme acid stress (pH 3). The second stage (post acid shock) is engaged once pH<sub>o</sub> falls below 4.5 (6). Below is an illustration to show this mechanism (Figure 5).

**Figure 5:** Schematic representation depicting the two stages of ATR

Adopted and modified from (18)





The acid protection is rendered largely due to a series of acid shock proteins (ASPs) induced during adaptation (1).

### **3.1 RpoS dependent ATR**

The transcriptional regulator  $\sigma^s$ , encoded by the *rpoS* gene, is an alternative sigma factor whose level in the cell dramatically increases upon entry into stationary phase (1). This increase in  $\sigma^s$  concentration stimulates the expression of a series of genes, called the acid shock proteins, that aids in cell survival under a variety of stress conditions such as low pH, high osmolarity, oxidation and heat (24).

Sudden acidification of exponential growing cells also triggers a sharp increase in  $\sigma^s$  levels (7). Mutants defective in *rpoS*, or that produce low levels of  $\sigma^s$ , are extremely acid sensitive (1).

### **3.2 Regulation of RpoS dependent ATR**

A key feature of the acid tolerance response is the induction of  $\sigma^s$  by rapid transitions to low pH. Production of  $\sigma^s$  is extensively regulated at the transcriptional, translational and proteolytic levels (27). Protein turnover and translation play the most important roles in its induction by acid shock (Figure 7).

Audia et al, (1) provide evidence that indicates that inorganic acid ( $H^+$ ) induces signals that trigger an increase in the proteolysis of  $\sigma^s$  while, in contrast, organic

acids like acetic and propionic acid not only increase  $\sigma^s$  stability but also stimulate the translation of the *rpoS* messages. The proteolytic turnover of  $\sigma^s$  is mediated by the protease ClpXP (41) and seems to be regulated by an unusual, putative response regulator called *mviA* (mouse virulence gene A, known in *E. coli* as *rssB* (35)) . Mutations in *mviA* (*rssB*) increase the level of  $\sigma^s$  in log-phase cells due to stabilization of the protein (1). The result is elevated expression of  $\sigma^s$  – dependent genes and increased resistance to acid stress (6). Based on the lack of an obvious DNA-binding domain, it was proposed that MviA (*rssB*) exerts its effects on  $\sigma^s$  not as a DNA-binding protein but through protein-protein interactions (6). The interaction between  $\sigma^s$  and MviA (*rssB*) has been demonstrated in vitro (8), but recent, bacterial 2-hybrid analysis confirms that MviA (*rssB*) interacts with  $\sigma^s$  and with ClpX, the ATPase recognition subunit of ClpXP protease (34).

*Salmonella* will also experience acid stress in the form of exposure to organic acids (volatile fatty acids, VFAs). VFAs are produced by commensal microbes in the cecum as a result of fermentation reactions. The major volatile fatty acids are acetate, propionate and butyrate (1). It should also be noted that adaptation to inorganic acids provides protection against VFAs and that adaptation to VFAs will protect the cell against inorganic acids (2). Inorganic acid stress leads to the accumulation of  $\sigma^s$  by down-regulating proteolysis, but stress imposed by organic acids will also stimulate translation. Translational control of *rpoS* is quite complex, involving a variety of proteins and small untranslated RNAs (9). Audia et al (1), recently discovered a new regulator of *rpoS* translation, called *DksA* (encodes for a protein of unknown

function) that may play a role in VFA acid shock induction of  $\sigma^S$ . Genetic analysis has revealed that *DksA* is a positive regulator of *rpoS* translation (49). *DksA* expression appears to be required for the optimal translation of *rpoS* based on *dksA* mutant effects on *rpoS* transcriptional and translational lacZ fusions. Furthermore, recent studies seek to determine the mechanism by which *DksA* regulates *rpoS* translation.

### **3.3 Regulation of RpoS independent ATR**

This regulatory system affects the log-phase ATR involved in the two-component *PhoPQ* system (17). The *PhoPQ* regulon is known to be important for macrophage survival, protection against antimicrobial peptides and virulence. In an elegant series of experiments, Groisman and colleagues have demonstrated that the membrane-bound *PhoQ* sensor-kinase senses periplasmic  $Mg^{2+}$  concentrations and phosphorylates the response regulator *PhoP*. *PhoP-P* will then activate the expression of a series of target genes. The first inkling that this system was involved with acid tolerance came when *PhoP* was identified as an ASP (17). Subsequently, mutations in *phoP* and *phoQ* proved to make cells sensitive to inorganic acids in an *rpoS* mutant, but *PhoPQ* proved nonessential for log-phase acid tolerance in the presence of  $\sigma^S$  (5). This study also found that *PhoQ*, in addition to sensing magnesium, can probably sense pH even in the presence of high magnesium concentrations. Therefore, in the macrophage phagolysosome environment where pH is acidic and  $Mg^{2+}$  levels are low, either one or both of these conditions may be involved in activating the *PhoPQ* system (17).

*PhoPQ* controls the expression of another two-component system (*PmrAB*) involved in activating polymyxin resistance (17). Consistent with *PhoPQ* being an acid shock protein, was the finding, that an acidic environment also induces the *PhoP*-dependent polymyxin resistance (23).

### **3.4 Role of the Ferric uptake regulator in ATR**

The iron-regulatory protein, Fur (ferric uptake regulator), was first discovered as a negative regulator of genes involved in the assimilation of exogenous iron (18). In addition, Fur appears to positively regulate ASP synthesis in an iron independent manner (19). While fur mutants are acid sensitive due to the loss of a subset of Fur-dependent ASPs, how Fur mediates induction of ASP synthesis is not known (17).

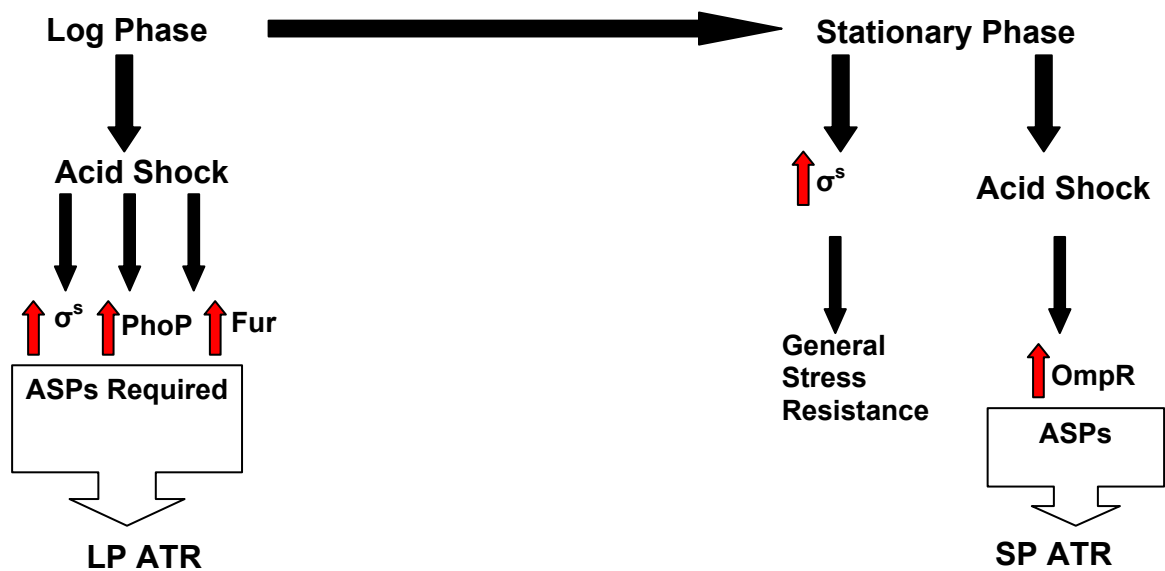
### **3.5 Acid Induced RpoS independent stationary-phase ATR**

*Salmonella typhimurim* possesses two independently regulated stationary phase acid tolerance systems. The first is part of a general stress response that is induced as cells enter stationary phase. This is pH-independent, but is dependent on the alternative sigma factor  $\sigma$  (1). The second ATR system, referred to as the stationary-phase ATR, is induced by exposing stationary-phase cells to low pH (28). The second ATR has been shown to provide a higher level of acid resistance than log-phase ATR and involves the synthesis of fewer proteins (28).

Recently, genetic studies have revealed that the two component response regulator, OmpR, is the master regulator of this stationary phase acid tolerance (3, 4). Insertion mutations within *ompR* gene render stationary-phase cells acid sensitive with almost complete ablation of the inducible ATR (3). OmpR is part of a classic two-component regulatory circuit initially discovered to respond to osmolarity (20). This system is composed of the membrane bound sensor kinase, *EnvA*, and the cytoplasmic response regulator, *ompR* (1). Normally *ompR* is phosphorylated by its cognate transmembrane sensor kinase (*EnvZ*) in response to changes in osmolarity. Interestingly, *ompR* regulates the stationary phase ATR system independently from its known sensor kinase, *EnvZ* (20).

**Figure 6:** Comparison of log-phase and stationary-phase acid tolerance responses of *S. enterica* serovar *Typhimurium*.

Modified and Adapted from (17).



#### **4 ATR response in *Campylobacter* spp.**

Studies in *Campylobacter* spp. have shown a large degree of variation in the survival kinetics obtained with different strains and in different media (14). Murphy et al. (36) have determined a considerable difference in the resistance of stationary phase cells of *C. jejuni* CI 120 to a challenge of pH 4.5, when grown in different media.

These results show that the medium used can have a major influence on growth under stressful conditions and on survival kinetics of *Campylobacter* spp. that are exposed to lethal challenge. They have also shown that ATR to acid was induced by early stationary phase cells but not by late stationary phase cells (37). In addition, stationary phase cells of *C. jejuni* are relatively more acid sensitive than mid-exponential phase cells (26). Other organisms, such as *E. coli* and *Salmonella*, induce an ATR in mid-exponential phase, but the stationary phase response is difficult to interpret since these organisms express the global stationary phase stress response mediated by RpoS. Analysis of the *C. jejuni* NCTC 11168 genome sequence shows that it does not contain the genes encoding for *rpoS* (39). Murphy et al (36) have also observed the production of an extracellular component that confers stress resistance against acid challenge in *C. jejuni*. The extracellular component appears to be phase-specific (36). These results suggest that stationary phase cells produce a slightly modified or different extracellular component than the mid-exponential phase cells or that cell receptors are different in each stage of growth (36). It is also interesting to note that of the strains tested only the natural isolates produced the protective effect (36). This raises the question as to whether the genome sequenced has lost the ability to produce the compound (36), or whether the natural

isolates have survived in the environment as a result of their ability to produce an extracellular protective compound (36). There appears to be a considerable amount of variation in the methodologies used as well as strain variation to study ATR response in *C. jejuni* so far.

Acid stress response in enteric organisms is critical for their successful passage through the gastrointestinal tract and for surviving other acidic environments. The overlapping regulatory circuits of acid resistance and the acid tolerance response are extremely complex (1). The systems discussed above represent models of metabolic interlock in which cells coordinate thousands of biological reactions in order to optimize maximal survival under conditions of extreme stress. Progress has been made in terms of identifying the genes and proteins that bear the brunt of pH stress.

Nevertheless, the importance of battling protons is widely recognized for the enteric organism's pathogenesis. Campylobacteriosis is one of the most common causes of bacterial food borne diseases and hence has a significant social and economic consequence worldwide. Thus, there is an urgent need to address the diseases caused by these organisms. The unusual sensitivity elicited by *C. jejuni* is a striking feature, since it does not obey many of the physiological paradigms established by model organisms such as *E.coli* and *Salmonella* (38). Intriguingly, even today after the publication of the complete genome sequence of NCTC 11168 (39), the study of the response to acid stress of this bacterium has hardly scratched the surface in understanding the mechanism used to surmount acid stress.

Explorative approaches such as DNA microarray technology are becoming increasingly important in microbial research. Despite these major technical advancements, approaches to study acid stress are still lacking for *C.jejuni*. The acid-stress responses of *E.coli* have been the focus of several microarray studies. Tucker and co-workers examined pH 5.5 acid-induced gene expression in exponential-phase minimal media-grown cells (47). The authors identified 28 genes that were induced under these conditions, 11 of which seemed to be under the control of GadX (48). Masuda and Church examined the transcriptional patterns that were produced when certain regulators were over expressed (47). The regulators selected were those that, when overproduced, lead to acid resistance (16). The results were indicative of both the power and limitations of microarray studies (16). Therefore, although microarray studies are beneficial in identifying potential regulators, physiological studies must be carried out to confirm their significance.



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**CHAPTER III**

**RESPONSE OF *C.JEJUNI* TO pH STRESS**

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## Chapter III

### **Abstract.**

Enteric pathogens, in order to transit through the gut and cause disease, must survive the acid pH of the stomach. Pathogenic species that prefer to grow at neutral pH display a wide range of mechanisms to survive extreme pH. *Campylobacter jejuni* is the leading cause of bacterial food-borne diarrhea throughout the developed world. In this study, we used DNA microarrays to measure the global change in transcript levels over time due to pH stress. The transcript levels of 325 genes were affected over a 20 min time period. Genes encoding heat shock proteins, chaperonins, cell signaling and oxidative stress defense were up-regulated. This gene profile suggests that *C. jejuni* quickly readjusts its transcript levels to a new steady state, thus allowing the bacterium to survive the stress. These findings provide new insights of the *C. jejuni* mechanisms for acid stress and how the bacterium survives and adapts its transcriptome to a new growth condition.

Prokaryotic organisms faced with constantly changing environments have the amazing ability to sense and respond to stressful situations (17). This ability to sense and respond to potentially lethal changes in the environment is a crucial trait required for the survival of these microorganisms (9). Sudden as well as gradual exposures to acid stress occur in a variety of ecological niches occupied by food-borne pathogens (1). Acid resistant food-borne pathogens can be capable of surviving in acidic and fermented food for extended periods of time and they can tolerate the very low pH of the human stomach (7). Some organisms are endowed with an adaptive stress response that gives them the ability to survive exposure to extreme acidic environments (17). Enteropathogenic microorganisms possess the ability to endure harsh conditions encountered in their natural environment and within a host organism during pathogenesis (3). Neutralophiles like *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri* have to endure extreme low pH in the stomach as well as volatile fatty acids present in the intestine and feces (5) during their travels through the gastrointestinal tract. Varying terminologies have been used to describe acid stress response systems (5). Acid resistance (AR), acid tolerance (AT) and acid habituation (21) are all terms used to describe survival to low pH stress under varying conditions (5). The acid resistance (AR) and acid-tolerance responses (AT) have been well studied in *Escherichia coli*, *Salmonella enterica* and *Shigella flexneri*, in which it has been shown that exposure to sublethal pH induces the expression of numerous acid-shock proteins (ASPs) that in turn promote bacterial survival in extreme acid environments (9). Such AT or AR systems function in both Gram-negative as well as Gram-positive bacteria, and an increasing amount of evidence supports the fact that this stress response is an important component of survival of bacterial pathogens within

the host niche (17). Several amino acid decarboxylases have been identified in *E.coli* and the presence of these glutamate, arginine and lysine decarboxylases are linked to its ATR (14). These amino acid decarboxylase-dependent AR systems require the availability of exogenous amino acid in the acidic growth media, and it is assumed that they protect the bacterial cell by alkalization of the cytoplasm during amino acid decarboxylation (9).

*Campylobacter jejuni* is the leading cause of bacterial gastroenteritis and food poisoning in developed countries, affecting more people than *Salmonella* and *Shigella* spp. combined (18). *C. jejuni* is most commonly transmitted to humans by consumption of contaminated poultry, cross contamination of other food matter with raw poultry, and other sources such as contaminated water and milk products (22). *C. jejuni* has very fastidious growth and survival requirements (10). Nevertheless, *C. jejuni* must possess mechanisms for surviving a wide range of environmental stresses, both inside and outside of its natural zoonotic hosts (10). In contrast to most enteric bacteria, *C. jejuni* does not become more stress resistant during stationary phase and has been described as lacking a “classical” stationary phase response (13). In addition, an analysis of the *C. jejuni* NCTC 11168 genome sequence indicates that it also lacks the stationary phase sigma factor RpoS (20), which modulates stress and stationary phase responses in many Gram-negative bacteria (12). Consequently, these observations raise the following question: “How does *C. jejuni* overcome the variable pH environments of the host in order to successfully transit through the harsh milieu of the stomach and colonize the distal end of the small intestine?”

## **Materials and Methods**

### ***Bacterial strain and growth conditions***

The bacterial strain used in this study was *Campylobacter jejuni* NCTC 11168. It was obtained from the National Collection of Type Culture (NCTC, England). *C. jejuni* was routinely maintained on Mueller Hinton (MH) agar plates at 37°C under microaerophilic conditions (83% N<sub>2</sub>, 4% H<sub>2</sub>, 8% O<sub>2</sub>, and 5% CO<sub>2</sub>) with the use of a MACS-VA500 microaerophilic workstation (Don Whitley, West Yorkshire, England).

### ***Microarray construction***

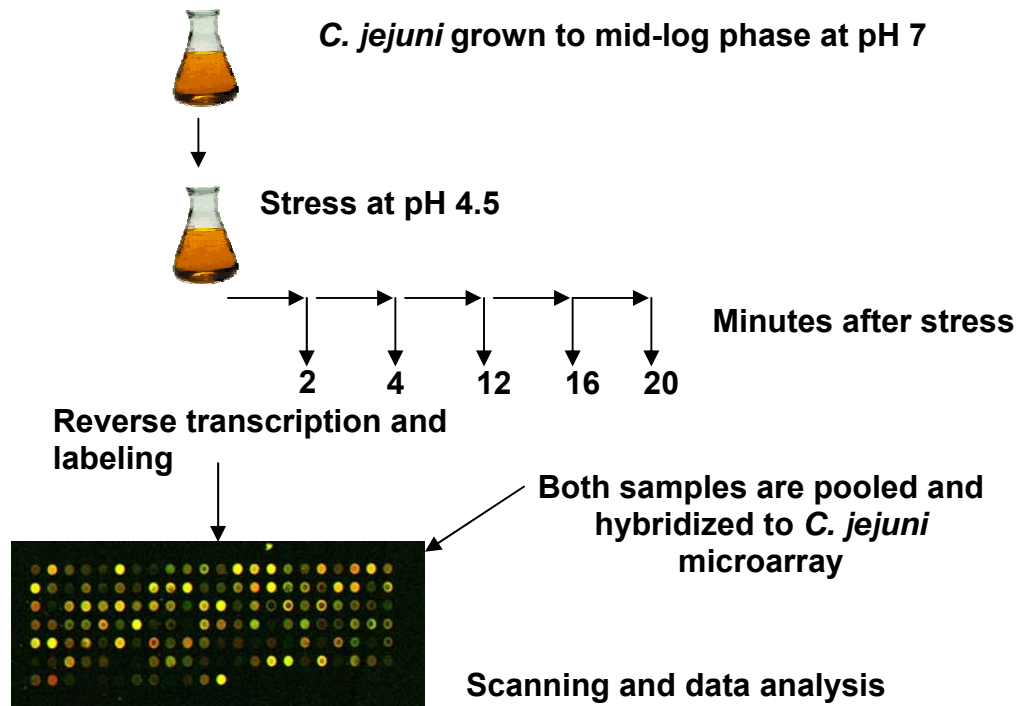
The *C. jejuni* NCTC 11168 microarray was constructed as described below. Of the 1654 ORFs identified from the genome sequence of NCTC 11168, 3,308 primers were designed using the PRIMER3 software (Code available at: [http://www.genomewi.mit.edu/genome\\_software/other/primer3.htm](http://www.genomewi.mit.edu/genome_software/other/primer3.htm)). These primers were selected using an optimum primer size of 20 nucleotides, a PCR product size between 200 and 600 bp, and an optimal T<sub>m</sub> of 60<sup>0</sup> C. About 98% of the ORFs were successfully amplified. 20 ng of genomic DNA was used as a template in the first round of PCR amplifications using standard methods in a 96-well plate format. The quality of the PCR products was analyzed by 3.4% agarose gel electrophoresis. Successful PCR products were re-amplified in order to reduce the amount of residual genomic DNA carried-over from the first PCR. Those PCR reactions without product or those with incorrectly sized products were performed again by modifying the reaction conditions or by designing new primers. PCR product purification was done using the Millipor PCR<sub>96</sub> cleanup kit and further quantified using the PicoGreen dsDNA quantitation reagent from Molecular Probes. The PCR products were diluted into a 50% DMSO solution at a concentration of

75 ng/ $\mu$ l, and re-arrayed into a 384-well format. These were then printed on aminosilane-coated glass microscope slides (CMT GAPS-II from Corning Inc., Corning, N.Y.) using an arrayer robot (OmniGrid Arrayer) in a repeating 20 $\times$ 10 spot pattern. Each probe was printed in triplicate. Lastly, the DNA fragments were immobilized by baking at 80°C for 4 hours. The quality of the microarray printing, the efficiency of the DNA binding to the slide and spot morphology was assessed by direct labeling of the spotted DNA with a fluorescent nucleic acid stain (POPO-3 iodide from Molecular Probes). Finally, the hybridization capacity of the bound DNA was confirmed by using fluorescently labeled genomic DNA.

***Mid-log Phase acid challenge experiment 1:***

We chose to study the *C. jejuni* response to pH challenge over a time frame of 20-min. *Campylobacter* spp. was grown microaerobically in a 25 ml biphasic flask at 37°C in Mueller-Hinton medium to an optical density at 600 nm of 0.9. Five ml of the growth medium was then added to 20 ml of an MH medium buffered to pH 4.5 using MES (2-(N-morpholino) ethane sulfonic acid). Total RNA was isolated at 2, 4, 12, 16, and 20 min after acid challenge. The figure below illustrates the experimental design (Figure 1)

**Figure 1.** Experimental design of experiment 1



***Sampling and isolation of total RNA.***

The acid challenged *Campylobacter* cells were grown in 20 ml of MH broth in biphasic flasks under microaerobic conditions at 37°C. At mid-log phase (optical density at 600 nm of approximately 0.9), a 5 ml sample was removed and added to the 20 ml of MH medium buffered to pH 4.5 using MES. Immediately after the 2, 4, 12, 16, and 20 min acid challenge, 2.5 ml of cold RNA degradation stop solution (10% buffer-saturated phenol in ethanol), which has been shown to keep the bacterial transcriptome intact (6), was added and samples were rapidly mixed and placed on ice. The acid challenged *Campylobacter* broth was centrifuged at 4°C (10min, 8,000 x g), and the pellet was

resuspended in lysozyme-TE buffer (50 mM Tris-Cl [pH 8], 1 mM EDTA, 0.5 mg/ml lysozyme). Total RNA was isolated by using a hot phenol-chloroform protocol (25). Upon ethanol precipitation, the RNA was resuspended in RNase-free water, and remaining traces of genomic DNA were removed by DNase I treatment. The absence of genomic DNA was confirmed by PCR. RNA was quantified by using the RiboGreen RNA quantitation reagent (Molecular Probes). RNA integrity was further checked by 1% agarose gel electrophoresis. Total purified RNA was stored at -80°C.

***Probe labeling and slide hybridization.***

Sixteen µg of total RNA from each time point was reverse transcribed as follows. 16 µg of total RNA was mixed with 10 µg of random hexamers (10 µg/µl; Amersham Pharmacia) in a 34.35 µl reaction mixture comprising of 8 µl of 5X Superscript II reverse transcriptase buffer (Invitrogen) and 2 µl of 0.1M aminoallyl-dTT at 65°C for 5 min. Immediately after the 5 min incubation, the reaction mixture was brought to a final volume of 40 µl by the addition of : 0.5 mM each of dGTP, dATP, and dCTP; 0.16mM dTTP; 0.34mM aminoallyl-dUTP; and 2 µl of the Superscript II enzyme (Invitrogen) and further incubated at 42 °C for 2 hours. Subsequently, the RNA was hydrolyzed by the addition of 4 µl of 50 mM EDTA and 2 µl of 10N NaOH and incubating at 65 °C for 20 min. This reaction mixture was neutralized by adding 4 µl of 5 M acetic acid. Free amines from the reaction mixture were removed by adding the mixture to 450 µl of water and spinning through a Microcon 30 (Millipore) for 8 min at 10,000 x g. This step was repeated four times. After the last wash, the labeled probes were concentrated to less than 8 µl using vacuum in a SpeedVac and adjusted to a final volume of 10 µl by adding 1 µl

of 1M sodium carbonate (pH 9.0, freshly made). The probe was coupled to monoreactive fluors (Amersham) by adding 10  $\mu$ l of dimethyl sulfoxide containing one-sixth of one reaction vial FluoroLink indocarbocyanine or indodicarbocyanine dye and incubating for 1 hour at room temperature in the dark. The Fluorescent indocarbocyanine- and indodicarbocyanine-labeled cDNAs were mixed and purified with Qiaquick PCR spin columns according to the manufacturer's instructions (Qiagen, Valencia, Calif.). The fluor-labeled cDNA mix was dried under vacuum with a SpeedVac and resuspended in 15.14  $\mu$ l of water, to which was added 2.5  $\mu$ l of salmon sperm DNA (10 mg/ml), 9  $\mu$ l of 20x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7), 0.36  $\mu$ l of 10% sodium dodecyl sulfate (SDS), and 9  $\mu$ l of formamide. Before hybridizing, microarray slides were prehybridized at 42°C for 1 hour in a prehybridization buffer (25% formamide, 5x SSC buffer, 0.1% SDS, and 1% bovine serum albumin), rinsed with water, and spin dried. The probe solution was denatured by boiling at 99 °C for 2 min, followed by cooling to 42°C. The probe was then applied to the microarray slide under a coverslip (Fisher), placed in a humidified chamber (ArrayIt), and incubated at 42°C for 16 hours. Following hybridization, slides were washed for 5 min with 2x SSC-0.1% SDS which was preheated to 42°C for 5 min. Next, the slides were washed at room temperature in 0.1x SSC-0.1% SDS for 10 min, followed by four washes for 1 min at room temperature in 0.1x SSC . Finally, slides were rinsed with distilled water and spun dried.

#### ***Data collection and analysis.***

The microarray slides were scanned using a laser-activated confocal scanner ( ScanArray 5000) at 532 and 635 nm, at 10- $\mu$ m resolution. Fluorescent signal values were obtained

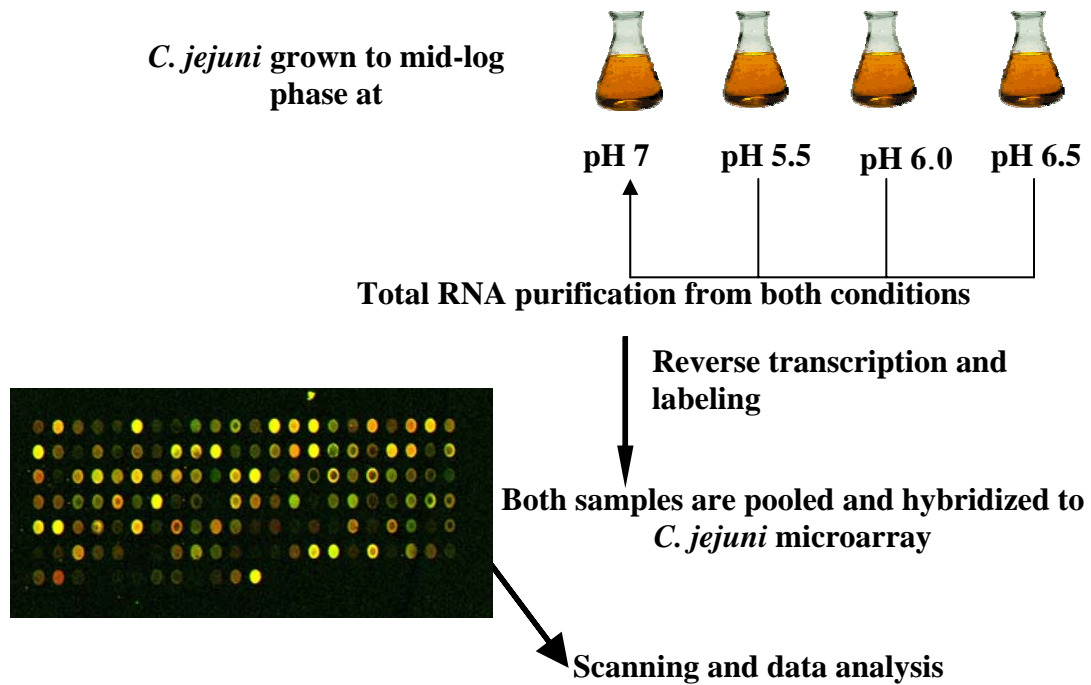


by using GenePix Pro 4 software (Axon Instruments, Foster City, Calif.). Spots were removed from further analysis if either of two criteria was met: (1) the spots were localized in a regions of hybridization abnormalities or slide abnormalities, (2) or the fluorescent mean intensities in both channels, 1 (Cy5) and 2 (Cy3), were below three times the standard deviation of the local background. From the second criterion, all negative controls were uniformly excluded from the microarray data. Upon normalization (using a Lowess function as previously described (19)), the ratio of channels 2 to 1 was  $\log_2$  transformed, and the data were statistically analyzed with the empirical Bayes method (15). The time course experiment was repeated twice (biological replicate), and up to three measurements were generated per experiment (technical replicate). Genes were selected as being differentially expressed if their p-value was below  $10^{-6}$  and their fold change in transcript abundance was above 2. Finally, genes were grouped by hierarchical clustering analysis using the Genesis software available from Graz University of Technology (<http://genome.tugraz.at>).

### ***Steady state experiment 2***

*C. jejuni* NCTC 11168 was cultivated in biphasic flasks until stationary phase. Then an aliquot of this was taken to start growth curves (Figure 6) of MH-MES broth buffered to pH 5.5, 6.0, and 6.5. The optical density at 600 nm of the inoculum was 0.05. The RNA was then isolated at mid-log phase with samples having an approximate optical density at 600 nm of 0.9. The figure below illustrates the experimental design (Figure 2)

**Figure 2.** Experimental design of steady state experiment 2



### ***Survival assay experiment 3***

The *Campylobacter* cells were grown at 37° C till mid-log phase in MH medium to OD<sub>600</sub> of 0.9. Two and half ml of this medium was then added to 10 mL of MH-MES broth buffered to pH 4.5. At the time points 0, 2, 4, 12, 16, and 20 minute, samples were collected, serial diluted and plated in triplicate. The plates were incubated for 48 hrs at 37°C under microaerophilic conditions. Finally, the cfu/ml (colony forming units) were

then counted and enumerated. To note, the data presented in this study are preliminary and need to be repeated for better statistical accuracy.

## **Results and Discussion**

### ***Experimental design, statistical analysis, and validation of the microarray data.***

The experiments presented here were designed to determine the global change in gene expression profile elicited by *C. jejuni* NCTC 11168 in response to acid stress at pH 4.5. Two sets of experiments were performed. The first experiment addressed the immediate response of *C. jejuni* gene expression to a sudden acid challenge. The changes in transcript levels were determined as a function of time after the acid shock.

*Campylobacter jejuni* was cultivated to mid-log phase in Mueller Hinton broth.

*Campylobacter* spp. was grown microaerobically in a 25 ml biphasic flask at 37°C in Mueller-Hinton medium to an optical density at 600 nm of 0.9. Five ml of the growth medium was then added to 20ml of an MH medium buffered to pH 4.5 using MES. Total RNA was isolated at 2, 4, 12, 16, and 20 min after acid challenge. All total RNA samples were reverse transcribed and fluorescently labeled as previously described (23). Finally, the relative abundance of gene transcripts at each time point after the acid challenge was compared with the level of transcripts at pH 7 by using the *C. jejuni* NCTC 11168 microarray. Each hybridization was repeated twice, yielding up to six technical replicates from each time point given that each microarray slide contained each gene in triplicate. In addition, another independent time course experiment was performed, constituting a biological replicate. A total of up to 12 measurements per gene were analyzed. The data was normalized, merged, and reported as the log<sub>2</sub> ratios of the transcript abundance of *C. jejuni* upon acid challenge. The significance of the differential abundance of transcripts

was analyzed using a regularized *t*-test based on a Bayesian statistical analysis of variance (4). This statistical method has been shown to be more reliable than a simple *t*-test by significantly reducing the number of false positives in the selected data set. Genes were selected as being differentially expressed if their p value was below  $10^{-6}$  (which corresponds to a significance level below 0.01 after Bonferroni correction) and if their change in transcript abundance was above 2 fold in at least one of the 5 time points following acid shock.

In the second experiment *C. jejuni* was grown in biphasic flasks till mid-log phase, and then an aliquot of this was taken to start growth curves (Figure 4) as well as samples for MH-MES broth buffered to pH 5.5, 6.0, and 6.5. Total RNA was isolated at mid-log phase from each of the growth media at OD<sub>600</sub> of approximately 0.9. The total RNAs were reverse transcribed and fluorescently labeled. Finally, the relative level of gene transcripts was monitored by competitive hybridization to our *C.jejuni* microarray cDNA obtained from *C.jejuni* growth at pH 7 (labeled with the green fluor) and at pH 6.5, 6.0, or 5.5 (labeled with the red fluor)

## **Global analysis and kinetics of *C. jejuni* gene expression in response to acid challenge**

To visualize the temporal expression of genes in response to acid challenge we subjected our microarray data to hierarchical clustering analysis.

From the time course experiment the transcript abundance profile was grouped into 5 major clusters, named A, B, C, D and E (Figure 3).

Globally, the transcript abundance of 324 genes was found to be significantly altered during this experiment.

Cluster A contains 4 genes that have their transcript abundance level most highly up-regulated at 15 and 20 min following acid challenge. As a consequence, these are likely required for *campylobacter* to overcome acid shock.

These genes are *metA* (putative homoserine O-succinyltransferase), *metY* (putative homoserine O-succinyltransferase), *Cj 0414* (putative oxidoreductase subunit), *Cj0876c* (a putative periplasmic protein) and *Cj 1586* (putative bacterial hemoglobin).

Interestingly, a *Cj 1586* deficient mutant of *C. jejuni* has been shown to display increased sensitivity towards nitrosative stress (8). While the expression of this gene has been previously shown to be induced by nitric oxide, our study suggests that it is also induced by acidic pH. The chemical production of nitric oxide from nitride in the stomach is thought to be a powerful defense against gut pathogens. Therefore, the up-regulation of *Cj 1586* in response to a low pH might allow the bacteria to deal with nitric oxide present in the stomach and to successfully transit towards the gut.

Cluster B displays genes that are most rapidly and highly up-regulated. The genes in this cluster were immediately up-regulated after the pH challenge. This cluster is dominated by genes encoding for chaperones, chaperonins, protein transport, periplasmic membrane, and heat shock regulators. Specifically, *dnaK* (heat shock protein), *groEL* (60 kD chaperonin), *hrcA* (putative heat shock regulator), *grpE* (heat shock protein) *clpB* (ATP-dependent CLP protease ATP-binding subunit) *pebC* (ABC-type amino-acid transporter ATP-binding protein), *exbB2* (putative exbB/tolQ family transport protein), *wlaI* (putative transferase), *katA* (catalase), *p19* (periplasmic protein). Genes from the heat shock regulon have been intensively studied and have been shown to be induced in response to stresses. These proteins act by repairing and preventing damage caused by accumulation of unfolded proteins. *DnaK* and *GroEL* also play an important role under normal physiological conditions by assisting in the proper folding of newly synthesized proteins. Consequently, these genes might allow *campylobacter* to cope with the changes caused by a sudden change or shift of pH.

Genes that are repressed at 2 min and then upregulated between 4-12min include *AcnB*, which encodes for aconitate hydratase and has recently been shown to initiate a regulatory cascade controlling flagella biosynthesis in *Salmonella enterica* by binding to the *ftsH* transcript and inhibiting the synthesis of the *FtsH* protease (24). This is in concordance with our data, which also shows that 3 flagellum genes (*flgI*, *flgD* and *flaC*) follow the same profile. In addition, genes encoding for the amino-acid *PebC* transport, (ABC-type amino-acid transporter ATP-binding protein), *Cj0919c-Cj920c* (putative ABC-type amino-acid transporter permease protein), *kgtP* (alpha-ketoglutarate permease),

*dcuB* (putative anaerobic C4-dicarboylate transporter) and *Cj1661* (putative ABC-type amino-acid transporter permease protein) are included in this cluster. One reason for this can be that these transporters are involved in the acquisition and uptake of amino acids enabling *campylobacter* to surmount the pH challenge. A group of genes from cluster B that are worth noting encode for proteins involved in the oxidative stress defense. These are the alkyl hydroperoxide reductase (Ahpc), the catalase (KatA), the ferritin (*cft*), a thioredoxin reductase (TrxB), and the peroxide stress regulator (PerR). The up-regulation of these genes indicates a strong correlation between the acid stress and oxidative stress responses. One possible explanation for this phenomenon could be that low pH increases the toxicity of oxygen radicals. This connection between these two functional groups of genes in *Campylobacter* is in close agreement with the recent observation that pH regulates the oxidative stress in E.coli K12 (16).

Cluster C contains genes that have their transcript abundance decreased at 2 and 4 min following acid challenge. The transient down-regulation of these genes during the first few minutes reflects the primary response of *campylobacter* to surmount the sudden pH change, allowing the bacteria to survive and ultimately adapt to the new growth conditions. This cluster contains genes encoding proteins of unknown function and ribosomal proteins. The down regulation of these genes in response to acid stress is unclear and requires further investigation.

Cluster D consists of genes whose expression increased immediately after the acid challenge and then decreased with time. Some of the genes to note are *napA*, which

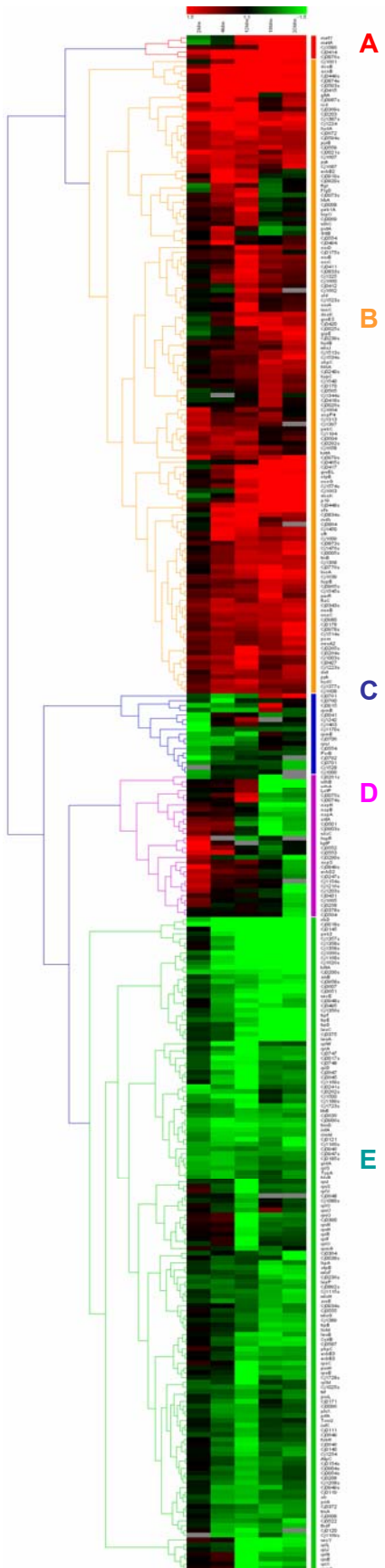
encodes for periplasmic nitrate reductase, *napB* which encodes for periplasmic nitrate reductase and *napH* which encodes for putative ferredoxin. A subgroup of noteworthy genes encode for succinate dehydrogenase (*sdhABC*). This enzyme complex is a membrane component of the tricarboxylic acid cycle, and it catalyzes the oxidation of succinate to fumarate. The observed differential expression of the *sdhABC* gene is puzzling. Indeed the expression of these genes has been shown to be induced in *E.coli* in response to acidic conditions. The consumption of acids by the tricarboxylic acid cycle is believed to alkalize the cytoplasm and thus contributes to acid survival and adaptation. Therefore, down-regulation of the genes encoding this enzyme in *C.jejuni* is an unexpected finding and requires further study.

Cluster E consists of transcripts that are constantly repressed. The most notable subgroup of genes with repressed expression includes a number of genes involved in ribosomal protein synthesis and modification ( *rplW* (50S ribosomal protein L23), *rplA* (50S ribosomal protein L1), *rplD* (50S ribosomal protein L4), *rpsS* (30S ribosomal protein S19), *rplV* (50S ribosomal protein L22), *rplQ* (50S ribosomal protein L17), *rpsQ* (30S ribosomal protein S17), *rpsN* (30S ribosomal protein S14), *rpsH* (30S ribosomal protein S8), *rplR* (50S ribosomal protein L18), *rplF* (50S ribosomal protein L6), *rplO* (50S ribosomal protein L15), *rplL* (50S ribosomal protein L7 /L12), *rplJ* (50S ribosomal protein L10), *rpsE* (30S ribosomal protein S5), *rplN* (50S ribosomal protein L14), and *rplX* (50S ribosomal protein L24)). The repression of these ribosomal genes demonstrates a brief growth arrest that might allow the bacterium to rechannel energy devoted to an increased expression of genes involved in protective responses and adaptation to the new

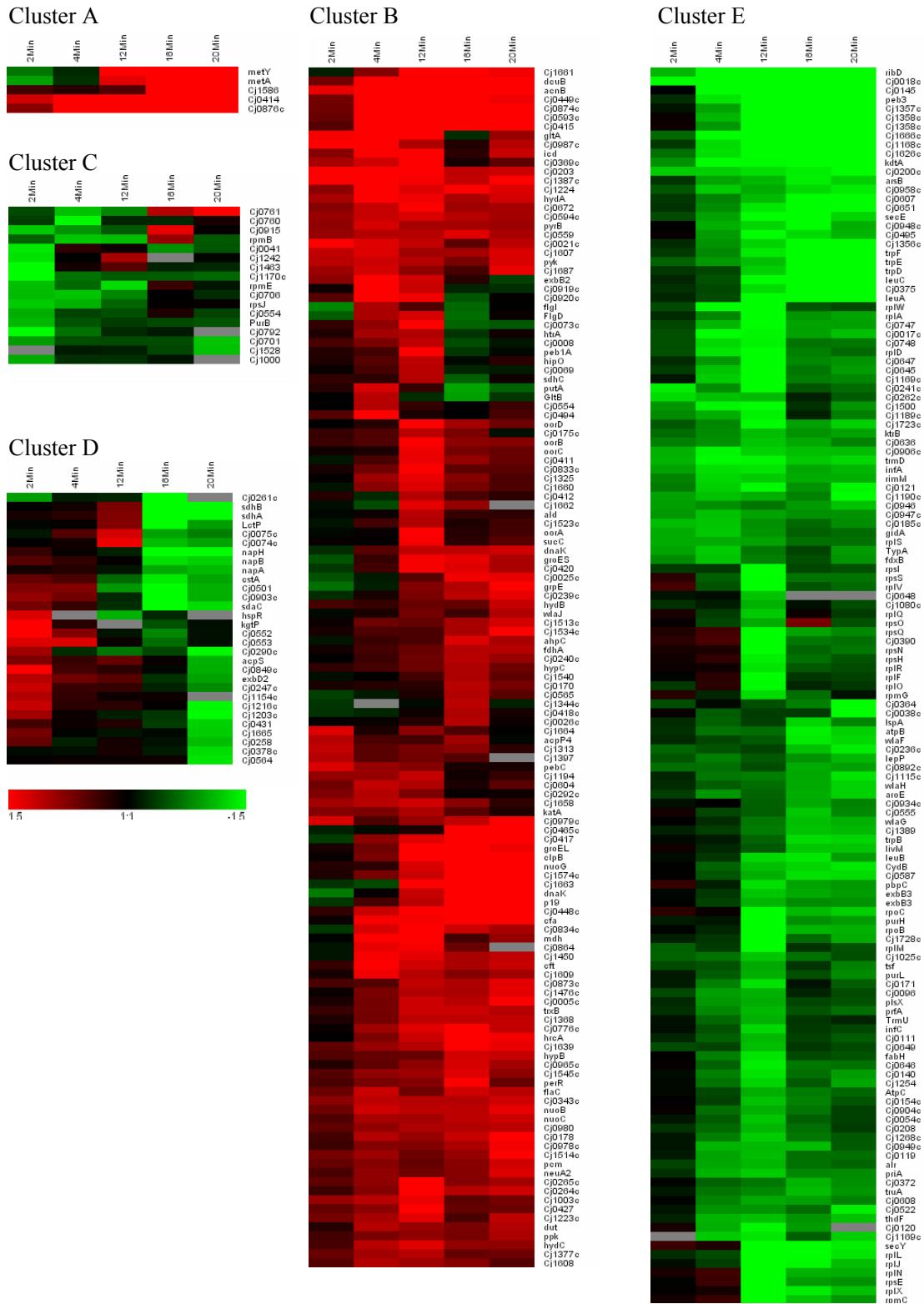


growth condition. The reduction in ribosomal gene expression reflects the energy-starved condition of the cell and the necessity for saving and reshuffling energy for the increased expression of proteins involved in repairing damage as a result of the pH change. This observation may provide insight into the mechanism of ribosomes as sensors of acid challenge.

**Figure 3** Hierarchical cluster analysis of the genes found to be significantly up- or down-regulated at mid-log phase. Going from left to right, the columns represent the transcriptome change after a pH 4.5 acid challenge. The intensity of the color is proportional to the fold change as represented by the scale at the bottom. Red is an indication of up-regulation while green is down-regulation.



**Figure 4.** Hierarchical cluster of the genes from Figure 3



### **Transcript profiling of *C. jejuni* at mid-log phase in steady-state growth conditions**

To identify coregulated patterns of gene expression, we classified all differentially expressed genes at mid-log phase as a function of the pH of the growth medium into three hierarchical clusters based on their expression log ratios (Figure 5).

Cluster A consists of genes that are significantly down-regulated at pH 5.5. This is an indication of a stress response, while at pH 6.0 and 6.5, the transcriptome profile is basically transiently expressed, or back to a baseline response. Most of the genes from cluster A (Figure 5) the transcriptome profile of *C.jejuni* genome at pH 5.5. One of the most notable genes of this subgroup includes the genes that encode for succinate dehydrogenase (*sdhABC*). These genes show repressed transcript abundance when pH challenged as discussed above.

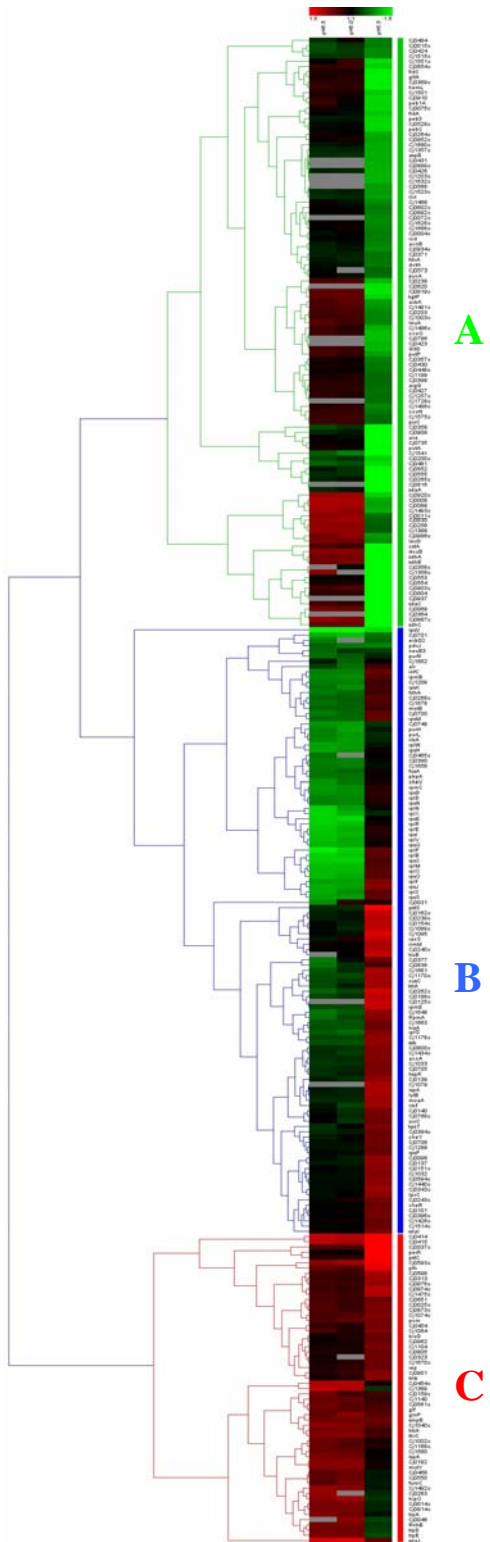
Cluster B is comprised of genes that are antagonistically expressed over the pH changes. While the genes from this cluster are up-regulated at pH 5.5, they are down-regulated at pH 6.0 and 6.5 as compared to pH 7.0. Most of the genes that are down regulated at pH 6.0 and 6.5 are the ribosomal genes, suggesting a slower growth rate at these pHs. Indeed the growth of campylobacter was found to be slightly affected at pH 6.0 and 6.5 (Figure 6).

In contrast, the same ribosomal genes are induced at pH 5.5. Yet, the growth rate of campylobacter is significantly reduced at this pH (Figure 6). The up-regulation of these proteins at pH 5.0 likely indicates that they are required to carry on an efficient translation at a pH below 6.0. A notable gene *ktrA*, which encodes for K<sup>+</sup> uptake proton, has increased transcript abundance. In other bacteria, such as *Bacillus subtilis*, the potassium transporter plays an important role in both sudden and prolonged osmotic

stress (11). Therefore, the up-regulation of the gene at pH 5.5 indicates a direct connection between the pH stress and the osmotic stress responses. KtrA, is similar to the Kup potassium uptake protein of *E.coli* (26) An interesting hypothesis would be that KtrA would allow *campylobacter* to cope with both osmotic and pH stress by functioning as H<sup>+</sup>-K<sup>+</sup> symporter. KtrA would probably extrude H<sup>+</sup> and accumulate K<sup>+</sup> in the cytoplasm.

Cluster C is composed of genes with transcript levels that are over-expressed during the pH shift. These genes likely provide the first line of defense in order to overcome the acid stress. A notable subgroup of genes, {*pstS* (putative phosphate transport system permease protein) and *pstC* (possible periplasmic phosphate binding protein)} are the genes encoding for the phosphate-specific transport system. Aguena et al. have shown that these genes are induced during phosphate starvation in *E.coli* (2). This is in agreement with our data, which suggests that during acid stress the transcript abundance is increased, since phosphates will be in short supply. The *wlaL* gene product has been shown to be involved in the glycosylation pathway. Interestingly, our microarray data suggest that the transcript abundance of the *wlaL* gene is also affected by acid stress. Consequently, the glycosylation pattern of the cell could vary as a function of pH. Since the glycosylation pathway has been shown to be associated with the pathogenesis of *campylobacter*, our data suggests that pH could modulate the virulence potential of *C.jejuni*.

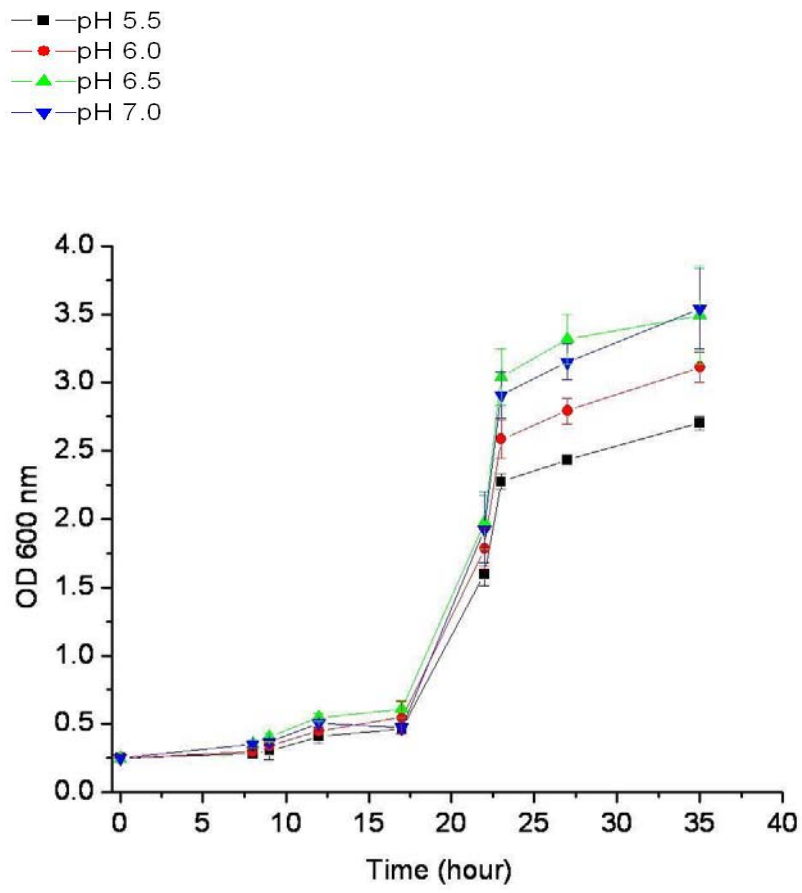
**Figure 5.** Hierarchical cluster analysis genes found to be significantly up- or down-regulated at mid-log phase. Going from left to right, the columns represent the transcriptome change from pH 7 at mid-log phase at pH of 5.5, 6.0 and 6.5. The intensity of the color is proportional to the fold change as represented by the scale at the bottom.



It is apparent from this data that the immediate response of *C. jejuni* to acid challenge is significantly similar to the bacterial transcriptome during steady-state growth at pH 5.5. The groups of transcripts whose abundance was affected in the time course and not in the steady-state experiment encode ribosomal proteins, surface structures, and proteins of unknown function. Some important points to note are 1) Up to 290 genes are differentially expressed in the steady state experiments 2) the number of genes differentially expressed during the acid challenge experiment is 325. It is evident from this data that the immediate response of *C. jejuni* to the acid challenge differs significantly from the bacterial transcriptome at pH 6.5 and 6.0 during the steady-state growth at mid-log phase. As a result, the observed transcriptome profile of *C. jejuni* at 2, 4, 12, 16, and 20 min after the acid challenge most likely represents the bacterial adaptation snap shot of new growth condition, while the transcriptome profile at mid-log phase represents the steady-state transcriptome.



**Figure 6:** Growth Curve of steady-state culture at different pH values



## **Conclusion**

In contrast to steady-state or single-time point studies, time course experiments are particularly valuable in rendering a deeper understanding of the mechanism involved in regulating a bacterial response to stress and in doing so provide useful data for generating computational models of stress response pathways. In our study, the temporal gene expression analysis indicated the global changes in mRNA levels upon inducing acid challenge were largely transient. *C. jejuni* responds with large changes in the expression level at pH 5.5, and then the percentage of genes decreased. This gene profile suggests that *C. jejuni* quickly readjusts its transcript levels to a new steady state, hence allowing the bacterium to survive the stress.

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## VITA

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Master of Science

**Thesis: Response of *C.Jejuni* to pH stress**

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Biographical:

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**Scope and Method of study:** Enteric pathogens, in order to transit through the gut and cause disease, must survive the acid pH of the stomach. Pathogenic species that prefer to grow at neutral pH, display a wide range of mechanisms to survive extreme pH. *Campylobacter jejuni* is the leading cause of bacterial food-borne diarrhea throughout the developed world. In this study, we used DNA microarrays to measure the global change in transcript levels over time due to pH stress. The transcript level of 325 genes was affected over a 20 min time period. Genes encoding heat shock proteins, chaperonins and oxidative stress defence were up-regulated. This gene profile suggests that *C. jejuni* quickly readjusts its transcript levels to a new steady state, thus allowing the bacterium to survive the stress. These findings provide new insights towards the *C.jejuni* mechanisms for acid stress and how to survive and adapt its transcriptome to a new growth condition.

**Findings and Conclusions:** It is apparent from this data that the immediate response of *C. jejuni* to acid challenge is significantly similar to the bacterial transcriptome during steady-state growth at pH 5.5. The groups of transcripts whose abundance was affected in the time course and not the steady-state experiment encode ribosomal proteins, surface structures, and proteins of unknown function. Some important points to note are 1) Up to 290 genes are differentially expressed in the steady state experiments 2) the number of genes differentially expressed during the acid challenge experiment are 325. It is evident from this data that the immediate response of *C. jejuni* to the acid challenge differs significantly from the bacterial transcriptome at pH 6.5 and 6.0 during the steady-state growth at mid-log phase. As a result, the observed transcriptome profile of *C. jejuni* at 2, 4, 12, 16, and 20 min after the acid challenge most likely represents the bacterial adaptation snap shot to the new growth condition, while the transcriptome profile at mid-log phase represents the steady-state transcriptome.

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