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Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni*

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Abstract

In this study we aimed to determine if Campylobacter had the ability to induce an adaptive tolerance response (ATR) to acid and/or aerobic conditions. *Campylobacter jejuni* CI 120 was grown to the appropriate phase in Brucella broth under microaerobic conditions. Cells were initially adapted to a mild stress (pH 5.5) for 5 h prior to challenge at pH 4.5, a lethal pH. Survival was examined by determining the numbers of viable cells on Campylobacter blood free selective agar base. Stationary phase cells adapted at pH 5.5 induced an ATR that enabled a 100-fold greater survival compared to an uninduced culture. Aerobic adaptation also protected the cells against acid challenge. The cross protection provided a 500-fold increase in survival when compared to unadapted cells. The incorporation of chloramphenicol during the induction period eliminated the ATR and resulted in death kinetics similar to an uninduced culture. These data suggest that *Campylobacter* spp. have the ability to induce an ATR to sublethal treatments, which increased their ability to withstand subsequent stresses.

Keywords: Adaptive tolerance response; ATR; Stress survival; Low pH adaptation; *Campylobacter jejuni*

1. Introduction

Campylobacter jejuni is one of the most common bacterial causes of diarrhoea in the developed world, as well as a major cause of diarrhoea in developing countries, and can, therefore, be considered a major public health burden. Most foodborne pathogens appear to be relatively robust and have the ability to survive environmental stress. However, *Campylobacter* spp. are considered to be very fragile organisms that lack the genes encoding the RpoS global stress response mechanism [1] and do not contain an oxidative stress defence mechanism [2].

Several methods of Campylobacter survival have been proposed. Entry into the viable but non-culturable (VBNC) state [3], the transition from rod to coccoid shape [4] and the high degree of genetic heterogeneity [5] have been suggested as potential survival mechanisms. However, the VBNC state in *C. jejuni* is still a matter of controversy as it is considered to be a degenerative state in addition to being a dormant state that provides the organism the ability to grow when favourable conditions are encountered [2]. There is a general lack of understanding of the physiology of *Campylobacter* spp. with regards to their ability to survive environmental stress.

Despite the significance of Campylobacter as foodborne pathogens, little is known of the response of these organisms to stressful environmental conditions, including aerobic and acidic stress. These conditions are especially relevant to their survival in the environment and during food processing. A sublethal stress induces an adaptive tolerance response in many bacteria and provides protection towards subsequent exposure to a lethal stress, a mechanism known as the ATR [6]. ATR has been identified and studied in *Escherichia coli*, *Listeria monocytogenes*, *Lactococci*, *Aeromonas*, *Propionibacterium freudenreichii* and *Helicobacter pylori* [7–11]. *E. coli* O157:H7 and *L. monocytogenes* have been shown to require de novo protein synthesis to induce an ATR. The aim of this present study was to determine whether *Campylobacter* spp. have the ability to induce an ATR that enables it to increase its survival to aerobic and acidic conditions.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

C. jejuni CI 120, CI 195, NCTC 11351 and NCTC 81116 were used during this study. CI 120 and CI 195 are chicken isolates (Department of Microbiology NUI, Galway, Ireland) obtained during and after processing, respectively. CI 120 and CI 195 were identified by API Campy (bioMerieux) and by a polymerase chain reaction PCR/DNA probe membrane-based colorimetric assay [12]. All strains were maintained at −20°C in Brucella broth (Difco) supplemented with 15% (w/v) glycerol. They were grown at 42°C in a multigas incubator under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2). Cultures were routinely subcultured from frozen stocks prior to subjecting them to test conditions.

2.2. Preparation of an aerotolerant culture

To obtain an aerotolerant culture, cells were inoculated onto Campylobacter blood free agar base (Oxoid) and incubated aerobically, initially for a prolonged incubation (3-day period). After several transfers under aerobic conditions the culture had a similar growth pattern to a microaerobically grown culture.

2.3. Assay of acid tolerance

Cultures were grown to the appropriate phase in Brucella broth (pH 7.0) at 42°C under microaerobic conditions. The populations of cells used were (i) mid-exponential phase cells (8 h), (ii) early stationary phase cells (16 h) and (iii) late stationary phase cells (48 h). Cultures were acid challenged at 42°C by direct addition of 0.5 M HCl to reduce the medium pH to 4.5. Viable cell counts were estimated immediately prior to the pH adjustment, immediately after the pH adjustment and at suitable time intervals thereafter. Plate counts were performed by serially diluting cell suspensions in maximum recovery diluent (Oxoid) and spread plating on Campylobacter blood free selective agar base. Plates were incubated at 42°C under microaerobic conditions for 48 h.

2.4. Induction of an ATR to acid

Cultures were grown to the appropriate phase in Brucella broth at pH 7.0. By direct addition of 0.5 M HCl the pH of the medium was reduced to the induction value (pH 5.5 or 5.75 for stationary and mid-exponential phase cells, respectively) for 5 h and the cells were subsequently challenged at pH 4.5. To determine the ATR induced by aerobic+acid conditions the cells were exposed to pH 5.5 for 5 h aerobically and then challenged at pH 4.5. Cell counts of surviving organisms were performed as described above.

2.5. Aerobic induced cross protection against acid

Cross protection was investigated by adapting the cells aerobically for 5 h at pH 7.0 before subjecting the cells to pH 4.5.

2.6. Contribution of protein synthesis to the induction of an ATR

Different concentrations (1–15 μg ml^{-1}) of the protein synthesis inhibitor, chloramphenicol, were incorporated into the medium during the induction period. This was followed by a lethal acid challenge at pH 4.5.

2.7. Reproducibility of results

Experiments were repeated at least in triplicate and the results expressed are the averages with the standard error of the mean shown as error bars.

3. Results

3.1. Use of a natural isolate

In preliminary studies, *C. jejuni* CI 120 (natural isolate) had increased acid resistance when compared to *C. jejuni* culture collection strains (NCTC 81116 and NCTC 11351) and *C. jejuni* CI 195 (natural isolate). As the increased acid resistance suggested that it should have more pronounced survival mechanisms, CI 120 was used in further studies. Challenge pH values of 3.5–4.5 showed that rapid death occurred at pH 3.5 and survival increased with increasing pH. A lethal challenge of pH 4.5 was used for subsequent experiments. Of the sublethal pH values tested, the maximum ATR was induced at pH 5.5 for stationary phase

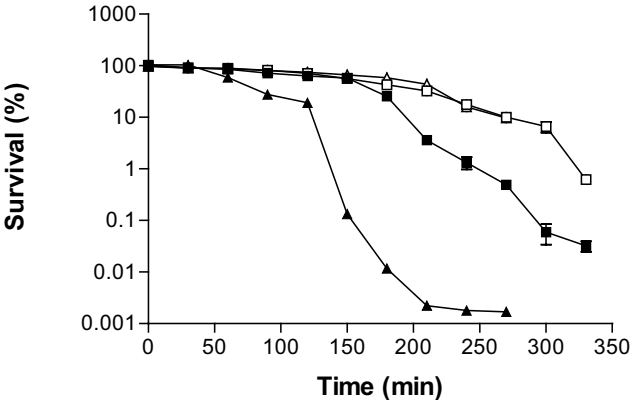

Fig. 1. Mid-exponential phase *C. jejuni* CI 120 were challenged at pH 4.5. Cells were either unadapted (open symbols) or adapted (solid symbols). Cells were adapted either at pH 5.75 for 5 h (solid squares) or pH 5.5 for 5 h under aerobic conditions (solid triangles). Experiments were undertaken in triplicate. Average values are shown with the standard error of the mean as error bars. Error bars are present but cannot be seen.

cells. Adaptation periods ranging from 1 to 6 h were examined. No increase in induction was achieved after 5 h.

3.2. ATR in mid-exponential phase cells

Cells were grown to mid-exponential phase in Brucella broth at pH 7.0 and were either acid adapted at pH 5.75 or unadapted. The unadapted culture showed almost a 100-fold greater survival when compared to the acid adapted culture (Fig. 1). Further to this no ATR was induced when mid-exponential phase cells were adapted under aerobic+acid conditions prior to challenge at pH 4.5 (Fig. 1). This suggests that mid-exponential phase cells do not induce an ATR under these conditions.

3.3. ATR in stationary phase cells

Early stationary phase cells adapted at pH 5.5 showed a 100-fold increase in tolerance to pH 4.5 in comparison with survival of unadapted cells (Fig. 2a). This compares

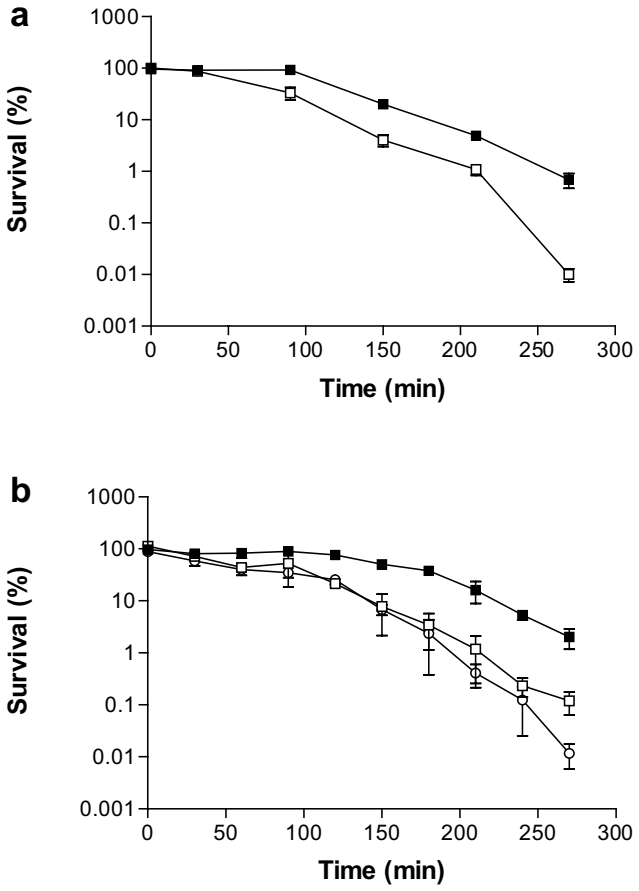

Fig. 2. Early stationary phase cells of *C. jejuni* CI 120 were challenged at pH 4.5. a: Cells were either unadapted (open squares) or adapted at pH 5.5 for 5 h (solid squares). b: Cells were either unadapted (open squares), adapted with the incorporation of chloramphenicol (open circles) or adapted at pH 5.5 for 5 h under aerobic conditions (solid squares). Experiments were undertaken in triplicate. Average values are shown with the standard error of the mean as error bars. Error bars are present but cannot be seen.

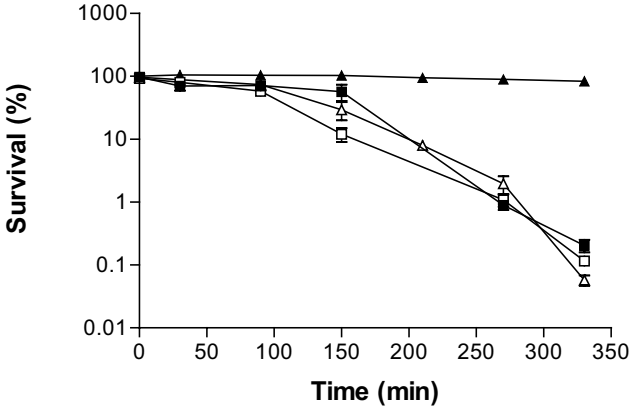

Fig. 3. Late stationary phase cells of *C. jejuni* CI 120. Survival of cells challenged at pH 4.5. Cells were either unadapted (open symbols) or adapted at pH 5.5 for 5 h (solid symbols). Cells were adapted either at pH 5.75 for 5 h (solid squares) or pH 5.5 for 5 h under aerobic conditions (solid triangles). Experiments were undertaken in triplicate. Average values are shown with the standard error of the mean as error bars.

to a 50-fold increase in survival when cells were adapted to aerobic+acid conditions (Fig. 2b).

Late stationary phase cells showed evidence of ATR induction to acid at 150 min, but this was not consistent over the death curve, while late stationary phase cells adapted to aerobic+acid conditions showed up to a 1000-fold increase in survival when compared to unadapted cells (Fig. 3). This indicates that stationary phase cells have the ability to induce an ATR to acid, but that this ability was variable.

3.4. Aerobic induced cross protection to acid

Aerobic adaptation of early stationary phase cells resulted in a 100-fold increase in survival of pH 4.5 when compared to unadapted cells (Fig. 4). Aerobic adaptation

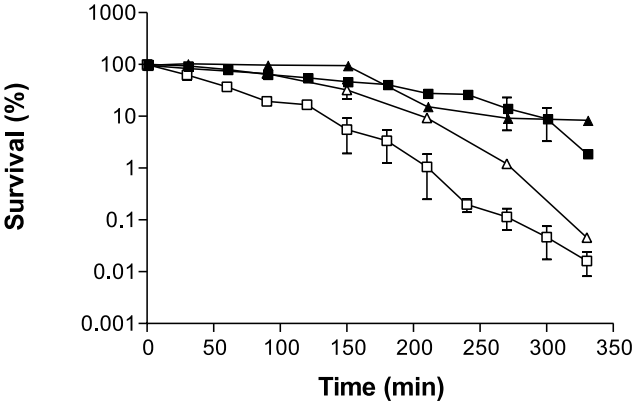

Fig. 4. Aerobic induced cross protection to acid. Survival of cells challenged at pH 4.5. Early stationary phase cells (squares) and late stationary phase cells (triangles) were either unadapted (open symbols) or adapted at pH 7.0 aerobically for 5 h (solid symbols). Experiments were undertaken in triplicate. Average values are shown with the standard error of the mean as error bars. Error bars are present but cannot be seen.

of late stationary phase cells resulted in a 500-fold increase in acid resistance when compared to an unadapted culture (Fig. 4). An aerotolerant culture of *C. jejuni* did not have increased acid tolerance (data not shown), indicating that the aerobic ATR is due to adaptation.

3.5. Chloramphenicol inhibits induction of the ATR

The protein synthesis inhibitor, chloramphenicol, was used to determine whether de novo protein synthesis was required for the induction of the ATR to acid. Various concentrations (1–15 μg ml^{-1}) of chloramphenicol were examined. A concentration of 7 μg ml^{-1} was used as no lethal effect was observed in *C. jejuni* CI 120 during adaptation. The incorporation of chloramphenicol during the induction period resulted in death kinetics similar to an uninduced culture (Fig. 2b). This confirms the requirement for protein synthesis during induction of the ATR to acid.

4. Discussion

The results presented here demonstrate that *C. jejuni* CI 120 has the ability to induce an ATR to acid or aerobic +acid conditions and confers resistance to a lethal pH. To our knowledge, this is the first time an ATR to these conditions has been shown in *Campylobacter* spp. The 100–1000-fold increase in survival as a result of the ATR is comparable if not greater than the response found in *E. coli*, *L. monocytogenes*, *Salmonella typhimurium*, *P. freudenreichii* and *H. pylori* [7–11]. The response appears to be primarily dependent on the growth phase of the cells. Mid-exponential phase cells do not induce an ATR under the conditions used (Fig. 1). In fact, the adapted cells had an increased death rate, indicating that the cells had become sensitised during the adaptation period. The ATR to acid was induced by early stationary phase cells, but not by late stationary phase cells, while the ATR to aerobic+acid conditions was induced by early and late stationary phase cells. Other organisms, such as *E. coli* and Salmonella, induce an ATR in mid-exponential phase, but the stationary phase stress response is difficult to interpret since these organisms express the global stationary phase stress response mechanism mediated by RpoS. Analysis of the *C. jejuni* 11168 genome sequence shows that it does not contain the genes encoding *rpoS* [1]. Therefore, it is feasible to study the stationary phase ATR in *Campylobacter* spp. The absence of the RpoS mechanism correlates with the known physiological data for *C. jejuni*. It has been observed that stationary phase cells of *C. jejuni* NCTC 11351 are more sensitive to heat and aeration than mid-exponential phase cells [13] and that mid-exponential phase cells are more acid tolerant than stationary phase cells (compare the open symbols from Figs. 1 and 2). In the natural environment, optimal growth conditions would rarely be encountered and Campylobacter would most likely be in stationary phase. Consequently, the ability to induce an ATR to acid and aerobic conditions in stationary phase may play a significant factor in the survival of this organism in the environment.

Other factors also influence the induction of the ATR to acid and aerobic conditions. These include the strain used, the period of adaptation, the induction pH and the medium used. While induction of ATR to acid and aerobic conditions by strain CI 120, a natural isolate, resulted in considerable degree of increased survival, induction of a similar ATR by several different strains of *C. jejuni* needs to be investigated.

During food processing mild pH under aerobic conditions are likely to be encountered by contaminating foodborne pathogens. ATR induction under aerobic+acidic conditions was observed in early and late stationary phase cells. Survival of the organism as a result of ATR induction was increased by up to 100-fold. Under these conditions it is not clear whether the adaptation is due to the mild pH or the aerobic stress. Since late stationary phase cells did not adapt to mild pH, it is likely that the ATR induced by late stationary phase cells in response to aerobic+acid conditions was due to the aerobic conditions. Induction of ATR under aerobic conditions resulted in cross protection to acid stress, for both early and late stationary phase cells. Therefore, it is clear that either sublethal aerobic or acid conditions can induce an ATR that increases the ability of *C. jejuni* CI 120 to survive a lethal pH of 4.5.

The existence of the ATR mechanism to acid and aerobic conditions has implications with regard to the pathogenesis of *C. jejuni*. A low infectious dose (500 cells or less) of this organism can be associated with illness. The involvement of an ATR mechanism that allows up to 500-fold increased survival of *C. jejuni* in food and the environment could be a significant factor in their ability to survive in numbers high enough to cause diarrhoeal illness in humans.

Other organisms exhibit inducible tolerance response systems which allow them to survive extreme acidity. It has been proposed that the inducible tolerance responses are dependent on intracellular [14] and extracellular mechanisms [10]. Considering that de novo protein synthesis was required for ATR to acid and aerobic conditions in Campylobacter (Fig. 2b) it is likely that this ATR is based on intracellular mechanisms, although further investigation of the mode of action is required.

In conclusion, the data demonstrate the ability of *C. jejuni* CI 120 to induce ATR that enhances its survival to aerobic and acid stress.

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