Adaptive acid tolerance response (ATR) in *Aeromonas hydrophila*

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***Aeromonas hydrophila*, a gastrointestinal pathogen of humans, was shown to exhibit a significant adaptive acid tolerance response (ATR) capable of protecting cells from severe acid at a pH of 3.5. The ATR was induced by exposure to a relatively mild pH level of 5.0 for 20 min. Adaptation required protein synthesis since treatment with chloramphenicol during adaptation to pH 5.0 prevented the development of acid tolerance. The adaptation to acid environment was found to be a non-transient phenomenon. Also, iron was not required for acid adaptation in *A. hydrophila*. Two-dimensional protein analyses revealed an increased production of 28 proteins and decreased synthesis of 10 following pH shifts from 7.2 to 5.0. The mild pH treatment must act as a signal to *A. hydrophila* to adapt and survive in acid environments by producing 'protective' proteins. The adaptation and survival of this pathogen in low pH may provide valuable information about its ability to withstand acid environments in nature and in the human gastrointestinal tract.**

Keywords: *Aeromonas hydrophila*, acid adaptation, acid tolerance response (ATR), low pH adaptation, gastrointestinal pathogen

INTRODUCTION

Bacterial survival in stressful environments is an intriguing biological problem with applications toward understanding pathogenic and environmentally important micro-organisms. A number of stress conditions such as starvation (Jenkins *et al.*, 1988, 1990; Siegle & Kolter, 1992), heat-shock (Allen *et al.*, 1992; Foster, 1992; Henry *et al.*, 1992), cold-shock (Goldstein *et al.*, 1990; Jones *et al.*, 1992; Willimsky *et al.*, 1992), and pH (Foster, 1992; Slonczewski, 1992; White *et al.*, 1992) have been documented for a number of microbial pathogens. In each case global physiological changes were shown to occur within the cell when they were exposed to the stress condition (Murray & Young, 1992; Pannekoek *et al.*, 1992; Nystrom & Neidhardt, 1992). Many of the stress conditions formed part of the host defence system used to prevent or limit bacterial infections (Gluskin *et al.*, 1992). An important stress condition that must be faced by many pathogenic micro-organisms is low pH. Upon ingestion, food- and water-borne microbial pathogens are exposed to an acid pH environment in the stomach and in the small intestine (Booth, 1985; Goodson & Rowbury, 1989a, b, c, 1990). Also, some of these microbial pathogens can be

exposed to acid conditions in the urinary tract and in the phagolysosome (Goodson & Rowbury, 1989b; Raja *et al.*, 1991). Adaptation and survival in a low pH environment may be an important prerequisite toward the production of disease by many gastrointestinal pathogens. The response to low pH environments has been studied in *Salmonella typhimurium* (Aliabadi *et al.*, 1988; Foster, 1991, 1992; Foster & Hall, 1990), *Escherichia coli* (Slonczewski, 1992; Raja *et al.*, 1991; Watson *et al.*, 1992), *Streptococcus mutans* (Hamilton & Buckley, 1991) and *Helicobacter pylori* (Mooney *et al.*, 1990).

Among a wide variety of potential water- and food-borne gastrointestinal microbial pathogens, *Aeromonas* spp. have recently drawn significant attention due to their role in causing acute diarrhoeal disease (Schubert, 1991). Various species of *Aeromonas* are widely distributed in polluted and unpolluted aquatic environments (Hazen *et al.*, 1978a). Among the *Aeromonas* spp. isolated from various aquatic environments, *A. hydrophila* is found to be predominant (approximately 70%) (Schubert, 1991). Although *A. hydrophila* is recognized as a primary pathogen of fish, reptiles, and amphibia (Hazen *et al.*, 1978b; Hird *et al.*, 1981) and is considered an opportunistic pathogen of immunocompromised humans (Gluskin *et al.*, 1992; Von Gravenitz & Mensch, 1968), numerous recent reports suggest that this pathogen can

Abbreviations: ATR, acid tolerance response; DTPA, dipyrilidyl.

cause serious infection in patients without known immunological abnormalities (Gluskin *et al.*, 1992; Schubert, 1991; Monfort & Baleux, 1991). During the last decade, *A. hydrophila* has been traced as a causative agent for acute diarrhoeal disease in humans following ingestion of contaminated water (Burke *et al.*, 1984; Cumberbatch *et al.*, 1979; Figura *et al.*, 1986; Kirov *et al.*, 1986; Pitarangsi *et al.*, 1982). As a result, *A. hydrophila* is now considered a significant enteric pathogen (Janda & Duffy, 1988).

Because *A. hydrophila* is a waterborne pathogen (Schubert, 1991) it must cope with environmental as well as host-associated acid stress (Schubert, 1991; Figura *et al.*, 1986; Kirov *et al.*, 1986). Consequently its ability to survive these acid encounters must contribute to its persistence in nature and its pathogenesis in man. This report initiates a study designed to dissect the cellular and physiological responses of *A. hydrophila* to severe external acid stress conditions.

METHODS

Bacterial strains and media. Two strains of *A. hydrophila* were used in this study. *A. hydrophila* ATCC 7965 was obtained from the American Type Culture Collection. A second strain, *A. hydrophila* SM 20, isolated from the Ohio river at Louisville, Kentucky, was a gift from S. McCarty of the Louisville Water Company. Both strains were grown and maintained on nutrient agar (Difco) media at 25 °C or in liquid Luria-Bertani (LB) broth (Miller, 1972) with gentle shaking (80–100 r.p.m.). In addition, M9 glucose salt minimal medium was used (per litre): 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, pH 7.2; after autoclaving filter (0.2 µm pore size Acrodisc, Gelman)-sterilized solutions of 10 ml 0.01 M CaCl₂, 1 ml 1 M MgSO₄·7H₂O, and 10 ml 20% (w/v) glucose were added (Miller, 1972).

Acid adaptation and viable plate counts. A 1 ml culture of *A. hydrophila* was inoculated into three sterile flasks each containing 100 ml LB broth or glucose salts minimal medium and grown on a rotary shaker at 25 °C until an OD₄₅₀ of 0.2 was reached (100% viability was estimated to be 2×10^8 cells ml⁻¹ by viable plate counts). The pH of one of the cultures (adapted) was adjusted to 5.0 by addition of a 50% (v/v) solution of HCl (Fisher Scientific), and the incubation of the cells was continued for 20 min at 25 °C with shaking. The cells in this culture were then challenged to pH 3.5 by adding additional HCl, and incubated for another 2.5 h. At the same time, another culture was grown at pH 7.2 to OD₄₅₀ 0.2 (2×10^8 cells ml⁻¹) and then challenged to pH 3.5 without an intermediate incubation at pH 5.0. As a control, a third culture was maintained at pH 7.2 without changing the pH during the entire period of the experiment.

For viable plate counts, aliquots from each culture were serially diluted in phosphate buffer (pH 7.2) (Miller, 1972), and plated onto LB agar plates using the spread plate method. The plates were incubated at 25 °C for 36–48 h.

Transient acid adaptation. A 1 ml overnight culture of *A. hydrophila* was inoculated into seven sterile flasks each containing 100 ml LB broth or glucose salts minimal medium and grown on a rotary shaker at 25 °C to an OD₄₅₀ of 0.2 (100% viability was estimated at 1.9×10^8 cells ml⁻¹ by viable plate counts). The pH of five of the seven cultures was adjusted to 5.0 by addition of a 50% solution of HCl. The pH of four of these five cultures was shifted to pH 3.5 following adaptation to pH 5.0 for

15 min, 30 min, 60 min, or 90 min, respectively and continued to grow for an additional 4 h. Concurrently, two cultures, at pH 7.2 and 5.0, were maintained throughout this experimentation as controls.

For viable plate counts, aliquots from each culture before and after the pH shifts were serially diluted in phosphate buffer (pH 7.2) (Miller, 1972), and plated on to LB agar plates using the spread plate method. The plates were incubated at 25 °C for 36–48 h.

Role of iron in acid adaptation. A 1 ml overnight culture of *A. hydrophila* was inoculated into three sterile flasks, each containing 100 ml LB broth or glucose salts minimal medium and grown on a rotary shaker at 25 °C, to an OD₄₅₀ of 0.3 (100% viability was estimated at 1.2×10^7 cells ml⁻¹ by viable plate counts). One of the three cultures was treated with 100 µM dipyrityl (DTPA) (Sigma) as an iron chelator for 15 min and the pH was shifted to 5.0. Simultaneously, a second culture was treated with 100 µM DTPA and 100 µM Fe(OH)₃ for 15 min, and the pH was changed to 5.0. A third culture was simply treated with the adaptation pH of 5.0. All cultures were incubated at pH 5.0 for 20 min and then challenged to pH 3.5.

For viable plate counts, aliquots from each culture before and after the DTPA, DTPA + Fe(OH)₃ treatments and the pH shifts, were serially diluted in phosphate buffer (pH 7.2) (Miller, 1972), and plated on to LB agar plates using the spread plate method. The plates were incubated at 25 °C for 36–48 h.

O'Farrell two-dimensional analysis of ATR proteins. Two-dimensional protein profiles were determined at different pH values, following growth in minimal glucose salts medium (pH 7.2) to exponential phase (OD₄₅₀ approximately 0.2). One culture was shifted to a pH of 5.0 for 20 min, and then labelled with 40 µCi ³⁵S-Trans label (ICN Biomedicals) ml⁻¹ for 15 min. A second culture was maintained at pH 7.2 and labelled for an equivalent period of time. Cells were then harvested by centrifugation in a refrigerated centrifuge (Beckman) at 8000 r.p.m. for 15 min, and the cell pellets were resuspended in 13 µl of SDS-lysing solution consisting of 50 µl 0.5 M Tris/HCl (pH 6.8), 80 µl 15% (w/v) SDS, 20 µl glycerol, 40 µl β-mercaptoethanol and 272 µl distilled water (Foster, 1991). The cells were lysed at 95 °C for 10 min, cooled to room temperature, and 10 µl 100% NP40 (Sigma) was added. A 150 µl aliquot of SDS-lysis buffer [5.7 g urea, 2.0 ml NP40, 0.5 ml β-mercaptoethanol, 0.4 ml pH 5–7 ampholines (BioRad) and 2.4 ml distilled water (Foster, 1991)] was added. The protocol for O'Farrell two-dimensional gel electrophoresis for total cellular proteins was performed as described elsewhere (Dunn *et al.*, 1989; Spector *et al.*, 1986). The first dimension electrophoresis was performed by isoelectric focusing in tube gels containing 1.6% (pH 5–7) and 0.4% (pH 3–10) (v/v) Bio-Lyte ampholyte (BioRad) and 0.8% (pH 5–7) and 0.2% (pH 3–10) Bio-Lyte ampholyte as sample overlay buffer. The first dimension tube gels were run at a constant voltage of 200 V for 2 h, followed by 500 V for 2 h and then 800 V for 16 h (overnight). The second dimension separation of the proteins was performed in 11.5% vertical SDS-polyacrylamide slab gels at 160 V for 10 h at 12 °C. The gels were dried on Whatman 3MM filter paper and autoradiography was performed for 72 h at room temperature.

RESULTS AND DISCUSSION

Acid adaptation

Fig. 1 shows that *A. hydrophila* undergoes rapid exponential death when subjected to pH 3.5. However, cultures adapted at pH 5.0 for 20 min with subsequent

Fig. 1. Viable plate counts of acid induction in *Aeromonas hydrophila* ATCC 7965. ●, *A. hydrophila* culture maintained at pH 7.2; ○, culture adapted to a mild pH of 5.0 prior to challenge at pH 3.5; ■, culture to which the pH was shifted from 7.2 to 3.5 without any intermediate pH treatment. The arrows indicate the times when the pH of each of the cultures was shifted.

Fig. 2. Viable plate counts of acid induction in chloramphenicol-treated cultures of *A. hydrophila*. ●, Culture which was not treated with chloramphenicol (CM) before acid pH shift from 7.2 to 5.0 followed by treatment with a much lower pH of 3.5; ○, culture which was treated with chloramphenicol before pH shift from 7.2 to 5.0 followed by challenge at pH 3.5. The arrows indicate the times when the chloramphenicol and acid pH treatments were conducted for each culture.

challenge to pH 3.5 showed > 5 log higher survival within 1.5 h post-acid challenge than cultures without prior adaptation at pH 5.0. The unadapted control culture at pH 7.2 continued to grow as expected. Acid adaptation was comparable in both complex and minimal media (data not shown). Thus, survival of *A. hydrophila* at pH 3.5 requires adaptation to an intermediate pH of 5.0.

ATR development requires protein synthesis

Acid tolerance could involve the physiological activation of a pre-existing protective system or the induction of specific ATR protective proteins. In order to determine whether synthesis of new proteins is required for acid adaptation, cultures were treated with chloramphenicol ($1 \mu\text{g ml}^{-1}$) immediately before and during adaptation to pH 5.0. Chloramphenicol treatment eliminated the ATR as shown in Fig. 2. This result suggests that proteins synthesized during adaptation at pH 5.0 are involved in the survival during subsequent exposure to pH 3.5.

Transient acid adaptation

The cultures that were adapted at pH 5.0 for 15, 30, 60 and 90 min and subsequently challenged to pH 3.5 showed survival and growth suggesting the acid adaptation in *A. hydrophila* is not a transient type. The growth of the cells in these cultures was comparable with the growth when the pH was shifted to 5.0 and then maintained at this pH (Fig. 3). However, longer exposure to pH 5.0 followed by challenge to pH 3.5 increased the viability of the cells (Fig. 3). Also, decreased survival was evident in the culture that was adapted to pH 5.0 for 15 min followed by challenge to pH 3.5 as compared to the cultures that were adapted for 30, 60, or 90 min (Fig. 3). In unadapted culture (pH 7.2→3.5), the viability of the cells declined within the first 1.5 h. The culture maintained at 7.2

Fig. 3. Viable plate counts of acid induction in cultures of *A. hydrophila* incubated at adaptation pH of 5.0 for 15, 30, 60 or 90 min followed by shift to challenge pH of 3.5. Following the shift to pH 3.5, an aliquot of cells at every 30 min interval was serially diluted and plated on LB agar for viable plate counts. Cells grown at pH 7.2, pH 7.2-5.0, and pH 7.2-3.5 were used as controls. Arrows indicate the times when the pH was shifted for each culture. The experiment was performed in triplicate. Comparable results were obtained in both LB and glucose minimal media.

throughout the experiment showed increased growth. The cultures in minimal salt medium and those in LB medium showed comparable results.

Fig. 4. Viable plate counts of acid induction in cultures of *A. hydrophila* incubated with dipyriddy (DTPA; $\square$) or DTPA+Fe(OH)₃ ($\blacksquare$). Following treatment, the cultures were adapted to pH 5.0 for 20 min followed by a shift to challenge pH of 3.5. Before and after the shift to challenge pH, at every 30 min interval, aliquot of each culture was serially diluted and plated on LB agar for viable plate counts. Arrows indicate the shift in all cultures. Control culture was pH 7.2–5.0–3.5 ($\diamond$). The experiment was performed in triplicate. Both LB and minimal salts media showed comparable results.

Role of iron in acid adaptation

The DTPA-treated culture showed survival at the challenge pH of 3.5 following adaptation at pH 5.0 indicating

that iron is not required for acid adaptation in *A. hydrophila*. The growth and survival of the DTPA culture was comparable with the culture treated with DTPA + Fe(OH)₃ and the culture adapted at pH 5.0 followed by challenge to pH 3.5 (Fig. 4).

Two-dimensional polyacrylamide gel analysis of ATR proteins

Since acid tolerance required new protein synthesis for adaptation, an attempt was made to identify changes in polypeptides that occur in response to acidification. Since labelling of proteins for gel analysis requires growth in minimal media, it was first crucial to confirm that *A. hydrophila* could mount an ATR in glucose salts minimal medium. Cells were subjected to the acid induction procedure described in Methods using minimal glucose medium. The results were similar to those in rich medium (LB), suggesting that the ATR proteins were being produced in glucose salts minimal medium and therefore could be radiolabelled by incorporation of radiolabelled amino acids (data not shown).

The two-dimensional protein profiles of *A. hydrophila* at pH 5.0 and pH 7.2 are shown in Fig. 5. Cells shifted to pH 5.0 exhibited a protein profile with marked differences from that displayed by cells grown at pH 7.2. An increase in production of at least 28 proteins was observed (arrows) upon the acid shift while a decrease in synthesis of 10 proteins (circles) was evident. One or more of these

Fig. 5. Autoradiograms of two-dimensional PAGE analyses of the ATR proteins in *A. hydrophila*. A, Total cellular protein profile of a culture which was maintained at pH 7.2; B, total cellular protein profile of a culture in which the pH was shifted from pH 7.2 to 5.0. The arrows indicate the increased synthesis of the proteins when the pH was shifted from 7.2 to 5.0. The circles represent the decreased synthesis of proteins when the pH was shifted from 7.2 to 5.0.

proteins are likely to be responsible for survival at low acid pH since in their absence (when treated with chloramphenicol) cells are extremely acid sensitive. This protein response is similar to that seen in *S. typhimurium* (Foster, 1991) and in *E. coli* (Raja *et al.*, 1991). Since this represents the first two-dimensional analysis of *A. hydrophila* polypeptides we do not know yet whether any of these protein changes may overlap those induced by other stress responses encountered by this organism.

ATR proteins induced by acid stress may protect cells by one or more possible mechanisms. First, newly synthesized proteins may bolster pH homeostasis enabling the cells to maintain internal pH close to neutral levels even at extremely low external pH. Second, chaparonin proteins could be involved in protection of proteins from acid denaturation or damage. It is significant to note in this regard that the heat shock proteins GroEL and DnaK have been identified as being induced in *S. typhimurium* under acidic conditions (Foster, 1991; Buchmeier & Heffron, 1990). Third, DNA binding proteins may play a role in habituation or adaptation to acid by preventing or repairing DNA damage as previously suggested in *E. coli* (Raja *et al.*, 1991).

CONCLUSIONS

In the present study, we have shown that *A. hydrophila* can adapt to survive severe acid environments. This adaptation requires prior exposure to a relatively mild pH (5.0) before challenge at a much lower pH (3.5). Also, acid adaptation in *A. hydrophila* requires new or increased synthesis of proteins since the addition of chloramphenicol prevents this phenomenon. *A. hydrophila* increases the synthesis of at least 28 proteins upon shifting cultures from pH 7.2 to 5.0, while simultaneously reducing the levels of 10 proteins. This suggests that the new proteins may have a significant role in protecting the cells in low pH conditions. The synthesis of additional proteins for adaptation to an acidic environment does not require iron. Also, the acid-adaptation proteins are not transient. The mild pH condition may act as a signal for the cell indicating potentially lethal pH changes in the external environment, allowing the cell to produce new 'protective' proteins required for survival at more acidic levels. This represents a global cellular response at both the physiological and genetic levels similar to those described in other bacterial species. Further studies on the genetic mechanism(s) whereby this bacterial pathogen can adapt and survive in harsh acid environments may provide insight into its ability to cause disease in humans.

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