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; Siegel & 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; Slonczeewski, 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 (Alaibadi et al., 1988; Foster, 1991, 1992; Foster & Hall, 1990), Escherichia coli (Slonczeewski, 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...
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 NaHPO₄, 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 OD₅₆₀ of 0.2 was reached (100% viability was estimated to be 2 × 10⁸ 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. The pH of four of these five cultures 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 OD₅₆₀ of 0.3 (100% viability was estimated at 1.2 × 10⁷ cells ml⁻¹ by viable plate counts). One of these three cultures was treated with 100 µM dipyridyl (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.

**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 OD₅₆₀ of 0.2 (100% viability was estimated at 1.2 × 10⁷ 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% (v/v) 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 experiment as controls.

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**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 (OD₅₆₀ 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 [57 g urea, 200 ml NP40, 0.15 M 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 16% (pH 5–7) and 04% (pH 3–10) (v/v) Bio-Lyte ampholines (BioRad) and 08% (pH 5–7) and 02% (pH 3–10) Bio-Lyte ampholines 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
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 µg 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 throughout the experiment showed increased growth. The cultures in minimal salt medium and those in LB medium showed comparable results.
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Fig. 4. Viable plate counts of acid induction in cultures of A. hydrophila incubated with dipyridyl (DTPA; [ ]) or DTPA + Fe(OH)$_3$ (■). 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 (○). 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, chaperonin 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.

**REFERENCES**


Received 1 October 1993; revised 15 February 1994; accepted 21 February 1994.