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Acid stress responses in enterobacteria

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Abstract

The enteric microorganisms *Salmonella typhimurium*, *Escherichia coli* and *Shigella flexneri* prefer to grow in neutral pH environments. They nevertheless experience dramatic pH fluctuations in nature and during pathogenesis. In response to environmental encounters with acid, these organisms have evolved complex, inducible acid survival strategies. Regulatory features include an alternative sigma factor (σ^S), 2-component signal transduction systems (PhoP/Q; MviA/?) and the major iron regulatory protein Fur. Specific survival mechanisms include emergency pH homeostasis by inducible amino acid decarboxylases and probable roles for DNA repair, chaparonins, membrane biogenesis as well as others that remain poorly defined. Continued study of acid survival in these organisms will provide general insights regarding stress management and will have a direct impact on our understanding of pathogenesis.

Keywords: *Salmonella typhimurium*; *Escherichia coli*; *Shigella flexneri*; Acid stress

1. Introduction

Bacteria periodically experience life-threatening stresses in a variety of pathogenic and natural situations. In order to increase the chance of surviving these encounters, the microorganism under siege will sense a deteriorating environment and undergo a programmed molecular response by which specific, stress-inducible proteins are synthesized. These proteins presumably act to prevent or repair macromolecular damage caused by the stress. While some stress proteins are induced under many different conditions (e.g., universal stress proteins), others are induced only in response to a specific stress. How organisms survive during environmental stress is a fundamental question of biology. Understanding these systems will be crucial to the development of biotechnologies where bacteria are asked to perform tasks under duress. In addition, the discovery that bacteria with impaired stress responses are also less virulent has provided new insight into microbial pathogenesis.

In their natural habitats, Enterobacteriaceae are constantly under assault by a wide array of environmental stresses. One of the most frequently encountered hostile conditions is acid stress. Neutralophiles like *Escherichia coli*, *Salmonella typhimurium* or *Shigella flexneri* while travelling through the gastrointestinal tract must endure extreme low pH in the stomach as well as volatile fatty acids present in the intestine and faeces. Facultative intracellular pathogens such as *Salmonella* also tolerate episodes of low pH within macrophage phagolysosomes. Even upon

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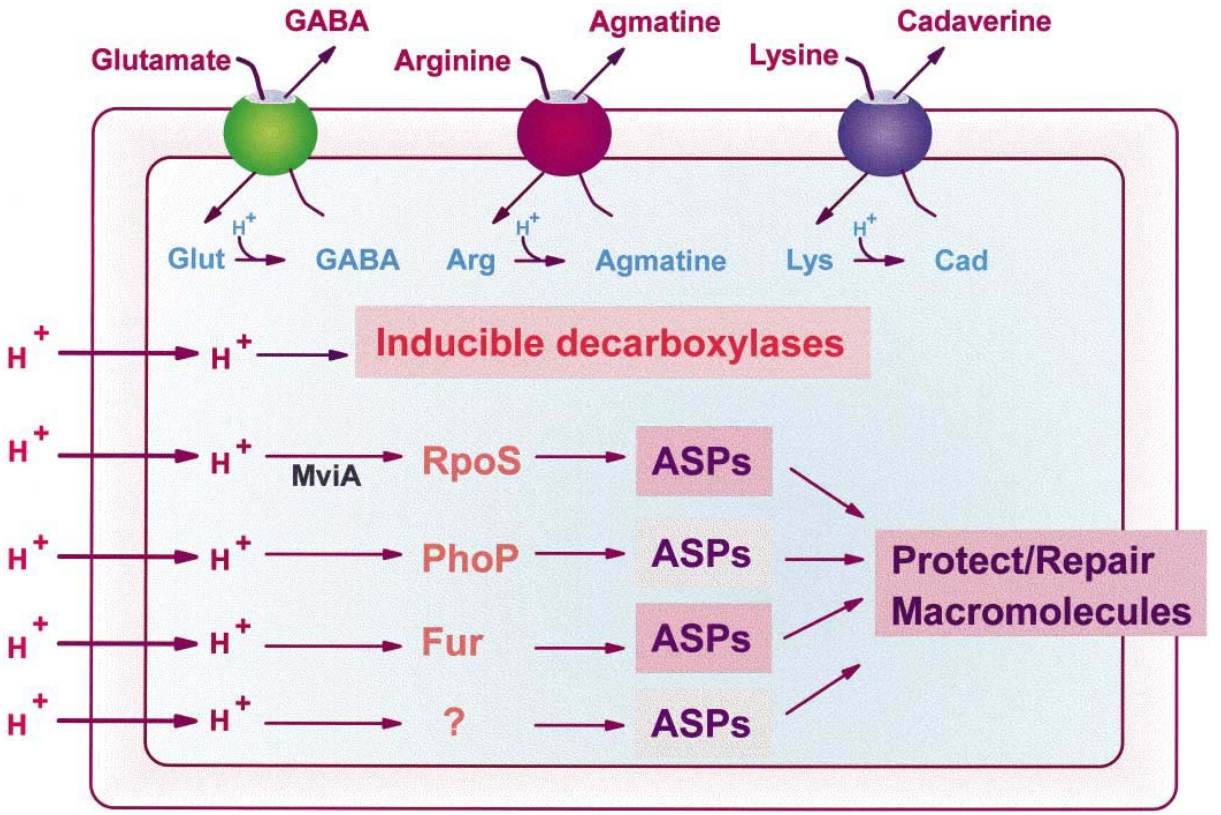

Fig. 1. Acid survival responses in enteric microorganisms. The figure represents a composite cell containing all the known components of inducible acid tolerance and acid resistance. The lowering of pH_i by proton leak at low pH_o will induce several amino acid decarboxylases, if they are present, in a given species. These systems appear to act as inducible pH homeostasis systems, elevating pH_i by consuming a proton during decarboxylation and then exchanging the decarboxylation endproduct for new substrate via a membrane-bound antiporter. In addition low pH will increase the accumulation of at least two important regulators; σ^S (RpoS), through the modulation of σ^S proteolysis by MviA; and PhoP via an unknown mechanism. These two regulators control distinct sets of ASPs defining partially redundant systems of acid tolerance. The Fur protein appears to sense acidic pH independently of iron and controls a third set of ASPs. The function of the ASPs is presumed to include the prevention and/or repair of acid-induced damage to macromolecules.

exiting a host, enteric organisms may confront acid stress in the form of industrial waste, acid mine drainage, or in decaying organic matter. Thus, the ability to sense and respond to potentially lethal changes in environmental pH is crucial to the survival of the Enterobacteriaceae.

Over the past 10 years, the existence of inducible acid survival mechanisms in enterobacteria has become apparent. The most extensively studied members in terms of acid stress responses are *Salmonella, Shigella* and *E. coli*. However, comparisons between laboratories is difficult due to variations in the assay conditions used (minimal vs. complex medium, log vs. stationary phase cells, different adaptive and challenge pH conditions, etc.). Different terminologies have also been used to describe acid stress response systems. Acid resistance (AR), acid tolerance (AT) and acid habituation (AH) are all terms used to describe survival to low pH stress under different conditions. It is difficult to determine from the literature if the systems described are truly different or simply reflect different ways of measuring the same system. One goal of this article is to define each system and point out where overlap may occur. A composite cell illustrating various acid survival systems and regulatory circuits is shown in Fig. 1.

2. Definition of acid stress

Before defining the responses to acid stress, acid stress itself must be defined. Acid stress can be described as the combined biological effect of low pH and weak (organic) acids present in the environment. Weak acids include volatile fatty acids (VFAs) like butyrate, propionate and acetate produced as a result of fermentation. Weak acids in their uncharged, protonated forms can diffuse across the cell membrane and dissociate inside the cell, lowering internal pH (pH_i) in the process. The lower the external pH (pH_o), the more undissociated weak acid will be available (based upon pK_a values) to cross the membrane and affect pH_i. This means that it takes less organic acid to kill a cell at pH_o 3.5 than is needed at pH_o 4.4. Intracellular accumulation of weak acids is also thought to have harmful effects on the cell beyond that of acidifying pH_i. It is important to remain cognizant of the relationship between pH and weak acid concentration when considering cellular acid survival strategies.

3. Acid tolerance responses of *Salmonella typhimurium*

S. typhimurium possesses different low pH inducible acid survival systems depending upon whether the cells are in exponential or stationary phase [1]. Most of our knowledge comes from studies with exponential phase cells grown in minimal glucose media. Cells actively growing at pH_o 7.7 rapidly die when shifted to conditions below pH_o 4. However, adapting these organisms to a mildly acidic pH (pH_o 5.8) for one generation increases their tolerance to more extreme acid conditions (pH_o 3) [2]. This inducible acid tolerance response (ATR) is a two-stage process involving overlapping acid protection systems triggered at different levels of acidity. Encounters with pH_o 6 invoke 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_o 3). The second stage (post-acid shock) is engaged once pH_o falls below 4.5. Approximately 50 acid shock proteins (ASP) that are believed to prevent or repair macromolecular damage are induced during this stage.

3.1. Inducible pH homeostasis

Emergency pH homeostasis systems induced by mild acid function to keep pH_i above 5 as the organism encounters severe acid pH_o. This, in turn, allows the synthesis of ASPs to occur at pH_o 3, an extreme pH in which unadapted cells cannot synthesize ASPs. Several inducible amino acid decarboxylases appear to contribute to emergency pH_i maintenance in *S. typhimurium*. One of these systems has been identified as lysine decarboxylase (CadA) working in collaboration with a lysine–cadaverine antiporter (CadB) [3]. CadA decarboxylates intracellular lysine to cadaverine consuming a proton in the process. Cadaverine is then exchanged for fresh lysine from the medium via the CadB antiporter. Inducing the *cadAB* operon by low pH and lysine elevates ΔpH (pH_i–pH_o) by 1 pH unit during extreme acid exposure (pH_o 3.5) providing a clear survival advantage over *cad* mutants. The variety of inducible amino acid decarboxylases available to *S. typhimurium* (lysine, ornithine, arginine) suggests this organism can survive many extreme acid pH situations depending on which amino acids are present in the surrounding environment. There is also evidence for an emergency pH homeostasis system, albeit ill-defined, that is not dependent on extracellular amino acids [4].

Emergency pH homeostasis alone, however, does not provide effective acid tolerance. ASP synthesis during the second stage of the ATR is an absolute requirement for exponential phase Salmonellae to survive acid challenge. Three regulatory proteins (RpoS, Fur and PhoP) that control various ATR systems have been identified. Each regulator governs the expression of a distinct subset of ASPs.

3.2. Control of acid tolerance by an alternative sigma factor

The alternative sigma factor σ^S, encoded by *rpoS*, regulates one aspect of acid tolerance. σ^S is known to be a critical regulator of stationary phase physiology but its importance to exponential phase cells is increasingly being recognized [5]. A connection to acid tolerance was discovered after noting that virulent strains of *Salmonella* (UK1, SL1344, and 14028s) exhibited an acid tolerance superior to that

of an avirulent laboratory strain of LT2. The cause was traced to a mutation in the *rpoS* allele of LT2 by showing that the acid tolerance phenotypes of virulent and avirulent stains, as well as virulence itself, could be exchanged simply by swapping *rpoS* alleles [6,7]. Western blot and 2-dimensional PAGE analysis of ASPs revealed that RpoS is itself an ASP and that it controls the expression of eight other ASPs [6]. The N-terminal sequences for four of the eight σ^S-dependent ASPs have been determined and the proteins are either unidentified (ASP71 and ASP72) or of unknown function (ASP73 (identical to orf3 of the *E. coli tonB–trpA* region) and ASP74 (identical to *E. coli OsmY*)).

The acid shock induction of σ^S appears to be controlled by a 38-kDa protein, encoded by the mouse virulence gene *mviA* [7,8]. MviA controls the accumulation of σ^S and of the σ^S-dependent ASPs by regulating the proteolytic turnover of σ^S. Hence, MviA stimulates σ^S turnover in the absence of stress while allowing σ^S to accumulate in the presence of stress. The N-terminal half of MviA exhibits sequence homology to the regulatory protein components of 2-component signal transduction systems [8]. Thus, MviA probably does not directly degrade σ^S, a function shown to be performed by the ClpXP protease [9], but rather indirectly influences σ^S turnover after sensing some perturbation of cellular physiology [8]. Whether the MviA influence is transcriptional or otherwise is not known.

Mutations in *rpoS* or *mviA* render *Salmonella* avirulent [10,11]. This suggests that either under or overproducing σ^S is detrimental to the pathogenic process. It would appear that the successful pathogen must be free to vary σ^S in response to changing environments encountered during infection. Interestingly, the presently used live oral typhoid vaccine, attenuated *S. typhi* strain Ty21a, is, in fact, an *rpoS* mutant [12].

3.3. RpoS-independent ATR

As noted above, acid stress is the combined effect of low pH and weak (organic) acids. Different systems of protection may be needed for each acid stress component. Recent evidence indicates that RpoS-dependent systems are required to survive the deleterious effects of weak acids [13]. A 1-h adaptation at pH 4.4 prior to challenge at the same pH with organic acids enhances survival compared to unadapted controls. RpoS was shown to be required for this protection. In fact, a primary function of the σ^S-dependent ATR system may be to provide protection against organic acids. Normally acid sensitive, *rpoS* mutants will nevertheless elicit a robust ATR if the organic acids produced by fermentation are removed from a culture prior to adaptation (pH_o 4.4) and acid challenge (pH_o 3.1). This indicates the existence of an RpoS-independent ATR primarily directed against the low pH component of acid stress.

At least one regulator of this σ^S-independent ATR is PhoP, the regulatory component of the PhoP/PhoQ 2-component system. The PhoP/Q regulon is known to be important for macrophage survival, protection against antimicrobial peptides and virulence [14]. The PhoP/Q system has been shown to sense external Mg^{2+} [15]. However, our laboratory has recently shown that PhoP is also an ASP used for protection against low pH (Bearson and Foster, unpublished).

Another regulator of acid tolerance is the ferric uptake regulator (Fur). In the presence of excess intracellular Fe^{2+}, this 17-kDa protein represses the expression of iron-regulated genes. Surprisingly, Fur also governs the expression of several ASPs as an activator in an iron-independent manner. This regulation occurs even when the iron-binding site of Fur is compromised leading to the hypothesis that Fur senses iron and pH separately [16]. Mutations in *fur* render the cell acid sensitive, but which component of acid stress (H^+ or weak acid concentration) is countered by the Fur-regulated ASPs is not known.

Other genes with demonstrable effects on acid tolerance include the *PolA* and *Ada* genes involved in DNA repair, FabF involved in fatty acid synthesis, the cAMP receptor protein CRP and the Mg^{2+}-dependent proton-translocating ATPase.

3.4. Cross-protection

The ability of one stress condition to provide protection against other stresses is referred to as cross-protection. Several studies have shown that acid adaptations confer resistance to a wide range of stress conditions including heat, salt, H_2O_2, crystal

violet and polymyxin B [6,17]. However, adaptation to other stresses does not typically induce significant acid tolerance. This implies that exposure to acid may be perceived by bacteria as a more general stress indicator whereas heat, salt and H_2O_2 may be more specific stress signals. Furthermore, the demonstration that acid shock induces cross-resistance to a variety of stresses suggests that cells undergoing acid shock in the stomach will be well prepared to endure the environmental stresses subsequently confronted in the intestine.

4. Acid resistance in *E. coli* and *Shigella* species

Acid resistance (AR) is a phenomenon that at first glance appears distinct from the ATR described above. AR measurements are done using stationary-phase cells and the complex medium Luria Broth (LB) rather than exponential phase cells and defined minimal media (as in the ATR). Survival is measured after a 2-h exposure to pH_o 2.5 (a more severe acid challenge than used in the ATR). Using a standard of 10% survival under these conditions, Gorden and Small demonstrated that 75% of *Shigella* and 80% of *E. coli* isolates are acid resistant [18] whereas all *Salmonella* species tested were acid sensitive. However, in a subsequent study using minimal medium, none of these species survived pH_o 2.5 exposure [19]. Even at pH_o 3, *Shigella* was more sensitive than either *E. coli* or *S. typhimurium*, with the later two species exhibiting about equal acid tolerance [19]. It was clear from this work that components of LB and stationary-phase cells are important to the phenomenon of AR at pH_o 2.5.

4.1. Amino acid-dependent acid resistance systems

As explanation for the observations noted above, three complex medium-dependent AR systems not present in *S. typhimurium* have been described for *E. coli* [19]. Two of these systems are also present in *Shigella* [19]. The activity of each system depends in part on whether cells have undergone oxidative or fermentative metabolism. Two fermentative AR systems involve the inducible amino acid decarboxylases, arginine decarboxylase and glutamate decarboxylase. They are presumed to function much like the lysine decarboxylase system described above for *S. typhimurium* where amino acid decarboxylation consumes a proton at low pH_i after which a membrane antiporter exchanges the product for more of the amino acid substrate in the medium. Mutations causing defects in each system have now been described. The *adi* operon encoding arginine decarboxylase is required for the arginine-dependent system in *E. coli* [20,21] and *gadC* encoding a putative glutamate/γ-amino butyrate antiporter is required for the glutamate-dependent AR system in *E. coli* [22] and *S. flexneri* [23]. The third AR system, found in both *E. coli* and *S. flexneri*, is induced by growth to stationary phase in LB, is repressed by glucose and, once induced, does not require the presence of amino acids in the medium during a subsequent challenge at pH_o 2.5. This is the so-called oxidative system. It is dependent upon σ^S in both organisms, whereas the arginine and glutamate AR systems (in *E. coli*) are only partially dependent on this alternative sigma factor [19]. The *S. flexneri* glutamate system, however, is very dependent on σ^S due to a σ^S requirement for *gadC* expression [23]. The partial σ^S dependence of the arginine and glutamate AR systems reported for *E. coli* is probably related to the loss of other components of AR required in addition to the decarboxylases rather than with the expressions of *adi* and *gadC*. This, again, would be analogous to the *S. typhimurium* situation where lysine decarboxylase fulfills only one aspect of a complex tolerance system.

Most of the initial observations concerning AR in *E. coli* and *S. flexneri* may be accounted for by the regulation of these three systems. For example, acid resistance in *E. coli* only showed modest acid induction when using LB for both adaptation and pH_o 2.5 challenge [24]. However, when a *gadC* mutant was similarly tested, a dramatic acid induction was observed [22]. This may be explained by the fact that the glutamate system is not acid inducible in contrast to the arginine and oxidative systems. An interesting observation by Small et al. [24] might also be explained by these systems. They discovered that *E. coli rpoS* mutants that were acid sensitive when grown aerobically could develop an effective, acid-inducible AR system when grown anaerobically. This might also be explained by the *adi* system which is not only acid-induced but requires anaerobiosis

for expression [21]. It is possible that other anaerobiosis and acid-inducible systems may become evident under these conditions.

4.2. Acid habituation in exponential phase E. coli

The AR studies of *E. coli* involved stationary-phase cells. Other investigators have studied acid survival of log-phase cultures but employed a testing strategy different from both the ATR and AR. Acid habituation (AH) occurs when *E. coli* is grown in nutrient broth at pH 5.0. These acid-habituated cells will survive challenge pH of 3.5 or pH 3.0 better than cells grown at pH 7.0 [25]. AH appears to involve protein synthesis-dependent and independent steps [26] with repair of DNA damage a key event [27]. A role for phosphate and the phosphate-specific porin PhoE in AH was implicated by showing that phosphate ions inhibit AH and finding that *phoE* mutants are acid resistant [28]. It is proposed that PhoE provides a conduit for H^+ influx allowing acidification of the periplasm and stimulation of a transmembrane sensory protein that would signal induction of AH. A high level of phosphate in the medium is proposed to block access of H^+ to the PhoE pore and thereby interfere with signal transduction. It appears that AH is separate from the ATR and AR since both of these acid survival systems occur in high phosphate media.

Using exponential phase *E. coli*, Guilfoyle and Hirshfield demonstrated that organic acids can induce acid resistance in complex media [29]. *E. coli* adapted with 0.1% butyrate or propionate at pH_o 6.5 survived a 30-min challenge at pH_o 3.5 between 50- to 200-fold better than cells adapted at pH_o 6.5 without the weak acid. They also suggest that arginine and lysine decarboxylases can contribute to survival in organic acids. Exponential phase *E. coli* adapted at pH 5.5 were able to survive a challenge with 0.5% butyric acid (also at pH_o 5.5). However, survival rates of *adi* and *cad* mutants in this system were significantly lower than control cultures.

5. Low pH and pathogenicity

Studies by several groups have underscored the importance of low pH in the pathogenesis of enteric bacteria. For example, the infectious dose for *Salmonella* species is significantly decreased if stomach acidity is buffered, suggesting that the better prepared the organism is to tolerate stomach acid, the more likely it will survive to cause disease [30,31]. In addition, systems associated with *Salmonella* pathogenesis are induced by the low pH present in phagocytic compartments following the invasion of epithelial cells and macrophages [32–34]. Various mutations that confer acid sensitivity also attenuate *S. typhimurium* [35]. In *E. coli*, Poynter et al. [36] have shown that surface attachment of the bacteria enhances acid resistance. In fact, the apparent low infectious dose of enterohemorrhagic *E. coli* disease has been correlated to its acid survival properties [37–39]. It is clearly evident that a correlation exists between the response of enterobacteria to acid stress and the pathogenic process.

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