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MINIREVIEW

Acid Stress Responses of *Salmonella* and *E. coli* : Survival Mechanisms, Regulation, and Implications for Pathogenesis

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Enteric pathogens, in order to gain entrance into the intestine and cause disease, must survive the acid pH of the stomach. Pathogenic species that prefer to grow at neutral pH exhibit widely varying abilities to survive pH extremes. The importance of these differences becomes evident when comparing the infectious doses of these organisms (6, 7). The most acid sensitive, *Vibrio cholerae*, requires the highest oral infectious dose, while only a few *E. coli* and *Shigella* must be ingested to cause disease. The enteric pathogens can protect themselves from acid in several basic ways. They can prevent protons from entering the cell, pump protons out of the cell, or, once internal pH enters a dangerous acid zone, they could protect or repair damage to macromolecules. Finally, they could synthesize isoforms of key enzymes with pH optima in the acid range so they can function when internal pH falls. Our laboratory has examined the basic systems of acid tolerance in *Salmonella* that protect the cell down to pH 3 and the even more effective systems of acid resistance in *E. coli* that build on acid tolerance but that protect cells to pH 2 or less.

Systems that protect cells in acid conditions between pH 4 and pH 3

During our studies with *Salmonella*, we noticed that exposing log phase cells to mild (pH 5.8) or moderate (pH 4.5) acid stress for 30 to 60 minutes would protect cells undergoing a subsequent, rapid transition to pH 3 (Fig. 1). Protection persisted for at least two hours and required protein synthesis during the adaptation period. The process of adaptation is called the acid tolerance response (ATR). A similar ATR adaptation phenomenon was observed using stationary phase cells which, even without acid adaptation, are more acid tolerant than log phase cells. The acid adaptation of stationary phase cells also requires protein synthesis. Thus, there are four levels of acid tolerance. Presented in increasing order of tolerance they are: log phase cells, acid-adapted log phase cells, stationary phase cells, and acid-adapted stationary phase cells.

Acid shock protein synthesis

Two-dimensional analysis of the proteome revealed that 60 acid shock proteins (ASPs) are induced during log phase ATR while 45 ASPs are induced following adaptation of stationary phase cells. However, only 5 of those proteins overlap, indicating separate systems of acid tolerance. We have identified four regulatory proteins that control acid induction of ASP subsets. The log phase ATR is regulated by the iron regulatory protein Fur (senses iron and acid stress separately), the two component regulatory system PhoPQ (senses magnesium and protons), and the alternative RNA polymerase sigma factor σ^S (2, 14, 16). A mutation in any one of these regulators prevents log phase acid tolerance but has little to no effect on stationary phase acid tolerance. Critical ASPs of stationary phase acid tolerance are regulated by the classic response regulator OmpR (1). Significantly, three of the regulators mentioned are themselves acid-induced. Since mutants

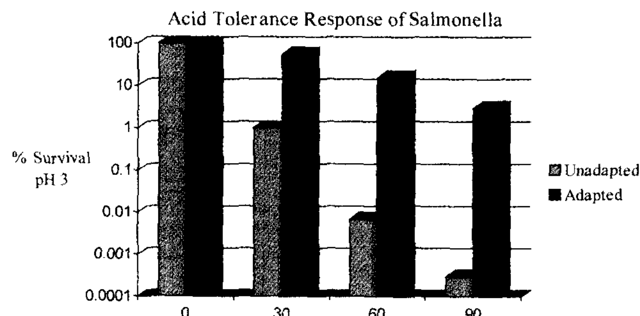

Fig. 1. *Salmonella* log phase acid tolerance. Cells grown to exponential phase at pH 7.7 in minimal glucose media and either directly shifted to pH 3 (unadapted) or adapted at pH 4.5 for 60 minutes prior to shifting to pH 3. Percent survival is measured over time.

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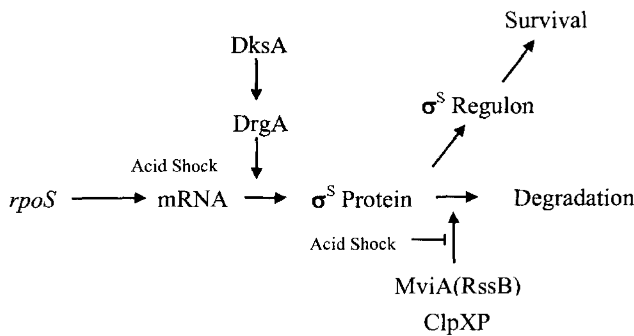

Fig. 2. Aspects of *rpoS* regulation affected by acid stress. Acid shock (short chain fatty acids like acetic acid) stimulates *rpoS* translation. The DksA protein activates *drgA* expression which is involved in *rpoS* translation. The MviA(RssB) adapter protein and ClpXP protease barrel degrade σ^S in rapidly growing cells. Acid shock prevents that degradation.

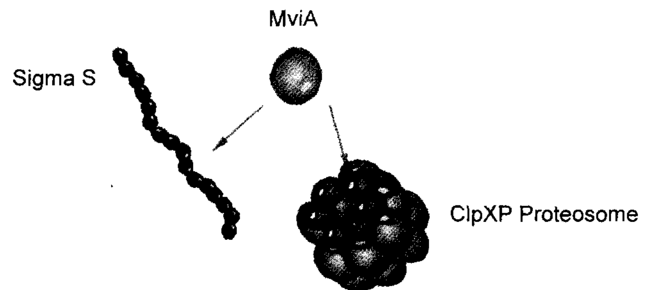

Fig. 3. MviA (RssB) binds to and presents σ^S to ClpXP for degradation.

lacking these regulators are acid sensitive, it is important to understand how acid stress signals an increase in the production of these regulators which translates into increased expression of their target genes. I will focus on two of these acid-induced regulators because they use different mechanisms to control their production.

The role of an alternative sigma factor

The sigma factor σ^S, encoded by the *rpoS* gene, was originally identified as being important to stationary phase physiology and its level increases during transition into stationary phase. But we have shown that the level of σ^S increases dramatically in log phase cells subjected to a brief acid shock (pH 4.5, 30 min) (16). Expression of *rpoS* and production of σ^S in stationary phase is regulated at all levels (transcription, translation, and protein stability, Fig. 2). Acid shock, however, only targets translation and protein turnover (3, 25). Acid shock in the form of short chain fatty acids (eg. acetate) increases the expression of translational but not transcriptional *rpoS-lacZ* fusions 10- to 15-fold. We have identified a regulatory cascade that is required for efficient translation of the *rpoS* message. The cascade begins with DksA, a zinc-finger protein with no known function and a gene regulated by DksA called *drgA* (25) (Webb and Foster, unpublished). The *drgA* product is required for *rpoS* translation but its role is unclear. Where acid shock modulates this cascade is not known. Curiously, Hfq, an RNA-binding protein important to *rpoS* translation in *E. coli*, has little, if any, effect on the translation of *Salmonella rpoS* (Webb and Foster, unpublished).

Acid shock control of σ^S degradation

Degradation of σ^S is very rapid in log phase cells (3 minute turnover rate) but slows considerably following acid shock. Degradation requires the ClpXP protease and a chaperone called MviA (RssB in *E. coli*) (3, 22). Bacterial two hybrid analysis indicates that MviA cross links σ^S and ClpX (the ATPase subunit of the protease) (18). MviA essentially expands the substrate specificity of ClpXP to include σ^S (Fig. 3). So, how does acid shock stop σ^S degradation? Initial focus was placed on acid shock influencing phosphorylation of MviA since the MviA N-terminus bears homology to the receiver domains of response regulators and is phosphorylated *in vitro* at residue aspartate-58. However, changing aspartate-58 to a residue that cannot be phosphorylated does not prevent the acid shock-mediated decrease in σ^S degradation (Audia and Foster, unpublished). The reason for acid shock control of σ^S turnover remains a mystery.

Autoinduction of ompR following acid shock

The stationary phase ATR is regulated, in part, by OmpR. Approximately 10 ASPs are not produced in an *ompR* mutant. OmpR is an important regulator of several aspects of cell physiology, not the least of which is its role in controlling expression of the SsrA/SsrB two component regulators whose genes reside in *Salmonella* pathogenicity island 2 (15). As a result of this regulation, *ompR* mutants are avirulent (5). We discovered that OmpR is itself induced by acid shock at the transcriptional level (1). Surprisingly, little attention has been given to whether environmental conditions can modulate expression of this regulator. We have used a variety of approaches to show that OmpR autoregulates itself. DNA footprint analysis revealed that OmpR protein binds to three sites in the *ompR* promoter region (Fig. 4, Bang and Foster, submitted). A centrally located site is protected on both strands while the upstream and downstream sites are protected only on the top and bottom strands, respectively. The evidence indicates that the EnvZ sensor-kinase phosphorylates OmpR, OmpR-P binds to the three sites and relieves repression of a second promoter by the nucleoid protein H-NS. Since DNA gyrase inhibitors also induce *ompR*, the working model is that acid alters the local topology of the *ompR* promoter, allowing OmpR-P to bind and prevent repression by H-NS. A possible effect of acid on OmpR phosphorylation has not totally been ruled out, however.

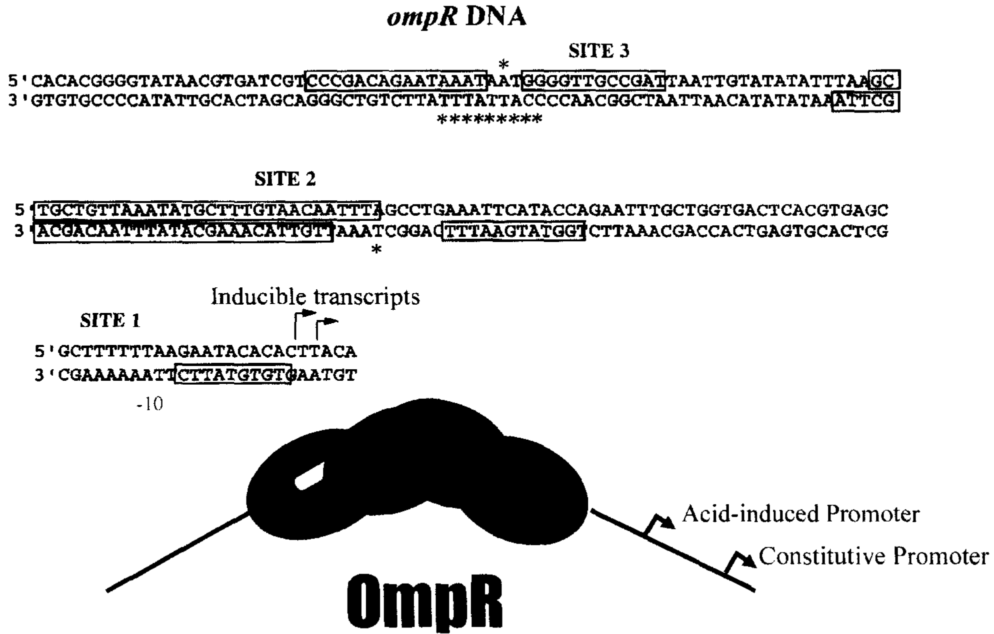

Fig. 4. DNA footprint of OmpR protein on *ompR* promoter region. Lower figure illustrates model for OmpR binding and autoinducing the *ompR* gene in response to acid shock.

Mechanisms of acid tolerance

The actual protective mechanisms of acid tolerance have not been defined but we do know that acid shock induces pH homeostasis and protein repair systems. Acid adapted log phase cells challenged at pH 3 possess a less acidic internal pH than unadapted cells similarly challenged. This inducible pH homeostasis appears to be the result of preventing net proton movement across the membrane of adapted cells rather than increased internal buffer capacity (Foster, in preparation). In addition, a protein repair pathway has been revealed using green fluorescent protein as a reporter of protein denaturation. Severe acid challenge only partially denatures GFP_{UV} in acid adapted cells. This partially denatured GFP will renature once the acid stress is removed. GFP in unadapted cells completely denatures at pH 3 and will not recover when cells (that are still >90% viable) are returned to neutral pH (Foster, in preparation). The components of these systems are under investigation.

Acid Resistance Systems protect between pH 2 to 3

The ATR systems protect well at pH values above pH 3

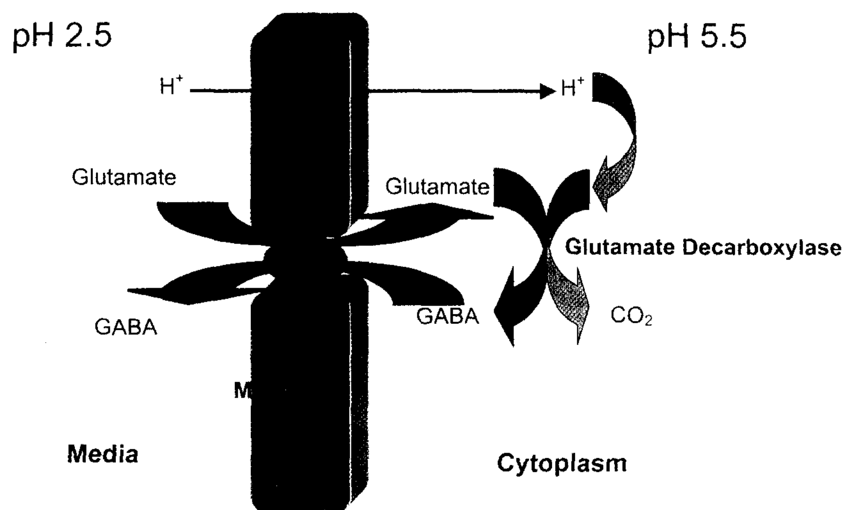

Fig. 5. Model of glutamate-dependent acid resistance in *E. coli*. GadC is a membrane-bound antiporter that exchanges glutamate and γ-amino butyric acid (GABA).

but have no effect below pH 3. *E. coli* possesses additional acid resistance (AR) systems that protect well at pH 2. These systems are not present in *Salmonella*. There are three AR systems, each of which is induced by transitions into stationary phase under acid conditions (10, 17). AR system 1 is σ^S and CRP-dependent and protects cells suspended in simple pH 2.5 buffer. AR system 2 depends on glutamate decarboxylase and will only protect at pH 2.5 if glutamate is present in the challenge medium. AR system 3 depends on arginine decarboxylase, meaning it will protect only if arginine is present at pH 2.5. These three systems form a pH stress "umbrella" that protects cells under a variety of different acid stress situations. The most effective is the glutamate decarboxylase system. Its working model (Fig. 5) has been that the intracellular decarboxylation of glutamate consumes a proton and produces gamma amino butyric acid (GABA). A dedicated antiporter, GadC, pumps out GABA while bringing in more glutamate. Repetitive cycles of decarboxylation and pumping were thought to keep intracellular pH less acid. However, log phase cells that overproduce GAD and GadC are not proportionately more acid resistant, indicating additional factors are required for acid resistance.

Regulation of glutamate-dependent acid resistance

Regulation of the GAD system is very complex. Two operons produce the GAD structural proteins (23, 24, 26). The *gadBC* operon encodes one of two glutamate decarboxylase isozymes and the GadC antiporter. The second transcriptional unit is *gadA*, the structural gene for the second GAD isozyme. Although there is only one transcriptional start site, the genes can be transcribed either by

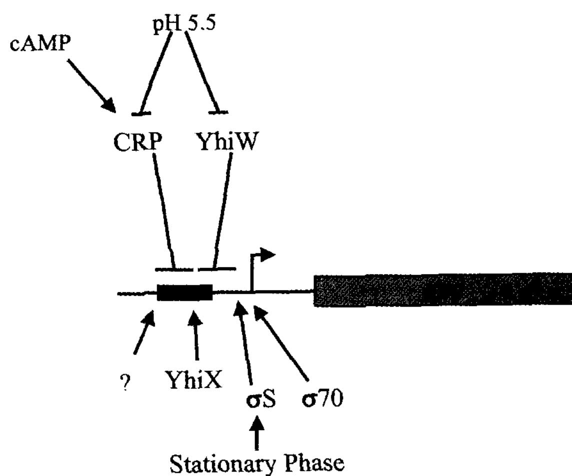

Fig. 6. Model for regulating *gad* gene expression. The black box located upstream of the *gad* transcriptional start represents a conserved 20 bp sequence required for expression. CRP and YhiW act as negative regulators whose repression is relieved by pH 5.5 exposure. Expression is positively controlled by YhiX and a hypothetical second activator. Either σ^S or σ^{70} can direct transcription and generate identical transcripts.

σ^S or σ^{70} RNA polymerase holoenzymes (10). There are three repressor systems and at least two activators controlling expression (Fig. 6). In complex media (LB), H-NS represses expression in log phase cells (12). However, H-NS appears unimportant to *gad* expression in minimal glucose media (Ma and Foster, unpublished). The cAMP receptor protein CRP and YhiW are the two other repressor systems that prevent *gad* expression in log phase under alkaline conditions (9) (Conway, Foster, Ma and Tucker, unpublished). No other repressors are likely to be involved since the *gad* genes are constitutively expressed in a *yhiX crp* double mutant, even in LB. YhiX(GadR) is an activator of the *gad* genes and is cotranscribed with *yhiW* (*yhiXW*) (Conway, Foster, Ma and Tucker, unpublished; Shin, Castanie-Cornet, Foster and Kaper, submitted). Since a *crp yhiW yhiX* triple mutant lacking the known repressors and activator still expresses large amounts of GAD, a second, unknown activator may be involved. There is only a single transcriptional start for each *gad* operon, but RNA polymerase complexed either to σ^S or σ^{70} will drive expression depending on the media conditions. What determines the choice between σ^S or σ^{70} RNP is not known.

E. coli O157:H7 Acid Resistance

Although several studies suggest that the enterohemorrhagic *E. coli* are more acid resistant than commensal organisms (4, 11, 13, 20, 21; 8), a side by side comparison of true human commensals versus clinical strains of O157 indicate little difference (17). Both groups of organisms exhibit outstanding levels of acid resistance, capable of withstanding pH 2 environments for several hours. Mutational analysis indicates that O157 possesses the same three *E. coli* K-12 systems of AR.

Acid resistance systems important to survival in the gastrointestinal tract

The main reservoir of O157 is the bovine gastrointestinal tract which includes a very acidic stomach, the abomasum. We have recently tested whether the AR systems help the organism survive in the bovine gastrointestinal tract. Competitive mixing experiments were performed where wild type and mutant O157, differentially tagged with antibiotic resistance markers, were administered to calves. The relative abilities of mutant and wild type to survive in the GI tract were determined by their relative presence in fecal contents. The O157 *rpoS* mutant survived and shed extremely poorly, relative to wild type, but since σ^s systems can protect against many stresses, one cannot conclude that poor survival was due to acid sensitivity (19). However, a *gadC* mutant also survived extremely poorly as compared to wild type (Price and Foster, submitted). Because GAD has no apparent effect on any other parameter of cell physiology, we conclude that AR system 2 is very important for gastrointestinal

survival. We suspect *gadC* mutants succumb to the extreme acidic environment in the abomasum of the bovine GI tract. Only a few survivors make their way to the intestine and feces.

Why multiple acid resistance systems?

Disease caused by O157 has also been linked to contaminated acidic foods like apple cider whose pH is about 3.5. Since our studies suggest there are different acid stress survival systems responsible for protection above and below pH 3, we tested the O157 mutants for survival in pH 3.5 apple cider at room temperature. The arginine and glutamate dependent systems were not required to survive in this environment. The σ^S-dependent AR system 1, however, was vitally important (Foster and Price, in preparation). In contrast to the bovine study, acid was clearly the relevant stress in this situation because neutralizing the pH of apple cider allowed the *rpoS* mutant to survive very well. It seems that *E. coli* has developed multiple acid stress survival mechanisms not just for the sake of redundancy, but to target specific stress situations.

Conclusions

Acid stress responses in enteric bacteria are critical for surviving passage through the gastrointestinal tract and for survival in other acidic environments. There are systems that function above, but not below, pH 3 (acid tolerance response) and separate systems that work best below pH 3 (acid resistance). Additional studies of these systems will yield important information regarding the basic physiology of the cell and the systems used to survive in harsh environments.

Acknowledgments

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